DESCRIPTION

CANCER TREATMENT METHODS USING THERAPEUTIC CONJUGATES THAT BIND TO AMINOPHOSPHOLIPIDS

BACKGROUND OF THE INVENTION

The present application claims priority to first provisional application Serial No. 60/092,589, filed July 13, 1998, and second provisional application Serial No. 60/110,600, filed December 02, 1998, the entire text and figures of which applications are incorporated herein by reference without disclaimer. The U.S. Government owns rights in the present invention pursuant to grant numbers 1RO1CA74951-01 and 5RO1CA54168-05 from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the fields of blood vessels and tumor biology. More particularly, it embodies the surprising findings that aminophospholipids, such as phosphatidylserine and phosphatidylethanolamine, are accessible, stable and specific markers of tumor vasculature. The invention thus provides therapeutic constructs and conjugates that bind to aminophospholipids for use in delivering toxins and coagulants to tumor blood vessels and for inducing thrombosis and tumor regression.

2. Description of the Related Art

Tumor cell resistance to chemotherapeutic agents represents a significant problem in clinical oncology. In fact, this is one of the main reasons why many of the most prevalent forms of human cancer still resist effective chemotherapeutic intervention, despite certain advances in the field of chemotherapy.

A significant problem to address in tumor treatment regimens is the desire for a "total cell kill". This means that the more effective treatment regimens come closer

to a total cell kill of all so-called "clonogenic" malignant cells, *i.e.*, cells that have the ability to grow uncontrolled and replace any tumor mass that might be removed by the therapy. Due to the goal of developing treatments that approach a total cell kill, certain types of tumors have been more amenable to therapy than others. For example, the soft tissue tumors, *e.g.*, lymphomas, and tumors of the blood and blood-forming organs, *e.g.*, leukemias, have generally been more responsive to chemotherapeutic therapy than have solid tumors, such as carcinomas.

One reason for the susceptibility of soft and blood-based tumors to chemotherapy is the greater accessibility of lymphoma and leukemic cells to chemotherapeutic intervention. Simply put, it is much more difficult for most chemotherapeutic agents to reach all of the cells of a solid tumor mass than it is the soft tumors and blood-based tumors, and therefore much more difficult to achieve a total cell kill. Increasing the dose of chemotherapeutic agents most often results in toxic side effects, which generally limits the effectiveness of conventional anti-tumor agents.

Another tumor treatment strategy is the use of an "immunotoxin", in which an anti-tumor cell antibody is used to deliver a toxin to the tumor cells. However, in common with the chemotherapeutic approaches described above, immunotoxin therapy also suffers from significant drawbacks. For example, antigen-negative or antigen-deficient cells can survive and repopulate the tumor or lead to further metastases. Also, in the treatment of solid tumors, the tumor mass is generally impermeable to molecules of the size of antibodies and immunotoxins. Both the physical diffusion distances and the interstitial pressure within the tumor are significant limitations to this type of therapy.

A more recent strategy has been to target the vasculature of solid tumors. Targeting the blood vessels of the tumors, rather than the tumor cells themselves, has certain advantages in that it is not likely to lead to the development of resistant tumor cells, and that the targeted cells are readily accessible. Moreover, destruction of the

blood vessels leads to an amplification of the anti-tumor effect, as many tumor cells rely on a single vessel for their oxygen and nutrients (Denekamp, 1990). Exemplary vascular targeting strategies are described in U.S. Patents Nos. 5,855,866 and 5,___,___ (U.S. Application 08/350,212, Issue Fee paid), which particularly describe the targeted delivery of anti-cellular agents and toxins to protein markers of tumor vasculature.

Another effective version of the vascular targeting approach is to target a coagulation factor to a protein marker expressed or adsorbed within the tumor vasculature (Huang *et al.*, 1997; U.S. Patents 5,877,289, 5,___,___ and 5,___,___ (U.S. Applications Serial Nos. 08/487,427 and 08/482,369; Issue Fees paid)). The delivery of coagulants, rather than toxins, to tumor vasculature has the further advantages of reduced immunogenicity and even lower risk of toxic side effects. As disclosed in U.S. Patent 5,877,289, a preferred coagulation factor for use in such tumor-specific thrombogens, or "coaguligands", is a truncated version of the human coagulation-inducing protein, Tissue Factor (TF). TF is the major initiator of blood coagulation (Ruf *et al.*, 1991; Edgington *et al.*, 1991; Ruf and Edgington, 1994). Treatment of tumor-bearing mice with such coaguligands results in significant tumor necrosis and even complete tumor regression in many animals (Huang *et al.*, 1997; U.S. Patents 5,877,289, 5,___,___ and 5,___,___ (U.S. Applications Serial Nos. 08/487,427 and 08/482,369; Issue Fees paid)).

Although the specific delivery of therapeutic agents, such as anti-cellular agents, toxins and coagulation factors, to protein markers of tumor vessels represents a significant advance in tumor treatment protocols, there is still room for additional vascular targeting therapies. The identification of additional stable targets to allow specific tumor vessel destruction *in vivo* would naturally be of benefit in expanding the number of targeting options. More particularly, the development of targeting agents for delivering therapeutics even closer to the tumor vascular endothelial cell membrane would represent an important advance.

SUMMARY OF THE INVENTION

The present invention addresses the needs of the prior art by providing new compositions and methods for tumor vasculature imaging and destruction. The invention is based, in part, on the finding that aminophospholipid membrane components, such as phosphatidylserine and phosphatidylethanolamine, are accessible, stable markers of tumor vasculature. The invention thus provides binding ligands and antibodies against aminophospholipids that are operatively attached to therapeutic agents, and methods of using constructs in the specific delivery of diagnostics and therapeutics to the actual surface of tumor vascular endothelial cell membranes.

Important aspects of the invention are that therapeutic agents can be delivered in intimate contact with the tumor vascular endothelial cell membrane, allowing either rapid entry into the target cell or rapid association with effector cells, components of the coagulation cascade, and such like. Certain surprising features of the invention include the discovery that translocation of aminophospholipids, such as phosphatidylserine (PS), to the surface of tumor vascular endothelial cells occurs, at least in a significant part, independently of cell damage and apoptopic or other cell-death mechanisms. Thus, PS surface expression in this environment is not a consequence of cell death, nor does it trigger immediate cell destruction.

The discovery of sufficiently stable PS expression on morphologically intact tumor-associated vascular endothelial cells is important to the targeting nature of the present invention. Should PS translocation to the outer surface of tumor vascular endothelium occur only in dying cells, or should it inevitably trigger cell death, then PS expression would be expected to be transient and PS would not likely be a good candidate target for therapeutic intervention. Surprisingly, the present invention shows that significant stable PS expression occurs in viable endothelial cells in a tumor environment, thus providing ample targeting opportunities.

The present invention therefore basically provides methods for delivering selected diagnostic and therapeutic agents to tumor or intratumoral vasculature, comprising administering to an animal having a vascularized tumor a biologically effective amount of a binding ligand that comprises a selected diagnostic or therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid, preferably one that binds to phosphatidylserine or phosphatidylethanolamine, on the luminal surface of blood vessels or intratumoral blood vessels of the vascularized tumor.

The methods of the invention provide for killing, or specifically killing, tumor or intratumoral vascular endothelial cells, and comprise administering to an animal or patient having a vascularized tumor a biologically effective amount of at least a first pharmaceutical composition comprising a binding ligand that comprises a selected therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid, preferably one that binds to phosphatidylserine or phosphatidylethanolamine, on the luminal surface of tumor or intratumoral vascular endothelial cells.

The "binding ligands" of the present invention are thus "aminophospholipid binding ligands", "therapeutic aminophospholipid binding ligand constructs", "aminophospholipid-targeted therapeutic agents", "aminophospholipid-targeted therapeutics", "aminophospholipid-targeted therapeutic agent constructs", or "therapeutic agent-aminophospholipid targeting agent constructs". For simplicity, these agents are referred to herein as "binding ligands" or "therapeutic agent-targeting agent constructs", with the understanding that such terms are used as a succinct way of referring to a conjugate or other operative association of a selected therapeutic agent and a targeting agent, antibody, binding protein or active fragment thereof, that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, expressed on the luminal surface of tumor or intratumoral vascular endothelial cells.

"Biologically effective amounts" are amounts of the therapeutic agent-targeting agent construct effective to specifically kill at least a portion, and preferably a significant portion, of the tumor or intratumoral vascular endothelial cells, as opposed to endothelial cells in normal vessels, upon binding to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, expressed on the luminal surface of the tumor or intratumoral vascular endothelial cells. As such, it is an "endothelial cell killing amount" or a "tumor vascular endothelial cell killing amount" of a therapeutic agent-targeting agent construct.

As used throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, except in instances wherein an upper limit is thereafter specifically stated. Therefore a "therapeutic agent-targeting agent construct" means "at least a first therapeutic agent-targeting agent construct". The operable limits and parameters of combinations, as with the amounts of any single agent, will be known to those of ordinary skill in the art in light of the present disclosure.

The "a" and "an" terms are also used to mean "at least one", "at least a first", "one or more" or "a plurality" of steps in the recited methods, except where specifically stated. This is particularly relevant to the administration steps in the treatment methods. Thus, not only may different doses be employed with the present invention, but different numbers of doses, *e.g.*, injections, may be used, up to and including multiple injections.

An "aminophospholipid", as used herein, means a phospholipid that includes within its structure at least a first primary amino group. Preferably, the term "aminophospholipid" is used to refer to a primary amino group-containing phospholipid that occurs naturally in mammalian cell membranes. However, this is not a limitation on the meaning of the term "aminophospholipid", as this term also extends to non-naturally occurring or synthetic aminophospholipids that nonetheless have uses in the invention, *e.g.*, as an immunogen in the generation of anti-aminophospholipid antibodies ("cross-reactive antibodies") that do bind to

aminophospholipids of mammalian plasma membranes. The aminophospholipids of U.S. Patent No. 5,767,298, incorporated herein by reference, are appropriate examples.

The prominent aminophospholipids found in mammalian biological systems are the negatively-charged phosphatidylserine ("PS") and the neutral or zwitterionic phosphatidylethanolamine ("PE"), which are therefore preferred aminophospholipids for targeting by the present invention. However, the invention is by no means limited to the targeting of phosphatidylserines and phosphatidylethanolamines, and any other aminophospholipid target may be employed (White *et al.*, 1978; incorporated herein by reference) so long as it is expressed, accessible or complexed on the luminal surface of tumor vascular endothelial cells.

All aminophospholipid-, phosphatidylserine- and phosphatidylethanolamine-based components are encompassed as targets of the invention irrespective of the type of fatty acid chains involved, including those with short, intermediate or long chain fatty acids, and those with saturated, unsaturated and polyunsaturated fatty acids. Preferred compositions for raising antibodies for use in the present invention may be aminophospholipids with fatty acids of C18, with C18:1 being more preferred (Levy *et al.*, 1990; incorporated herein by reference). To the extent that they are accessible on tumor vascular endothelial cells, aminophospholipid degradation products having only one fatty acid (lyso derivatives), rather than two, may also be targeted (Qamar *et al.*, 1990; incorporated herein by reference).

Another group of potential aminophospholipid targets include, for example, phosphatidal derivatives (plasmalogens), such as phosphatidalserine and phosphatidalethanolamine (having an ether linkage giving an alkenyl group, rather than an ester linkage giving an acyl group). Indeed, the targets for therapeutic intervention by the present invention include any substantially lipid-based component that comprises a nitrogenous base and that is present, expressed, translocated, presented or otherwise complexed in a targetable form on the luminal surface of tumor

vascular endothelial cells, not excluding phosphatidylcholine ("PC"). Lipids not containing glycerol may also form appropriate targets, such as the sphingolipids based upon sphingosine and derivatives.

The biological basis for including a range of lipids in the group of targetable components lies, in part, with the observed biological phenomena of lipids and proteins combining in membranous environments to form unique lipid-protein complexes. Such lipid-protein complexes extend to antigenic and immunogenic forms of lipids such as phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine with, *e.g.*, proteins such as β_2-glycoprotein I, prothrombin, kininogens and prekallikrein. Therefore, as proteins and polypeptides can have one or more free primary amino groups, it is contemplated that a range of effective "aminophospholipid targets" may be formed *in vivo* from lipid components that are not aminophospholipids in the strictest sense. Nonetheless, all such targetable complexes that comprise lipids and primary amino groups constitute an "aminophospholipid" within the scope of the present invention.

The inventive methods also act to arrest blood flow, or specifically arrest blood flow, in tumor vasculature. This is achieved by administering to an animal or patient having a vascularized tumor at least one dose of at least a first pharmaceutical composition comprising a coagulation-inducing amount, or a vessel-occluding amount, of at least a first cytotoxic or coagulative agent operatively attached to a targeting agent that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, translocated to the luminal surface of tumor vasculature.

The "coagulation-inducing amount" or "vessel-occluding amount" is an amount of the therapeutic agent-targeting agent construct effective to specifically promote coagulation in, and hence occlude, at least a portion, and preferably a significant portion, of tumor or intratumoral blood vessels, as opposed to normal blood vessels, upon binding to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, translocated to the luminal surface of tumor or

intratumoral blood vessels. The "vessel-occluding amount" is therefore a functionally effective amount, and is not a physical mass of therapeutic agent-targeting agent construct sufficient to span the breadth of a vessel.

Methods for destroying, or specifically destroying, tumor vasculature are provided that comprise administering to an animal or patient having a vascularized tumor one or more doses of at least a first pharmaceutical composition comprising a tumor-destructive amount of at least a first occluding or destructive agent operatively attached to a targeting agent that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, presented on the luminal surface of tumor or intratumoral vasculature. The "tumor-destructive amount" is an amount of the therapeutic agent-targeting agent construct effective to specifically destroy or occlude at least a portion, and preferably a significant portion, of tumor or intratumoral blood vessels, as opposed to normal blood vessels, upon binding to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, presented on the luminal surface of the vascular endothelial cells of the tumor or intratumoral blood vessels.

The invention further encompasses methods for treating cancer and solid tumors, comprising administering to an animal or patient having a vascularized tumor a tumor necrosis-inducing amount or amounts of at least a first pharmaceutical composition comprising at least a first therapeutic or necrotic agent operatively attached to a targeting agent that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, on the luminal surface of blood vessels or intratumoral blood vessels of the vascularized tumor. The "tumor necrosis-inducing amount" is an amount of the therapeutic agent-targeting agent construct effective to specifically induce hemorrhagic necrosis in at least a portion, and preferably a significant portion, of the tumor upon binding to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, complexed at the luminal surface of the vascular endothelial cells of the tumor or intratumoral blood vessels, while exerting little adverse side effects on normal, healthy tissues.

The methods of the invention may thus be summarized as methods for treating an animal or patient having a vascularized tumor, comprising administering to the animal or patient a therapeutically effective amount of at least a first pharmaceutical composition comprising at least a first therapeutic agent-targeting agent construct that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of blood transporting vessels of the vascularized tumor.

The essence of the invention may also be defined as a composition comprising at least a first diagnostic agent-targeting agent construct, or preferably a therapeutic agent-targeting agent construct, preferably that binds to phosphatidylserine or phosphatidylethanolamine, for use in the preparation of a medicament for use in tumor vasculature imaging and/or destruction and for human tumor diagnosis and/or treatment. This can also be defined as a composition comprising at least a first diagnostic agent-targeting agent construct, or preferably a therapeutic agent-targeting agent construct, for use in the preparation of a medicament for use in binding to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of blood transporting vessels of a vascularized tumor and for use in forming an image of tumor vasculature and/or for use in inducing tumor vasculature destruction and for human tumor diagnosis and/or treatment.

In the methods, medicaments and uses of the present invention, one of the advantages lies in the fact that the provision of the diagnostic or therapeutic agent-targeting agent construct, preferably one that binds to phosphatidylserine or phosphatidylethanolamine, into the systemic circulation of an animal or patient results in the preferential or specific localization to the tumor vascular surface membranes themselves, and not to some protein complex more distant from the membrane. The invention thus provides for more intimate cell contact than the methods and anti-vascular agents of the prior art.

In the context of the present invention, the term "a vascularized tumor" most preferably means a vascularized, malignant tumor, solid tumor or "cancer". The invention is particularly advantageous in treating vascularized tumors of at least about intermediate size, and in treating large vascularized tumors - - although this is by no means a limitation on the invention. The invention may therefore be used in the treatment of any tumor that exhibits aminophospholipid-positive blood vessels, preferably phosphatidylserine- and/or phosphatidylethanolamine-positive blood vessels.

In preferred embodiments, the tumors to be treated by the present invention will exhibit a killing effective number of aminophospholipid-positive blood vessels. "A killing effective number of aminophospholipid-positive blood vessels", as used herein, means that at least about 3% of the total number of blood vessels within the tumor will be positive for aminophospholipid expression, preferably phosphatidylserine and/or phosphatidylethanolamine expression. Preferably, at least about 5%, at least about 8%, or at least about 10% or so, of the total number of blood vessels within the tumor will be positive for aminophospholipid expression. Given the aminophospholipid-negative, particularly PS-negative, nature of the blood vessels within normal tissues, the tumor vessels will act as sink for the administered antibodies. Furthermore, as destruction of only a minimum number of tumor vessels can cause widespread thrombosis, necrosis and an avalanche of tumor cell death, antibody localization to all, or even a majority, of the tumor vessels is not necessary for effective therapeutic intervention.

Nonetheless, in more preferred embodiments, tumors to be treated by this invention will exhibit a significant number of aminophospholipid-positive blood vessels. "A significant number of aminophospholipid-positive blood vessels", as used herein, means that at least about 10-12% of the total number of blood vessels within the tumor will be positive for aminophospholipid expression, preferably phosphatidylserine and/or phosphatidylethanolamine expression. Even more preferably, the % of aminophospholipid-expressing tumor vessels will be at least about

15%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, or at least about 80% or so of the total number of blood vessels within the tumor, up to and including even at least about 90% or 95% of the vessels.

The "therapeutically effective amounts" for use in the invention are amounts of therapeutic agent-targeting agent constructs, preferably PS- or PE-binding constructs, effective to specifically kill at least a portion of tumor or intratumoral vascular endothelial cells; to specifically promote coagulation in at least a portion of tumor or intratumoral blood vessels; to specifically occlude or destroy at least a portion of blood transporting vessels of the tumor; to specifically induce necrosis in at least a portion of a tumor; and/or to induce tumor regression or remission upon administration to selected animals or patients. Such effects are achieved while exhibiting little or no binding to, or little or no killing of, vascular endothelial cells in normal, healthy tissues; little or no coagulation in, occlusion or destruction of blood vessels in healthy, normal tissues; and exerting negligible or manageable adverse side effects on normal, healthy tissues of the animal or patient.

The terms "preferentially" and "specifically", as used herein in the context of promoting coagulation in, or destroying, tumor vasculature, and/or in the context of causing tumor necrosis, thus mean that the therapeutic agent-targeting agent constructs function to achieve coagulation, destruction and/or tumor necrosis that is substantially confined to the tumor vasculature and tumor site, and does not substantially extend to causing coagulation, destruction and/or tissue necrosis in normal, healthy tissues of the animal or subject. The structure and function of healthy cells and tissues is therefore maintained substantially unimpaired by the practice of the invention.

Although understanding the mechanism of action is not necessary to the practice of the present invention, the methods will generally operate on the basis of the mode of action of the particular therapeutic agent or agents chosen for attachment to the targeting agent. As such, the aminophospholipid binding agents that are

conjugated to, or operatively associated with, cytotoxic or anticellular agents ("anti-aminophospholipid immunotoxins") will act initially via cellular destruction. Likewise, aminophospholipid binding agents that are conjugated to, or operatively associated with, coagulation factors ("anti-aminophospholipid coaguligands") will act initially via coagulation. However, these mechanisms will have some cross-over, as cell destruction exposes basement membranes and results in coagulation, and as coagulation deprives the cells of oxygen and nutrients and results in cell destruction.

Naked or unconjugated antibodies against aminophospholipid components are also capable of specifically inducing tumor blood vessel destruction and tumor necrosis *in vivo*. Such methods of tumor treatment are also contemplated by the present inventors, and are disclosed and claimed in first and second provisional applications Serial Nos. 60/092,672 (filed July 13, 1998) and 60/110,608 (filed December 02, 1998) and in co-filed U.S. and PCT patent applications (Attorney Docket Nos. 4001.002200, 4001.002282 and 4001.002210), each specifically incorporated herein by reference. In light of the beneficial effects of naked anti-aminophospholipid antibodies, the mechanism of action of the present conjugates may extend beyond the mode of action of the particular therapeutic agent or agents employed.

Therefore, the following mechanisms may contribute to the success of the invention: cell-mediated cytotoxicity, complement-mediated lysis, apoptosis, antibody-induced cell signaling (direct signaling), or mimicking or altering signal transduction pathways (indirect signaling).

The treatment methods thus include administering to an animal or patient having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first therapeutic agent-targeting agent construct effective to induce, or specifically induce, cell-mediated cytotoxicity of at least a portion of the tumor or intratumoral vascular endothelial cells. Herein, the first therapeutic agent-targeting agent construct binds to an aminophospholipid, preferably

phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of tumor or intratumoral vascular endothelial cells and induces cell-mediated cytotoxicity of at least a portion of the tumor or intratumoral vascular endothelial cells, as opposed to endothelial cells in normal vessels. As used herein, "cell-mediated cytotoxicity or destruction" includes ADCC (antibody-dependent, cell-mediated cytotoxicity) and NK (natural killer) cell killing.

The methods further include administering to an animal or patient having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first therapeutic agent-targeting agent construct effective to induce, or specifically induce, complement-mediated lysis of at least a portion of the tumor or intratumoral vascular endothelial cells. Herein, the first therapeutic agent-targeting agent construct binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of tumor or intratumoral vascular endothelial cells and induces complement-mediated lysis of at least a portion of the tumor or intratumoral vascular endothelial cells, as opposed to endothelial cells in normal vessels.

As used herein, "complement-mediated or complement-dependent lysis or cytotoxicity" means the process by which the complement-dependent coagulation cascade is activated, multi-component complexes are assembled, ultimately generating a lytic complex that has direct lytic action, causing cell permeabilization. Therapeutic agent-targeting agents for use in inducing complement-mediated lysis will generally include an antibody Fc portion.

The complement-based mechanisms by which the present invention may operate further include "complement-activated ADCC". In such aspects, the administered therapeutic agent-targeting agent contains an antibody, or fragment thereof, that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed

at the luminal surface of tumor or intratumoral vascular endothelial cells and induces complement-activated ADCC of at least a portion of the tumor or intratumoral vascular endothelial cells, as opposed to endothelial cells in normal vessels. "Complement-activated ADCC" is used to refer to the process by which complement, not an antibody Fc portion *per se*, holds a multi-component complex together and in which cells such as neutrophils lyse the target cell.

In other embodiments, the methods include administering to an animal or patient having a vascularized tumor at least a first pharmaceutical composition comprising an amount of at least a first therapeutic agent-targeting agent construct effective to induce, or specifically induce, apoptosis in at least a portion of the tumor or intratumoral vascular endothelial cells. Herein, the first therapeutic agent-targeting agent construct binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of tumor or intratumoral vascular endothelial cells and induces apoptosis in least a portion of the tumor or intratumoral vascular endothelial cells, as opposed to endothelial cells in normal vessels.

As used herein, "induces apoptosis" means induces the process of programmed cell death that, during the initial stages, maintains the integrity of the cell membrane, yet transmits the death-inducing signals into the cell. This is opposed to the mechanisms of cell necrosis, during which the cell membrane loses its integrity and becomes leaky at the onset of the process.

Therapeutic benefits may be realized by the administration of at least two, three or more therapeutic agent-targeting agent constructs. The therapeutic agent-targeting agent constructs may also be combined with other therapies to provide combined therapeutically effective amounts, as disclosed herein.

The treatment methods of the present invention will generally involve the administration of the pharmaceutically effective composition to the animal

systemically, such as via intravenous injection. However, any route of administration that allows the therapeutic agent-targeting agent construct to localize to the tumor or intratumoral vascular endothelial cells will be acceptable.

"Administration", as used herein, therefore means provision or delivery of therapeutic agent-targeting agent constructs in an amount(s) and for a period of time(s) effective to allow binding to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of blood transporting vessels of the vascularized tumor, and to exert a tumor vasculature destructive and tumor-regressive effect. The passive administration of proteinaceous therapeutic agent-targeting agent constructs is generally preferred, in part, for its simplicity and reproducibility.

However, the term "administration" is herein used to refer to any and all means by which therapeutic agent-targeting agent constructs are delivered or otherwise provided to the tumor vasculature. "Administration" therefore includes the provision of cells that produce the therapeutic agent-targeting agent constructs in a manner effective to result in the delivery of the therapeutic agent-targeting agent constructs to the tumor vasculature, and/or their localization to such vasculature. In such embodiments, it may be desirable to formulate or package the cells in a selectively permeable membrane, structure or implantable device, generally one that can be removed to cease therapy. Exogenous therapeutic agent-targeting agent administration will still generally be preferred, as this represents a non-invasive method that allows the dose to be closely monitored and controlled.

The "therapeutic agent-targeting agent administration methods" of the invention also extend to the provision of nucleic acids that encode therapeutic agent-targeting agent constructs in a manner effective to result in the expression of the therapeutic agent-targeting agent constructs in the vicinity of the tumor vasculature, and/or in the expression of therapeutic agent-targeting agent constructs that can localize to the tumor vasculature. Any gene therapy technique may be employed, such as naked DNA

delivery, recombinant genes and vectors, cell-based delivery, including *ex vivo* manipulation of patients' cells, and the like.

One of the benefits of the present invention is that aminophospholipids, particularly phosphatidylserine and phosphatidylethanolamine, are generally expressed or available throughout the tumor vessels. Aminophospholipid expression on established, intratumoral blood vessels is advantageous as targeting and destroying such vessels will rapidly lead to anti-tumor effects. However, so long as the administered therapeutic agent-targeting agent constructs bind to at least a portion of the blood transporting vessels, significant anti-tumor effects will ensue. This will not be problematical as aminophospholipids, such as phosphatidylserine and phosphatidylethanolamine, are expressed on the large, central vessels, and also on veins, venules, arteries, arterioles and blood transporting capillaries in all regions of the tumor.

In any event, the ability of the therapeutic agent-targeting agent constructs to destroy the tumor vasculature means that tumor regression can be achieved, rather than only tumor stasis. Tumor stasis is most often the result of anti-angiogenic therapies that target only the budding vessels at the periphery of a solid tumor and stop the vessels proliferating. Even if the present invention targeted more of the peripheral regions of the tumor in certain tumor types, which is not currently believed to be the case, destruction of the blood transporting vessels in such areas would still lead to widespread thrombosis and tumor necrosis.

The targeting portions of the diagnostic and/or therapeutic agent-targeting agent constructs of the present invention, whether binding to phosphatidylethanolamine or phosphatidylserine, may be either antibody-based or binding ligand or binding protein based. Any aminophospholipid binding ligand or protein known in the art may thus now be advantageously used in the delivery of therapeutic agents to tumor vasculature.

By way of example only, suitable aminophospholipid binding ligands and proteins include low and high molecular weight kininogens and other rat, bovine, monkey or human phosphatidylethanolamine binding proteins; and any one or more of a number of phosphatidylserine-serine binding annexins. The protein and DNA sequences for such binding ligands are known in the art and incorporated herein by reference, facilitating the production of recombinant fusion proteins for use in the present invention.

Aminophospholipid binding reagents encompassed by the term "aminophospholipid binding ligands or binding proteins" extend to all aminophospholipid binding ligands and proteins from all species, and aminophospholipid binding fragments thereof, including dimeric, trimeric and multimeric ligands and proteins; bispecific ligands and proteins; chimeric ligands and proteins; human ligands and proteins; recombinant and engineered ligands and proteins, and fragments thereof.

Where antibody-based targeting portions are employed, whether binding to phosphatidylethanolamine or phosphatidylserine, the term "anti-aminophospholipid antibody", as used herein, refers broadly to any immunologic binding agent, such as polyclonal and monoclonal IgG, IgM, IgA, IgD and IgE antibodies. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

Polyclonal anti-aminophospholipid antibodies, obtained from antisera, may be employed in the invention. However, the use of monoclonal anti-aminophospholipid antibodies (MAbs) will generally be preferred. MAbs are recognized to have certain advantages, *e.g.*, reproducibility and large-scale production, that makes them suitable for clinical treatment. The invention thus provides monoclonal antibodies of the murine, human, monkey, rat, hamster, rabbit and even frog or chicken origin. Due to the ease of preparation and ready availability of reagents, murine monoclonal antibodies will be used in certain embodiments.

As will be understood by those in the art, the immunologic binding reagents encompassed by the term "anti-aminophospholipid antibody" extend to all anti-aminophospholipid antibodies from all species, and antigen binding fragments thereof, including dimeric, trimeric and multimeric antibodies; bispecific antibodies; chimeric antibodies; human and humanized antibodies; recombinant and engineered antibodies, and fragments thereof.

The term "anti-aminophospholipid antibody" is thus used to refer to any anti-aminophospholipid antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art.

In certain embodiments, the antibodies employed in the therapeutic agent-targeting agent constructs will be "humanized" or human antibodies. "Humanized" antibodies are generally chimeric monoclonal antibodies from mouse, rat, or other non-human species, bearing human constant and/or variable region domains ("part-human chimeric antibodies"). Mostly, humanized monoclonal antibodies for use in the present invention will be chimeric antibodies wherein at least a first antigen binding region, or complementarity determining region (CDR), of a mouse, rat or other non-human anti-aminophospholipid monoclonal antibody is operatively attached to, or "grafted" onto, a human antibody constant region or "framework".

"Humanized" monoclonal antibodies for use herein may also be anti-aminophospholipid monoclonal antibodies from non-human species wherein one or more selected amino acids have been exchanged for amino acids more commonly observed in human antibodies. This can be readily achieved through the use of routine recombinant technology, particularly site-specific mutagenesis.

Entirely human, rather than "humanized", anti-aminophospholipid antibodies may also be prepared and used in the therapeutic agent-targeting agent constructs of the present invention. Such human antibodies may be polyclonal antibodies, as obtained from human patients that have any one or more of a variety of diseases, disorders or clinical conditions associated with the production of anti-aminophospholipid antibodies. Such antibodies may be concentrated, partially purified or substantially purified for use herein.

A range of techniques are also available for preparing human monoclonal antibodies. As human patients with anti-aminophospholipid antibody-producing diseases exist, the anti-aminophospholipid antibody-producing cells from such patients may be obtained and manipulated *in vitro* to provide a human monoclonal antibody for use in a therapeutic agent-targeting agent construct. The *in vitro* manipulations or techniques include fusing to prepare a monoclonal antibody-producing hybridoma, and/or cloning the gene(s) encoding the anti-aminophospholipid antibody from the cells ("recombinant human antibodies").

Human anti-aminophospholipid antibody-producing cells may also be obtained from human subjects without an anti-aminophospholipid antibody-associated disease, *i.e.* "healthy subjects" in the context of the present invention. To achieve this, one would simply obtain a population of mixed peripheral blood lymphocytes from a human subject, including antigen-presenting and antibody-producing cells, and stimulate the cell population *in vitro* by admixing with an immunogenically effective amount of an aminophospholipid sample. Again, the human anti-aminophospholipid antibody-producing cells, once obtained, could be used in hybridoma and/or recombinant antibody production prior to therapeutic agent-targeting agent construct preparation.

Further techniques for human monoclonal antibody production include immunizing a transgenic animal, preferably a transgenic mouse, that comprises a human antibody library with an immunogenically effective amount of an aminophospholipid

sample. This also generates human anti-aminophospholipid antibody-producing cells for further manipulation in hybridoma and/or recombinant antibody production, with the advantage that spleen cells, rather than peripheral blood cells, can be readily obtained from the transgenic animal or mouse.

Preferred anti-aminophospholipid antibodies for use in the therapeutic agent-targeting agent constructs of the present invention are anti-phosphatidylserine (anti-PS) and anti-phosphatidylethanolamine (anti-PE) antibodies. Anti-PS antibodies will generally recognize, bind to or have immunospecificity for the PS molecule present, expressed, translocated, presented or complexed at the luminal surface of tumor vascular endothelial cells. Suitable antibodies will thus bind to phosphatidyl-L-serine (Umeda *et al.*, 1989; incorporated herein by reference). Anti-PE antibodies will generally recognize, bind to or have immunospecificity for the PE molecule present, expressed, translocated, presented or complexed at the luminal surface of tumor vascular endothelial cells.

Administering diagnostic and/or therapeutic agent-targeting agent constructs to an animal with a tumor will result in specific binding to the aminophospholipid molecules present, expressed or translocated to the luminal surface of the tumor blood vessels, *i.e.*, the therapeutic agent-targeting agent constructs will bind to the aminophospholipid molecules in a natural, biological environment. Therefore, no particular manipulation will be necessary to ensure binding.

However, in terms of antibody binding, it is of scientific interest to note that aminophospholipids may be most frequently recognized, or bound, by anti-aminophospholipid antibodies when the aminophospholipid molecules are associated with one or more proteins or other non-lipid biological components. For example, anti-PS antibodies that occur as a sub-set of anti-phospholipid (anti-PL) antibodies in patients with certain diseases and disorders are now believed to bind to PS in combination with proteins such as β_2-glycoprotein I (β_2-GPI or apolipoprotein H, apoH) and prothrombin (U.S. Patent No. 5,344,758; Rote, 1996;

each incorporated herein by reference). Similarly, anti-PE antibodies that occur in disease states are now believed to bind to PE in combination with proteins such as low and high molecular weight kininogen (HK), prekallikrein and even factor XI (Sugi and McIntyre, 1995; 1996a; 1996b; each incorporated herein by reference).

This is the meaning of the terms "presented" and "complexed at" the luminal surface of tumor blood vessels, as used herein, which mean that the aminophospholipid molecules are present at the surface of tumor blood vessels in a binding competent state, or antibody-binding competent state, irrespective of the molecular definition of that particular state. PS may even be targeted as a complex with factor II/IIa, VII/VIIa, IX/IXa and X/Xa. Moreover, the nature of the aminophospholipid target may change during practice of the invention, as the initial aminophospholipid antibody binding, anti-endothelial cell and anti-tumor effects may result in biological changes that alter the number, conformation and/or type of the aminophospholipid target epitope(s).

The term "anti-aminophospholipid antibody", as used in the context of the present invention, therefore means any antibody, immunological binding agent or antisera; monoclonal, human, humanized, dimeric, trimeric, multimeric, chimeric, bispecific, recombinant or engineered antibody; or Fab', Fab, $F(ab')_2$, DABs, Fv or scFv antigen binding fragment thereof; that at least binds to a lipid and amino group-containing complex or aminophospholipid target, preferably a phosphatidylserine- or phosphatidylethanolamine-based target.

The requirement that the antibody "at least bind to an aminophospholipid target" is met by the antibody binding to any and/or all physiologically relevant forms of aminophospholipids, including so-called "hexagonal" and "hexagonal phase II" PS and PE (HexII PS and HexII PE) (Rauch *et al.*, 1986; Rauch and Janoff, 1990; Berard *et al.*, 1993; each incorporated herein by reference) and PS and PE in combination with any other protein, lipid, membrane component, plasma or serum component, or any combination thereof. Thus, an "anti-aminophospholipid antibody" is an antibody

that binds to an aminophospholipid in the tumor blood vessels, notwithstanding the fact that bilayer or micelle aminophospholipids may be considered to be immunogenically neutral.

The anti-aminophospholipid antibodies may recognize, bind to or have immunospecificity for aminophospholipid molecules, or an immunogenic complex thereof (including hexagonal aminophospholipids and protein combinations), to the exclusion of other phospholipids or lipids. Such antibodies may be termed "aminophospholipid-specific or aminophospholipid-restricted antibodies", and their use in the therapeutic agent-targeting agent constructs of the invention will often be preferred. "Aminophospholipid-specific or aminophospholipid-restricted antibodies" will generally exhibit significant binding to aminophospholipids, while exhibiting little or no significant binding to other lipid components, such as phosphatidylinositol (PI), phosphatidylglycerol (PG) and even phosphatidylcholine (PC) in certain embodiments.

"PS-specific or PS-restricted antibodies" will generally exhibit significant binding to PS, while exhibiting little or no significant binding to lipid components such as phosphatidylethanolamine and cardiolipin (CL), as well as PC, PI and PG. "PE-specific or PE-restricted antibodies" will generally exhibit significant binding to PE, while exhibiting little or no significant binding to lipid components such as phosphatidylserine and cardiolipin, as well as PC, PI and PG. The preparation of specific anti-aminophospholipid antibodies is readily achieved, *e.g.*, as disclosed by Rauch *et al.* (1986); Umeda *et al.* (1989); Rauch and Janoff (1990); and Rote *et al.* (1993); each incorporated herein by reference.

"Cross-reactive anti-aminophospholipid antibodies" that recognize, bind to or have immunospecificity for an aminophospholipid molecule, or an immunogenic complex thereof (including hexagonal aminophospholipids and protein combinations), in addition to exhibiting lesser but detectable binding to other phospholipid or lipid components are by no means excluded from use in the invention. Such "cross-

reactive anti-aminophospholipid antibodies" may be employed so long as they bind to an aminophospholipid present, expressed, translocated, presented or complexed at the luminal surface of tumor vascular endothelial cells *in vivo*.

Further suitable aminophospholipid-specific or aminophospholipid-restricted antibodies are those anti-aminophospholipid antibodies that bind to both PS and PE. While clearly being specific or restricted to aminophospholipids, as opposed to other lipid components, antibodies exist that bind to each of the preferred targets of the present invention. Examples of such antibodies for use in the therapeutic agent-targeting agent constructs of the invention include, but are not limited to, PS3A, PSF6, PSF7, PSB4, PS3H1 and PS3E10 (Igarashi *et al.*, 1991; incorporated herein by reference)

Further exemplary anti-PS antibodies for use in the therapeutic agent-targeting agent constructs include, but are not limited to BA3B5C4, PS4A7, PS1G3 and 3SB9b; with PS4A7, PS1G3 and 3SB9b generally being preferred. Monoclonal antibodies, humanized antibodies and/or antigen-binding fragments based upon the 3SB9b antibody (Rote *et al.*, 1993; incorporated herein by reference) are currently most preferred.

Although aminophospholipids, such as PS and PE, in bilayer or micelle form have been reported to be non- or weakly antigenic, or non- or weakly-immunogenic, the scientific literature has reported no difficulties in generating anti-aminophospholipid antibodies, such as anti-PS and anti-PE antibodies. Anti-aminophospholipid antibodies or monoclonal antibodies may therefore be readily prepared by preparative processes and methods that comprise:

(a) preparing an anti-aminophospholipidantibody-producingcell; and

(b) obtaining an anti-aminophospholipid antibody or monoclonal antibody from the antibody-producing cell.

The processes of preparing anti-aminophospholipid antibody-producing cells and obtaining anti-aminophospholipid antibodies therefrom may be conduced *in situ* in a given patient. That is, simply providing an immunogenically effective amount of an immunogenic aminophospholipid sample to a patient will result in anti-aminophospholipid antibody generation. Thus, the anti-aminophospholipid antibody is still "obtained" from the antibody-producing cell, but it does not have to be isolated away from a host and subsequently provided to a patient, being able to spontaneously localize to the tumor vasculature and exert its biological anti-tumor effects.

As disclosed herein, anti-aminophospholipid antibody-producing cells may be obtained, and antibodies subsequently isolated and/or purified, from human patients with anti-aminophospholipid antibody-producing diseases, from stimulating peripheral blood lymphocytes with aminophospholipids *in vitro*, and also by immunization processes and methods. The latter of which generally comprise:

(a) immunizing an animal by administering to the animal at least one dose, and optionally more than one dose, of an immunogenically effective amount of an immunogenic aminophospholipid sample (such as a hexagonal, or hexagonal phase II form of an aminophospholipid), preferably an immunogenic PS or PE sample; and

(b) obtaining an anti-aminophospholipid antibody-producing cell from the immunized animal.

The immunogenically effective amount of the aminophospholipid sample or samples may be a *Salmonella*-coated aminophospholipid sample (Umeda *et al.*, 1989; incorporated herein by reference); an aminophospholipid micelle, liposome, lipid complex or lipid formulation sample; or an aminophospholipid sample fabricated with

SDS. Any such aminophospholipid sample may be administered in combination with any suitable adjuvant, such as Freund's complete adjuvant (Rote *et al.*, 1993; incorporated herein by reference). Any empirical technique or variation may be employed to increase immunogenicity, and/or hexagonal or hexagonal phase II forms of the aminophospholipids may be administered.

The immunization may be based upon one or more intrasplenic injections of an immunogenically effective amount of an aminophospholipid sample (Umeda *et al.*, 1989; incorporated herein by reference).

Irrespective of the nature of the immunization process, or the type of immunized animal, anti-aminophospholipid antibody-producing cells are obtained from the immunized animal and, preferably, further manipulated by the hand of man. "An immunized animal", as used herein, is a non-human animal, unless otherwise expressly stated. Although any antibody-producing cell may be used, most preferably, spleen cells are obtained as the source of the antibody-producing cells. The anti-aminophospholipid antibody-producing cells may be used in a preparative process that comprises:

(a) fusing an anti-aminophospholipid antibody-producing cell with an immortal cell to prepare a hybridoma that produces an anti-aminophospholipid monoclonal antibody and

(b) obtaining an anti-aminophospholipid monoclonal antibody from the hybridoma.

Hybridoma-based monoclonal antibody preparative methods thus include those that comprise:

(a) immunizing an animal by administering to the animal at least one dose, and optionally more than one dose, of an immunogenically effective amount of an immunogenic aminophospholipid sample (such as a hexagonal, or hexagonal phase II form of an aminophospholipid), preferably an immunogenic PS or PE sample;

(b) preparing a collection of monoclonal antibody-producing hybridomas from the immunized animal;

(c) selecting from the collection at least a first hybridoma that produces at least a first anti-aminophospholipid monoclonal antibody, and preferably, at least a first aminophospholipid-specific monoclonal antibody; and

(d) culturing the at least a first anti-aminophospholipid-producing or aminophospholipid-specific hybridoma to provide the at least a first anti-aminophospholipid monoclonal antibody or aminophospholipid-specific monoclonal antibody; and preferably

(e) obtaining the at least a first anti-aminophospholipid monoclonal antibody or aminophospholipid-specific monoclonal antibody from the cultured at least a first hybridoma.

As non-human animals are used for immunization, the anti-aminophospholipid monoclonal antibodies obtained from such a hybridoma will often have a non-human make up. Such antibodies may be optionally subjected to a humanization process, grafting or mutation, as known to those of skill in the art and further disclosed herein. Alternatively, transgenic animals, such as mice, may be used that comprise a human antibody gene library. Immunization of such animals will therefore directly result in the generation of human anti-aminophospholipid antibodies.

After the production of a suitable antibody-producing cell, most preferably a hybridoma, whether producing human or non-human antibodies, the monoclonal antibody-encoding nucleic acids may be cloned to prepare a "recombinant" monoclonal antibody. Any recombinant cloning technique may be utilized, including the use of PCR to prime the synthesis of the antibody-encoding nucleic acid sequences. Therefore, yet further appropriate monoclonal antibody preparative methods include those that comprise using the anti-aminophospholipid antibody-producing cells as follows:

(a) obtaining at least a first anti-aminophospholipid antibody-encoding nucleic acid molecule or segment from an anti-aminophospholipid antibody-producing cell, preferably a hybridoma; and

(b) expressing the nucleic acid molecule or segment in a recombinant host cell to obtain a recombinant anti-aminophospholipid monoclonal antibody.

However, other powerful recombinant techniques are available that are ideally suited to the preparation of recombinant monoclonal antibodies. Such recombinant techniques include the phagemid library-based monoclonal antibody preparative methods comprising:

(a) immunizing an animal by administering to the animal at least one dose, and optionally more than one dose, of an immunogenically effective amount of an immunogenic aminophospholipid sample (such as a hexagonal, or hexagonal phase II form of an aminophospholipid), preferably an immunogenic PS or PE sample;

(b) preparing a combinatorial immunoglobulin phagemid library expressing RNA isolated from the antibody-producing cells, preferably from the spleen, of the immunized animal;

(c) selecting from the phagemid library at least a first clone that expresses at least a first anti-aminophospholipid antibody, and preferably, at least a first aminophospholipid-specific antibody;

(d) obtaining anti-aminophospholipid antibody-encoding nucleic acids from the at least a first selected clone and expressing the nucleic acids in a recombinant host cell to provide the at least a first anti-aminophospholipid antibody or aminophospholipid-specific antibody; and preferably

(e) obtaining the at least a first anti-aminophospholipid antibody or aminophospholipid-specific antibody expressed by the nucleic acids obtained from the at least a first selected clone.

Again, in such phagemid library-based techniques, transgenic animals bearing human antibody gene libraries may be employed, thus yielding recombinant human monoclonal antibodies.

Irrespective of the manner of preparation of a first anti-aminophospholipid antibody nucleic acid segment, further suitable anti-aminophospholipid antibody nucleic acid segments may be readily prepared by standard molecular biological techniques. In order to confirm that any variant, mutant or second generation anti-aminophospholipid antibody nucleic acid segment is suitable for use in the present invention, the nucleic acid segment will be tested to confirm expression of an antibody that binds to an aminophospholipid. Preferably, the variant, mutant or second generation anti-aminophospholipid antibody nucleic acid segment will also be tested to confirm hybridization to an anti-aminophospholipid antibody nucleic acid segment under standard, more preferably, standard stringent hybridization conditions. Exemplary suitable hybridization conditions include hybridization in about 7%

sodium dodecyl sulfate (SDS), about 0.5 M $NaPO_4$, about 1 mM EDTA at about 50°C; and washing with about 1% SDS at about 42°C.

As a variety of recombinant monoclonal antibodies, whether human or non-human in origin, may be readily prepared, the treatment methods of the invention may be executed by providing to the animal or patient at least a first nucleic acid segment that expresses a biologically effective amount of at least a first therapeutic agent-targeting agent construct in the patient. The "nucleic acid segment that expresses a therapeutic agent-targeting agent construct" will generally be in the form of at least an expression construct, and may be in the form of an expression construct comprised within a virus or within a recombinant host cell. Preferred gene therapy vectors of the present invention will generally be viral vectors, such as comprised within a recombinant retrovirus, herpes simplex virus (HSV), adenovirus, adeno-associated virus (AAV), cytomegalovirus (CMV), and the like.

Once a targeting agent has been selected, whether antibody-based or binding ligand-based, and whether binding to phosphatidylethanolamine and/or phosphatidylserine, the targeting agent is operatively attached to one or more diagnostic and/or therapeutic agents or "effector" portions. The therapeutic agents of the present constructs will generally be either anti-cellular, cytotoxic or anti-angiogenic agents, or coagulation factors (coagulants).

The use of anti-cellular, cytotoxic and/or anti-angiogenic agents results in "aminophospholipid immunotoxins" (or anti-aminophospholipid immunotoxins), whereas the use of coagulation factors results in "aminophospholipid coaguligands" (or anti-aminophospholipid coaguligands). These terms are again used for simplicity and succinctly refer to aminophospholipid binding ligands or therapeutic agent-aminophospholipid targeting agent constructs in terms of their attached therapeutic moiety.

The present invention further provides binding ligands, and methods of use, comprising at least two therapeutic agents operatively attached to a targeting agent comprising a single aminophospholipid binding site. The binding ligands may comprise at least two therapeutic agents operatively attached to a targeting agent that comprises at least two aminophospholipid binding sites; or a plurality of therapeutic agents operatively attached to a targeting agent that comprises a plurality of aminophospholipid binding sites, generally at regions distinct from the aminophospholipid binding sites.

Combinations of anti-cellular and cytotoxic agents with coagulation factors are also contemplated, irrespective of the number of aminophospholipid binding sites. The combined use of therapeutic agents of different classes, such as cytotoxins and coagulants, is also contemplated in embodiments where two or more binding ligands are administered to the animal, each containing a single type of therapeutic agent. Different cytotoxins may also be employed in one or more binding ligands or methods, such as DNA synthesis inhibitors combined with classic cytotoxins, such as ricin.

In certain applications, the aminophospholipid-targeted constructs will be operatively attached to cytotoxic, cytostatic or otherwise anti-cellular agents that have the ability to kill or suppress the growth or cell division of endothelial cells. Suitable anti-cellular agents include chemotherapeutic agents, as well as cytotoxins and cytostatic agents. Cytostatic agents are generally those that disturb the natural cell cycle of a target cell, preferably so that the cell is taken out of the cell cycle.

Exemplary chemotherapeutic agents include: steroids; cytokines; anti-metabolites, such as cytosine arabinoside, fluorouracil, methotrexate or aminopterin; anthracyclines; mitomycin C; vinca alkaloids; antibiotics; demecolcine; etoposide; mithramycin; and anti-tumor alkylating agents, such as chlorambucil or melphalan. Indeed, any of the agents disclosed herein in Table C could be used. Certain preferred anti-cellular agents are DNA synthesis inhibitors, such as daunorubicin, doxorubicin, adriamycin, and the like.

In other embodiments, aminophospholipid-targeted constructs of the invention may be operatively attached to anti-angiogenic agents that, acting either alone or in concert with other host factors, or administered therapeutic agents, have the ability to prevent or inhibit vascularization and/or to induce regression of blood vessels. Suitable anti-angiogenic agents include those listed in Table D, as well as other anti-angiogenic agents known to those of skill in the art. By way of example only, one may mention the angiopoietins, preferably, angiopoietin-2 (Ang-2; SEQ ID NO:3 and SEQ ID NO:4), but also angiopoictin-1 (Ang-1; SEQ ID NO:1 and SEQ ID NO:2), angiopoietin fusion proteins (for example, as in SEQ ID NO:5), and even angiopoietin-3 and angiopoietin-4.

In certain therapeutic applications, toxin moieties will be preferred, due to the much greater ability of most toxins to deliver a cell killing effect, as compared to other potential agents. Therefore, certain preferred anti-cellular agents for aminophospholipid-targeted constructs are plant-, fungus- or bacteria-derived toxins. Exemplary toxins include epipodophyllotoxins; bacterial endotoxin or the lipid A moiety of bacterial endotoxin; ribosome inactivating proteins, such as saporin or gelonin; α-sarcin; aspergillin; restrictocin; ribonucleases, such as placental ribonuclease; diphtheria toxin and *pseudomonas* exotoxin.

Preferred toxins for certain embodiments are gelonin and/or the A chain toxins, such as ricin A chain. The most preferred toxin moiety is often ricin A chain that has been treated to modify or remove carbohydrate residues, so called "deglycosylated A chain" (dgA). Deglycosylated ricin A chain is preferred because of its extreme potency, longer half-life, and because it is economically feasible to manufacture it a clinical grade and scale. Recombinant and/or truncated ricin A chain may also be used.

For tumor targeting and treatment with immunotoxins, the following patents and patent applications are specifically incorporated herein by reference for the purposes of even further supplementing the present teachings regarding anticellular

and cytotoxic agents: U.S. Patents Nos. 5,855,866; 5,776,427; 5,863,538; and 5,660,827; and U.S. Applications Serial No. 07/846,349; 08/295,868 (Patent No. 5,___,___; Issue Fee paid); 08/350,212 (Patent No. 5,___,___; Issue Fee paid); and 08/457,869 (Notice of Allowance Received).

The aminophospholipid-targeted constructs of the invention may comprise a component that is capable of promoting coagulation, *i.e.*, a coagulant. Here, the targeting antibody or ligand may be directly or indirectly, *e.g.*, via another antibody, linked to a factor that directly or indirectly stimulates coagulation.

Preferred coagulation factors for such uses are Tissue Factor (TF) and TF derivatives, such as truncated TF (tTF), dimeric, trimeric, polymeric/multimeric TF, and mutant TF deficient in the ability to activate Factor VII. Other suitable coagulation factors include vitamin K-dependent coagulants, such as Factor II/IIa, Factor VII/VIIa, Factor IX/IXa and Factor X/Xa; vitamin K-dependent coagulation factors that lack the Gla modification; Russell's viper venom Factor X activator; platelet-activating compounds, such as thromboxane A_2 and thromboxane A_2 synthase; and inhibitors of fibrinolysis, such as α2-antiplasmin.

Tumor targeting and treatment with coaguligands is described in the following patents and patent applications, each of which are specifically incorporated herein by reference for the purposes of even further supplementing the present teachings regarding coaguligands and coagulation factors: U.S. Patent Nos. 5,855,866 and 5,877,289; U.S. Applications Serial Nos. 07/846,349; 08/350,212 (Patent No. 5,___,___; Issue Fee paid); 08/482,369 (U.S. Patent 5,___,___; Issue Fee paid); 08/487,427 (U.S. Patent 5,___,___; Issue Fee paid); and 08/479,727 (U.S. Patent 5,___,___; Issue Fee paid).

As somewhat wider distribution of a coagulating agent will not be associated with severe side effects, there is a less stringent requirement imposed on the targeting element of coaguligands than with immunotoxins. Therefore, to achieve specific

targeting means that coagulation is promoted in the tumor vasculature relative to the vasculature in non-tumor sites. Thus, specific targeting of a coaguligand is a functional term, rather than a purely physical term relating to the biodistribution properties of the targeting agent.

The preparation of immunotoxins is generally well known in the art (see, *e.g.*, U.S. Patent 4,340,535, incorporated herein by reference). Each of the following patents and patent applications are further incorporated herein by reference for the purposes of even further supplementing the present teachings regarding immunotoxin generation, purification and use: U.S. Patents Nos. 5,855,866; 5,776,427; 5,863,538; and 5,660,827; and U.S. Applications Serial No. 07/846,349; 08/295,868 (Patent No. 5,___,___; Issue Fee paid); 08/350,212 (Patent No. 5,___,___; Issue Fee paid); and 08/457,869 (Notice of Allowance Received).

In the preparation of immunotoxins, advantages may be achieved through the use of certain linkers. For example, linkers that contain a disulfide bond that is sterically "hindered" are often preferred, due to their greater stability *in vivo*, thus preventing release of the toxin moicty prior to binding at the site of action. It is generally desired to have a conjugate that will remain intact under conditions found everywhere in the body except the intended site of action, at which point it is desirable that the conjugate have good "release" characteristics.

Depending on the specific toxin compound used, it may be necessary to provide a peptide spacer operatively attaching the targeting agent and the toxin compound, wherein the peptide spacer is capable of folding into a disulfide-bonded loop structure. Proteolytic cleavage within the loop would then yield a heterodimeric polypeptide wherein the targeting agent and the toxin compound are linked by only a single disulfide bond.

When certain other toxin compounds are utilized, a non-cleavable peptide spacer may be provided to operatively attach the targeting agent and the toxin

compound. Toxins that may be used in conjunction with non-cleavable peptide spacers are those that may, themselves, be converted by proteolytic cleavage, into a cytotoxic disulfide-bonded form. An example of such a toxin compound is a *Pseudonomas* exotoxin compound.

A variety of chemotherapeutic and other pharmacological agents can also be successfully conjugated to aminophospholipid antibodies or targeting ligands. Exemplary antineoplastic agents that have been conjugated to antibodies include doxorubicin, daunomycin, methotrexate and vinblastine. Moreover, the attachment of other agents such as neocarzinostatin, macromycin, trenimon and α-amanitin has been described (see U.S. Patent No. 5,855,866; and Patent No. 5,___,___ (Application Serial No. 08/350,212, Issue Fee paid) and references incorporated therein.

In light of one of the present inventors earlier work, the preparation of coaguligands is now also easily practiced. The operable association of one or more coagulation factors with an aminophospholipid targeting agent may be a direct linkage, such as those described above for the immunotoxins. Alternatively, the operative association may be an indirect attachment, such as where the targeting agent is operatively attached to a second binding region, preferably and antibody or antigen binding region of an antibody, that binds to the coagulation factor. The coagulation factor should be attached to the targeting agent at a site distinct from its functional coagulating site, particularly where a covalent linkage is used to join the molecules.

Indirectly linked coaguligands are often based upon bispecific antibodies. The preparation of bispecific antibodies is also well known in the art. One preparative method involves the separate preparation of antibodies having specificity for the targeted tumor component, on the one hand, and the coagulating agent on the other. Peptic $F(ab'\gamma)_2$ fragments from the two chosen antibodies are then generated, followed by reduction of each to provide separate $Fab'\gamma_{SH}$ fragments. The SH groups on one of the two partners to be coupled are then alkylated with a cross-linking reagent, such as o-phenylenedimaleimide, to provide free maleimide groups on one partner. This

partner may then be conjugated to the other by means of a thioether linkage, to give the desired $F(ab'\gamma)_2$ heteroconjugate (Glennie *et al.*, 1987; incorporated herein by reference). Other approaches, such as cross-linking with SPDP or protein A may also be carried out.

Another method for producing bispecific antibodies is by the fusion of two hybridomas to form a quadroma. As used herein, the term "quadroma" is used to describe the productive fusion of two B cell hybridomas. Using now standard techniques, two antibody producing hybridomas are fused to give daughter cells, and those cells that have maintained the expression of both sets of clonotype immunoglobulin genes are then selected.

A preferred method of generating a quadroma involves the selection of an enzyme deficient mutant of at least one of the parental hybridomas. This first mutant hybridoma cell line is then fused to cells of a second hybridoma that had been lethally exposed, *e.g.*, to iodoacetamide, precluding its continued survival. Cell fusion allows for the rescue of the first hybridoma by acquiring the gene for its enzyme deficiency from the lethally treated hybridoma, and the rescue of the second hybridoma through fusion to the first hybridoma. Preferred, but not required, is the fusion of immunoglobulins of the same isotype, but of a different subclass. A mixed subclass antibody permits the use if an alternative assay for the isolation of a preferred quadroma.

Microtiter identification embodiments, FACS, immunofluorescence staining, idiotype specific antibodies, antigen binding competition assays, and other methods common in the art of antibody characterization may be used to identify preferred quadromas. Following the isolation of the quadroma, the bispecific antibodies are purified away from other cell products. This may be accomplished by a variety of antibody isolation procedures, known to those skilled in the art of immunoglobulin purification (see, *e.g.*, Antibodies: A Laboratory Manual, 1988; incorporated herein by reference). Protein A or protein G sepharose columns are preferred.

In the preparation of both immunotoxins and coaguligands, recombinant expression may be employed. The nucleic acid sequences encoding the chosen targeting agent, and toxin or coagulant, are attached in-frame in an expression vector. Recombinant expression thus results in translation of the nucleic acid to yield the desired targeting agent-toxin/coagulant compound. Chemical cross-linkers and avidin:biotin bridges may also join the therapeutic agent(s) to the targeting agent(s).

The following patents and patent applications are each incorporated herein by reference for the purposes of even further supplementing the present teachings regarding coaguligand preparation, purification and use, including bispecific antibody coaguligands: U.S. Patent Nos. 5,855,866 and 5,877,289; U.S. Applications Serial Nos. 07/846,349; 08/350,212 (Patent No. 5,___,___; Issue Fee paid); 08/273,567; 08/482,369; 08/485,482; and 08/472,631 (U.S. Patent 5,___,___; Issue Fee paid); 08/487,427 (U.S. Patent 5,___,___; Issue Fee paid); and 08/479,727 and 08/481,904 (U.S. Patent 5,___,___; Issue Fee paid).

In certain embodiments, the vasculature of the vascularized tumor of the animal or patient to be treated may be first imaged. Generally this is achieved by first administering to the animal or patient a diagnostically effective amount of at least a first pharmaceutical composition comprising at least a first detectably-labeled aminophospholipid binding construct that binds to and identifies an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of blood vessels or intratumoral blood vessels of the vascularized tumor. The invention thus further provides compositions for use in, and methods of, distinguishing between tumor and/or intratumoral blood vessels and normal blood vessels. The "distinguishing" is achieved by administering one or more of the detectably-labeled aminophospholipid binding constructs described.

The detectably-labeled aminophospholipid binding construct may comprise an X-ray detectable compound, such as bismuth (III), gold (III), lanthanum (III) or lead (II); a radioactive ion, such as copper^{67}, gallium^{67}, gallium^{68}, indium^{111}, indium^{113}, iodine^{123}, iodine^{125}, iodine^{131}, mercury^{197}, mercury^{203}, rhenium^{186}, rhenium^{188}, rubidium^{97}, rubidium^{103}, technetium^{99m} or yttrium^{90}; a nuclear magnetic spin-resonance isotope, such as cobalt (II), copper (II), chromium (III), dysprosium (III), erbium (III), gadolinium (III), holmium (III), iron (II), iron (III), manganese (II), neodymium (III), nickel (II), samarium (III), terbium (III), vanadium (II) or ytterbium (III); or rhodamine or fluorescein .

Pre-imaging before tumor treatment may thus be carried out by:

(a) administering to the animal or patient a diagnostically effective amount of a pharmaceutical composition comprising at least a first detectably-labeled aminophospholipid binding construct that comprises a diagnostic agent operatively attached to an antibody, binding protein or ligand, or aminophospholipid binding fragment thereof, that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, present, expressed, translocated, presented or complexed at the luminal surface of blood vessels or intratumoral blood vessels of the vascularized tumor; and

(b) subsequently detecting the detectably-labeled aminophospholipid binding construct bound to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, on the luminal surface of tumor or intratumoral blood vessels, thereby obtaining an image of the tumor vasculature.

Cancer treatment may also be carried out by:

(a) forming an image of a vascularized tumor by administering to an animal or patient having a vascularized tumor a diagnostically minimal amount of at least a first detectably-labeled aminophospholipid binding construct comprising a diagnostic agent operatively attached to an antibody, binding protein or ligand, or aminophospholipid binding fragment thereof, that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, on the luminal surface of tumor or intratumoral blood vessels of the vascularized tumor, thereby forming a detectable image of the tumor vasculature; and

(b) subsequently administering to the same animal or patient a therapeutically optimized amount of at least a first therapeutic agent-targeting agent construct that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, on the tumor or intratumoral blood vessel luminal surface and thereby destroys the tumor vasculature.

Imaging and treatment formulations or medicaments are thus provided, which generally comprise:

(a) a first pharmaceutical composition comprising a diagnostically effective amount of a detectably-labeled aminophospholipid binding construct that comprises a detectable agent operatively attached to an antibody, binding protein or ligand, or aminophospholipid binding fragment thereof, that binds to an aminophospholipid, preferably phosphatidylserine or phosphatidylethanolamine, on the luminal surface of tumor or intratumoral blood vessels of the vascularized tumor; and

(b) a second pharmaceutical composition comprising a therapeutically effective amount of at least one therapeutic agent-targeting agent construct, preferably one that binds to phosphatidylserine or phosphatidylethanolamine.

In such methods and medicaments, advantages will be realized wherein the first and second pharmaceutical compositions comprise the same targeting agents, *e.g.*, anti-aminophospholipid antibodies, or fragments thereof, from the same antibody preparation, or preferably, from the same antibody-producing hybridoma. The foregoing medicaments may also further comprise one or more anti-cancer agents.

In the vasculature imaging aspects of the invention, it is recognized that the administered detectably-labeled aminophospholipid binding construct, or anti-aminophospholipid antibody-detectable agent, may itself have a therapeutic effect. Whilst this would not be excluded from the invention, the amounts of the detectably-labeled constructs to be administered would generally be chosen as "diagnostically effective amounts", which are typically lower than the amounts required for therapeutic benefit.

In the imaging embodiments, as with the therapeutics, the targeting agent may be either antibody-based or binding ligand- or binding protein-based. Although not previously connected with tumors or tumor vasculature, detectably labeled aminophospholipid binding ligand compositions are known in the art and can now, in light of this motivation and the present disclosure, be used in the present invention. The detectably-labeled annexins of U.S. Patent No. 5,627,036; WO 95/19791; WO 95/27903; WO 95/34315; WO 96/17618; and WO 98/04294; each incorporated herein by reference; may thus be employed.

In still further embodiments, the animals or patients to be treated by the present invention are further subjected to surgery or radiotherapy, or are provided with

a therapeutically effective amount of at least a first anti-cancer agent. The "at least a first anti-cancer agent" in this context means "at least a first anti-cancer agent in addition to the therapeutic agent-targeting agent construct of the invention. The "at least a first anti-cancer agent" may thus be considered to be "at least a second anti-cancer agent", where the therapeutic agent-targeting agent construct is a first anti-cancer agent. However, this is purely a matter of semantics, and the practical meaning will be clear to those of ordinary skill in the art.

The at least a first anti-cancer agent may be administered to the animal or patient substantially simultaneously with the therapeutic agent-targeting agent construct; such as from a single pharmaceutical composition or from two pharmaceutical compositions administered closely together.

Alternatively, the at least a first anti-cancer agent may be administered to the animal or patient at a time sequential to the administration of the at least a first therapeutic agent-targeting agent construct. "At a time sequential", as used herein, means "staggered", such that the at least a first anti-cancer agent is administered to the animal or patient at a time distinct to the administration of the at least a first therapeutic agent-targeting agent construct. Generally, the two agents are administered at times effectively spaced apart to allow the two agents to exert their respective therapeutic effects, *i.e.*, they are administered at "biologically effective time intervals".

The at least a first anti-cancer agent may be administered to the animal or patient at a biologically effective time prior to the therapeutic agent-targeting agent construct, or at a biologically effective time subsequent to the therapeutic agent-targeting agent construct. Administration of a non-aminophospholipid targeted anti-cancer agent at a therapeutically effective time subsequent to the therapeutic agent-targeting agent construct may be particularly desired wherein the anti-cancer agent is an anti-tumor cell immunotoxin designed to kill tumor cells at the outermost rim of the tumor, and/or wherein the anti-cancer agent is an anti-angiogenic agent designed

to prevent micrometastasis of any remaining tumor cells. Such considerations will be known to those of skill in the art.

Administration of one or more non-aminophospholipid targeted anti-cancer agents at a therapeutically effective time prior to a therapeutic agent-targeting agent construct may be particularly employed where the anti-cancer agent is designed to increase aminophospholipid expression. This may be achieved by using anti-cancer agents that injure, or induce apoptosis in, the tumor endothelium. Exemplary anti-cancer agent include, *e.g.*, taxol, vincristine, vinblastine, neomycin. combretastatin(s), podophyllotoxin(s), TNF-α, angiostatin, endostatin, vasculostatin, $\alpha_v\beta_3$ antagonists, calcium ionophores, calcium-flux inducing agents, any derivative or prodrug thereof.

The one or more additional anti-cancer agents may be chemotherapeutic agents, radiotherapeutic agents, cytokines, anti-angiogenic agents, apoptosis-inducing agents or anti-cancer immunotoxins or coaguligands. "Chemotherapeutic agents", as used herein, refer to classical chemotherapeutic agents or drugs used in the treatment of malignancies. This term is used for simplicity notwithstanding the fact that other compounds may be technically described as chemotherapeutic agents in that they exert an anti-cancer effect. However, "chemotherapeutic" has come to have a distinct meaning in the art and is being used according to this standard meaning.

A number of exemplary chemotherapeutic agents are described herein. Those of ordinary skill in the art will readily understand the uses and appropriate doses of chemotherapeutic agents, although the doses may well be reduced when used in combination with the present invention. A new class of drugs that may also be termed "chemotherapeutic agents" are agents that induce apoptosis. Any one or more of such drugs, including genes, vectors and antisense constructs, as appropriate, may also be used in conjunction with the present invention.

Anti-cancer immunotoxins or coaguligands are further appropriate anti-cancer agents. "Anti-cancer immunotoxins or coaguligands", or targeting-agent/therapeutic

agent constructs, are based upon targeting agents, including antibodies or antigen binding fragments thereof, that bind to a targetable component of a tumor cell, tumor vasculature or tumor stroma, and that are operatively attached to a therapeutic agent, generally a cytotoxic agent (immunotoxin) or coagulation factor (coaguligand). A "targetable component" of a tumor cell, tumor vasculature or tumor stroma, is preferably a surface-expressed, surface-accessible or surface-localized component, although components released from necrotic or otherwise damaged tumor cells or vascular endothelial cells may also be targeted, including cytosolic and/or nuclear tumor cell antigens.

Both antibody and non-antibody targeting agents may be used, including growth factors, such as VEGF and FGF; peptides containing the tripeptide R-G-D, that bind specifically to the tumor vasculature; and other targeting components such as annexins and related ligands.

Anti-tumor cell immunotoxins or coaguligands may comprise antibodies exemplified by the group consisting of B3 (ATCC HB 10573), 260F9 (ATCC HB 8488), D612 (ATCC HB 9796) and KS1/4, said KS1/4 antibody obtained from a cell comprising the vector pGKC2310 (NRRL B-18356) or the vector pG2A52 (NRRL B-18357).

Anti-tumor stroma immunotoxins or coaguligands will generally comprise antibodies that bind to a connective tissue component, a basement membrane component or an activated platelet component; as exemplified by binding to fibrin, RIBS or LIBS.

Anti-tumor vasculature immunotoxins or coaguligands may comprise ligands, antibodies, or fragments thereof, that bind to a surface-expressed, surface-accessible or surface-localized component of the blood transporting vessels, preferably the intratumoral blood vessels, of a vascularized tumor. Such antibodies include those that bind to surface-expressed components of intratumoral blood vessels of a

vascularized tumor, including aminophospholipids themselves, and intratumoral vasculature cell surface receptors, such as endoglin (TEC-4 and TEC-11 antibodies), a TGFβ receptor, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA, a VEGF/VPF receptor, an FGF receptor, a TIE, $\alpha_v\beta_3$ integrin, pleiotropin, endosialin and MHC Class II proteins. The antibodies may also bind to cytokine-inducible or coagulant-inducible components of intratumoral blood vessels.

Other anti-tumor vasculature immunotoxins or coaguligands may comprise antibodies, or fragments thereof, that bind to a ligand or growth factor that binds to an intratumoral vasculature cell surface receptor. Such antibodies include those that bind to VEGF/VPF (GV39 and GV97 antibodies), FGF, TGFβ, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor/hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP. The antibodies, or fragments thereof, may also bind to a ligand:receptor complex or a growth factor:receptor complex, but not to the ligand or growth factor, or to the receptor, when the ligand or growth factor or the receptor is not in the ligand:receptor or growth factor:receptor complex.

Anti-tumor cell, anti-tumor stroma or anti-tumor vasculature antibody-therapeutic agent constructs may comprise cytotoxic agents such as plant-, fungus- or bacteria-derived toxins (immunotoxins). Ricin A chain and deglycosylated ricin A chain will often be preferred, and gelonin and angiopoietins are also contemplated. Anti-tumor cell, anti-tumor stroma or anti-tumor vasculature antibody-therapeutic agent constructs may comprise coagulation factors or second antibody binding regions that bind to coagulation factors (coaguligands). The operative association with Tissue Factor or Tissue Factor derivatives, such as truncated Tissue Factor, will often be preferred.

The invention still further provides a series of novel therapeutic binding ligands, binding ligand compositions and pharmaceutical compositions, each of which comprise at least a first targeting agent that binds to an aminophospholipid, operatively

attached to at least a first therapeutic agent, such as a cytotoxin, anti-angiogenic agent or coagulant. Radiolabels are generally excluded from the binding ligands and binding ligand compositions; although not from the diagnostic methods, or even from the therapeutic methods described above.

The targeting agents of the binding ligands preferably bind to phosphatidylethanolamine and/or phosphatidylserine. The entire range of binding ligands described above in the context of the therapeutic and combined methods may be employed in the present compositions. Annexin conjugates and constructs; anti-PS, anti-PE, human, humanized and monoclonal antibody conjugates and constructs; ricin conjugates; and Tissue Factor conjugates and constructs are currently preferred. Compositions comprising one or more anti-PS antibodies operatively attached to one or more Tissue Factor derivatives, preferably, truncated Tissue Factor, are currently particularly preferred.

Direct or indirect attachment and linkages may be employed in the binding ligand compositions, including all variations of bispecific antibodies. Operative combinations of a first antigen-binding region of an antibody that binds to an aminophospholipid, with a second antigen-binding region of an antibody that binds Tissue Factor or a Tissue Factor derivative are also preferred. In the aminophospholipid binding protein constructs or conjugates, annexins are preferred, with Annexin V being more preferred, and Annexin V operatively attached to truncated Tissue Factor currently being most preferred.

Components of the invention therefore include an antibody construct, comprising at least a first anti-aminophospholipid antibody, or antigen-binding fragment thereof, operatively attached to at least a first therapeutic agent; and a bispecific antibody, comprising a first antigen-binding region that binds to an aminophospholipid operatively attached to a second antigen-binding region that binds to a therapeutic agent.

The compositions and pharmaceutical compositions may comprise at least a first and second binding ligand that each comprise at least a first targeting agent operatively attached to at least a first therapeutic agent; wherein each targeting agent binds to an aminophospholipid. Compositions and pharmaceutical compositions that comprise at least a first binding ligand that binds to phosphatidylethanolamine and at least a second binding ligand that binds to phosphatidylserine are exemplary combined compositions.

The present invention yet further provides a series of novel therapeutic kits, medicaments and/or cocktails for use in conjunction with the methods of the invention. The kits, medicaments and/or cocktails generally comprise a combined effective amount of an anti-cancer agent and a therapeutic agent-targeting agent construct, preferably one that binds to phosphatidylserine or phosphatidylethanolamine. Imaging components may also be included.

The kits and medicaments will comprise, preferably in suitable container means, a biologically effective amount of at least a first therapeutic agent-targeting agent construct, preferably binding to phosphatidylserine or phosphatidylethanolamine; in combination with a biologically effective amount of at least a first anti-cancer agent. The components of the kits and medicaments may be comprised within a single container or container means, or comprised within distinct containers or container means. The cocktails will generally be admixed together for combined use.

The entire range of therapeutic agent-targeting agent construct, as described above, may be employed in the kits, medicaments and/or cocktails, with annexin conjugates and constructs; anti-PS, anti-PE, human, humanized and monoclonal antibody conjugates and constructs; ricin conjugates; and Tissue Factor conjugates and constructs being preferred. The anti-cancer agents are also those as described above, including chemotherapeutic agents, radiotherapeutic agents, anti-angiogenic agents, apoptopic agents, immunotoxins and coaguligands. Agents formulated for intravenous administration will often be preferred.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and **FIG. 1B**. Activity of cell-bound anti-VCAM-1•tTF *in vitro*. **FIG. 1A**. Binding of anti-VCAM-1•tTF coaguligand to unstimulated (control) and IL-1α-activated bEnd.3 cells. **FIG. 1B.** Generation of factor Xa by cell-bound anti-VCAM-1•tTF coaguligand.

FIG. 2. Retardation of growth of L540 tumors in mice treated with anti-VCAM-1•tTF. L540 tumor bearing mice were injected i.v. with either saline, 20 μg of anti-VCAM-1•tTF, 4 μg of unconjugated tTF or 20 μg of control IgG•tTF. Injections were repeated on day 4 and 8 after the first treatment. Tumors were measured daily. Mean tumor volume and SD of 8 mice per group is shown.

FIG. 3. Annexin V blocks coaguligand activation of Factor X *in vitro*. IL-1α-stimulated bEnd.3 cells were incubated with anti-VCAM-•tTF coaguligand in 96-well microtiter plates, as described in Example V. Annexin V was added at concentrations ranging from 0.1 to 10 μg/ml (as shown) and cells were incubated for 30 min. before addition of diluted Proplex T. The amount of Factor Xa generated in the presence or absence of Annexin V was determined using a chromogenic substrate, as described in Example V.

FIG. 4A and **FIG. 4B.** Anti-tumor effects of naked anti-PS antibodies in animals with syngeneic and xenogeneic tumors. $1x10^7$ cells of murine colorectal carcinoma Colo 26 (**FIG. 4A**) or human Hodgkin's lymphoma L540 (**FIG. 4B**) were injected subcutaneously into the right flank of Balb/c mice (**FIG. 4A**) or male CB17 SCID mice (**FIG. 4B**), respectively. Tumors were allowed to grow to a size of about

0.6-0.9 cm^3 and then the mice (4 animals per group) were injected i.p. with 20 μg of naked anti-PS antibody (open squares) or saline (open circles) (control mouse IgM gave similar results to saline.). Treatment was repeated 3 times with a 48 hour interval. Animals were monitored daily for tumor measurements and body weight. Tumor volume was calculated as described in Example VII. Mice were sacrificed when tumors had reached 2 cm^3, or earlier if tumors showed signs of necrosis or ulceration.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

A. Tumor Destruction using VCAM-1 Coaguligand

Solid tumors and carcinomas account for more than 90% of all cancers in man. Although the use of monoclonal antibodies and immunotoxins has been investigated in the therapy of lymphomas and leukemias (Vitetta *et al.*, 1991), these agents have been disappointingly ineffective in clinical trials against carcinomas and other solid tumors (Abrams and Oldham, 1985). A principal reason for the ineffectiveness of antibody-based treatments is that macromolecules are not readily transported into solid tumors. Even once within a tumor mass, these molecules fail to distribute evenly due to the presence of tight junctions between tumor cells, fibrous stroma, interstitial pressure gradients and binding site barriers (Dvorak *et al.*, 1991).

In developing new strategies for treating solid tumors, the methods that involve targeting the vasculature of the tumor, rather than the tumor cells, offer distinct advantages. An effective destruction or blockade of the tumor vessels arrests blood flow through the tumor and results in an avalanche of tumor cell death. Antibody-toxin and antibody-coagulant constructs have already been effectively used in the specific targeting and destruction of tumor vessels, resulting in tumor necrosis (Burrows *et al.*, 1992; Burrows and Thorpe, 1993; WO 93/17715; WO 96/01653; U.S. Patents 5,855,866; 5,877,289; 5,___,___; 5,___,___; and 5,___,___ (corresponding to U.S. Serial No. 08/350,212; 08/487,427; and 08/482,369, Issue Fees paid); each incorporated herein by reference).

Where antibodies, growth factors or other binding ligands are used to specifically deliver a coagulant to the tumor vasculature, such agents are termed "coaguligands". A currently preferred coagulant for use in coaguligands is truncated Tissue Factor (tTF) (Huang *et al.*, 1997; WO 96/01653; U.S. Patent 5,877,289). TF is the major initiator of blood coagulation (Ruf *et al.*, 1991). At sites of injury, Factor VII/VIIa in the blood comes into contact with, and binds to, TF on cells in the perivascular tissues. The TF:VIIa complex, in the presence of the phospholipid surface, activates factors IX and X. This, in turn, leads to the formation of thrombin and fibrin and, ultimately, a blood clot (Ruf and Edgington, 1994).

The recombinant, truncated form of tissue factor (tTF), lacking the cytosolic and transmembrane domains, is a soluble protein that has about five orders of magnitude lower coagulation inducing ability than native TF (Stone *et al.*, 1995; Huang *et al.*, 1997). This is because TF needs to be associated with phospholipids for the complex with VIIa to activate IXa or Xa efficiently. However, when tTF is delivered to tumor vascular endothelium by means of a targeting antibody or agent, it is brought back into proximity to a lipid surface and regains thrombogenic activity (Huang *et al.*, 1997; U.S. Patents 5,___,___; 5,___,___ and 5,___,___). A coaguligand is thus created that selectively thromboses tumor vasculature.

Truncated TF has several advantages that commend its use in vascular targeted coaguligands: human tTF is readily available, and the human protein will have negligible or low immunogenicity in man; human tTF is fully functional in experimental animals, including mice; and targeted tTF is highly potent because it triggers the activation of a cascade of coagulation proteins, giving a greatly amplified effect (U.S. Patents 5,___,___; 5,___,___ and 5,___,___).

A range of suitable target molecules that are available on tumor endothelium, but largely absent from normal endothelium, have been described. For example, expressed targets may be utilized, such as endoglin, E-selectin, P-selectin, VCAM-1,

ICAM-1, PSMA, a TIE, a ligand reactive with LAM-1, a VEGF/VPF receptor, an FGF receptor, $\alpha_v\beta_3$ integrin, pleiotropin or endosialin (U.S. Patents 5.855,866; 5,5,877,289 and 5,___,___; corresponding to U.S. Serial No. 08/487,427; Burrows *et al.*, 1992; Burrows and Thorpe, 1993; Huang *et al.*, 1997; Liu *et al.*, 1997; Ohizumi *et al.*, 1997; each incorporated herein by reference).

Adsorbed targets are another suitable group, such as VEGF, FGF, TGFβ, HGF, PF4, PDGF, TIMP, a ligand that binds to a TIE or a tumor-associated fibronectin isoform (U.S. Patents 5,877,289; 5,___,___ and 5,___,___; corresponding to U.S. Serial No. 08/350,212 and 08/487,427, Issue Fees paid; each incorporated herein by reference). Fibronectin isoforms are ligands that bind to the integrin family of receptors. Tumor-associated fibronectin isoforms are targetable components of both tumor vasculature and tumor stroma. The monoclonal antibody BC-1 (Carnemolla *et al.*, 1989) specifically binds to tumor-associated fibronectin isoforms.

Other targets inducible by the natural tumor environment or following intervention by man are also targetable entities, as described in U.S. Patent Nos. 5,776,427, 5,863,538 and 5,___,___ (U.S. Serial No. 08/479,727, Issue Fee paid; each incorporated herein by reference). When used in conjunction with prior suppression in normal tissues and tumor vascular induction, MHC Class II antigens may also be employed as targets (U.S. Patent Nos. 5,776,427; 5,863,538; 5,___,___ and 5,___,___ (U.S. Serial Nos. 08/295,868 and 08/479,727, Issue Fees paid); each incorporated herein by reference).

One currently preferred target for clinical applications is vascular endothelial adhesion molecule-1 (VCAM-1) (U.S. Patents 5,855,866, 5,877,289, 5,___,___ and 5,___,___ (corresponding to U.S. Serial Nos. 08/482,369 and 08/487,427, Issue Fees paid); each incorporated herein by reference). VCAM-1 is a cell adhesion molecule that is induced by inflammatory cytokines IL-1α, IL-4 (Thornhill *et al.*, 1990) and TNFα (Munro, 1993) and whose role *in vivo* is to recruit leukocytes to sites of acute inflammation (Bevilacqua, 1993).

VCAM-1 is present on vascular endothelial cells in a number of human malignant tumors including neuroblastoma (Patey *et al.*, 1996), renal carcinoma (Droz *et al.*, 1994), non-small lung carcinoma (Staal-van den Brekel *et al.*, 1996), Hodgkin's disease (Patey *et al.*, 1996), and angiosarcoma (Kuzu *et al.*, 1993), as well as in benign tumors, such as angioma (Patey *et al.*, 1996) and hemangioma (Kuzu *et al.*, 1993). Constitutive expression of VCAM-1 in man is confined to a few vessels in the thyroid, thymus and kidney (Kuzu *et al.*, 1993; Bruijn and Dinklo, 1993), and in the mouse to vessels in the heart and lung (Fries *et al.*, 1993).

Certain of the data presented herein even further supplement those provided in U.S. Patents 5,855,866, 5,877,289 and 5,___,___ (corresponding to U.S. Serial No 08/487,427, Issue Fee paid; each incorporated herein by reference) and show the selective induction of thrombosis and tumor infarction resulting from administration of an anti-VCAM-1•tTF coaguligand. The results presented were generated using mice bearing L540 human Hodgkin lymphoma. When grown as a xenograft in SCID mice, this tumor shows close similarity to the human disease with respect to expression of inflammatory cytokines (Diehl *et al.*, 1985) and the presence of VCAM-1 and other endothelial cell activation molecules on its vasculature.

Using a covalently-linked anti-VCAM-1•tTF coaguligand, in which tTF was directly linked to the anti-VCAM-1 antibody, it is shown herein that the coaguligand localizes selectively to tumor vessels, induces thrombosis of those vessels, causes necrosis to develop throughout the tumor and retards tumor growth in mice bearing solid L540 Hodgkin tumors. Tumors generally needed to be at least about 0.3 cm in diameter to respond to the coaguligand, because VCAM-1 was absent from smaller tumors. Presumably, in small tumors, the levels of cytokines secreted by tumor cells or host cells that infiltrate the tumor are too low for VCAM-1 induction. This is in accordance with the studies in U.S. Patents 5,855,866, 5,877,289, 5,776,427, 5,___,___, and 5,___,___ (Serial Nos. 08/487,427 and 08/479,727, Issue Fees paid), where the inventions were shown to be most useful in larger solid tumors.

Although VCAM-1 staining was initially observed more in the periphery of the tumor, the coaguligand evidently bound to and occluded blood transporting vessels - - as it was capable of curtailing blood flow in all tumor regions. Furthermore, one of the inventors contemplates that the thrombin generation caused by the initial administration of the coaguligand likely leads to further VCAM-1 induction on central vessels (Sluiter *et al.*, 1993), resulting in an amplified signal and evident destruction of the intratumoral region. This type of coagulant-induced expression of further targetable markers, and hence signal amplification, is also disclosed in U.S. Serial No. 08/479,727 and 08/481,904 (U.S. Patent 5,___,___; Issue Fee paid).

B. Mechanism of VCAM-1-Targeted Tumor Destruction

As shown herein, although localization to VCAM-1-expressing vessels in the heart and lungs of mice was observed upon administration of an anti-VCAM-1 coaguligand, this construct did not induce thrombosis in such non-tumor sites. Furthermore, the anti-VCAM-1 coaguligand was no more toxic to mice than was a control coaguligand of irrelevant specificity, again indicating that the constitutive expression of VCAM-1 on heart and lung vessels did not lead to toxicity. This data is important to the immediate clinical progress of coaguligand therapy, given that VCAM-1 is a naturally occurring marker of tumor vascular endothelium in humans. However, this phenomenon also provided the inventors with a unique insight, leading to other approaches for tumor vasculature destruction.

The inventors sought to understand the mechanism behind the ability of the anti-VCAM-1 coaguligand to bind to the VCAM-1 constitutively expressed on blood vessels in the heart and lungs, and yet not to cause thrombosis in those vessels. There are numerous scientific possibilities for this empirical observation, generally connected with the prothrombotic nature of the tumor environment any fibrinolytic predisposition in the heart and lungs.

Generally, there is a biological equilibrium between the coagulation system (fibrin deposition) and the fibrinolytic system (degradation of fibrin by enzymes). However, in malignant disease, particularly carcinomas, this equilibrium is disrupted, resulting in the abnormal activation of coagulation (hypercoagulability or the "prothrombotic state"). Evidence also indicates that various components of these pathways may contribute to the disorderly characteristics of malignancy, such as proliferation, invasion, and metastasis (Zacharski *et al.*, 1993).

Donati (1995) reviewed the complex interplay between the original clinical observations of thrombotic complications of malignant diseases, and the subsequent progress in the cell biology and biochemistry of tumor cell activities. However, despite extensive research, a clear molecular explanation for the prothrombotic nature of the tumor environment could not be provided (Donati, 1995). Donati did emphasize, though, the role of tumor cells in this process. It was explained that tumor cells express procoagulant activities, such as tissue thromboplastin and cancer procoagulant (CP) (Donati, 1995). WO 91/07187 also reported a procoagulant activity of tumor cells.

Numerous other studies have also identified the tumor cells themselves as being responsible for the prothrombotic state within a tumor. For example, Nawroth *et al.* (1988) reported that factor(s) elaborated by sarcoma cells enhance the procoagulant response of nearby endothelium to TNF. These authors reported that fibrin formation occurred throughout the tumor vascular bed 30 minutes after TNF infusion, but that fibrin deposition and platelet aggregates were not observed in normal vasculature (Nawroth *et al.*, 1988). TNF was later shown to enhance the expression of tissue factor on the surface of endothelial cells (Murray *et al.*, 1991). This was proposed to explain earlier studies showing that cultured endothelial cells incubated with recombinant TNF have enhanced procoagulant activity, tissue factor, and concomitant suppression of the protein C pathway, an anti-thrombotic mechanism that functions on the surface of quiescent endothelial cells (Nawroth *et al.*, 1985; Nawroth and Stern, 1986).

Data from Sugimura *et al.* (1994) also implicated tumor cells as the key components of the procoagulant activity of the tumor. It was reported that four tumor cell lines were able to support different stages of the extrinsic pathway of coagulation (Sugimura *et al.*, 1994). Another study reported that a human ovarian carcinoma cell line, OC-2008, constitutively expressed surface membrane Tissue Factor activity and exhibited cell surface-dependent prothrombinase complex activity (Rao *et al.*, 1992). Connor *et al.* (1989) further suggested that it is the pathologic cells that control coagulation. Their results indicated that tumorigenic, undifferentiated murine erythroleukemic cells exhibit a 7- to 8-fold increase in the potency of their procoagulant activity (Connor *et al.*, 1989).

Zacharski *et al.* (1993) also focused on tumor cells and sought to define the mode of interaction of ovarian carcinoma cells with the coagulation (procoagulant-initiated) and fibrinolysis (urokinase-type plasminogen activator-initiated, u-PA) pathways. They reported that tumor cells expressed Tissue Factor and coagulation pathway intermediates that resulted in local thrombin generation - - as evidenced by the conversion of fibrinogen, present in tumor connective tissue, to fibrin that was found to hug the surfaces of tumor nodules and individual tumor cells. Detected fibrin could not be accounted for on the basis of necrosis or a local inflammatory cell infiltrate (Zacharski *et al.*, 1993). These authors concluded that there exists a dominant tumor cell-associated procoagulant pathway that leads to thrombin generation and hypercoagulability.

Other hypotheses have proposed that it is changes in the tumor blood vessels that render these vessels better able to support the formation of thrombi and/or less able to dissolve fibrin. For example, tumor vessels have been reported to exhibit upregulation of Tissue Factor, down-regulation of plasminogen activators and/or upregulation of the inhibitor of plasminogen activators, PAI-1 (Nawroth and Stern, 1986; Nawroth *et al.*, 1988). Such effects are believed to be magnified by tumor derived factors (Murray *et al.*, 1991; Ogawa *et al.*, 1990), possibly VEGF.

For example, Ogawa *et al.* (1990) reported that hypoxia caused endothelial cell surface coagulant properties to be shifted to promote activation of coagulation. This was accompanied by suppression of the anticoagulant cofactor, thrombomodulin, and induction of an activator of factor X, distinct from the classical extrinsic and intrinsic systems (Ogawa *et al.*, 1990). Also, there could be an increase in the local concentration of Factors VIIa, IXa, Xa, or other molecules that interact with TF, within the tumor vessels, thus encouraging thrombosis.

Additionally, platelets are a major component of any procoagulant state. Recently, the procoagulant potential of platelets has been linked to their ability to shed procoagulant microparticles from the plasma membrane (Zwaal *et al.*, 1989; 1992; Dachary-Prigent *et al.*, 1996). It has been proposed that an increased proportion of circulating microparticles, vesicles or membrane fragments from platelets contributes to 'prethrombotic' (prothrombotic) states in various pathological conditions (Zwaal *et al.*, 1989; 1992; Dachary-Prigent *et al.*, 1996, pp. 159 and references cited therein). McNeil *et al.* (1990) also reported that β_2-GPI exerts multiple inhibitory effects on coagulation and platelet aggregation. Tumor platelet biology could thus explain the effectiveness of the anti-VCAM-1 coaguligand.

Further tenable explanations include the simple possibility that VCAM-1 is expressed at higher levels in tumor vessels than on blood vessels in the heart and lungs, probably due to induction by tumor-derived cytokines, and that binding to the healthy vessels cannot tip the balance into sustained thrombosis. Also the fibrinolytic mechanisms could be upregulated in the heart, as exemplified by increased Tissue Factor pathway inhibitor (TFPI), increased plasminogen activators, and/or decreased plasminogen activator inhibitors. Should the fibrinolytic physiology of the heart and lung vessels prove to be the major reason underlying the tumor-specific effects of the anti-VCAM-1 coaguligand, this would generally preclude the development of additional anti-tumor therapies targeted to unique aspects of tumor biology.

Despite all the possible options, the inventors reasoned that the failure of the anti-VCAM-1 coaguligand to cause thrombosis in vessels of normal tissues was due to the absence of the aminophospholipid, phosphatidylserine (PS), from the luminal surface of such vessels. To complete the theory, therefore, not only would phosphatidylserine have to be shown to be absent from these normal vessels, but its presence on the luminal side of tumor-associated vessels would have to be conclusively demonstrated.

The inventors therefore used immunohistochemical staining to evaluate the distribution of a monoclonal anti-phosphatidylserine (anti-PS) antibody injected intravenously into tumor-bearing mice. These studies revealed that the VCAM-1 expressing vessels in the heart and lungs lacked PS, whereas the VCAM-1 expressing vessels in the tumor expressed PS. The need for surface PS expression in coaguligand action is further indicated by the inventors' finding that annexin V, which binds to PS, blocks anti-VCAM-1•tTF coaguligand action, both *in vitro* and *in vivo*.

The lack of thrombotic effect of the anti-VCAM-1 coaguligand on normal heart and lung vessels can thus be explained, at least in part: the absence of the aminophospholipid, phosphatidylserine, means that the normal vessels lack a procoagulant surface upon which coagulation complexes can assemble. In the absence of surface PS, anti-VCAM-1•tTF binds to VCAM-1 expressing heart and lung vessels, but cannot induce thrombosis. In contrast, VCAM-1 expressing vessels in the tumor show coincident expression of surface PS. The coaguligand thus binds to tumor vessels and activates coagulation factors locally to form an occlusive thrombus.

In addition to delineating the tumor-specific thrombotic effects of anti-VCAM-1 coaguligands, the specific expression of the aminophospholipid, phosphatidylserine, on the luminal surface of tumor blood vessels also allowed the inventors to explain the prothrombotic phenotype observed, but not understood, in earlier studies (Zacharski *et al.*, 1993; Donati, 1995). Rather than being predominantly due to tumor cells or elaborated factors; platelets, procoagulant

microparticles or membrane fragments; or due to imbalances in thromboplastin, thrombomodulin, cancer procoagulant, Tissue Factor, protein C pathway, plasminogen activators or plasminogen activator inhibitors (*e.g.*, PAI-1), the inventors' studies indicate that it is PS expression that plays a significant role in the prothrombotic state of tumor vasculature.

C. Aminophospholipids as Markers of Tumor Vasculature

Following their discovery that the representative aminophospholipid, phosphatidylserine, was specifically expressed on the luminal surface of tumor blood vessels, but not in normal blood vessels, the inventors reasoned that aminophospholipids had potential as targets for therapeutic intervention. The present invention therefore provides compositions and methods for the targeted delivery of therapeutic agents to aminophospholipid membrane constituents, particularly phosphatidylserine (PS) and phosphatidylethanolamine (PE). Although anti-tumor effects from aminophospholipid-targeted delivery are demonstrated herein, using art-accepted animal models, the ability of aminophospholipids to act as safe and effective targetable markers of tumor vasculature could not have been predicted from previous studies.

For example, although tumor vessels are generally prothrombotic in nature, as opposed to other blood vessels, it is an inherent property of the tumor to maintain a network of blood vessels in order to deliver oxygen and nutrients to the tumor cells. Evidently, tumor-associated blood vessels cannot be so predisposed towards thrombosis that they spontaneously and readily support coagulation, as such coagulation would necessarily cause the tumor to self-destruct. It is thus unexpected that any thrombosis-associated tumor vessel marker, such as the presently identified phosphatidylserine, could be discovered that is expressed in quantities sufficient to allow effective therapeutic intervention by targeting, and yet is expressed at levels low enough to ordinarily maintain blood flow through the tumor.

The present identification of aminophospholipids as safe and effective tumor vasculature targets is even more surprising given (1) the previous speculations regarding the role of other cell types and/or various factors, activators and inhibitors underlying the complex, prothrombotic state of the tumor (as discussed above); and (2) the confusing and contradictory state of the art concerning aminophospholipid biology, in terms of both expression and function in various cell types.

Phosphatidylserine and phosphatidylethanolamine are normally segregated to the inner surface of the plasma membrane bilayer in different cells (Gaffet *et al.*, 1995; Julien *et al.*, 1995). In contrast, the outer leaflet of the bilayer membrane is rich in phosphatidylcholine analogs (Zwaal *et al.*, 1989; Gaffet *et al.*, 1995). This lipid segregation creates an asymmetric transbilayer. Although the existence of membrane asymmetry has been discussed for some time, the reason for its existence and the mechanisms for its generation and control are poorly understood (Williamson and Schlegel, 1994), particularly in cells other than platelets.

There are even numerous conflicting reports regarding the presence or absence of PS and PE in different cells and tissues, let alone concerning the likely role that these aminophospholipids may play. For example, the many PS studies conducted with platelets, key components in blood coagulation (Dachary-Prigent *et al.*, 1996), have yielded highly variable results. Bevers *et al.* (1982) measured the platelet prothrombin-converting activity of non-activated platelets after treatment with various phospholipases or proteolytic enzymes. They concluded that negatively charged phosphatidylserine, and possibly phosphatidylinositol, were involved in the prothrombin-converting activity of non-activated platelets (Bevers *et al.*, 1982).

Bevers *et al.* (1983) then reported an increased exposure of phosphatidylserine, and a decreased exposure of sphingomyelinase, in activated platelets. However, these alterations were much less apparent in platelets activated either by thrombin or by collagen alone, in contrast to collagen plus thrombin, diamide, or a calcium ionophore (Bevers *et al.*, 1983). The surface expression of PS in

response to diamide was contradicted by studies in erythrocytes, which showed no diamide-stimulated PS exposure (de Jong *et al.*, 1997). While echoing their earlier results, Bevers and colleagues then later reported that changes in the plasma membrane-cytoskeleton interaction, particularly increased degradation of cytoskeletal actin-binding protein, was important to platelet surface changes (Bevers *et al.*, 1985; pages 368-369).

Maneta-Peyret *et al.* (1989) also reported the detection of PS on human platelets. These authors noted that the platelet procoagulant surface could be formed by negatively charged phospholipids, such as phosphatidylserine and phosphatidylethanolamine (generally neutral or zwitterionic), or both. The role of phosphatidylserine in the process of coagulation has been questioned in favor of phosphatidylethanolamine (Maneta-Peyret *et al.*, 1989; Schick *et al.*, 1976; 1978). For example, studies have reported that 18% of phosphatidylethanolamine becomes surface-accessible after 2 hours, in contrast to zero phosphatidylserine (Schick *et al.*, 1976).

Ongoing studies with platelets were also reported as showing a further 16% increase in phosphatidylethanolamine exposure after thrombin treatment, with no increase in the phosphatidylserine levels (Schick *et al.*, 1976). Therefore, PS was said not to be a component of the functional surface of the platelet plasma membrane (Schick *et al.*, 1976; 1978). Nonetheless, current evidence does seem to indicate that both PS and PE are involved in the phospholipid asymmetry observed in the outer membrane of platelets and erythrocytes, and that PS is involved in the procoagulant activity of platelets (Gaffet *et al.*, 1995; de Jong *et al.*, 1997; U.S. Patent No. 5,627,036).

The mechanisms for achieving and maintaining differential aminophospholipid distribution, let alone the functional significance of doing so, have long been the subject of controversial speculations. In reviewing the regulation of transbilayer phospholipid movement, Williamson and Schlegel (1994) indicated that

elevating intracellular Ca^{2+} allows the major classes of phospholipids to move freely across the bilayer, scrambling lipids and dissipating asymmetry. de Jong *et al.* (1997) also reported that an increase of intracellular calcium leads to a rapid scrambling of the lipid bilayer and the exposure of PS, which could be partially inhibited by cellular oxidation. The interaction of aminophospholipids with cytoskeletal proteins has also been proposed as a mechanism for regulating membrane phospholipid asymmetry (Zwaal *et al.*, 1989).

Gaffet *et al.* (1995) stated that the transverse redistribution of phospholipids during human platelet activation is achieved by a vectorial outflux of aminophospholipids, not counterbalanced by a rapid reciprocal influx of choline head phospholipids, *i.e.* not scrambling. They suggested that the specific vectorial outflux of aminophospholipids could be catalyzed by a "reverse aminophospholipid translocase" activity (Gaffet *et al.*, 1995). An alternative hypothesis would be that the activity of an inward translocase was inhibited. Zwaal *et al.* (1989) proposed the involvement of a phospholipid-translocase that catalyzed both the outward and inward movement of aminophospholipids.

The presence of an energy- and protein-dependent aminophospholipid translocase activity that transports phosphatidylethanolamine from the outer to the inner leaflet of the lipid bilayer was reported by Julien *et al.* (1993). They then showed that the aminophospholipid translocase activity could also transfer phosphatidylserine, and that the activity could be maintained, suppressed and restored depending on the conditions of cell incubation (Julien *et al.*, 1993), and inhibited by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Julien *et al.*, 1997).

A 35 kD phospholipid scramblase that promotes the Ca^{2+}-dependent bidirectional movement of phosphatidylserine and other phospholipids was recently cloned from a cDNA library (Zhou *et al.*, 1997). This "PL scramblase" protein is a proline-rich, type II plasma membrane protein with a single transmembrane segment

near the C terminus. Subsequent studies confirmed that this protein was responsible for the rapid movement of phospholipids from the inner to the outer plasma membrane leaflets in cells exposed to elevated cytosolic calcium concentrations (Zhao *et al.*, 1998).

The aminophospholipid translocase activity reported by Julien *et al.* (1993; 1997), which transports PS and PE from the outer to the inner leaflet, is different to the bidirectional Ca^{2+}-dependent scramblase (Zhou *et al.*, 1997; Zhao *et al.*, 1998). The scramblase is activated by Ca^{2+}, and mostly functions to move PS from the inner to the outer leaflet in response to increased Ca^{2+} levels. It is now generally believed that the aminophospholipid translocase maintains membrane asymmetry during normal conditions, but that the scramblase is activated by Ca^{2+} influx, over-riding the translocase and randomizing aminophospholipid distribution.

The normal segregation of PS and PE to the inner surface of the plasma membrane is thus now generally accepted, and certain membrane components involved in the asymmetric processes have even been identified. However, doubts remain about the conditions, mechanisms and cell types that are capable of re-locating aminophospholipids to the outer leaflet of the membrane, and the biological implications of such events.

Contradictory reports concerning aminophospholipid expression are not limited to studies of platelets. Phosphatidylserine and phosphatidylethanolamine are generally about 7% and about 10%, respectively, of the phospholipid composition of cultured human endothelial cells from human artery, saphenous and umbilical vein (7.1% and 10.2%, respectively; Murphy *et al.*, 1992). However, an important example of the contradictions in the literature concerns the ability of anti-PS antibodies to bind to endothelial cells (Lin *et al.*, 1995).

The anti-PS antibodies present in recurrent pregnancy loss (Rote *et al.*, 1995; Rote, 1996; Vogt *et al.*, 1996; Vogt *et al.*, 1997) were believed to modulate

endothelial cell function, without evidence of binding to endothelial cells. In an attempt to explain this discrepancy, Lin *et al.* (1995) tried but failed to demonstrate anti-PS antibody binding to resting endothelial cells. They concluded that PS antigenic determinants are not expressed on the surface of resting endothelial cells, although a PS-dependent antigenic determinant was associated with cytoskeletal-like components in acetone-fixed cells (Lin *et al.*, 1995).

Van Heerde *et al.* (1994) reported that vascular endothelial cells *in vitro* can catalyze the formation of thrombin by the expression of binding sites at which procoagulant complexes can assemble. In contrast to other studies with activated platelets (Bevers *et al.*, 1982; 1983; 1985; Maneta-Peyret *et al.*, 1989; Schick *et al.*, 1976; 1978), stimulated HUVEC endothelial cells did not exhibit an increase in PS binding sites as compared to quiescent cells (Van Heerde *et al.*, 1994). Phosphatidylserine was reported to be necessary for Factor Xa formation via the extrinsic as well as the intrinsic route (Van Heerde *et al.*, 1994). Nonetheless, Brinkman *et al.* (1994) published contradictory results, indicating that other membrane constituents besides negatively charged phospholipids are involved in endothelial cell mediated, intrinsic activation of factor X.

Ravanat *et al.* (1992) also studied the catalytic potential of phospholipids in pro- and anti-coagulant reactions in purified systems and at the surface of endothelial cells in culture after stimulation. Their seemingly contradictory results were proposed to confirm a role for phospholipid-dependent mechanisms in both procoagulant Tissue-Factor activity and anticoagulant activities (activation of protein C by the thrombin-thrombomodulin complex and by Factor Xa) (Ravanat *et al.*, 1992). The Ravanat *et al.* (1992) results were also said to provide evidence of phospholipid exposure during activation of human endothelial cells, which was not observed by Van Heerde *et al.* (1994) or Brinkman *et al.* (1994). However, they did note that anionic phospholipids are of restricted accessibility in the vicinity of cellular Tissue Factor. The situation is further complicated as, even after Tissue Factor induction,

other events are likely necessary for coagulation, as the Tissue Factor remains inaccessible, being under the cell.

Ravanat *et al.* (1992) went on to suggest that the different extent of inhibition of Tissue Factor and thrombomodulin activities on stimulated endothelial cells means that the cofactor environments differ for the optimal expression of these opposite cellular activities. However, the acknowledged difficulties in trying to reproduce exact cellular phospholipid environments (Ravanat *et al.*, 1992), raise the possibility of artifactual data from these *in vitro* studies. Indeed, irrespective of the Ravanat *et al.* (1992) data, it is generally acknowledged that meaningful information regarding tumor biology, and particularly therapeutic intervention, can only be gleaned from *in vivo* studies in tumor-bearing animals, such as those conducted by the present inventors.

In addition to the disagreements regarding aminophospholipid expression, as discussed above, there are also conflicting reports concerning the function of aminophospholipids in various cell types. Although it is now generally accepted that PS expression on activated platelets is connected with the procoagulant surface, in discussing the physiological significance of membrane phospholipid asymmetry in platelets and red blood cells, Zwaal *et al.* (1989) highlighted other important functions. Moreover, Toti *et al.* (1996) stated that the physiological implications of a loss of asymmetric phospholipid distribution remain poorly understood in cell types other than blood cells.

Zwaal *et al.* (1989) stated that the membrane phospholipid asymmetry of platelets and red cells is undone when the cells are activated in various ways, presumably mediated by the increased transbilayer movement of phospholipids. These changes, coupled with the release of shed microparticles, were explained to play a role in local blood clotting reactions. A similar phenomenon was described to occur in sickled red cells: phospholipid vesicles breaking off from reversibly sickled

cells contribute to intravascular clotting in the crisis phase of sickle cell disease (Zwaal *et al.*, 1989).

Both Zwaal *et al.* (1989) and Williamson and Schlegel (1994) have indicated that the physiological significance of surface phospholipid changes is not restricted to hemostasis. In fact, the surface exposure of PS by blood cells was said to significantly alter their recognition by the reticuloendothelial system, and was to likely represent at least part of the homeostatic mechanism for the clearance of blood cells from the circulation (Zwaal *et al.*, 1989). Thus, PS acts as a signal for the elimination of activated platelets after bleeding has stopped. Recognition of PS exposed on sickle cells and malarially infected cells by phagocytes and macrophages explains their counter-pathophysiological effects (Zwaal *et al.*, 1989). Furthermore, PS-dependent phagocytosis marks virally infected cells for phagocytic uptake (WO 97/17084). The surface expression of aminophospholipids could also confer "fusion competence" to a cell (Williamson and Schlegel, 1994).

Williamson and Schlegel (1994) also speculated that there is a more general *raison d'être* for lipid asymmetry. For example, although the different head groups have received most attention, it could well be that fatty acid asymmetry is the important factor (Williamson and Schlegel, 1994). A further hypothesis is that the asymmetric distribution of transbilayer phospholipids has no function in itself, but that it is the dynamic process of lipid movement that is important to biological systems (Williamson and Schlegel, 1994).

Many groups have reported that tumor cells are responsible for the prothrombinase activity of the tumor (Connor *et al.*, 1989; Rao *et al.*, 1992; Zacharski *et al.*, 1993; Sugimura *et al.*, 1994; Donati, 1995). This could have been reasoned to be due to PS (WO 91/07187). However, the results of Sugimura *et al.* (1994) argue against this: they reported that although both the prothrombinase activity and total procoagulant activity of the tumorigenic cells, HepG2 and MKN-28, fell on reaching confluency, the PS levels remained constant.

Rather than supporting a role for tumor cell PS in prothrombinase activity, Connor *et al.* (1989) suggested that the increased expression of PS in tumorigenic cells is relevant to their ability to be recognized and bound by macrophages. Utsugi *et al.* (1991) similarly proposed that the presence of PS in the outer membrane of human tumor cells explains their recognition by monocytes.

Jamasbi *et al.* (1994) suggested a totally different role for lipid components in tumorigenic cells, proposing that the lipids interfere with tumor antigen accessibility. Thus, tumor cell lipids would act to modify the tumor cell surface antigen(s), thus protecting the tumor cells from host immune destruction (Jamasbi *et al.*, 1994). This hypothesis is not unlike that proposed by Qu *et al.* (1996), in terms of endothelial cells. These authors showed that T cells adhered to thrombin-treated human umbilical endothelial cells by virtue of binding to PS (Qu *et al.*, 1996).

It has thus been proposed that PS-mediated T cell adhesion to endothelial cells *in vivo* is important to both immune surveillance, and also to the disease processes of atherosclerosis (Qu *et al.*, 1996; Moldovan *et al.*, 1994). Bombeli *et al.* (1997) and Flynn *et al.* (1997) also suggested that cells within atherosclerotic plaques may contribute to disease progression by exposing PS, although this was based solely on *in vitro* studies. Qu *et al.* (1996) and Moldovan *et al.* (1994) even hinted at an approach opposite to that of the present invention, *i.e.*, the manipulation of phosphatidylserine interactions as an anticoagulant approach. U.S. Patent No. 5,658,877 and No. 5,296,467 have proposed annexin (or "annexine") for use as anti-endotoxins and anti-coagulants. U.S. Patent No. 5,632,986 (incorporated herein by reference) suggests the use of the phosphatidylserine-binding ligand, annexin V, as a conjugate with a component, such as urokinase, that lyses thrombi.

Toti *et al.* (1996) suggested that Scott syndrome, an inherited bleeding disorder, may reflect the deletion or mutation of a putative outward phosphatidylserine translocase or "scramblase". Although an interesting notion, Stout

et al. (1997) later isolated a membrane protein from Scott erythrocytes that exhibited normal PL scramblase activity when reconstituted in vesicles with exogenous PLs. It was suggested that the defect in Scott syndrome is related to an altered interaction of Ca^{2+} with PL scramblase on the endofacial surface of the cell membrane, due either to an intrinsic constraint upon the protein, preventing interaction with Ca^{2+} *in situ*, or due to an unidentified inhibitor or cofactor in the Scott cell that is dissociated by detergent (Stout *et al.*, 1997).

More variable results have been reported in connection with the possible role of PS in apoptosis. Williamson and Schlegel (1994) discussed the theme of PS as a marker of programmed cell death (PCD or apoptosis). It is generally accepted that programmed cell death, at least in the hematopoietic system, requires the phagocytic sequestration of the apoptopic cells before the loss of membrane integrity or "rupture". The loss of membrane asymmetry in apoptopic cells, and particularly the appearance of PS in the external leaflet, was proposed to be the trigger for their recognition by phagocytic macrophages (Williamson and Schlegel, 1994).

Martin *et al.* (1995) further reported PS externalization to be an early and widespread event during apoptosis of a variety of murine and human cell types, regardless of the initiating stimulus. They also indicated that, under conditions in which the morphological features of apoptosis were prevented (macromolecular synthesis inhibition, overexpression of Bcl-2 or Abl), the appearance of PS on the external leaflet of the plasma membrane was similarly prevented (Martin *et al.*, 1995).

However, other analyses argue against the Williamson and Schlegel (1994) and Martin *et al.* (1995) proposals to some extent (Vermes *et al.*, 1995). Although these authors indicate that the translocation of PS to the outer membrane surface is a marker of apoptosis, they reason that this is not unique to apoptosis, but also occurs during cell necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs the cell membrane loses its integrity and becomes

leaky. Therefore, according to this reasoning, PS expression at the cell surface does not indicate apoptosis unless a dye exclusion assay has been conducted to establish cell membrane integrity (Vermes *et al.*, 1995).

Nonetheless, the body of literature prior to the present invention does seem to indicate that the appearance of PS on the outer surface of a cell identifies an apoptotic cell and signals that cell's ingestion (Hampton *et al.*, 1996; WO 95/27903). Hampton *et al.* (1996) concluded that while an elevation of intracellular Ca^{2+} was an ineffective trigger of apoptosis in the cells investigated, extracellular Ca^{2+} was required for efficient PS exposure during apoptosis. In contrast, the proposal of Martin *et al.* (1995) that activation of an inside-outside PS translocase is an early widespread event during apoptosis would seem to require at least some intracellular Ca^{2+} (Zhou *et al.*, 1997; Zhao *et al.*, 1998).

Blankenberg *et al.* (1998) very recently reported that annexin V, an endogenous human protein with a high affinity for PS, can be used to concentrate at sites of apoptotic cell death *in vivo*. Radiolabeled annexin V localized to sites of apoptosis in three models, including acute cardiac allograft rejection (Blankenberg *et al.*, 1998). Staining of cardiac allografts for exogenously administered annexin V revealed myocytes at the periphery of mononuclear infiltrates, of which only a few demonstrated positive apoptotic nuclei.

Finally, the transbilayer movement of phospholipids in the plasma membrane has even been analyzed in ram sperm cells, where the existence of a transverse segregation of phospholipids has been implicated in the fertilization process (Müller *et al.*, 1994). Phospholipid asymmetry has thus been receiving increasing attention, although a clear understanding of this phenomenon, or its relationship to health or disease, has not been realized.

Irrespective of the confusing state of the art regarding aminophospholipid biology, the present inventors discovered, in controlled *in vivo* studies, that

aminophospholipids, such as PS and PE, were specific markers of tumor blood vessels. This is surprising in light of the earlier studies of aminophospholipid function, particularly those indicating that the cell surface expression of PS is accompanied by binding of circulating cells, such as T cells (Qu *et al.*, 1996), macrophages (Connor *et al.*, 1989), monocytes (Utsugi *et al.*, 1991) or phagocytes (Zwaal *et al.*, 1989; Williamson and Schlegel, 1994) and is a marker of apoptopic cells (Hampton *et al.*, 1996; Martin *et al.*, 1995; Zhou *et al.*, 1997; Zhao *et al.*, 1998).

Thus, prior to this invention, the possibility of using aminophospholipids as targetable markers of any disease, let alone of tumor vasculature, would be unlikely to be contemplated, due to the perceived masking of these molecules by the binding of one or more cell types. In fact, speculative suggestions have concerned the disruption of PS-cellular interactions, such as in preventing leukocyte binding, an initial event in atherosclerosis (Qu *et al.*, 1996).

Other surprising aspects of this discovery are evident in a comparison to earlier work concerning the shedding of procoagulant microparticles from plasma membranes and the demarcation of cells for phagocytosis (WO 97/17084). Zwaal *et al.* (1989; 1992) and Dachary-Prigent *et al.* (1996) explained that PS translocation to the plasma membrane is followed by release of microparticles, microvesicles or microspheres from the cells. Zwaal *et al.* (1989) and Williamson and Schlegel (1994) indicated that PS surface expression prompts clearance by the reticuloendothelial system. In light of these fates of PS-expressing cells, and the various documented bilayer translocase activities (Julien *et al.*, 1995; Zhou *et al.*, 1997; Zhao *et al.*, 1998), it is surprising that cell surface aminophospholipids such as PS and PE can form static and stable enough markers to allow antibody localization and binding.

Prior to the present invention, there was mounting evidence that surface PS appears as part of the apoptopic process, marking cells for rapid destruction (Hampton *et al.*, 1996; Martin *et al.*, 1995). Therefore, although reasonable for use as a diagnostic marker for certain disease states, such as graft rejection (Blankenberg *et al.*,

1998), the apparently limited life time of surface PS would also advise against its use as a viable marker for targeting in therapeutic intervention.

Nonetheless, the present study did indeed discover aminophospholipids to be markers of tumor vascular endothelial cells suitable for targeting. After postulating that PS expression was necessary for VCAM coaguligand action, the presence of PS on tumor blood vessels, but normal vessels, was demonstrated *in vivo*. The *in vivo* observations allowed the inventors to explain the safety and effectiveness of the anti-VCAM coaguligands. This is due to the requirement for coincident expression of a targeted marker (e.g., VCAM) and PS on tumor endothelium. Even if the target molecule is present on endothelium in normal or pathological conditions, thrombosis will not result if surface PS expression is lacking.

The value of the present invention is not limited to explaining coaguligand action, nor to the surprising development of naked antibody therapies (provisional applications Serial Nos. 60/092,672 and 60/110,608, each incorporated herein by reference). In fact, the present discoveries have allowed the inventors to show, for the first time, that PS translocation in endothelial cells can occur without significant cell damage or cell death (Example XIV). In the inventors' new model of tumor biology, the translocation of PS to the surface of tumor blood vessel endothelial cells occurs, at least in a significant part, independently of apoptopic or other cell-death mechanisms. Thus, PS surface expression in the tumor environment is not a consequence of cell death, nor does it trigger immediate cell destruction. This is of fundamental importance and represents a breakthrough in the scientific understanding of PS biology, membrane translocation, cell signaling and apoptosis pathways.

The separation of endothelial cell PS translocation from apoptosis (Example XIV) is also integral to methods of therapeutic intervention based upon PS surface expression. Should PS translocation to the outer membrane in tumor vascular endothelial cells occur only in dying cells, or should it inevitably trigger cell death, then the PS marker would not likely be sufficiently available to serve as a target for

the delivery of therapeutic agents. That is not to say that PS expression on certain tumor vascular endothelial cells is not transient, and that turnover and cell death do not occur in this endothelial cell population, but the finding that significant stable PS expression can be achieved without cell death is a landmark discovery important to various fields of biology and to the new targeted therapeutics described below.

D. Aminophospholipid-Targeted Therapeutics

The *in vivo* aminophospholipid tumor vasculature expression studies further support the use of coaguligands directed against previously identified tumor vasculature markers, *e.g.*, VCAM-1 and E-selectin, as selective thrombotic agents for the treatment of solid tumors. However, these observations also led the inventors to develop additional tumor treatment methods. For example, naked or unconjugated antibodies against aminophospholipid components were surprisingly found to be capable of specifically inducing tumor blood vessel destruction and tumor necrosis *in vivo* in the absence of additional effector moieties. Such uses are disclosed and claimed in first and second provisional applications Serial Nos. 60/092,672 (filed July 13, 1998) and 60/110,608 (filed December 02, 1998) and in co-filed U.S. and PCT patent applications (Attorney Docket Nos. 4001.002200, 4001.002282 and 4001.002210), each specifically incorporated herein by reference.

The studies of first and second provisional applications Serial Nos. 60/092,672 (filed July 13, 1998) and 60/110,608 (filed December 02, 1998) are in contrast to those recently reported by Nakamura *et al.* (1998). These authors analyzed antibody fractions from patients with lupus anticoagulant (LAC), a disorder associated with arterial and venous thrombosis, thrombocytopenia, and recurrent fetal loss. Plasma with LAC activity was initially reported to induce apoptosis in endothelial cells (Nakamura *et al.*, 1994). The apoptotic activities of LAC antisera were then reported to be localized in an annexin V-binding antibody fraction in 10/10 patients studied (Nakamura *et al.*, 1998). As annexin binds to PS, the apparent ability of anti-annexin antibodies to induce apoptosis would be the opposite of the ability of an anti-PS antibody to induce apoptosis.

The ability of LAC antibody fractions to induce apoptosis was further reported to be inhibited by preincubation with annexin V (Nakamura *et al.*, 1998). In contrast, removal of anti-phospholipid antibodies from the patients' IgG fractions with phospholipid liposomes did not abolish the apoptosis-inducing activities or annexin V binding (Nakamura *et al.*, 1998). These results reasonably implied that patients with LAC often have antibodies that do not bind phospholipids and yet are responsible for the induction of apoptosis in endothelial cells (Nakamura *et al.*, 1998).

Without needing to equate the Nakamura *et al.* (1998) LAC data with the inventors' observations from *in vivo* studies of tumors and tumor vasculature, due to the evidently disparate nature of these clinical conditions, the inventors nonetheless have certain unifying theories. Nakamura *et al.* (1998) attempted to remove anti-phospholipid antibodies from patients' antisera using phospholipid liposomes, and observed that this did not abolish the apoptosis-inducing activity. These results led Nakamura *et al.* (1998) to conclude that the anti-phospholipids antibodies cannot be responsible for apoptopic activity. However, the present inventors now have the insight to suggest that the incubation with phospholipid liposomes may not have removed the anti-phospholipids antibodies from the antisera, as phospholipids are antigenically neutral in bilayer and liposomal form, and largely only bind antibodies in hexagonal form (Rauch *et al.*, 1986; Rauch and Janoff, 1990; Berard *et al.*, 1993; each incorporated herein by reference) or in association with membrane proteins. Thus, anti-phospholipids antibodies may remain in the LAC antisera and may cause, or contribute to, the observed apoptopic activity.

The invention disclosed herein is directed to the use of aminophospholipids as targets for anti-tumor vasculature immunotoxin and/or coaguligand therapy. Although the identification of any additional target to allow specific tumor vessel localization in vascular targeting therapies is valuable, the present discovery of aminophospholipids as suitable targets is particularly important as it brings another entire group of targets into the picture: lipids rather than the proteins previously

preferred. The aminophospholipid discovery is also functionally significant as it allows therapeutic agents to be delivered into even more intimate contact with the target cell membrane, rather than binding to a protein complex more distant from the membrane.

One of the most surprising aspects of the present discovery is that PS expression on intact tumor-associated endothelial cells is sufficently stable to allow targeting. The present *in vivo* and *in vitro* data definitively show that PS is expressed on viable tumor-associated endothelial cells with normal morphology and intact cytoskeletons. As PS expression is not limited to cells undergoing cell death or about to enter an apoptopic pathway, targeting with diagnostic and therapeutic agents is both practicable and surprising (given that PS expression was thought to be associated only with cell destruction).

A precise molecular understanding of exactly how and why aminophospholipid-targeted therapeutic agents are suitable for use in tumor treatment is not necessary in order to practice the present invention. Given that the administration of aminophospholipid-directed therapeutic agents is herein shown to advantageously result in specific anti-tumor effects *in vivo*, the invention can be utilized irrespective of the molecular mechanisms that underlie the aminophospholipid expression in tumor vasculature.

However, it is interesting to note that a review of the scientific literature to date reveals features that argue against the present surprisingly effective uses, and even proposes directly opposite uses for distinct aminophospholipid binding agent-conjugates. For example, annexin, a phosphatidylserine binding protein, has itself been proposed for use as an anticoagulant (WO 91/07187; U.S. patent No. 5,296,467; each incorporated herein by reference). This use of annexin was said to be based upon the inhibition of the procoagulant activity of tumor cells (WO 91/07187).

Even more telling is the disclosure of U.S. Patent No. 5,632,986 which, in complete contrast to the present invention, proposes the use of annexin as a conjugate with compounds that lyse thrombi, or precursors of such thrombolytic compounds. The referenced combination of an aminophospholipid binding protein, annexin, with a lytic agent is, evidently, the opposite of the present invention, which concerns the combination of annexin and other aminophospholipid binding proteins with agents that induce thrombosis, either directly or indirectly.

In the preparation of both immunotoxins and coaguligands based upon aminophospholipid binding agents and antibodies, recombinant expression may be employed to create a fusion protein, as is known to those of skill in the art and further disclosed herein. Equally, immunotoxins and coaguligands may be generated using avidin:biotin bridges or any of the chemical conjugation and cross-linker technologies, mostly developed in reference to antibody conjugates. Therefore, any of the following aminophospholipid binding proteins and ligands may be conjugated to a toxin or coagulant in the same manner as used for antibody conjugates, described herein.

D1. Aminophospholipid Binding Proteins

In addition to antibodies (see below), aminophospholipid binding ligands or binding proteins may be used in the therapeutic agent-targeting agent constructs of the present invention. Naturally occurring proteins are known that bind to both phosphatidylethanolamineand phosphatidylserinewith specificity.

A series of studies by Sugi and McIntyre revealed that kininogens can bind to membrane-exposed PE, at least in platelets (Sugi and McIntyre 1995; 1996a; 1996b; each incorporated herein by reference). Kininogens are naturally occurring proteins that normally have anti-thrombotic effects. The present inventors propose that low or high molecular weight kininogens may therefore be attached to therapeutic agents and used in the delivery of therapeutics to phosphatidylethanolamine, newly discovered to be a marker of tumor vasculature.

Various mammalian and human kininogen genes have now been cloned, and such genes and proteins can be used in the various recombinant and/or chemical embodiments of the present invention. For example, the complete nucleotide and amino acid sequences of the genes and proteins described in Nakanishi *et al.*, 1983, are incorporated herein by reference for such purposes.

Nawa *et al.* (1983; incorporated herein by reference) reported cDNA and protein sequences for bovine low molecular weight kininogens. Fig. 2 of Nawa *et al.* (1983) is specifically incorporated herein by reference for purposes of providing these complete nucleotide and amino acid sequences. Kitamura *et al.* (1983; incorporated herein by reference) then reported that a single gene encodes the bovine high molecular weight and low molecular weight kininogens. Fig. 2 of Kitamura *et al.* (1983) is again incorporated herein by reference to provide the referenced gene and protein sequences. Kitamura *et al.* (1987) is also specifically incorporated herein by reference for purposes of providing further information concerning the bovine, rat and human kininogens, including low molecular weight, high molecular weight and T-kininogens.

Preferred high and low molecular weight kininogens for use in these aspects of the invention will be the human genes and proteins, as described by Takagaki *et al.* (1985), Kitamura *et al.* (1985) and Kellermann *et al.* (1986), each incorporated herein by reference. Each of Fig. 2 and Fig. 3 of Takagaki *et al.* (1985) are specifically incorporated herein by reference to provide the complete nucleotide and amino acid sequences of human low and high molecular weight prekininogens, respectively. Figs. 1 and 8 of the protein analysis paper of Kellermann *et al.* (1986) are similarly incorporated herein.

Kitamura *et al.* (1985) is also specifically incorporated herein by reference for purposes of providing further information regarding the structural organization of the human kininogen gene, as may be used, *e.g.*, to design particular expression constructs for use herewith. Kitamura *et al.* (1988) is further incorporated by

reference for purposes of providing detailed information regarding the cloning of cDNAs and genomic kininogens, such that any desired kininogen may be cloned.

In addition to the T-kininogens described by Kitamura *et al.* (1987; incorporated herein by reference), Anderson *et al.* (1989) is also specifically incorporated herein by reference for purposes of providing the gene and protein sequences of T-kininogen. Fig. 3 of Anderson *et al.* (1989) is specifically incorporated.

Other phosphatidylethanolamine binding proteins are known that can be used in such embodiments. A number of studies, particularly by Jones and Hall, and Bernier and Jolles, have concerned the purification, characterization and cloning of phosphatidylethanolamine binding proteins. For example, Bernier and Jolles (1984; incorporated herein by reference) first reported the purification and characterization of a basic ~23 kDa cytosolic protein from bovine brain that was later characterized as a phosphatidylethanolamine-binding protein (Bernier *et al.*, 1986; incorporated herein by reference). Schoentgen *et al.* (1987; incorporated herein by reference) reported the complete amino acid sequence of this bovine protein, then shown to be 21 kDa. Fig. 2 of Schoentgen *et al.* (1987) is specifically incorporated herein by reference for purposes of providing the complete amino acid sequence of this bovine phosphatidylethanolamine binding protein.

Jones and Hall (1991; incorporated herein by reference) later purified and partially sequenced a ~23 kDa protein from rat sperm plasma membranes that showed sequence similarity and phospholipid binding properties similar to the bovine brain cytosolic protein of Bernier and Jolles (Bernier and Jolles, 1984; Bernier *et al.*, 1986; Schoentgen *et al.*, 1987). The rat 23 kDa protein of Jones and Hall (1991; incorporated herein by reference) also showed selective affinity for phosphatidylethanolamine ($Kd = 1.6 \times 10^{-5}$ M).

Perry *et al.* (1994; incorporated herein by reference) then cloned and sequenced rat and monkey versions of the phosphatidylethanolamine binding protein

of Jones and Hall (1991). Figures, 4, 5 and 6 of Perry *et al.* (1994) are specifically incorporated herein by reference for purposes of providing the complete DNA and amino acid sequences of the rat and monkey phosphatidylethanolamine binding proteins, and comparison to the bovine protein sequence. Any of the foregoing mammalian phosphatidylethanolamine binding proteins, or their human counterparts, may be attached to therapeutic agents and used in the present invention. These mammalian sequences have EMBL Nucleotide Sequence Database Accession Numbers X71873 (rat) and X73137 (monkey), and are each incorporated herein by reference.

To counterpart human phosphatidylethanolamine binding protein has also been cloned (Hori *et al.*, 1994; incorporated herein by reference). Both Fig. 1 of Hori *et al.* (1994) and GenBank, EMBL and DDBJ Accession Number D16111 are incorporated herein by reference for purposes of providing the complete DNA and amino acid sequences of the human phosphatidylethanolamine binding proteins. The mammalian and human sequences, as incorporated herein, may be employed in well-known expression techniques, either to express the proteins themselves or therapeutic agent-fusions thereof. Phosphatidylethanolamine binding proteins and genes from other sources, such as yeast, Drosophila, simian, *T. canis* and *O. volvulus* may also be employed in these embodiments (Gems *et al.*, 1995; incorporated herein by reference).

Variant, mutant or second generation phosphatidylethanolamine binding protein nucleic acids may also be readily prepared by standard molecular biological techniques, and may optionally be characterized as hybridizing to any of the phosphatidylethanolamine binding protein nucleotide sequences set forth in any one or more of Nakanishi *et al.* (1983); Nawa *et al.* (1983); Kitamura *et al.* (1983; 1985; 1987; 1988); Takagaki *et al.* (1985); Kellermann *et al.* (1986); Anderson *et al.* (1989); Bernier and Jolles (1984); Bernier *et al.* (1986); Schoentgen *et al.* (1987); Jones and Hall (1991); Perry *et al.* (1994); and Hori *et al.* (1994); each incorporated herein by reference. Exemplary suitable hybridization conditions include hybridization in about

7% sodium dodecyl sulfate (SDS), about 0.5 M $NaPO_4$, about 1 mM EDTA at about 50°C; and washing with about 1% SDS at about 42°C.

In addition to the foregoing phosphatidylethanolamine binding proteins or "ligands", naturally occurring proteins exist that specifically bind phosphatidylserine. Preferred amongst these are annexins (sometimes spelt "annexines"), a group of calcium-dependent phospholipid binding proteins. At least nine members of the annexin family have been identified in mammalian tissues (Annexin I through Annexin IX). Most preferred amongst these is annexin V (also known as PAP-I).

U.S. Patent No. 5,658,877, incorporated herein by reference, describes Annexin I, effective amounts of Annexin I and pharmaceutical compositions thereof. Also described are methods of treating an animal to prevent or alleviate the adverse effects of endotoxin in the lung that comprise administering into the airway of an animal a safe amount of 33 kDa Annexin I fragment.

Annexin V contains one free sulfhydryl group and does not have any attached carbohydrate chains. The primary structure of annexin V deduced from the cDNA sequence shows that annexin V comprises four internal repeating units (U.S. Patent No. 4,937,324; incorporated herein by reference).

U.S. Patent No. 5,296,467 and WO 91/07187 are also each incorporated herein by reference as they provide pharmaceutical compositions comprising 'annexine' (annexin). Although proposed for use as anticoagulants, the annexins of U.S. Patent No. 5,296,467 and WO 91/07187 may now be used as part of the conjugates of the present invention.

WO 91/07187 provides natural, synthetic or genetically prepared derivatives and analogues of 'annexine' (annexin), which may now be used in the conjugates of the present invention. Particular annexins are provided of 320 amino acids,

containing variant amino acids and, optionally, a disulphide bridge between the 316-Cys and the 2-Ala.

U.S. Patent No. 5,296,467 is incorporated herein by reference in its entirety, including all figures and sequences, for purposes of even further describing annexins and pharmaceutical compositions thereof. U.S. Patent No. 5,296,467 describes annexin cloning, recombinant expression and preparation. Aggregates of two or more annexines, *e.g.*, linked by disulfide bonds between one or more cysteine groups on the respective annexine, are also disclosed. Yet a further example of suitable annexin starting materials is provided by WO 95/27903 (incorporated herein by reference), which provides annexins for use in detecting apoptotic cells.

WO 97/17084 is also incorporated herein by reference for purposes of describing annexin starting materials for preparing constructs of the present invention. WO 97/17084 particularly concerns the use of Annexin V to alter phosphatidylserine-dependent phagocytosis. It is said that blocking PS-dependent phagocytosis means that PS-carrying cells undergo phagocytosis by other pathways, leading to greater immune responses, such that Annexin V may be used as an adjuvant to increase immunogenicity of vaccines. The treatment of sickle cell anemia and malaria is also described. WO 97/17084 also provides certain expression vector systems that may be adapted for use herein.

To the extent that they clearly describe appropriate annexin starting materials for preparing therapeutic constructs of the present invention, each of the diagnostic approaches of U.S. Patent No. 5,627,036; WO 95/19791; WO 95/27903; WO 95/34315; WO 96/17618; and WO 98/04294; are also specifically incorporated herein by reference. Various of these documents also concern recombinant expression vectors useful for adaptation into the present invention.

Although totally counter-intuitive prior to the present invention, the annexin conjugation technology of U.S. Patent No. 5,632,986 may now be adapted for use in

the present tumor treatment methods. U.S. Patent No. 5,632,986 (incorporated herein by reference) provides annexin conjugates using compounds that lyse thrombi, or precursors of such compounds. Annexin-plasminogen activator conjugates and annexin-urokinase conjugates were particularly provided for thrombolysis and for treating disorders resulting from thrombosis. By switching the thrombolytic compounds of U.S. Patent No. 5,632,986 for the toxic and coagulative compounds disclosed herein, the basic conjugate technology of U.S. Patent No. 5,632,986 can be easily adapted for use in the present invention.

U.S. Patent No. 5,632,986 is thus provided for purposes of further describing annexin isolation from tissue extracts (U.S. Patent No. 4,937,324; also incorporated herein by reference) and annexin production by recombinant methods. Each of the cDNA clones and expression vectors of U.S. Patent No. 5,632,986 are thus specifically incorporated herein by reference.

U.S. Patent No. 5,632,986 is also specifically incorporated herein by reference for purposes of further describing mutants and variants of the annexin molecule that are subdivided or altered at one or more amino acid residues so long as the phospholipid binding capability is not reduced substantially. Appropriate annexins for use in the present invention can thus be truncated, for example, to include one or more domains or contain fewer amino acid residues than the native protein, or can contain substituted amino acids. Any changes are acceptable within the scope of the invention so long as the mutein or second generation annexin molecule does not contain substantially lower affinity for aminophospholipid. Such guidance can also be applied to phosphatidylethanolamine binding proteins.

Second generation, variant and mutant annexin-encoding nucleic acids may also be readily prepared by standard molecular biological techniques, and may optionally be characterized as hybridizing to any of the foregoing annexin-encoding nucleic acid sequences under hybridization conditions such as those including

hybridization in about 7% sodium dodecyl sulfate (SDS), about 0.5 M $NaPO_4$, about 1 mM EDTA at about 50°C; and washing with about 1% SDS at about 42°C.

The chemical cross-linking of annexins and other agents is also described in U.S. Patent No. 5,632,986, incorporated herein by reference. All such techniques can be adapted for use herewith simply by substituting the thrombolytic agents for those described herein. Aliphatic diamines; succinimide esters; hetero-bifunctional coupling reagents, such as SPDP; maleimide compounds; linkers with spacers; and the like, may thus be used.

U.S. Patent No. 5,632,986 is yet further specifically incorporated herein by reference for purposes of describing the recombinant production of annexin-containing conjugates. Appropriate nucleic acid sequences are thus joined to produce chimeric coding sequences that, in turn, produce chimeric proteins. Exemplary expression vectors are said to be pKK233-2 (*E. coli*), DPOT (yeast) and pDSP1.1BGH (mammalian). Such teaching is supplemented by further information provided herein.

D2. Biologically Functional Equivalents

Equivalents, or even improvements, of aminophospholipid binding proteins can now be made, generally using the materials provided above as a starting point. Modifications and changes may be made in the structure of an aminophospholipid binding protein and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity, such as, binding to the aminophospholipids, PS and PE. These considerations also apply to toxins and coagulants.

Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or of course, the underlying DNA sequence) and nevertheless obtain a protein with like (agonistic) properties. It is thus contemplated that various changes may be made in the sequence of known aminophospholipid binding proteins or peptides (or underlying DNA sequences) without appreciable loss of their biological utility or activity. Biological functional equivalents made from mutating an underlying DNA sequence can be made using the codon information provided herein in Table A, and the supporting technical details on site-specific mutagenesis.

It also is well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent" protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent proteins and peptides are thus defined herein as those proteins and peptides in which certain, not most or all, of the amino acids may be substituted. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

In making more quantitative changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is thus understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein. As detailed in U.S. Patent No. 4,554,101 (incorporated herein by reference), the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

D3. Toxic and Anti-Cellular Agents

For certain applications, the therapeutic agents will be cytotoxic or pharmacological agents, particularly cytotoxic, cytostatic, anti-cellular or anti-angiogenic agents having the ability to kill or suppress the growth or cell division of endothelial cells. In general, these aspects of the invention contemplate the use of any pharmacological agent that can be conjugated to a targeting agent, and delivered in active form to the targeted endothelium.

Exemplary anti-cellular agents include chemotherapeutic agents, as well as cytotoxins. Chemotherapeutic agents that may be used include: hormones, such as steroids; anti-metabolites, such as cytosine arabinoside, fluorouracil, methotrexate or aminopterin; anthracyclines; mitomycin C; vinca alkaloids; demecolcine; etoposide; mithramycin; anti-tumor alkylating agents, such as chlorambucil or melphalan. Other embodiments may include agents such as cytokines. Basically, any anti-cellular agent may be used, so long as it can be successfully conjugated to, or associated with, a targeting agent or antibody in a manner that will allow its targeting, internalization, release and/or presentation to blood components at the site of the targeted endothelial cells.

There may be circumstances, such as when the target antigen does not internalize by a route consistent with efficient intoxication by the toxic compound, where one will desire to target chemotherapeutic agents, such as anti-tumor drugs, cytokines, antimetabolites, alkylating agents, hormones, and the like. A variety of chemotherapeutic and other pharmacological agents have now been successfully conjugated to antibodies and shown to function pharmacologically, including doxorubicin, daunomycin, methotrexate, vinblastine, neocarzinostatin, macromycin, trenimon and α-amanitin.

In other circumstances, any potential side-effects from cytotoxin-based therapy may be eliminated by the use of DNA synthesis inhibitors, such as daunorubicin, doxorubicin, adriamycin, and the like. These agents are therefore preferred examples

of anti-cellular agents for use in the present invention. In terms of cytostatic agents, such compounds generally disturb the natural cell cycle of a target cell, preferably so that the cell is taken out of the cell cycle. Exemplary cytostatic agents include.

A wide variety of cytotoxic agents are known that may be conjugated to anti-aminophospholipid antibodies or binding ligands. Examples include numerous useful plant-, fungus- or bacteria-derived toxins, which, by way of example, include various A chain toxins, particularly ricin A chain; ribosome inactivating proteins, such as saporin or gelonin; α-sarcin; aspergillin; restrictocin; ribonucleases, such as placental ribonuclease; diphtheria toxin; and *pseudomonas* exotoxin, to name just a few.

Of the toxins, ricin A chains are preferred. The most preferred toxin moiety for use herewith is toxin A chain that has been treated to modify or remove carbohydrate residues, so-called deglycosylated A chain (dgA). Deglycosylated ricin A chain is preferred because of its extreme potency, longer half-life, and because it is economically feasible to manufacture it in a clinical grade and scale.

It may be desirable from a pharmacological standpoint to employ the smallest molecule possible that nevertheless provides an appropriate biological response. One may thus desire to employ smaller A chain peptides that will provide an adequate anti-cellular response. To this end, it has been discovered that ricin A chain may be "truncated" by the removal of 30 N-terminal amino acids by Nagarase (Sigma), and still retain an adequate toxin activity. It is proposed that where desired, this truncated A chain may be employed in conjugates in accordance with the invention.

Alternatively, one may find that the application of recombinant DNA technology to the toxin A chain moiety will provide additional benefits in accordance the invention. In that the cloning and expression of biologically active ricin A chain has been achieved, it is now possible to identify and prepare smaller or otherwise variant peptides which nevertheless exhibit an appropriate toxin activity. Moreover, the fact that ricin A chain has now been cloned allows the application of site-directed

mutagenesis, through which one can readily prepare and screen for A chain-derived peptides and obtain additional useful moieties for use in connection with the present invention.

Other agents for use in immunoconjugate targeting of PS expressed on tumor vasculature are the angiopoietins. The angiopoietins, like the members of the VEGF family, are growth factors largely specific for vascular endothelium (Davis and Yancopoulos, 1999; Holash *et al.*, 1999; incorporated herein by reference). The angiopoietins first described were a naturally occurring agonist, angiopoietin-1 (Ang-1; SEQ ID NO:1 and SEQ ID NO:2), and a naturally occurring antagonist, angiopoietin-2 (Ang-2; SEQ ID NO:3 and SEQ ID NO:4), both of which act by means of the endothelial cell tyrosine kinase receptor, Tie2.

Two new angiopoietins, angiopoietin-3 (mouse) and angiopoietin-4 (human) have also been identified (Valenzuela *et al.*, 1999). Angiopoietin-3 appears to act as an antagonist, whereas angiopoietin-4 appears to function as an agonist (Valenzuela *et al.*, 1999). A protein termed angiopoietin-3 was also cloned from human heart and reported not to have mitogenic effects on endothelial cells (Kim *et al.*, 1999).

Whereas VEGF is necessary for the early stages of vascular development, angiopoietin-1 is generally required for the later stages of vascular remodeling. Angiopoietin-1 is thus a maturation or stabilization factor, which converts immature vessels to mature vessels.

Angiopoietin-1 has been shown to augment revascularization in ischemic tissue (Shyu *et al.*, 1998) and to increase the survival of vascular networks exposed to either VEGF or a form of aFGF (Papapetropoulos *et al.*, 1999). These authors also showed that angiopoietin-1 prevents apoptotic death in HUVEC triggered by withdrawal of the same form of aFGF (Papapetropoulos *et al.*, 1999). Such data are consistent with the direct role of angiopoietin-1 on human endothelial cells and its

interaction with other angiogenic molecules to stabilize vascular structures by promoting the survival of differentiated endothelial cells.

Of the angiopoietins, angiopoietin-2 is a preferred agent for use in PS-targeted therapy, particularly in tumors with low VEGF levels and/or in combination with VEGF inhibition. Angiopoietin-2 is also a ligand for Tie2, but generally counteracts blood vessel maturation/stability mediated by angiopoietin-1. It is thus an antagonist of angiopoietin-1, and acts to disturb capillary structure. However, as angiopoietin-2 renders endothelial cells responsive to angiogenic stimuli, it can initiate neovascularization in combination with other appropriate signals, particularly VEGF (Asahara *et al.*, 1998; Holash *et al.*, 1999; incorporated herein by reference).

In the absence of another angiogenic signal, angiopoietin-2 causes vessels to destabilize and become immature. In the presence of a stimulus, such as VEGF, angiopoietin-2 promotes angiogenesis. Indeed, the angiogenic effects of a number of regulators are believed to be achieved, at least in part, through the regulation of an autocrine loop of angiopoietin-2 activity in microvascular endothelial cells (Mandriota and Pepper, 1998).

Angiopoietin-2 expression in tumor tissue has been reported (Tanaka *et al.*, 1999), where it presumably acts in combination with VEGF to promote angiogenesis (Stratmann *et al.*, 1998). However, as angiopoietin-2 provides a negative signal when VEGF is low or absent, provision of angiopoietin-2 can be a useful therapeutic approach. In addition to tumor-targeted forms, angiopoietin-2 can also be administered as a protein or gene therapy therapeutic (see combination therapies described herein). Fusion proteins of angiopoietins are also envisioned for use in this invention, such as the stable Ang-1-Ang-2 fusion protein included herein as SEQ ID NO:5.

D4. Coagulation Factors

The antibody and ligand targeting agents of the invention may be linked to a component that is capable of directly or indirectly stimulating coagulation, to form a coaguligand. Here, the targeting agents may be directly linked to the coagulant or coagulation factor, or may be linked to a second binding region that binds and then releases the coagulant or coagulation factor. As used herein, the terms "coagulant" and "coagulation factor" are each used to refer to a component that is capable of directly or indirectly stimulating coagulation under appropriate conditions, preferably when provided to a specific *in vivo* environment, such as the tumor vasculature.

Preferred coagulation factors are Tissue Factor compositions, such as truncated TF (tTF), dimeric, multimeric and mutant TF molecules. "Truncated TF" (tTF) refers to TF constructs that are rendered membrane-binding deficient by removal of sufficient amino acid sequences to effect this change in property. A "sufficient amount" in this context is an amount of transmembrane amino acid sequence originally sufficient to enter the TF molecule in the membrane, or otherwise mediate functional membrane binding of the TF protein. The removal of such a "sufficient amount of transmembrane spanning sequence" therefore creates a truncated Tissue Factor protein or polypeptide deficient in phospholipid membrane binding capacity, such that the protein is substantially a soluble protein that does not significantly bind to phospholipid membranes. Truncated TF thus substantially fails to convert Factor VII to Factor VIIa in a standard TF assay, and yet retains so-called catalytic activity including activating Factor X in the presence of Factor VIIa.

U.S. Patent No. 5,504,067 is specifically incorporated herein by reference for the purposes of further describing such truncated Tissue Factor proteins. Preferably, the Tissue Factors for use in these aspects of the present invention will generally lack the transmembrane and cytosolic regions (amino acids 220-263) of the protein. However, there is no need for the truncated TF molecules to be limited to molecules of the exact length of 219 amino acids.

Tissue Factor compositions may also be useful as dimers. Any of the truncated, mutated or other Tissue Factor constructs may be prepared in a dimeric form for use in the present invention. As will be known to those of ordinary skill in the art, such TF dimers may be prepared by employing the standard techniques of molecular biology and recombinant expression, in which two coding regions are prepared in-frame and expressed from an expression vector. Equally, various chemical conjugation technologies may be employed in connection with the preparation of TF dimers. The individual TF monomers may be derivatized prior to conjugation. All such techniques would be readily known to those of skill in the art.

If desired, the Tissue Factor dimers or multimers may be joined via a biologically-releasable bond, such as a selectively-cleavable linker or amino acid sequence. For example, peptide linkers that include a cleavage site for an enzyme preferentially located or active within a tumor environment are contemplated. Exemplary forms of such peptide linkers are those that are cleaved by urokinase, plasmin, thrombin, Factor IXa, Factor Xa, or a metalloproteinase, such as collagenase, gelatinase or stromelysin.

In certain embodiments, the Tissue Factor dimers may further comprise a hindered hydrophobic membrane insertion moiety, to later encourage the functional association of the Tissue Factor with the phospholipid membrane, but only under certain defined conditions. As described in the context of the truncated Tissue Factors, hydrophobic membrane-association sequences are generally stretches of amino acids that promote association with the phospholipid environment due to their hydrophobic nature. Equally, fatty acids may be used to provide the potential membrane insertion moiety.

Such membrane insertion sequences may be located either at the N-terminus or the C-terminus of the TF molecule, or generally appended at any other point of the molecule so long as their attachment thereto does not hinder the functional properties of the TF construct. The intent of the hindered insertion moiety is that it remains non-

functional until the TF construct localizes within the tumor environment, and allows the hydrophobic appendage to become accessible and even further promote physical association with the membrane. Again, it is contemplated that biologically-releasable bonds and selectively-cleavable sequences will be particularly useful in this regard, with the bond or sequence only being cleaved or otherwise modified upon localization within the tumor environment and exposure to particular enzymes or other bioactive molecules.

In other embodiments, the tTF constructs may be multimeric or polymeric. In this context a "polymeric construct" contains 3 or more Tissue Factor constructs. A "multimeric or polymeric TF construct" is a construct that comprises a first TF molecule or derivative operatively attached to at least a second and a third TF molecule or derivative. The multimers may comprise between about 3 and about 20 such TF molecules. The individual TF units within the multimers or polymers may also be linked by selectively-cleavable peptide linkers or other biological-releasable bonds as desired. Again, as with the TF dimers discussed above, the constructs may be readily made using either recombinant manipulation and expression or using standard synthetic chemistry.

Even further TF constructs useful in context of the present invention are those mutants deficient in the ability to activate Factor VII. Such "Factor VII activation mutants" are generally defined herein as TF mutants that bind functional Factor VII/VIIa, proteolytically activate Factor X, but are substantially free from the ability to proteolytically activate Factor VII. Accordingly, such constructs are TF mutants that lack Factor VII activation activity.

The ability of such Factor VII activation mutants to function in promoting tumor-specific coagulation is based upon their specific delivery to the tumor vasculature, and the presence of Factor VIIa at low levels in plasma. Upon administration of such a Factor VII activation mutant-targeting agent conjugate, the mutant will be localized within the vasculature of a vascularized tumor. Prior to

localization, the TF mutant would be generally unable to promote coagulation in any other body sites, on the basis of its inability to convert Factor VII to Factor VIIa. However, upon localization and accumulation within the tumor region, the mutant will then encounter sufficient Factor VIIa from the plasma in order to initiate the extrinsic coagulation pathway, leading to tumor-specific thrombosis. Exogenous Factor VIIa could also be administered to the patient.

Any one or more of a variety of Factor VII activation mutants may be prepared and used in connection with the present invention. There is a significant amount of scientific knowledge concerning the recognition sites on the TF molecule for Factor VII/VIIa. It will thus be understood that the Factor VII activation region generally lies between about amino acid 157 and about amino acid 167 of the TF molecule. However, it is contemplated that residues outside this region may also prove to be relevant to the Factor VII activating activity, and one may therefore consider introducing mutations into any one or more of the residues generally located between about amino acid 106 and about amino acid 209 of the TF sequence (WO 94/07515; WO 94/28017; each incorporated herein by reference).

A variety of other coagulation factors may be used in connection with the present invention, as exemplified by the agents set forth below. Thrombin, Factor V/Va and derivatives, Factor VIII/VIIIa and derivatives, Factor IX/IXa and derivatives, Factor X/Xa and derivatives, Factor XI/XIa and derivatives, Factor XII/XIIa and derivatives, Factor XIII/XIIIa and derivatives, Factor X activator and Factor V activator may be used in the present invention.

Russell's viper venom Factor X activator is contemplated for use in this invention. Monoclonal antibodies specific for the Factor X activator present in Russell's viper venom have also been produced, and could be used to specifically deliver the agent as part of a bispecific binding ligand.

Thromboxane A_2 is formed from endoperoxides by the sequential actions of the enzymes cyclooxygenase and thromboxane synthetase in platelet microsomes. Thromboxane A_2 is readily generated by platelets and is a potent vasoconstrictor, by virtue of its capacity to produce platelet aggregation. Both thromboxane A_2 and active analogues thereof are contemplated for use in the present invention.

Thromboxane synthase, and other enzymes that synthesize platelet-activating prostaglandins, may also be used as "coagulants" in the present context. Monoclonal antibodies to, and immunoaffinity purification of, thromboxane synthase are known; as is the cDNA for human thromboxane synthase.

α2-antiplasmin, or α2-plasmin inhibitor, is a proteinase inhibitor naturally present in human plasma that functions to efficiently inhibit the lysis of fibrin clots induced by plasminogen activator. α2-antiplasmin is a particularly potent inhibitor, and is contemplated for use in the present invention.

As the cDNA sequence for α2-antiplasmin is available, recombinant expression and/or fusion proteins are preferred. Monoclonal antibodies against α2-antiplasmin are also available that may be used in the bispecific binding ligand embodiments of the invention. These antibodies could both be used to deliver exogenous α2-antiplasmin to the target site or to garner endogenous α2-antiplasmin and concentrate it within the targeted region.

D5. Fusion Proteins and Recombinant Expression

The therapeutic agent-targeting agent compositions of the present invention may be readily prepared as fusion proteins using molecular biological techniques. The use of recombinant DNA techniques to achieve such ends is now standard practice to those of skill in the art. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. DNA and RNA synthesis may, additionally, be performed using an

automated synthesizers (see, for example, the techniques described in Sambrook *et al.*, 1989).

The preparation of such a fusion protein generally entails the preparation of a first and second DNA coding region and the functional ligation or joining of such regions, in frame, to prepare a single coding region that encodes the desired fusion protein. In the present context, the targeting agent DNA sequence will be joined in frame with a DNA sequence encoding a therapeutic agent. It is not generally believed to be particularly relevant which portion of the therapeutic agent-targeting agent is prepared as the N-terminal region or as the C-terminal region.

Once the desired coding region has been produced, an expression vector is created. Expression vectors contain one or more promoters upstream of the inserted DNA regions that act to promote transcription of the DNA and to thus promote expression of the encoded recombinant protein. This is the meaning of "recombinant expression".

To obtain a so-called "recombinant" version of the therapeutic agent-targeting agent protein, it is expressed in a recombinant cell. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of the therapeutic agent-targeting agent constructs.

Such proteins may be successfully expressed in eukaryotic expression systems, *e.g.*, CHO cells, however, it is envisioned that bacterial expression systems, such as *E. coli* pQE-60 will be particularly useful for the large-scale preparation and subsequent purification of the therapeutic agent-targeting agent constructs. cDNAs may also be expressed in bacterial systems, with the encoded proteins being expressed as fusions with β-galactosidase, ubiquitin, *Schistosoma japonicum* glutathione S-transferase, and

the like. It is believed that bacterial expression will have advantages over eukaryotic expression in terms of ease of use and quantity of materials obtained thereby.

In terms of microbial expression, U.S. Patent Nos. 5,583,013; 5,221,619; 4,785,420; 4,704,362; and 4,366,246 are incorporated herein by reference for the purposes of even further supplementing the present disclosure in connection with the expression of genes in recombinant host cells.

Recombinantly produced therapeutic agent-targeting agent constructs may be purified and formulated for human administration. Alternatively, nucleic acids encoding the therapeutic agent-targeting agent constructs may be delivered via gene therapy. Although naked recombinant DNA or plasmids may be employed, the use of liposomes or vectors is preferred. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into the host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells. Preferred genc therapy vectors for use in the present invention will generally be viral vectors.

Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines. Other viruses, such as adenovirus, herpes simplex viruses (HSV), cytomegalovirus (CMV), and adeno-associated virus (AAV), such as those described by U.S. Patent 5,139,941 (incorporated herein by reference), may also be engineered to serve as vectors for gene transfer.

Although some viruses that can accept foreign genetic material are limited in the number of nucleotides they can accommodate and in the range of cells they infect, these viruses have been demonstrated to successfully effect gene expression. However, adenoviruses do not integrate their genetic material into the host genome and therefore do not require host replication for gene expression, making them ideally

suited for rapid, efficient, heterologous gene expression. Techniques for preparing replication-defective infective viruses are well known in the art.

In certain further embodiments, the gene therapy vector will be HSV. A factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (*e.g.*, temporal, strength) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations. HSV also is relatively easy to manipulate and can be grown to high titers.

Of course, in using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

E. Anti-Aminophospholipid Antibodies and Conjugates

E1. Polyclonal Anti-Aminophospholipid Antibodies

Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference). To prepare polyclonal antisera an animal is immunized with an immunogenic aminophospholipid composition, and antisera collected from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, mouse, rat, hamster, guinea pig or goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the present aminophospholipid immunogen; subcutaneous, intramuscular, intradermal, intravenous, intraperitoneal and intrasplenic. The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired titer level is obtained, the immunized animal can be bled and the serum isolated and stored. The animal can also be used to generate monoclonal antibodies.

As is well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary adjuvants include complete Freund's adjuvant, a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*; incomplete Freund's adjuvant; and aluminum hydroxide adjuvant.

It may also be desired to boost the host immune system, as may be achieved by associating aminophospholipids with, or coupling aminophospholipids to, a carrier. Exemplary carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

As is also known in the art, a given composition may vary in its immunogenicity. However, the generation of antibodies against aminophospholipids is not particularly difficult. For example, highly specific anti-phosphatidylserine antibodies were raised in rabbits immunized by intramuscular injections of phosphatidylserine-containing polyacrylamide gels and with phosphatidylserine-cytochrome *c* vesicles (Maneta-Peyret *et al.*, 1988; 1989; each incorporated herein by reference). The use of acrylamide implants enhanced the production of antibodies

(Maneta-Peyret *et al.*, 1988; 1989). The anti-phosphatidylserine antibodies raised in this manner are able to detect phosphatidylserine *in situ* on human platelets (Maneta-Peyret *et al.*, 1988). The groups of Inoue, Rote and Rauch have also developed anti-PS and anti-PE antibodies (see below).

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E2. Monoclonal Anti-Aminophospholipid Antibodies

Various methods for generating monoclonal antibodies (MAbs) are also now very well known in the art. The most standard monoclonal antibody generation techniques generally begin along the same lines as those for preparing polyclonal 10 antibodies (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference). A polyclonal antibody response is initiated by immunizing an animal with an immunogenic aminophospholipid composition and, when a desired titer level is obtained, the immunized animal can be used to generate MAbs.

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MAbs may be readily prepared through use of well-known techniques, such as those exemplified in US. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with the selected aminophospholipid immunogen composition. The immunizing composition is 20 administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep and frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61; incorporated herein by reference), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher 25 percentage of stable fusions.

Following immunization, somatic cells with the potential for producing aminophospholipid antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied 30 spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of

antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 10^7 to 2 X 10^8 lymphocytes.

The anti-aminophospholipid antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984; each incorporated herein by reference). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F, 4B210 or one of the above listed mouse cell lines; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6, are all useful in connection with human cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 4:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976; each incorporated herein by reference), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by

Gefter *et al.* (1977; incorporated herein by reference). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986; incorporated herein by reference).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired anti-aminophospholipid reactivity. The assay should be sensitive, simple and rapid, such

as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual anti-aminophospholipid antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

MAbs produced by either means will generally be further purified, *e.g.*, using filtration, centrifugation and various chromatographic methods, such as HPLC or affinity chromatography, all of which purification techniques are well known to those of skill in the art. These purification techniques each involve fractionation to separate the desired antibody from other components of a mixture. Analytical methods particularly suited to the preparation of antibodies include, for example, protein A-Sepharose and/or protein G-Sepharose chromatography.

Umeda *et al.* (1989; incorporated herein by reference) reported the effective production of monoclonal antibodies recognizing stereo-specific epitopes of phosphatidylserine. The Umeda system is based on the direct immunization of phosphatidylserine into mouse spleen using a *Salmonella*-coated aminophospholipid sample (Umeda *et al.*, 1989; incorporated herein by reference). The Umeda protocol gives a high frequency of anti-PS MAbs, which exhibit three distinct reactivity profiles ranging from highly specific to broadly cross-reactive. Umeda is therefore also incorporated herein by reference for purposes of further describing screening

assays to identify MAbs that bind specifically to PS, *e.g.*, and do not bind to phosphatidylcholine.

Any of the 61 hybridomas generated by Umeda could potentially be employed in the therapeutic agent-targeting agent constructs of the present invention. Examples are PSC8, PSF11, PSG3, PSD11, PSF10, PS1B, PS3D12, PS2C11; PS3A, PSF6, PSF7, PSB4, PS3H1; PS4A7 and PS1G3. More preferred are PS3A, PSF6, PSF7, PSB4 and PS3H1 as they bind only to phosphatidylserine and phosphatidylethanolamine. Preferred anti-PS antibodies are PS4A7 (IgM) and PS1G3 (IgG_3), as they are highly specific for PS and exhibit no cross-reaction with other phospholipids. PS4A7 recognizes the stereo-specific configuration of the serine residue in PS (Figure 1 Umeda *et al.*, 1989; incorporated herein by reference).

Igarashi *et al.* (1991; incorporated herein by reference) also reported the effective induction of anti-PS antibodies of the IgG isotype by intrasplenic immunization. Only a slight increase of the titer was observed when the antigen was again injected intravenously. A high frequency of anti-PS MAbs of the IgG isotype was also observed even when MAbs were produced 10 days after the intrasplenic injection of the antigen. These antibodies were also employed by Schuurmans Stekhoven *et al.* (1994).

The other significant anti-PS antibody production has been by Rote and colleagues. Rote *et al.* (1993; incorporated herein by reference) particularly employed PS micelles in combination with Freund's complete adjuvant to generate specific anti-PS antibodies. Rote *et al.* (1993) also generated monoclonal antibodies that differentiate between cardiolipin (CL) and PS. Rote *et al.* (1993) is therefore also incorporated herein by reference for purposes of further describing screening assays to identify MAbs that bind specifically to PS by testing against resting and thrombin-activated platelets using flow cytometry.

The 3SB9b antibody produced by Rote *et al.* (1993) reacted with only with PS, and is a preferred antibody for use in the therapeutic agent-targeting agent constructs of the present invention. BA3B5C4 may also be used as it reacts with both PS and CL. These antibodies are also described in Lin *et al.* (1995), Obringer *et al.* (1995) and Katsuragawa *et al.* (1997).

E3. Anti-Aminophospholipid Antibodies from Phagemid Libraries

Recombinant technology now allows the preparation of antibodies having the desired specificity from recombinant genes encoding a range of antibodies (Van Dijk *et al.*, 1989; incorporated herein by reference). Certain recombinant techniques involve the isolation of the antibody genes by immunological screening of combinatorial immunoglobulin phage expression libraries prepared from RNA isolated from the spleen of an immunized animal (Morrison *et al.*, 1986; Winter and Milstein, 1991; each incorporated herein by reference).

For such methods, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination, which further increases the percentage of appropriate antibodies generated.

One method for the generation of a large repertoire of diverse antibody molecules in bacteria utilizes the bacteriophage lambda as the vector (Huse *et al.*, 1989; incorporated herein by reference). Production of antibodies using the lambda vector involves the cloning of heavy and light chain populations of DNA sequences into separate starting vectors. The vectors are subsequently combined randomly to form a single vector that directs the co-expression of heavy and light chains to form antibody fragments. The heavy and light chain DNA sequences are obtained by

amplification, preferably by PCR™ or a related amplification technique, of mRNA isolated from spleen cells (or hybridomas thereof) from an animal that has been immunized with a selected antigen. The heavy and light chain sequences are typically amplified using primers that incorporate restriction sites into the ends of the amplified DNA segment to facilitate cloning of the heavy and light chain segments into the starting vectors.

Another method for the generation and screening of large libraries of wholly or partially synthetic antibody combining sites, or paratopes, utilizes display vectors derived from filamentous phage such as M13, fl or fd. These filamentous phage display vectors, referred to as "phagemids", yield large libraries of monoclonal antibodies having diverse and novel immunospecificities. The technology uses a filamentous phage coat protein membrane anchor domain as a means for linking gene-product and gene during the assembly stage of filamentous phage replication, and has been used for the cloning and expression of antibodies from combinatorial libraries (Kang *et al.*, 1991; Barbas *et al.*, 1991; each incorporated herein by reference).

This general technique for filamentous phage display is described in U.S. Patent 5,658,727, incorporated herein by reference. In a most general sense, the method provides a system for the simultaneous cloning and screening of pre-selected ligand-binding specificities from antibody gene repertoires using a single vector system. Screening of isolated members of the library for a pre-selected ligand-binding capacity allows the correlation of the binding capacity of an expressed antibody molecule with a convenient means to isolate the gene that encodes the member from the library.

Linkage of expression and screening is accomplished by the combination of targeting of a fusion polypeptide into the periplasm of a bacterial cell to allow assembly of a functional antibody, and the targeting of a fusion polypeptide onto the coat of a filamentous phage particle during phage assembly to allow for convenient screening of the library member of interest. Periplasmic targeting is provided by the

presence of a secretion signal domain in a fusion polypeptide. Targeting to a phage particle is provided by the presence of a filamentous phage coat protein membrane anchor domain (*i.e.*, a cpIII- or cpVIII-derived membrane anchor domain) in a fusion polypeptide.

The diversity of a filamentous phage-based combinatorial antibody library can be increased by shuffling of the heavy and light chain genes, by altering one or more of the complementarity determining regions of the cloned heavy chain genes of the library, or by introducing random mutations into the library by error-prone polymerase chain reactions. Additional methods for screening phagemid libraries are described in U.S. Patent No. 5,580,717; 5,427,908; 5,403,484; and 5,223,409, each incorporated herein by reference.

Another method for the screening of large combinatorial antibody libraries has been developed, utilizing expression of populations of diverse heavy and light chain sequences on the surface of a filamentous bacteriophage, such as M13, fl or fd (U.S. Patent No. 5,698,426; incorporated herein by reference). Two populations of diverse heavy (Hc) and light (Lc) chain sequences are synthesized by polymerase chain reaction (PCR™). These populations are cloned into separate M13-based vector containing elements necessary for expression. The heavy chain vector contains a gene VIII (gVIII) coat protein sequence so that translation of the heavy chain sequences produces gVIII-Hc fusion proteins. The populations of two vectors are randomly combined such that only the vector portions containing the Hc and Lc sequences are joined into a single circular vector.

The combined vector directs the co-expression of both Hc and Lc sequences for assembly of the two polypeptides and surface expression on M13 (U.S. Patent No. 5,698,426; incorporated herein by reference). The combining step randomly brings together different Hc and Lc encoding sequences within two diverse populations into a single vector. The vector sequences donated from each independent vector are necessary for production of viable phage. Also, since the pseudo gVIII sequences are

contained in only one of the two starting vectors, co-expression of functional antibody fragments as Lc associated gVIII-Hc fusion proteins cannot be accomplished on the phage surface until the vector sequences are linked in the single vector.

Surface expression of the antibody library is performed in an amber suppressor strain. An amber stop codon between the Hc sequence and the gVIII sequence unlinks the two components in a non-suppressor strain. Isolating the phage produced from the non-suppressor strain and infecting a suppressor strain will link the Hc sequences to the gVIII sequence during expression. Culturing the suppressor strain after infection allows the coexpression on the surface of M13 of all antibody species within the library as gVIII fusion proteins (gVIII-Fab fusion proteins). Alternatively, the DNA can be isolated from the non-suppressor strain and then introduced into a suppressor strain to accomplish the same effect.

The surface expression library is screened for specific Fab fragments that bind preselected molecules by standard affinity isolation procedures. Such methods include, for example, panning (Parmley and Smith, 1988; incorporated herein by reference), affinity chromatography and solid phase blotting procedures. Panning is preferred, because high titers of phage can be screened easily, quickly and in small volumes. Furthermore, this procedure can select minor Fab fragments species within the population, which otherwise would have been undetectable, and amplified to substantially homogenous populations. The selected Fab fragments can be characterized by sequencing the nucleic acids encoding the polypeptides after amplification of the phage population.

Another method for producing diverse libraries of antibodies and screening for desirable binding specificities is described in U.S. Patent No. 5,667,988 and U.S. Patent No. 5,759,817, each incorporated herein by reference. The method involves the preparation of libraries of heterodimeric immunoglobulin molecules in the form of phagemid libraries using degenerate oligonucleotides and primer extension reactions to incorporate the degeneracies into the CDR regions of the immunoglobulin variable

heavy and light chain variable domains, and display of the mutagenized polypeptides on the surface of the phagemid. Thereafter, the display protein is screened for the ability to bind to a preselected antigen.

The method for producing a heterodimeric immunoglobulin molecule generally involves (1) introducing a heavy or light chain V region-coding gene of interest into the phagemid display vector; (2) introducing a randomized binding site into the phagemid display protein vector by primer extension with an oligonucleotide containing regions of homology to a CDR of the antibody V region gene and containing regions of degeneracy for producing randomized coding sequences to form a large population of display vectors each capable of expressing different putative binding sites displayed on a phagemid surface display protein; (3) expressing the display protein and binding site on the surface of a filamentous phage particle; and (4) isolating (screening) the surface-expressed phage particle using affinity techniques such as panning of phage particles against a preselected antigen, thereby isolating one or more species of phagemid containing a display protein containing a binding site that binds a preselected antigen.

A further variation of this method for producing diverse libraries of antibodies and screening for desirable binding specificities is described in U.S. Patent No. 5,702,892, incorporated herein by reference. In this method, only heavy chain sequences are employed, the heavy chain sequences are randomized at all nucleotide positions which encode either the CDRI or CDRIII hypervariable region, and the genetic variability in the CDRs is generated independent of any biological process.

In the method, two libraries are engineered to genetically shuffle oligonucleotide motifs within the framework of the heavy chain gene structure. Through random mutation of either CDRI or CDRIII, the hypervariable regions of the heavy chain gene were reconstructed to result in a collection of highly diverse sequences. The heavy chain proteins encoded by the collection of mutated gene

sequences possessed the potential to have all of the binding characteristics of an immunoglobulin while requiring only one of the two immunoglobulin chains.

Specifically, the method is practiced in the absence of the immunoglobulin light chain protein. A library of phage displaying modified heavy chain proteins is incubated with an immobilized ligand to select clones encoding recombinant proteins that specifically bind the immobilized ligand. The bound phage are then dissociated from the immobilized ligand and amplified by growth in bacterial host cells. Individual viral plaques, each expressing a different recombinant protein, are expanded, and individual clones can then be assayed for binding activity.

E4. Anti-Aminophospholipid Antibodies from Human Patients

Antibodies against aminophospholipids, particularly phosphatidylserine and phosphatidylethanolamine, occur in the human population, where they are correlated with certain disease states. Anti-aminophospholipid antibodies are part of the heterogeneous anti-phospholipid antibodies (aPL), observed to have families of different specificities and classes. Primary anti-phospholipid syndrome (APS) has even been separated from other forms of autoimmune disease associated with anti-phospholipid antibody production.

Anti-PS antibodies are particularly associated with recurrent pregnancy loss (Rote *et al.*, 1995; Rote, 1996; Vogt *et al.*, 1996; Vogt *et al.*, 1997; incorporated herein by reference) and with the autoimmune disease, systemic lupus erythematosus (SLE or "lupus") (Branch *et al.*, 1987; incorporated herein by reference). Anti-PE antibodies have also been reported in human patients, particularly those with autoimmune diseases (Staub *et al.*, 1989). Branch *et al.* (1987) reported that 80% of patients with lupus anticoagulant (LA or LAC) had autoantibodies that recognized PE; with Drouvalakis and Buchanan (1998) increasing this number to 95% PE-positives from autoimmune LAC sera.

Anti-phospholipid antibodies are not to be confused with anti-endothelial cell antibodies (AECA), although they can be found in the same patient. The existence of AECA has been documented in a variety of clinical settings associated with vasculitis, such as systemic sclerosis (SS). To study AECA, antibodies are obtained from patients that do not have anti-phospholipid antibodies (aPL-negative sera).

The pathogenic role of AECA remains unclear, although Bordron *et al.* (1998) very recently suggested that AECA may initiate apoptosis in endothelial cells, which would be followed by PS transfer to the outer face of the membrane. They proposed that this would account for the subsequent generation of the anti-phospholipid antibodies that are sometimes seen in conjunction with AECA in patients with skin lesions or connective tissue disease (Bordron *et al.*, 1998). However, although AECA binding to an apoptosis-inducing antigen was postulated, these studies did not lead to the further characterization of AECA, still said to represent an extremely heterogeneous family of antibodies reacting with different (non-lipid) structures on endothelial cells (Bordron *et al.*, 1998).

Anti-phosphatidylserine antibodies are closely associated with pregnancy loss, pregnancy-induced hypertension and intrauterine growth retardation. A phosphatidylserine-dependent antigen has been shown to be expressed on the surface of a choriocarcinoma model (BeWo) of differentiating cytotrophoblastic cells, indicating that it should be accessible *in vivo* to circulating anti-phosphatidylserine antibodies (Rote *et al.*, 1995). Indeed, Vogt *et al.* (1996) showed that the monoclonal antibody 3SB9b, which reacts with phosphatidylserine but not cardiolipin, induced a significant reduction in both fetal and placental weights in a mouse model for the anti-phospholipid antibody syndrome

These authors developed a model for explaining miscarriages associated with anti-phospholipid antibodies: anti-phosphatidylserine antibody reveals sites for prothrombin binding on the surface of the trophoblast, most likely by removing Annexin V (Vogt *et al.*, 1997). Trophoblast differentiation is associated with

externalization of phosphatidylserine from the inner to the outer surface of the plasma membrane. Normally, externalization of phosphatidylserine is concurrent with binding of Annexin V, which prevents the phosphatidylserine-rich surface from acting as a site for activation of coagulation. Thus, when anti-phospholipid antibodies are present, they prevent Annexin V binding and lead to a procoagulant state (Vogt *et al.*, 1997).

Anti-PE antibodies are frequently associated with lupus anticoagulants (LAC sera). The role of PE and anti-PE in LAC is extremely complex, see, *e.g.*, Smirnov *et al.* (1995; incorporated herein by reference), where various hypotheses are set forth. Smirnov *et al.* (1995) report that, in the presence of activated protein C and PE, LAC plasma clots faster than normal plasma. Rauch *et al.* (1986) characterize LAC anti-phospholipid antibodies as prolonging the clotting time in *in vitro* coagulation assays.

Vlachoyiannopoulos *et al.* (1993; incorporated herein by reference) tested SLE and APS sera by ELISA for antibodies to phosphatidylethanolamine and cardiolipin, as compared to healthy blood donors. Both SLE and APS patients were reported to present a higher titer of IgM anti-PE antibodies than normal subjects, while the IgG and IgA anti-PE reactivity reportedly did not differ. It was suggested that IgA and IgG anti-PE antibodies may occur in low titers as natural autoantibodies in normal subjects (Vlachoyiannopoulos *et al.*, 1993; incorporated herein by reference).

Rauch *et al.* (1986; incorporated herein by reference) produced hybridomas by fusing lymphocytes from 13 systemic lupus erythematosus patients with a lymphoblastoid line. They demonstrated that the autoantibodies that prolonged clotting time bound to hexagonal phase phospholipids, including natural and synthetic forms of phosphatidylethanolamine (Rauch *et al.*, 1986; incorporated herein by reference). In contrast, lamellar phospholipids, such as phosphatidylcholine and synthetic lamellar forms of phosphatidylethanolamine, had no effect on the anticoagulant activity (Rauch *et al.*, 1986).

Rauch and Janoff (1990; incorporated herein by reference) went on to show that immunization of mice with phosphatidylethanolamine in the hexagonal II phase, but not in the bilayer phase, resulted in the induction of anti-phospholipid antibodies. These antibodies were strongly reactive with phosphatidylethanolamine and had functional lupus anticoagulant activity characteristic of autoantibodies from patients with autoimmune disease (Rauch and Janoff, 1990).

The hexagonal II phase form of aminophospholipids should thus be advantageously used to generate antibodies for use in the present invention. Indeed, Trudell reported that antibodies raised against TFA- (trifluoroacetyl-) protein adducts bind to TFA-phosphatidylethanolamine in hexagonal phase phospholipid micelles, but not in lamellar liposomes (Trudell *et al.*, 1991a; incorporated herein by reference). The authors suggested that TFA-phosphatidylethanolamine adducts that reside in non-lamellar domains on the hepatocyte surface could be recognition sites for anti-TFA-adduct antibodies and potentially participate in immune-mediated halothane hepatotoxicity (Trudell *et al.*, 1991a). It was later shown that these same antibodies cross-react with TFA-dioleoylphosphatidylethanolamine when this adduct is incorporated into the surface of hepatocytes (Trudell *et al.*, 1991b; incorporated herein by reference), thus supporting this hypothesis.

Berard further explained the hexagonal II phase form of aminophospholipids, such as PE (Berard *et al.*, 1993; incorporated herein by reference). In bilayers, phospholipids generally adopt a gel structure, crystalline lattice or lamellar phase (Berard *et al.*, 1993). However, depending on the cholesterol content, protein and ionic environments, phospholipids can easily change phases, adopting a hexagonal II phase (Berard *et al.*, 1993; incorporated herein by reference). It is this hexagonal II phase of aminophospholipids that is believed to be immunogenic, as initial proposed for autoantibody generation in disease situations (Berard *et al.*, 1993; incorporated herein by reference).

Qamar *et al.* (1990; incorporated herein by reference) have developed a variation on the hexagonal aminophospholipid recognition theme. Using phosphatidylethanolamine as a model, these authors reported that anti-PE antibodies from aPL-positive SLE sera do not bind to PE, but in fact are directed to lysophosphatidylethanolamine (lPE), a natural PE degradation product and a likely contaminant of most PE preparations (Qamar *et al.*, 1990; incorporated herein by reference).

Other recent data indicate that most anti-phospholipid antibodies recognize phospholipid in the context of nearby proteins (Rote, 1996; Chamley *et al.*, 1991). In plasma membranes, the majority of the phospholipid appears to be naturally in non-antigenic bilaminar form (Rote, 1996). Accessory molecules may help facilitate the transition to hexagonal antigenic forms and stabilize their expression (Galli *et al.*, 1993). For example, naturally occurring anti-phospholipid antibodies were first reported to recognize complexes of cardiolipin or phosphatidylserine with β_2-glycoprotein I (β_2-GPI or apolipoprotein H, apoH) (Galli *et al.*, 1990; 1993). β_2-GPI is believed to stabilize phospholipids in antigenic conformations that do not exist in pure phospholipids (McNeil *et al.*, 1990; U.S. Patent No. 5,344,758; Chamley *et al.*, 1991; Matsuura *et al.*, 1994). Prothrombin has also been implicated in the phospholipid stabilization process (Bevers *et al.*, 1991).

Phospholipid-binding plasma proteins are also generally necessary for antibody recognition of the electrically neutral or zwitterionic phospholipid, phosphatidylethanolamine. Sugi and McIntyre (1995; incorporated herein by reference) identified two prominent PE-binding plasma proteins as high molecular weight kininogen (HMWK or HK) and low molecular weight kininogen (LMWK or LK). Anti-PE antibodies from patients with SLE and/or recurrent spontaneous abortions were shown not to recognize PE, HMWK or LMWK when they were presented independently as sole antigens on ELISA plates (Sugi and McIntyre, 1995). Other anti-PE-positive sera that did not react with PE-HMWK or PE-LMWK were

suggested to recognize factor XI or prekallikrein, which normally bind to HMWK (Sugi and McIntyre, 1995; incorporated herein by reference).

The validity of these results was confirmed by showing that intact HMWK binds to various phospholipids, such as cardiolipin, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine; but that anti-PE antibodies recognize only a kininogen-PE complex, and do not recognize kininogens presented with other phospholipid substrates (Sugi and McIntyre, 1996a; incorporated herein by reference). This indicates that PE induces unique antigenic conformational changes in the kininogens that are not induced when the kininogens bind to other phospholipids (Sugi and McIntyre, 1996a).

It has further been suggested that kininogens can bind to platelets by virtue of exposed PE in the platelet membrane (Sugi and McIntyre, 1996b; incorporated herein by reference). Exogenously added kininogen-dependent anti-PE was shown to increase thrombin-induced platelet aggregation *in vitro*, but not to alter ADP-induced aggregation (Sugi and McIntyre, 1996b; incorporated herein by reference). In contrast, kininogen independent anti-PE, which recognized PE *per se*, was reported not augment thrombin-induced platelet aggregation. It was thus proposed that kininogen dependent anti-PE may disrupt the normal anti-thrombotic effects of kininogen (Sugi and McIntyre, 1996b; incorporated herein by reference).

Anti-aminophospholipid antibodies from human patients are therefore a mixture of antibodies that generally recognize aminophospholipids stabilized by protein interactions (Rote, 1996). The antibodies may bind to stabilized phospholipid epitopes, or may bind to an epitope formed from the interaction of the phospholipid and amino acids on the stabilizing protein (Rote, 1996). Either way, such antibodies clearly recognize aminophospholipids in natural membranes in the human body, probably associated with plasma proteins (McNeil *et al.*, 1990; Bevers *et al.*, 1991). These antibodies would thus be appropriate as starting materials for generating an

antibody for use in the therapeutic agent-targeting agent constructs of the present invention.

To prepare an anti-aminophospholipid antibody from a human patient, one would simply obtain human lymphocytes from an individual having anti-aminophospholipid antibodies, for example from human peripheral blood, spleen, lymph nodes, tonsils or the like, utilizing techniques that are well known to those of skill in the art. The use of peripheral blood lymphocytes will often be preferred.

Human monoclonal antibodies may be obtained from the human lymphocytes producing the desired anti-aminophospholipid antibodies by immortalizing the human lymphocytes, generally in the same manner as described above for generating any monoclonal antibody. The reactivities of the antibodies in the culture supernatants are generally first checked, employing one or more selected aminophospholipid antigen(s), and the lymphocytes that exhibit high reactivity are grown. The resulting lymphocytes are then fused with a parent line of human or mouse origin, and further selection gives the optimal clones.

The recovery of monoclonal antibodies from the immortalized cells may be achieved by any method generally employed in the production of monoclonal antibodies. For instance, the desired monoclonal antibody may be obtained by cloning the immortalized lymphocyte by the limiting dilution method or the like, selecting the cell producing the desired antibody, growing the selected cells in a medium or the abdominal cavity of an animal, and recovering the desired monoclonal antibody from the culture supernatant or ascites.

Such techniques have been used, for example, to isolate human monoclonal antibodies to *Pseudomonas aeruginosa* epitopes (U.S. Patent No. 5,196,337 and 5,252,480, each incorporated herein by reference); polyribosylribitol phosphate capsular polysaccharides (U.S. Patent No. 4,954,449, incorporated herein by reference); the Rh(D) antigen (U.S. Patent No. 5,665,356, incorporated herein by

reference); and viruses, such as human immunodeficiency virus, respiratory syncytial virus, herpes simplex virus, varicella zoster virus and cytomegalovirus (U.S. Patent No. 5,652,138; 5,762,905; and 4,950,595, each incorporated herein by reference).

The applicability of the foregoing techniques to the generation of human anti-aminophospholipid antibodies is clear. Rauch *et al.* (1986; incorporated herein by reference) generally used such methods to produce hybridomas by fusing lymphocytes from SLE patients with a lymphoblastoid line. This produced human antibodies that bound to hexagonal phase phospholipids, including natural and synthetic forms of phosphatidylethanolamine (Rauch *et al.*, 1986; incorporated herein by reference).

Additionally, the methods described in U.S. Patent 5,648,077 (incorporated herein by reference) can be used to form a trioma or a quadroma that produces a human antibody against a selected aminophospholipid. In a general sense, a hybridoma cell line comprising a parent rodent immortalizing cell, such as a murine myeloma cell, *e.g.* SP-2, is fused to a human partner cell, resulting in an immortalizing xenogeneic hybridoma cell. This xenogeneic hybridoma cell is fused to a cell capable of producing an anti-aminophospholipid human antibody, resulting in a trioma cell line capable of generating human antibody effective against such antigen in a human. Alternately, when greater stability is desired, a trioma cell line which preferably no longer has the capability of producing its own antibody is made, and this trioma is then fused with a further cell capable of producing an antibody useful against the aminophospholipid antigen to obtain a still more stable hybridoma (quadroma) that produces antibody against the antigen.

E5. Anti-Aminophospholipid Antibodies from Human Lymphocytes

In vitro immunization, or antigen stimulation, may also be used to generate a human anti-aminophospholipid antibody. Such techniques can be used to stimulate peripheral blood lymphocytes from both anti-aminophospholipid antibody-producing human patients, and also from normal, healthy subjects. Indeed, Vlachoyiannopoulos *et al.* (1993; incorporated herein by reference) reported that low titer anti-

aminophospholipid antibodies occur in normal subjects. Even if this were not the case, anti-aminophospholipid antibodies can be prepared from healthy human subjects, simply by stimulating antibody-producing cells with aminophospholipids *in vitro*.

Such "*in vitro* immunization" involves antigen-specific activation of non-immunized B lymphocytes, generally within a mixed population of lymphocytes (mixed lymphocyte cultures, MLC). *In vitro* immunizations may also be supported by B cell growth and differentiation factors and lymphokines. The antibodies produced by these methods are often IgM antibodies (Borrebaeck *et al.*, 1986; incorporated herein by reference).

Another method has been described (U.S. Patent No. 5,681,729, incorporated herein by reference) wherein human lymphocytes that mainly produce IgG (or IgA) antibodies can be obtained. The method involves, in a general sense, transplanting human lymphocytes to an immunodeficient animal so that the human lymphocytes "take" in the animal body; immunizing the animal with a desired antigen, so as to generate human lymphocytes producing an antibody specific to the antigen; and recovering the human lymphocytes producing the antibody from the animal. The human lymphocytes thus produced can be used to produce a monoclonal antibody by immortalizing the human lymphocytes producing the antibody, cloning the obtained immortalized human-originated lymphocytes producing the antibody, and recovering a monoclonal antibody specific to the desired antigen from the cloned immortalized human-originated lymphocytes.

The immunodeficient animals that may be employed in this technique are those that do not exhibit rejection when human lymphocytes are transplanted to the animals. Such animals may be artificially prepared by physical, chemical or biological treatments. Any immunodeficient animal may be employed. The human lymphocytes may be obtained from human peripheral blood, spleen, lymph nodes, tonsils or the like.

The "taking" of the transplanted human lymphocytes in the animals can be attained by merely administering the human lymphocytes to the animals. The administration route is not restricted and may be, for example, subcutaneous, intravenous or intraperitoneal. The dose of the human lymphocytes is not restricted, and can usually be 10^6 to 10^8 lymphocytes per animal. The immunodeficient animal is then immunized with the desired aminophospholipid antigen.

After the immunization, human lymphocytes are recovered from the blood, spleen, lymph nodes or other lymphatic tissues by any conventional method. For example, mononuclear cells can be separated by the Ficoll-Hypaque (specific gravity: 1.077) centrifugation method, and the monocytes removed by the plastic dish adsorption method. The contaminating cells originating from the immunodeficient animal may be removed by using an antiserum specific to the animal cells. The antiserum may be obtained by, for example, immunizing a second, distinct animal with the spleen cells of the immunodeficient animal, and recovering serum from the distinct immunized animal. The treatment with the antiserum may be carried out at any stage. The human lymphocytes may also be recovered by an immunological method employing a human immunoglobulin expressed on the cell surface as a marker.

By these methods, human lymphocytes mainly producing IgG and IgA antibodies specific to one or more selected aminophospholipid(s) can be obtained. Monoclonal antibodies are then obtained from the human lymphocytes by immortalization, selection, cell growth and antibody production.

E6. Transgenic Mice Containing Human Antibody Libraries

Recombinant technology is now available for the preparation of antibodies. In addition to the combinatorial immunoglobulin phage expression libraries disclosed above, another molecular cloning approach is to prepare antibodies from transgenic

mice containing human antibody libraries. Such techniques are described in U.S. Patent No. 5,545,807, incorporated herein by reference.

In a most general sense, these methods involve the production of a transgenic animal that has inserted into its germline genetic material that encodes for at least part of an immunoglobulin of human origin or that can rearrange to encode a repertoire of immunoglobulins. The inserted genetic material may be produced from a human source, or may be produced synthetically. The material may code for at least part of a known immunoglobulin or may be modified to code for at least part of an altered immunoglobulin.

The inserted genetic material is expressed in the transgenic animal, resulting in production of an immunoglobulin derived at least in part from the inserted human immunoglobulin genetic material. It is found the genetic material is rearranged in the transgenic animal, so that a repertoire of immunoglobulins with part or parts derived from inserted genetic material may be produced, even if the inserted genetic material is incorporated in the germline in the wrong position or with the wrong geometry.

The inserted genetic material may be in the form of DNA cloned into prokaryotic vectors such as plasmids and/or cosmids. Larger DNA fragments are inserted using yeast artificial chromosome vectors (Burke *et al.*, 1987; incorporated herein by reference), or by introduction of chromosome fragments (Richer and Lo, 1989; incorporated herein by reference). The inserted genetic material may be introduced to the host in conventional manner, for example by injection or other procedures into fertilized eggs or embryonic stem cells.

In preferred aspects, a host animal that initially does not carry genetic material encoding immunoglobulin constant regions is utilized, so that the resulting transgenic animal will use only the inserted human genetic material when producing immunoglobulins. This can be achieved either by using a naturally occurring mutant host lacking the relevant genetic material, or by artificially making mutants *e.g.*, in

cell lines ultimately to create a host from which the relevant genetic material has been removed.

Where the host animal carries genetic material encoding immunoglobulin constant regions, the transgenic animal will carry the naturally occurring genetic material and the inserted genetic material and will produce immunoglobulins derived from the naturally occurring genetic material, the inserted genetic material, and mixtures of both types of genetic material. In this case the desired immunoglobulin can be obtained by screening hybridomas derived from the transgenic animal, *e.g.*, by exploiting the phenomenon of allelic exclusion of antibody gene expression or differential chromosome loss.

Once a suitable transgenic animal has been prepared, the animal is simply immunized with the desired immunogen. Depending on the nature of the inserted material, the animal may produce a chimeric immunoglobulin, *e.g.* of mixed mouse/human origin, where the genetic material of foreign origin encodes only part of the immunoglobulin; or the animal may produce an entirely foreign immunoglobulin, *e.g.* of wholly human origin, where the genetic material of foreign origin encodes an entire immunoglobulin.

Polyclonal antisera may be produced from the transgenic animal following immunization. Immunoglobulin-producing cells may be removed from the animal to produce the immunoglobulin of interest. Preferably, monoclonal antibodies are produced from the transgenic animal, *e.g.*, by fusing spleen cells from the animal with myeloma cells and screening the resulting hybridomas to select those producing the desired antibody. Suitable techniques for such processes are described herein.

In an alternative approach, the genetic material may be incorporated in the animal in such a way that the desired antibody is produced in body fluids such as serum or external secretions of the animal, such as milk, colostrum or saliva. For example, by inserting *in vitro* genetic material encoding for at least part of a human

immunoglobulin into a gene of a mammal coding for a milk protein and then introducing the gene to a fertilized egg of the mammal, *e.g.*, by injection, the egg may develop into an adult female mammal producing milk containing immunoglobulin derived at least in part from the inserted human immunoglobulin genetic material. The desired antibody can then be harvested from the milk. Suitable techniques for carrying out such processes are known to those skilled in the art.

The foregoing transgenic animals are usually employed to produce human antibodies of a single isotype, more specifically an isotype that is essential for B cell maturation, such as IgM and possibly IgD. Another preferred method for producing human anti-aminophospholipid antibodies is to use the technology described in U.S. Patent No. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and 5,770,429; each incorporated by reference, wherein transgenic animals are described that are capable of switching from an isotype needed for B cell development to other isotypes.

In the development of a B lymphocyte, the cell initially produces IgM with a binding specificity determined by the productively rearranged V_H and V_L regions. Subsequently, each B cell and its progeny cells synthesize antibodies with the same L and H chain V regions, but they may switch the isotype of the H chain. The use of mu or delta constant regions is largely determined by alternate splicing, permitting IgM and IgD to be coexpressed in a single cell. The other heavy chain isotypes (gamma, alpha, and epsilon) are only expressed natively after a gene rearrangement event deletes the C mu and C delta exons. This gene rearrangement process, termed isotype switching, typically occurs by recombination between so called switch segments located immediately upstream of each heavy chain gene (except delta). The individual switch segments are between 2 and 10 kb in length, and consist primarily of short repeated sequences.

For these reasons, it is preferable that transgenes incorporate transcriptional regulatory sequences within about 1-2 kb upstream of each switch region that is to be utilized for isotype switching. These transcriptional regulatory sequences preferably

include a promoter and an enhancer element, and more preferably include the 5' flanking (*i.e.*, upstream) region that is naturally associated (*i.e.*, occurs in germline configuration) with a switch region. Although a 5' flanking sequence from one switch region can be operably linked to a different switch region for transgene construction, in some embodiments it is preferred that each switch region incorporated in the transgene construct have the 5' flanking region that occurs immediately upstream in the naturally occurring germline configuration. Sequence information relating to immunoglobulin switch region sequences is known (Mills *et al.*, 1990; Sideras *et al.*, 1989; each incorporated herein by reference).

In the method described in U.S. Patent No. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and 5,770,429, the human immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B-cell development, leading to isotype switching. Accordingly, in this method, these transgenes are constructed so as to produce isotype switching and one or more of the following: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

An important requirement for transgene function is the generation of a primary antibody repertoire that is diverse enough to trigger a secondary immune response for a wide range of antigens. The rearranged heavy chain gene consists of a signal peptide exon, a variable region exon and a tandem array of multi-domain constant region regions, each of which is encoded by several exons. Each of the constant region genes encode the constant portion of a different class of immunoglobulins. During B-cell development, V region proximal constant regions are deleted leading to the expression of new heavy chain classes. For each heavy chain class, alternative patterns of RNA splicing give rise to both transmembrane and secreted immunoglobulins.

The human heavy chain locus consists of approximately 200 V gene segments spanning 2 Mb, approximately 30 D gene segments spanning about 40 kb, six J segments clustered within a 3 kb span, and nine constant region gene segments spread out over approximately 300 kb. The entire locus spans approximately 2.5 Mb of the distal portion of the long arm of chromosome 14. Heavy chain transgene fragments containing members of all six of the known V_H families, the D and J gene segments, as well as the mu, delta, gamma 3, gamma 1 and alpha 1 constant regions are known (Berman *et al.*, 1988; incorporated herein by reference). Genomic fragments containing all of the necessary gene segments and regulatory sequences from a human light chain locus is similarly constructed.

The expression of successfully rearranged immunoglobulin heavy and light transgenes usually has a dominant effect by suppressing the rearrangement of the endogenous immunoglobulin genes in the transgenic nonhuman animal. However, in certain embodiments, it is desirable to effect complete inactivation of the endogenous Ig loci so that hybrid immunoglobulin chains comprising a human variable region and a non-human (*e.g.*, murine) constant region cannot be formed, for example by trans-switching between the transgene and endogenous Ig sequences. Using embryonic stem cell technology and homologous recombination, the endogenous immunoglobulin repertoire can be readily eliminated. In addition, suppression of endogenous Ig genes may be accomplished using a variety of techniques, such as antisense technology.

In other aspects of the invention, it may be desirable to produce a trans-switched immunoglobulin. Antibodies comprising such chimeric trans-switched immunoglobulins can be used for a variety of applications where it is desirable to have a non-human (*e.g.*, murine) constant region, *e.g.*, for retention of effector functions in the host. The presence of a murine constant region can afford advantages over a human constant region, for example, to provide murine effector functions (*e.g.*, ADCC, murine complement fixation) so that such a chimeric antibody may be tested in a mouse disease model. Subsequent to the animal testing, the human variable

region encoding sequence may be isolated, *e.g.*, by PCR amplification or cDNA cloning from the source (hybridoma clone), and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic use.

E7. Humanized Anti-Aminophospholipid Antibodies

Human antibodies generally have at least three potential advantages for use in human therapy. First, because the effector portion is human, it may interact better with the other parts of the human immune system, *e.g.*, to destroy target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). Second, the human immune system should not recognize the antibody as foreign. Third, the half-life in the human circulation will be similar to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

Various methods for preparing human anti-aminophospholipids are provided herein. In addition to human antibodies, "humanized" antibodies have many advantages. "Humanized" antibodies are generally chimeric or mutant monoclonal antibodies from mouse, rat, hamster, rabbit or other species, bearing human constant and/or variable region domains or specific changes. Techniques for generating a so-called "humanized" anti-aminophospholipid antibody are well known to those of skill in the art.

Humanized antibodies also share the foregoing advantages. First, the effector portion is still human. Second, the human immune system should not recognize the framework or constant region as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody. Third, injected humanized antibodies, as opposed to injected mouse antibodies, will presumably have a half-life more similar to naturally occurring human antibodies, also allowing smaller and less frequent doses.

A number of methods have been described to produce humanized antibodies. Controlled rearrangement of antibody domains joined through protein disulfide bonds to form new, artificial protein molecules or "chimeric" antibodies can be utilized (Konieczny *et al.*, 1981; incorporated herein by reference). Recombinant DNA technology can also be used to construct gene fusions between DNA sequences encoding mouse antibody variable light and heavy chain domains and human antibody light and heavy chain constant domains (Morrison *et al.*, 1984; incorporated herein by reference).

DNA sequences encoding the antigen binding portions or complementarity determining regions (CDR's) of murine monoclonal antibodies can be grafted by molecular means into the DNA sequences encoding the frameworks of human antibody heavy and light chains (Jones *et al.*, 1986; Riechmann *et al.*, 1988; each incorporated herein by reference). The expressed recombinant products are called "reshaped" or humanized antibodies, and comprise the framework of a human antibody light or heavy chain and the antigen recognition portions, CDR's, of a murine monoclonal antibody.

Another method for producing humanized antibodies is described in U.S. Patent No. 5,639,641, incorporated herein by reference. The method provides, via resurfacing, humanized rodent antibodies that have improved therapeutic efficacy due to the presentation of a human surface in the variable region. In the method: (1) position alignments of a pool of antibody heavy and light chain variable regions is generated to give a set of heavy and light chain variable region framework surface exposed positions, wherein the alignment positions for all variable regions are at least about 98% identical; (2) a set of heavy and light chain variable region framework surface exposed amino acid residues is defined for a rodent antibody (or fragment thereof); (3) a set of heavy and light chain variable region framework surface exposed amino acid residues that is most closely identical to the set of rodent surface exposed amino acid residues is identified; (4) the set of heavy and light chain variable region framework surface exposed amino acid residues defined in step (2) is substituted with

the set of heavy and light chain variable region framework surface exposed amino acid residues identified in step (3), except for those amino acid residues that are within 5Å of any atom of any residue of the complementarity determining regions of the rodent antibody; and (5) the humanized rodent antibody having binding specificity is produced.

A similar method for the production of humanized antibodies is described in U.S. Patent No. 5,693,762; 5,693,761; 5,585,089; and 5,530,101, each incorporated herein by reference. These methods involve producing humanized immunoglobulins having one or more complementarity determining regions (CDR's) and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. Each humanized immunoglobulin chain usually comprises, in addition to the CDR's, amino acids from the donor immunoglobulin framework that are capable of interacting with the CDR's to effect binding affinity, such as one or more amino acids that are immediately adjacent to a CDR in the donor immunoglobulin or those within about 3Å as predicted by molecular modeling. The heavy and light chains may each be designed by using any one, any combination, or all of the various position criteria described in U.S. Patent No. 5,693,762; 5,693,761; 5,585,089; and 5,530,101, each incorporated herein by reference. When combined into an intact antibody, the humanized immunoglobulins are substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the original antigen.

An additional method for producing humanized antibodies is described in U.S. Patent 5,565,332 and 5,733,743, each incorporated herein by reference. This method combines the concept of humanizing antibodies with the phagemid libraries also described in detail herein. In a general sense, the method utilizes sequences from the antigen binding site of an antibody or population of antibodies directed against an antigen of interest. Thus for a single rodent antibody, sequences comprising part of the antigen binding site of the antibody may be combined with diverse repertoires of

sequences of human antibodies that can, in combination, create a complete antigen binding site.

The antigen binding sites created by this process differ from those created by CDR grafting, in that only the portion of sequence of the original rodent antibody is likely to make contacts with antigen in a similar manner. The selected human sequences are likely to differ in sequence and make alternative contacts with the antigen from those of the original binding site. However, the constraints imposed by binding of the portion of original sequence to antigen and the shapes of the antigen and its antigen binding sites, are likely to drive the new contacts of the human sequences to the same region or epitope of the antigen. This process has therefore been termed "epitope imprinted selection" (EIS).

Starting with an animal antibody, one process results in the selection of antibodies that are partly human antibodies. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or after alteration of a few key residues. Sequence differences between the rodent component of the selected antibody with human sequences could be minimized by replacing those residues that differ with the residues of human sequences, for example, by site directed mutagenesis of individual residues, or by CDR grafting of entire loops. However, antibodies with entirely human sequences can also be created. EIS therefore offers a method for making partly human or entirely human antibodies that bind to the same epitope as animal or partly human antibodies respectively. In EIS, repertoires of antibody fragments can be displayed on the surface of filamentous phase and the genes encoding fragments with antigen binding activities selected by binding of the phage to antigen.

Additional methods for humanizing antibodies contemplated for use in the present invention are described in U.S. Patent No. 5,750,078; 5,502,167; 5,705,154; 5,770,403; 5,698,417; 5,693,493; 5,558,864; 4,935,496; and 4,816,567, each incorporated herein by reference.

E8. Mutagenesis by PCR

Site-specific mutagenesis is a technique useful in the preparation of individual antibodies through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, whether humanizing or not, by introducing one or more nucleotide sequence changes into the DNA.

Although many methods are suitable for use in mutagenesis, the use of the polymerase chain reaction (PCR™) is generally now preferred. This technology offers a quick and efficient method for introducing desired mutations into a given DNA sequence. The following text particularly describes the use of PCR™ to introduce point mutations into a sequence, as may be used to change the amino acid encoded by the given sequence. Adaptations of this method are also suitable for introducing restriction enzyme sites into a DNA molecule.

In this method, synthetic oligonucleotides are designed to incorporate a point mutation at one end of an amplified segment. Following PCR™, the amplified fragments are blunt-ended by treating with Klenow fragments, and the blunt-ended fragments are then ligated and subcloned into a vector to facilitate sequence analysis.

To prepare the template DNA that one desires to mutagenize, the DNA is subcloned into a high copy number vector, such as pUC19, using restriction sites flanking the area to be mutated. Template DNA is then prepared using a plasmid miniprep. Appropriate oligonucleotide primers that are based upon the parent sequence, but which contain the desired point mutation and which are flanked at the 5' end by a restriction enzyme site, are synthesized using an automated synthesizer. It is generally required that the primer be homologous to the template DNA for about 15 bases or so. Primers may be purified by denaturing polyacrylamide gel electrophoresis, although this is not absolutely necessary for use in PCR™. The 5' end of the oligonucleotides should then be phosphorylated.

The template DNA should be amplified by PCR™, using the oligonucleotide primers that contain the desired point mutations. The concentration of $MgCl_2$ in the amplification buffer will generally be about 15 mM. Generally about 20-25 cycles of PCR™ should be carried out as follows: denaturation, 35 sec. at 95°C; hybridization, 2 min. at 50°C; and extension, 2 min. at 72°C. The PCR™ will generally include a last cycle extension of about 10 min. at 72°C. After the final extension step, about 5 units of Klenow fragments should be added to the reaction mixture and incubated for a further 15 min. at about 30°C. The exonuclease activity of the Klenow fragments is required to make the ends flush and suitable for blunt-end cloning.

The resultant reaction mixture should generally be analyzed by nondenaturing agarose or acrylamide gel electrophoresis to verify that the amplification has yielded the predicted product. One would then process the reaction mixture by removing most of the mineral oils, extracting with chloroform to remove the remaining oil, extracting with buffered phenol and then concentrating by precipitation with 100% ethanol. Next, one should digest about half of the amplified fragments with a restriction enzyme that cuts at the flanking sequences used in the oligonucleotides. The digested fragments are purified on a low gelling/melting agarose gel.

To subclone the fragments and to check the point mutation, one would subclone the two amplified fragments into an appropriately digested vector by blunt-end ligation. This would be used to transform *E. coli*, from which plasmid DNA could subsequently be prepared using a miniprep. The amplified portion of the plasmid DNA would then be analyzed by DNA sequencing to confirm that the correct point mutation was generated. This is important as Taq DNA polymerase can introduce additional mutations into DNA fragments.

The introduction of a point mutation can also be effected using sequential PCR™ steps. In this procedure, the two fragments encompassing the mutation are annealed with each other and extended by mutually primed synthesis. This fragment

is then amplified by a second PCR™ step, thereby avoiding the blunt-end ligation required in the above protocol. In this method, the preparation of the template DNA, the generation of the oligonucleotide primers and the first PCR™ amplification are performed as described above. In this process, however, the chosen oligonucleotides should be homologous to the template DNA for a stretch of between about 15 and about 20 bases and must also overlap with each other by about 10 bases or more.

In the second PCR™ amplification, one would use each amplified fragment and each flanking sequence primer and carry PCR™ for between about 20 and about 25 cycles, using the conditions as described above. One would again subclone the fragments and check that the point mutation was correct by using the steps outlined above.

In using either of the foregoing methods, it is generally preferred to introduce the mutation by amplifying as small a fragment as possible. Of course, parameters such as the melting temperature of the oligonucleotide, as will generally be influenced by the GC content and the length of the oligo, should also be carefully considered. The execution of these methods, and their optimization if necessary, will be known to those of skill in the art, and are further described in various publications, such as *Current Protocols in Molecular Biology, 1995*, incorporated herein by reference.

When performing site-specific mutagenesis, **Table A** can be employed as a reference.

TABLE A

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

E9. Antibody Fragments

Irrespective of the source of the original anti-aminophospholipid antibody, either the intact antibody, antibody multimers, or any one of a variety of functional, antigen-binding regions of the antibody may be used in the present invention. Exemplary functional regions include scFv, Fv, Fab', Fab and $F(ab')_2$ fragments of the anti-aminophospholipid antibodies. Techniques for preparing such constructs are well known to those in the art and are further exemplified herein.

The choice of antibody construct may be influenced by various factors. For example, prolonged half-life can result from the active readsorption of intact antibodies within the kidney, a property of the Fc piece of immunoglobulin. IgG based antibodies, therefore, are expected to exhibit slower blood clearance than their Fab' counterparts. However, Fab' fragment-based compositions will generally exhibit better tissue penetrating capability.

Fab fragments can be obtained by proteolysis of the whole immunoglobulin by the non-specific thiol protease, papain. Papain must first be activated by reducing the sulphydryl group in the active site with cysteine, 2-mercaptoethanol or dithiothreitol. Heavy metals in the stock enzyme should be removed by chelation with EDTA (2 mM) to ensure maximum enzyme activity. Enzyme and substrate are normally mixed together in the ratio of 1:100 by weight. After incubation, the reaction can be stopped by irreversible alkylation of the thiol group with iodoacetamide or simply by dialysis. The completeness of the digestion should be monitored by SDS-PAGE and the various fractions separated by protein A-Sepharose or ion exchange chromatography.

The usual procedure for preparation of $F(ab')_2$ fragments from IgG of rabbit and human origin is limited proteolysis by the enzyme pepsin. The conditions, 100x antibody excess w/w in acetate buffer at pH 4.5, 37°C, suggest that antibody is cleaved at the C-terminal side of the inter-heavy-chain disulfide bond. Rates of digestion of mouse IgG may vary with subclass and it may be difficult to obtain high yields of active $F(ab')_2$ fragments without some undigested or completely degraded

IgG. In particular, IgG_{2b} is highly susceptible to complete degradation. The other subclasses require different incubation conditions to produce optimal results. all of which is known in the art.

Digestion of rat IgG by pepsin requires conditions including dialysis in 0.1 M acetate buffer, pH 4.5, and then incubation for four hours with 1% w/w pepsin; IgG_1 and IgG_{2a} digestion is improved if first dialyzed against 0.1 M formate buffer, pH 2.8, at 4°C, for 16 hours followed by acetate buffer. IgG_{2b} gives more consistent results with incubation in staphylococcal V8 protease (3% w/w) in 0.1 M sodium phosphate buffer, pH 7.8, for four hours at 37°C.

The following patents and patent applications are specifically incorporated herein by reference for the purposes of even further supplementing the present teachings regarding the preparation and use of functional, antigen-binding regions of antibodies, including scFv, Fv, Fab', Fab and $F(ab')_2$ fragments of the anti-aminophospholipid antibodies: U.S. Patent Nos. 5,855,866 and 5,877,289; and U.S. Applications Serial Nos. 08/350,212 (Patent No. 5,___,___; Issue Fee paid); 08/482,369 (U.S. Patent 5,___,___; Issue Fee paid); and 08/487,427 (U.S. Patent 5,___,___; Issue Fee paid).

E10. Antibody Conjugates

Anti-aminophospholipid antibodies may be conjugated to anti-cellular or cytotoxic agents, to prepare "immunotoxins"; or operatively associated with components that are capable of directly or indirectly stimulating coagulation, thus forming a "coaguligand". In coaguligands, the targeting agents may be directly linked to a direct or indirect coagulation factor, or may be linked to a second binding region that binds and then releases a direct or indirect coagulation factor. The 'second binding region' approach generally uses a coagulant-binding antibody as a second binding region, thus resulting in a bispecific antibody construct. The preparation and use of bispecific antibodies in general is well known in the art, and is further disclosed herein.

In the preparation of immunotoxins, coaguligands and bispecific antibodies, recombinant expression may be employed. The nucleic acid sequences encoding the chosen antibody-based targeting agent are attached, in-frame, to nucleic acid sequences encoding the chosen toxin, coagulant, or second binding region to create an expression unit or vector. Recombinant expression results in translation of the new nucleic acid, to yield the desired protein product. Although antibody-encoding nucleic acids are employed, rather than protein binding ligands, the recombinant approach is essentially the same as those described hereinabove.

Returning to conjugate technology, the preparation of immunotoxins is generally well known in the art. However, certain advantages may be achieved through the application of certain preferred technology, both in the preparation of the immunotoxins and in their purification for subsequent clinical administration. For example, while IgG based immunotoxins will typically exhibit better binding capability and slower blood clearance than their Fab' counterparts, Fab' fragment-based immunotoxins will generally exhibit better tissue penetrating capability as compared to IgG based immunotoxins.

Additionally, while numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate the toxin moiety to the targeting agent, certain linkers will generally be preferred over other linkers, based on differing pharmacological characteristics and capabilities. For example, linkers that contain a disulfide bond that is sterically "hindered" are to be preferred, due to their greater stability *in vivo*, thus preventing release of the toxin moiety prior to binding at the site of action.

A wide variety of cytotoxic agents are known that may be conjugated to anti-aminophospholipid antibodies, including plant-, fungus- and bacteria-derived toxins, such as ricin A chain or deglycosylated A chain. The cross-linking of a toxin A chain to a targeting agent, in certain cases, requires a cross-linker that presents disulfide

functions. The reason for this is unclear, but is likely due to a need for certain toxin moieties to be readily releasable from the targeting agent once the agent has "delivered" the toxin to the targeted cells.

Each type of cross-linker, as well as how the cross-linking is performed, will tend to vary the pharmacodynamics of the resultant conjugate. Ultimately, in cases where a releasable toxin is contemplated, one desires to have a conjugate that will remain intact under conditions found everywhere in the body except the intended site of action, at which point it is desirable that the conjugate have good "release" characteristics. Therefore, the particular cross-linking scheme, including in particular the particular cross-linking reagent used and the structures that are cross-linked, will be of some significance.

Depending on the specific toxin compound used as part of the fusion protein, it may be necessary to provide a peptide spacer operatively attaching the targeting agent and the toxin compound which is capable of folding into a disulfide-bonded loop structure. Proteolytic cleavage within the loop would then yield a heterodimeric polypeptide wherein the targeting agent and the toxin compound are linked by only a single disulfide bond. An example of such a toxin is a Ricin A-chain toxin.

When certain other toxin compounds are utilized, a non-cleavable peptide spacer may be provided to operatively attach the targeting agent and the toxin compound of the fusion protein. Toxins which may be used in conjunction with non-cleavable peptide spacers are those which may, themselves, be converted by proteolytic cleavage, into a cytotoxic disulfide-bonded form. An example of such a toxin compound is a *Pseudonomas* exotoxin compound.

There may be circumstances, such as when the target antigen does not internalize by a route consistent with efficient intoxication by targeting agent/toxin compounds, such as immunotoxins, where one will desire to target chemotherapeutic agents such as anti-tumor drugs, other cytokines, antimetabolites, alkylating agents,

hormones, and the like. A variety of chemotherapeutic and other pharmacological agents have now been successfully conjugated to antibodies and shown to function pharmacologically. Exemplary antineoplastic agents that have been investigated include doxorubicin, daunomycin, methotrexate, vinblastine, and various others. Moreover, the attachment of other agents such as neocarzinostatin, macromycin, trenimon and α-amanitin has been described.

Where coagulation factors are used in connection with the present invention, any covalent linkage to the antibody or targeting agent should be made at a site distinct from its functional coagulating site. The compositions are thus "linked" in any operative manner that allows each region to perform its intended function without significant impairment. Thus, the targeting agents bind to aminophospholipids, and the coagulation factor promotes blood clotting.

E11. Biochemical Cross-linkers

In additional to the general information provided above, anti-aminophospholipid antibodies may be conjugated to anti-cellular or cytotoxic agents using certain preferred biochemical cross-linkers. Cross-linking reagents are used to form molecular bridges that tie together functional groups of two different molecules. To link two different proteins in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation. Exemplary hetero-bifunctional cross-linkers are referenced in **Table B**.

TABLE B

HETERO-BIFUNCTIONAL CROSS-LINKERS

linker	Reactive Toward	Advantages and Applications	Spacer Arm Length after cross-linking
SMPT	Primary amines Sulfhydryls	· Greater stability	11.2 A
SPDP	Primary amines Sulfhydryls	· Thiolation · Cleavable cross-linking	6.8 A
LC-SPDP	Primary amines Sulfhydryls	· Extended spacer arm	15.6 A
Sulfo-LC-SPDP	Primary amines Sulfhydryls	· Extended spacer arm · Water-soluble	15.6 A
SMCC	Primary amines Sulfhydryls	· Stable maleimide reactive group · Enzyme-antibody conjugation · Hapten-carrier protein conjugation	11.6 A
Sulfo-SMCC	Primary amines Sulfhydryls	· Stable maleimide reactive group · Water-soluble · Enzyme-antibody conjugation	11.6 A
MBS	Primary amines Sulfhydryls	· Enzyme-antibody conjugation · Hapten-carrier protein conjugation	9.9 A
Sulfo-MBS	Primary amines Sulfhydryls	· Water-soluble	9.9 A
SIAB	Primary amines Sulfhydryls	· Enzyme-antibody conjugation	10.6 A
Sulfo-SIAB	Primary amines Sulfhydryls	· Water-soluble	10.6 A
SMPB	Primary amines Sulfhydryls	· Extended spacer arm · Enzyme-antibody conjugation	14.5 A
Sulfo-SMPB	Primary amines Sulfhydryls	· Extended spacer arm · Water-soluble	14.5 A
EDC/Sulfo-NHS	Primary amines Carboxyl groups	· Hapten-Carrier conjugation	0
ABH	Carbohydrates Nonselective	· Reacts with sugar groups	11.9 A

Hetero-bifunctional cross-linkers contain two reactive groups: one generally reacting with primary amine group (*e.g.*, N-hydroxy succinimide) and the other generally reacting with a thiol group (*e.g.*, pyridyl disulfide, maleimides, halogens, *etc.*). Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one protein (*e.g.*, the selected antibody or fragment) and through the thiol reactive group, the cross-linker, already tied up to the first protein, reacts with the cysteine residue (free sulfhydryl group) of the other protein (*e.g.*, the coagulant).

Compositions therefore generally have, or are derivatized to have, a functional group available for cross-linking purposes. This requirement is not considered to be limiting in that a wide variety of groups can be used in this manner. For example, primary or secondary amine groups, hydrazide or hydrazine groups, carboxyl alcohol, phosphate, or alkylating groups may be used for binding or cross-linking.

The spacer arm between the two reactive groups of a cross-linkers may have various length and chemical compositions. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (*e.g.*, benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (*e.g.*, disulfide bond resistant to reducing agents). The use of peptide spacers, such as L-Leu-L-Ala-L-Leu-L-Ala, is also contemplated.

It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and toxic or coagulating agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability *in vivo*, preventing release of the agent prior to binding at the site of action. These linkers are thus one preferred group of linking agents.

One of the most preferred cross-linking reagents for use in immunotoxins is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that steric hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions such as glutathione which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to the delivery of the attached agent to the tumor site. It is contemplated that the SMPT agent may also be used in connection with the bispecific ligands of this invention.

The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to cross-link functional groups such as the SH of cysteine or primary amines (*e.g.*, the epsilon amino group of lysine). Another possible type of cross-linker includes the hetero-bifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(p-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.

In addition to hindered cross-linkers, non-hindered linkers can also be employed in accordance herewith. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP and 2-iminothiolane. The use of such cross-linkers is well understood in the art.

Once conjugated, the conjugate is separated from unconjugated targeting and therapeutic agents and from other contaminants. A large a number of purification techniques are available for use in providing conjugates of a sufficient degree of purity to render them clinically useful. Purification methods based upon size separation, such as gel filtration, gel permeation or high performance liquid chromatography, will generally be of most use. Other chromatographic techniques, such as Blue-Sepharose separation, may also be used.

E12. Bispecific Antibodies

Bispecific antibodies are particularly useful in the coaguligand aspects of the present invention. However, bispecific antibodies in general may be employed, so long as one arm binds to an aminophospholipid and the bispecific antibody is attached to a therapeutic agent, generally at a site distinct from the antigen binding sites. Bispecific antibodies that bind to both PS and PE may also be used.

In general, the preparation of bispecific antibodies is also well known in the art. One method involves the separate preparation of antibodies having specificity for the targeted antigen, on the one hand, and (as herein) a coagulating agent on the other. Peptic $F(ab'\gamma)_2$ fragments are prepared from the two chosen antibodies, followed by reduction of each to provide separate $Fab'\gamma_{SH}$ fragments. The SH groups on one of the two partners to be coupled are then alkylated with a cross-linking reagent such as o-phenylenedimaleimide to provide free maleimide groups on one partner. This partner may then be conjugated to the other by means of a thioether linkage, to give the desired $F(ab'\gamma)_2$ heteroconjugate. Other techniques are known wherein cross-linking with SPDP or protein A is carried out, or a trispecific construct is prepared.

Another method for producing bispecific antibodies is by the fusion of two hybridomas to form a quadroma. As used herein, the term "quadroma" is used to describe the productive fusion of two B cell hybridomas. Using now standard techniques, two antibody producing hybridomas are fused to give daughter cells, and those cells that have maintained the expression of both sets of clonotype immunoglobulin genes are then selected.

A preferred method of generating a quadroma involves the selection of an enzyme deficient mutant of at least one of the parental hybridomas. This first mutant hybridoma cell line is then fused to cells of a second hybridoma that had been lethally exposed, *e.g.*, to iodoacetamide, precluding its continued survival. Cell fusion allows for the rescue of the first hybridoma by acquiring the gene for its enzyme deficiency from the lethally treated hybridoma, and the rescue of the second hybridoma through

fusion to the first hybridoma. Preferred, but not required, is the fusion of immunoglobulins of the same isotype, but of a different subclass. A mixed subclass antibody permits the use if an alternative assay for the isolation of a preferred quadroma.

In more detail, one method of quadroma development and screening involves obtaining a hybridoma line that secretes the first chosen MAb and making this deficient for the essential metabolic enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRT). To obtain deficient mutants of the hybridoma, cells are grown in the presence of increasing concentrations of 8-azaguanine (1×10^{-7}M to 1×10^{-5}M). The mutants are subcloned by limiting dilution and tested for their hypoxanthine/ aminopterin/ thymidine (HAT) sensitivity. The culture medium may consist of, for example, DMEM supplemented with 10% FCS, 2 mM L-Glutamine and 1 mM penicillin-streptomycin.

A complementary hybridoma cell line that produces the second desired MAb is used to generate the quadromas by standard cell fusion techniques. Briefly, 4.5×10^7 HAT-sensitive first cells are mixed with 2.8×10^7 HAT-resistant second cells that have been pre-treated with a lethal dose of the irreversible biochemical inhibitor iodoacetamide (5 mM in phosphate buffered saline) for 30 minutes on ice before fusion. Cell fusion is induced using polyethylene glycol (PEG) and the cells are plated out in 96 well microculture plates. Quadromas are selected using HAT-containing medium. Bispecific antibody-containing cultures are identified using, for example, a solid phase isotype-specific ELISA and isotype-specific immunofluorescence staining.

In one identification embodiment to identify the bispecific antibody, the wells of microtiter plates (Falcon, Becton Dickinson Labware) are coated with a reagent that specifically interacts with one of the parent hybridoma antibodies and that lacks cross-reactivity with both antibodies. The plates are washed, blocked, and the supernatants (SNs) to be tested are added to each well. Plates are incubated at room temperature

for 2 hours, the supernatants discarded, the plates washed, and diluted alkaline phosphatase-anti-antibody conjugate added for 2 hours at room temperature. The plates are washed and a phosphatase substrate, *e.g.*, P-Nitrophenyl phosphate (Sigma, St. Louis) is added to each well. Plates are incubated, 3N NaOH is added to each well to stop the reaction, and the OD_{410} values determined using an ELISA reader.

In another identification embodiment, microtiter plates pre-treated with poly-L-lysine are used to bind one of the target cells to each well, the cells are then fixed, *e.g.* using 1% glutaraldehyde, and the bispecific antibodies are tested for their ability to bind to the intact cell. In addition, FACS, immunofluorescence staining, idiotype specific antibodies, antigen binding competition assays, and other methods common in the art of antibody characterization may be used in conjunction with the present invention to identify preferred quadromas.

Following the isolation of the quadroma, the bispecific antibodies are purified away from other cell products. This may be accomplished by a variety of protein isolation procedures, known to those skilled in the art of immunoglobulin purification. Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, 1988).

For example, supernatants from selected quadromas are passed over protein A or protein G sepharose columns to bind IgG (depending on the isotype). The bound antibodies are then eluted with, *e.g.* a pH 5.0 citrate buffer. The elute fractions containing the BsAbs, are dialyzed against an isotonic buffer. Alternatively, the eluate is also passed over an anti-immunoglobulin-sepharose column. The BsAb is then eluted with 3.5 M magnesium chloride. BsAbs purified in this way are then tested for binding activity by, *e.g.*, an isotype-specific ELISA and immunofluorescence staining assay of the target cells, as described above.

Purified BsAbs and parental antibodies may also be characterized and isolated by SDS-PAGE electrophoresis, followed by staining with silver or Coomassie. This

is possible when one of the parental antibodies has a higher molecular weight than the other, wherein the band of the BsAbs migrates midway between that of the two parental antibodies. Reduction of the samples verifies the presence of heavy chains with two different apparent molecular weights.

F. Pharmaceutical Compositions

The most basic pharmaceutical compositions of the present invention will generally comprise an effective amount of at least a first therapeutic agent-targeting agent construct, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Combined therapeutics are also contemplated, and the same type of underlying pharmaceutical compositions may be employed for both single and combined medicaments.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards. Supplementary active ingredients can also be incorporated into the compositions.

F1. Parenteral Formulations

The therapeutic agent-targeting agent constructs of the present invention will most often be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, sub-cutaneous, transdermal, or other such routes, including peristaltic administration and direct instillation into a tumor or disease site

(intracavity administration). The preparation of an aqueous composition that contains a therapeutic agent-targeting agent construct as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form should be sterile and fluid to the extent that syringability exists. It should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The therapeutic agent-targeting agent compositions can be formulated into a sterile aqueous composition in a neutral or salt form. Solutions of the therapeutic agent-targeting agents as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein), and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, trifluoroacetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Suitable carriers include solvents and dispersion media containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. In many cases, it will be preferable to include isotonic agents, for example, sugars or

sodium chloride. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants.

Under ordinary conditions of storage and use, all such preparations should contain a preservative to prevent the growth of microorganisms. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Prior to or upon formulation, the therapeutic agent-targeting agent constructs should be extensively dialyzed to remove undesired small molecular weight molecules, and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. Sterile injectable solutions are prepared by incorporating the active therapeutic agent-targeting agents in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as desired, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above.

In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques that yield a powder of the active therapeutic agent-targeting agent ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of the therapeutic agent-targeting agent construct admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a range of final concentrations, depending on the intended use. The

techniques of preparation are generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

Upon formulation, therapeutic agent-targeting agent solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. Formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but other pharmaceutically acceptable forms are also contemplated, *e.g.*, tablets, pills, capsules or other solids for oral administration, suppositories, pessaries, nasal solutions or sprays, aerosols, inhalants, liposomal forms and the like. Pharmaceutical "slow release" capsules or compositions may also be used. Slow release formulations are generally designed to give a constant drug level over an extended period and may be used to deliver therapeutic agent-targeting agent constructs in accordance with the present invention.

F2. Liposomes and Nanocapsules

In certain embodiments, liposomes and/or nanoparticles may also be employed with the therapeutic agent-targeting agent constructs. The formation and use of liposomes is generally known to those of skill in the art, as summarized below.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one may operate at the same time.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be are easily made.

G. Therapeutic Kits

This invention also provides therapeutic kits comprising therapeutic agent-targeting agent constructs for use in the present treatment methods. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of at least one therapeutic agent-targeting agent construct. The kits may also contain other pharmaceutically acceptable formulations, either for diagnosis/imaging or combined therapy. For example, such kits may contain any one or more of a range of chemotherapeutic or radiotherapeutic drugs; anti-angiogenic agents; anti-tumor cell antibodies; and/or anti-tumor vasculature or anti-tumor stroma immunotoxins or coaguligands.

The kits may have a single container (container means) that contains the therapeutic agent-targeting agent construct, with or without any additional components, or they may have distinct containers for each desired agent. Where combined therapeutics are provided, a single solution may be pre-mixed, either in a molar equivalent combination, or with one component in excess of the other. Alternatively, each of the therapeutic agent-targeting agent construct and other anti-cancer agent components of the kit may be maintained separately within distinct containers prior to administration to a patient.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is preferably an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container.

The containers of the kit will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the therapeutic agent-targeting agent construct, and any other desired agent, may be placed and, preferably, suitably aliquoted. Where separate components are included, the kit will also

generally contain a second vial or other container into which these are placed, enabling the administration of separated designed doses. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits may also contain a means by which to administer the therapeutic agent-targeting agent construct to an animal or patient, *e.g.*, one or more needles or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected into the animal or applied to a diseased area of the body. The kits of the present invention will also typically include a means for containing the vials, or such like, and other component, in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained.

H. Tumor Treatment

The most important use of the present invention is in the treatment of vascularized, malignant tumors; with the treatment of benign tumors, such as BPH, also being contemplated. The invention may also be used in the therapy of other diseases and disorders having, as a component of the disease, prothrombotic blood vessels. Such vasculature-associated diseases include diabetic retinopathy, macular degeneration, vascular restenosis, including restenosis following angioplasty, arteriovenous malformations (AVM), meningioma, hemangioma, neovascular glaucoma and psoriasis; and also angiofibroma, arthritis, rheumatoid arthritis, atherosclerotic plaques, corneal graft neovascularization, hemophilic joints, hypertrophic scars, osler-weber syndrome, pyogenic granuloma retrolental fibroplasia, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, various other inflammatory diseases and disorders, and even endometriosis.

The therapeutic agent-targeting agent construct treatment of the invention is most preferably exploited for the treatment of solid tumors. Such uses may employ therapeutic agent-targeting agent constructs alone or in combination with

chemotherapeutic, radiotherapeutic, apoptopic, anti-angiogenic agents and/or immunotoxins or coaguligands. The therapeutic agent-targeting agent construct methods provided by this invention are broadly applicable to the treatment of any malignant tumor having a vascular component. Typical vascularized tumors are the solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients. Exemplary solid tumors that may be treated using the invention include, but are not limited to, carcinomas of the lung, breast, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, neuroblastomas, and the like.

The present invention is contemplated for use in the treatment of any patient that presents with a solid tumor. However, in that this invention is particularly successful in the treatment of solid tumors of moderate or large sizes, patients in these categories are likely to receive more significant benefits from treatment in accordance with the methods and compositions provided herein.

Therefore, in general, the invention can be used to treat tumors of about 0.3-0.5 cm and upwards, although it is a better use of the invention to treat tumors of greater than 0.5 cm in size. From the studies already conducted in acceptable animal models, it is believed that patients presenting with tumors of between about 1.0 and about 2.0 cm in size will be in the preferred treatment group of patients for therapeutic agent-targeting agent therapy, although tumors up to and including the largest tumors found in humans may also be treated.

Although the present invention is not generally intended as a preventative or prophylactic treatment, use of the invention is certainly not confined to the treatment of patients having tumors of only moderate or large sizes. There are many reasons underlying this aspect of the breadth of the invention. For example, a patient presenting with a primary tumor of moderate size or above may also have various

other metastatic tumors that are considered to be small-sized or even in the earlier stages of metastatic tumor seeding. Given that the therapeutic agent-targeting agent constructs, or combinations, of the invention are generally administered into the systemic circulation of a patient, they will naturally have effects on the secondary, smaller and metastatic tumors, although this may not be the primary intent of the treatment. Furthermore, even in situations where the tumor mass as a whole is a single small tumor, certain beneficial anti-tumor effects will result from the use of the present therapeutic agent-targeting agent treatment.

The guidance provided herein regarding the most suitable patients for use in connection with the present invention is intended as teaching that certain patient's profiles may assist with the selection of patients for treatment by the present invention. The pre-selection of certain patients, or categories of patients, does not in any way negate the basic usefulness of the present invention in connection with the treatment of all patients having a vascularized tumor. A further consideration is the fact that the assault on the tumor provided by the therapeutic agent-targeting agent construct of the invention may predispose the tumor to further therapeutic treatment, such that the subsequent treatment results in an overall synergistic effect or even leads to total remission or cure.

It is not believed that any particular type of tumor should be excluded from treatment using the present invention. However, the type of tumor cells may be relevant to the use of the invention in combination with secondary therapeutic agents, particularly chemotherapeutics and anti-tumor cell immunotoxins. As the effect of the present therapy is to destroy the tumor vasculature, and as the vasculature is substantially or entirely the same in all solid tumors, it will be understood that the present therapeutic agent-targeting agent methodology is widely or entirely applicable to the treatment of all solid tumors, irrespective of the particular phenotype or genotype of the tumor cells themselves.

Therapeutically effective doses of therapeutic agent-targeting agent constructs are readily determinable using data from an animal model, as shown in the studies detailed herein. Experimental animals bearing solid tumors are frequently used to optimize appropriate therapeutic doses prior to translating to a clinical environment. Such models are known to be very reliable in predicting effective anti-cancer strategies. For example, mice bearing solid tumors, such as used in the Examples, are widely used in pre-clinical testing. The inventors have used such art-accepted mouse models to determine working ranges of therapeutic agent-targeting agent constructs that give beneficial anti-tumor effects with minimal toxicity.

As is known in the art, there are realistic objectives that may be used as a guideline in connection with pre-clinical testing before proceeding to clinical treatment. However, due to the safety already demonstrated in accepted models, pre-clinical testing of the present invention will be more a matter of optimization, rather than to confirm effectiveness. Thus, pre-clinical testing may be employed to select the most advantageous therapeutic agent-targeting agent constructs, doses or combinations.

Any therapeutic agent-targeting agent dose, or combined medicament, that results in any consistent detectable tumor vasculature destruction, thrombosis and anti-tumor effects will still define a useful invention. Destructive, thrombotic and necrotic effects should be observed in between about 10% and about 40-50% of the tumor blood vessels and tumor tissues, upwards to between about 50% and about 99% of such effects being observed. The present invention may also be effective against vessels downstream of the tumor, *i.e.*, target at least a sub-set of the draining vessels, particularly as cytokines released from the tumor will be acting on these vessels, changing their antigenic profile.

It will also be understood that even in such circumstances where the anti-tumor effects of the therapeutic agent-targeting agent dose, or combined therapy, are towards the low end of this range, it may be that this therapy is still equally or even more effective than all other known therapies in the context of the particular tumor

targets. It is unfortunately evident to a clinician that certain tumors cannot be effectively treated in the intermediate or long term, but that does not negate the usefulness of the present therapy, particularly where it is at least about as effective as the other strategies generally proposed.

In designing appropriate doses of therapeutic agent-targeting agent constructs, or combined therapeutics, for the treatment of vascularized tumors, one may readily extrapolate from the animal studies described herein in order to arrive at appropriate doses for clinical administration. To achieve this conversion, one would account for the mass of the agents administered per unit mass of the experimental animal and, preferably, account for the differences in the body surface area between the experimental animal and the human patient. All such calculations are well known and routine to those of ordinary skill in the art.

For example, in taking the successful doses of annexin-TF constructs in the mouse studies, and applying standard calculations based upon mass and surface area, effective doses for use in human patients would be between about 1 mg and about 500 mgs antibody per patient, and preferably, between about 10 mgs and about 100 mgs antibody per patient.

Accordingly, using this information, the inventors contemplate that useful low doses of therapeutic agent-targeting agent constructs for human administration will be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or about 30 mgs or so per patient; and useful high doses of therapeutic agent-targeting agent constructs for human administration will be about 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 or about 500 mgs or so per patient. Useful intermediate doses of therapeutic agent-targeting agent constructs for human administration are contemplated to be about 35, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200 or about 225 mgs or so per patient.

Any particular range using any of the foregoing recited exemplary doses or any value intermediate between the particular stated ranges is contemplated. It will

also be understood that therapeutic agent-targeting agent constructs with coagulants can generally be used at higher doses than those with toxins.

In general, dosage ranges of between about 5-100 mgs, about 10-80 mgs, about 20-70 mgs, about 25-60 mgs, or about 30-50 mgs or so of therapeutic agent-targeting agent construct per patient will be preferred. Notwithstanding these stated ranges, it will be understood that, given the parameters and detailed guidance presented herein, further variations in the active or optimal ranges will be encompassed within the present invention. Although doses in and around about 5 or 10 to about 70, 80, 90 or 100 mgs per patient are currently preferred, it will be understood that lower doses may be more appropriate in combination with other agents, and that high doses can still be tolerated, particularly given the enhanced safety of the coagulant constructs. The use of human or humanized antibodies or binding proteins renders the present invention even safer for clinical use, further reducing the chances of significant toxicity or side effects in healthy tissues.

The intention of the therapeutic regimens of the present invention is generally to produce significant anti-tumor effects whilst still keeping the dose below the levels associated with unacceptable toxicity. In addition to varying the dose itself, the administration regimen can also be adapted to optimize the treatment strategy. A currently preferred treatment strategy is to administer between about 1-500 mgs, and preferably, between about 10-100 mgs of the therapeutic agent-targeting agent construct, or therapeutic cocktail containing such, about 3 times within about a 7 day period. For example, doses would be given on about day 1, day 3 or 4 and day 6 or 7.

In administering the particular doses themselves, one would preferably provide a pharmaceutically acceptable composition (according to FDA standards of sterility, pyrogenicity, purity and general safety) to the patient systemically. Intravenous injection is generally preferred, and the most preferred method is to employ a continuous infusion over a time period of about 1 or 2 hours or so. Although it is not required to determine such parameters prior to treatment using the present invention,

it should be noted that the studies detailed herein result in at least some thrombosis being observed specifically in the blood vessels of a solid tumor within about 12-24 hours of injection, and that widespread tumor necrosis is also observed in this period.

Naturally, before wide-spread use, clinical trials will be conducted. The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in establishing such trials.

Patients chosen for the first therapeutic agent-targeting agent construct treatment studies will have failed to respond to at least one course of conventional therapy, and will have objectively measurable disease as determined by physical examination, laboratory techniques, and/or radiographic procedures. Any chemotherapy should be stopped at least 2 weeks before entry into the study. Where murine monoclonal antibodies or antibody portions are employed, the patients should have no history of allergy to mouse immunoglobulin.

Certain advantages will be found in the use of an indwelling central venous catheter with a triple lumen port. The therapeutic agent-targeting agent constructs should be filtered, for example, using a 0.22 μ filter, and diluted appropriately, such as with saline, to a final volume of 100 ml. Before use, the test sample should also be filtered in a similar manner, and its concentration assessed before and after filtration by determining the A_{280}. The expected recovery should be within the range of 87% to 99%, and adjustments for protein loss can then be accounted for.

The therapeutic agent-targeting agent constructs may be administered over a period of approximately 4-24 hours, with each patient receiving 2-4 infusions at 2-7 day intervals. Administration can also be performed by a steady rate of infusion over a 7 day period. The infusion given at any dose level should be dependent upon any toxicity observed. Hence, if Grade II toxicity was reached after any single infusion, or

at a particular period of time for a steady rate infusion, further doses should be withheld or the steady rate infusion stopped unless toxicity improved. Increasing doses of therapeutic agent-targeting agent constructs should be administered to groups of patients until approximately 60% of patients showed unacceptable Grade III or IV toxicity in any category. Doses that are 2/3 of this value are defined as the safe dose.

Physical examination, tumor measurements, and laboratory tests should, of course, be performed before treatment and at intervals up to 1 month later. Laboratory tests should include complete blood counts, serum creatinine, creatine kinase, electrolytes, urea, nitrogen, SGOT, bilirubin, albumin, and total serum protein. Serum samples taken up to 60 days after treatment should be evaluated by radioimmunoassay for the presence of the administered therapeutic agent-targeting agent constructs, and antibodies against any portions thereof. Immunological analyses of sera, using any standard assay such as, for example, an ELISA or RIA, will allow the pharmacokinetics and clearance of the anti-aminophospholipid therapeutic agent to be evaluated.

To evaluate the anti-tumor responses, the patients should be examined at 48 hours to 1 week and again at 30 days after the last infusion. When palpable disease was present, two perpendicular diameters of all masses should be measured daily during treatment, within 1 week after completion of therapy, and at 30 days. To measure nonpalpable disease, serial CT scans could be performed at 1-cm intervals throughout the chest, abdomen, and pelvis at 48 hours to 1 week and again at 30 days. Tissue samples should also be evaluated histologically, and/or by flow cytometry, using biopsies from the disease sites or even blood or fluid samples if appropriate.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable tumor 1 month after treatment. Whereas a partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable tumor nodules 1 month after treatment, with no tumor sites showing enlargement. Similarly,

a mixed response may be defined by a reduction of the product of perpendicular diameters of all measurable lesions by 50% or greater 1 month after treatment, with progression in one or more sites.

In light of results from clinical trials, such as those described above, an even more precise treatment regimen may be formulated. Even so, some variation in dosage may later be necessary depending on the condition of the subject being treated. The physician responsible for administration will, in light of the present disclosure, be able to determine the appropriate dose for the individual subject. Such optimization and adjustment is routinely carried out in the art and by no means reflects an undue amount of experimentation.

I. Tumor Imaging

The present invention further provides combined tumor treatment and imaging methods, based upon anti-aminophospholipid binding ligands. Anti-aminophospholipid binding proteins or antibodies that are linked to one or more detectable agents are envisioned for use in pre-imaging the tumor, forming a reliable image prior to the treatment, which itself targets the aminophospholipid markers.

The anti-aminophospholipid imaging ligands or antibodies, or conjugates thereof, will generally comprise an anti-aminophospholipid antibody or binding ligand operatively attached, or conjugated to, a detectable label. "Detectable labels" are compounds or elements that can be detected due to their specific functional properties, or chemical characteristics, the use of which allows the component to which they are attached to be detected, and further quantified if desired. Preferably, the detectable labels are those detectable *in vivo* using non-invasive methods.

Antibody and binding protein conjugates for use as diagnostic agents generally fall into two classes, those for use in *in vitro* diagnostics, such as in a variety of immunoassays, and those for use *in vivo* diagnostic protocols. It is the *in vivo* imaging methods that are particularly intended for use with this invention.

Many appropriate imaging agents are known in the art, as are methods for their attachment to antibodies and binding ligands (see, *e.g.*, U.S. patents 5,021,236 and 4,472,509, both incorporated herein by reference). Certain attachment methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such a DTPA attached to the antibody (U.S. Patent 4,472,509). Monoclonal antibodies may also be reacted with an enzyme in the presence of a coupling agent such as glutaraldchyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate.

An example of detectable labels are the paramagnetic ions. In this case, suitable ions include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being particularly preferred.

Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III). Fluorescent labels include rhodamine, fluorescein and renographin. Rhodamine and fluorescein are often linked via an isothiocyanate intermediate.

In the case of radioactive isotopes for diagnostic applications, suitable examples include ^{14}carbon, ^{51}chromium, ^{36}chlorine, ^{57}cobalt, ^{58}cobalt, copper^{67}, ^{152}Eu, gallium^{67}, ^{3}hydrogen, iodine^{123}, iodine^{125}, iodine^{131}, indium^{111}, ^{59}iron, ^{32}phosphorus, rhenium^{186}, rhenium^{188}, ^{75}selenium, ^{35}sulphur, technetium^{99m} and yttrium^{90}. ^{125}I is often being preferred for use in certain embodiments, and technicium^{99m} and indium^{111} are also often preferred due to their low energy and suitability for long range detection.

Radioactively labeled anti-aminophospholipid antibodies and binding ligands for use in the present invention may be produced according to well-known methods in

the art. For instance, intermediary functional groups that are often used to bind radioisotopic metallic ions to antibodies are diethylenetriaminepentaacetic acid (DTPA) and ethylene diaminetetracetic acid (EDTA).

Monoclonal antibodies can also be iodinated by contact with sodium or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Anti-aminophospholipid antibodies according to the invention may be labeled with tcchnetium-^{99}m by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the antibody to this column; or by direct labeling techniques, *e.g.*, by incubating pertechnate, a reducing agent such as $SNCl_2$, a buffer solution such as sodium-potassium phthalate solution, and the antibody.

Any of the foregoing type of detectably labeled anti-aminophospholipid antibodies and aminophospholipid binding ligands may be used in the imaging aspects of the present invention. Although not previously proposed for use in combined tumor imaging and treatment, the detectably-labeled annexins of U.S. Patent No. 5,627,036; WO 95/19791; WO 95/27903; WO 95/34315; WO 96/17618; and WO 98/04294; each incorporated herein by reference; may also be employed.

WO 95/27903 (incorporated herein by reference) provides annexins for use in detecting apoptotic cells. Any of the annexin-detectable agent markers of WO 95/27903 may be used herein, although it will be known that certain of these are more suitable for *in vitro* uses. WO 95/27903 is also specifically incorporated herein by reference for purposes of providing detectable kits that may be adapted for combined use with the therapeutics of the present invention.

Each of WO 95/19791; WO 95/34315; WO 96/17618; and WO 98/04294; are also incorporated herein by reference for purposes of further describing radiolabelled annexin conjugates for diagnostic imaging. The intent of each of the foregoing

documents is to provide radiolabelled annexins for use in imaging vascular thromboses, particularly in or near the heart, such as in deep vein thrombosis, pulmonary embolism, myocardial infarction, atrial fibrillation, problems with prosthetic cardiovascular materials, stroke, and the like. These radiolabelled annexins were also proposed for use in imaging activated platelets, *e.g.*, in conditions such as abscesses, restenosis, inflammation of joints, clots in cerebral arteries, *etc.*

U.S. Patent No. 5,627,036 (incorporated herein by reference) also generally concerns 'annexine' (annexin) binding ligands for use in analyzing platelet phosphatidylserine. It is explained in U.S. Patent No. 5,627,036 that hemostatic disorders, such as arterial, coronary and venous thrombosis, are usually idiopathic, which makes prediction and prevention difficult. To recognize such hemostatic disorders earlier, the detection of activated platelets is proposed. The detectably labeled annexins compositions are thus disclosed in order to detect activated platelets in hemostatic disorders (U.S. Patent No. 5,627,036).

Although proposing a wide range of diagnostic uses, none of WO 95/19791; WO 95/34315; WO 96/17618; or WO 98/04294 make reference to imaging the vasculature of solid tumors. Neither does U.S. Patent No. 5,627,036 make any such suggestions. Nonetheless, the disclosed detectable and radiolabelled annexin compositions *per se* may now be used to advantage in this regard, in light of the surprising discoveries disclosed herein.

In particular, U.S. Patent No. 5,627,036 (incorporated herein by reference) discloses annexins detectably labeled with fluorescein isothiocyanate; radioisotopes of halogens, technetium, lead, mercury, thallium or indium; and paramagnetic contrast agents.

WO 95/19791 (incorporated herein by reference) provides conjugates of annexin bonded to an N_2S_2 chelate that can be radiolabelled by complexing a radionuclide to the chelate. WO 95/34315 (incorporated herein by reference) provides

annexin conjugates comprising one or more galactose residues with the N_2S_2 chelate. The galactose moiety is said to facilitate the rapid elimination of the radiolabelled conjugate from the circulation, reducing radiation damage to non-target tissues and background 'noise.'

WO 96/17618 (incorporated herein by reference) in turn provides annexin conjugates suitable for radiolabeling with diagnostic imaging agents that comprise an annexin with a cluster of galactose residues and an N_2S_2 chelate. These are reported to have a shorter circulating half-life and a higher binding affinity for target sites than the foregoing radiolabeled annexin-galactose conjugates.

Still further radiolabeled annexin conjugates are provided by WO 98/04294 (incorporated herein by reference). These conjugates comprise an annexin that is modified to provide an accessible sulphydryl group conjugated to a hexose moiety that is recognized by a mammalian liver receptor. Annexin multimer conjugates and chelating compounds conjugated via esterase-sensitive bonds are also provided.

Each of WO 95/19791; WO 95/34315; WO 96/17618; and WO 98/04294; are also specifically incorporated herein by reference for purposes of providing annexin conjugate components for radiolabelling that are amenable to packaging in "cold kits", *i.e.*, wherein the components are provided in separate vials. U.S. Patent No. 5,627,036 similarly provides kits comprising a carrier being compartmentalized to receive detectably labeled annexins that may be adapted for use herewith.

Although suitable for use in *in vitro* diagnostics, the present aminophospholipid detection methods are more intended for forming an image of the tumor vasculature of a patient prior to treatment with therapeutic agent-targeting agent constructs. The *in vivo* diagnostic or imaging methods generally comprise administering to a patient a diagnostically effective amount of an anti-aminophospholipid antibody or binding ligand that is conjugated to a marker that is detectable by non-invasive methods. The antibody- or binding ligand-marker

conjugate is allowed sufficient time to localize and bind to the aminophospholipid expressed on the luminal surface of the tumor vasculature. The patient is then exposed to a detection device to identify the detectable marker, thus forming an image of the tumor vasculature.

The nuclear magnetic spin-resonance isotopes, such as gadolinium, are detected using a nuclear magnetic imaging device; and radioactive substances, such as technicium^{99m} or indium^{111}, are detected using a gamma scintillation camera or detector. U.S. Patent No. 5,627,036 is also specifically incorporated herein by reference for purposes of providing even further guidance regarding the safe and effective introduction of such detectably labeled constructs into the blood of an individual, and means for determining the distribution of the detectably labeled annexin extracorporally, *e.g.*, using a gamma scintillation camera or by magnetic resonance measurement.

Dosages for imaging embodiments are generally less than for therapy, but are also dependent upon the age and weight of a patient. A one time dose of between about 0.1, 0.5 or about 1 mg and about 9 or 10 mgs, and more preferably, of between about 1 mg and about 5-10 mgs of anti-aminophospholipid antibody- or aminophospholipid binding ligand-conjugate per patient is contemplated to be useful. U.S. Patent No. 5,627,036; and WO 95/19791, each incorporated herein by reference, are also instructive regarding doses of detectably-labeled annexins.

J. Combination Therapies

The therapeutic agent-targeting agent treatment methods of the present invention may be combined with any other methods generally employed in the treatment of the particular tumor, disease or disorder that the patient exhibits. So long as a particular therapeutic approach is not known to be detrimental to the patient's condition in itself, and does not significantly counteract the therapeutic agent-targeting agent treatment, its combination with the present invention is contemplated.

In connection solid tumor treatment, the present invention may be used in combination with classical approaches, such as surgery, radiotherapy, chemotherapy, and the like. The invention therefore provides combined therapies in which therapeutic agent-targeting agent constructs are used simultaneously with, before, or after surgery or radiation treatment; or are administered to patients with, before, or after conventional chemotherapeutic, radiotherapeutic or anti-angiogenic agents, or targeted immunotoxins or coaguligands.

Combination therapy for other vascular diseases is also contemplated. A particular example of such is benign prostatic hyperplasia (BPH), which may be treated with therapeutic agent-targeting agent constructs in combination other treatments currently practiced in the art. For example, targeting of immunotoxins to markers localized within BPH, such as PSA.

When one or more agents are used in combination with the therapeutic agent-targeting agent therapy, there is no requirement for the combined results to be additive of the effects observed when each treatment is conducted separately. Although at least additive effects are generally desirable, any increased anti-tumor effect above one of the single therapies would be of benefit. Also, there is no particular requirement for the combined treatment to exhibit synergistic effects, although this is certainly possible and advantageous.

To practice combined anti-tumor therapy, one would simply administer to an animal a therapeutic agent-targeting agent construct in combination with another anti-cancer agent in a manner effective to result in their combined anti-tumor actions within the animal. The agents would therefore be provided in amounts effective and for periods of time effective to result in their combined presence within the tumor vasculature and their combined actions in the tumor environment. To achieve this goal, the therapeutic agent-targeting agent constructs and anti-cancer agents may be administered to the animal simultaneously, either in a single composition, or as two distinct compositions using different administration routes.

Alternatively, the therapeutic agent-targeting agent treatment may precede, or follow, the anti-cancer agent treatment by, *e.g.*, intervals ranging from minutes to weeks. In certain embodiments where the anti-cancer agent and therapeutic agent-targeting agent construct are applied separately to the animal, one would ensure that a significant period of time did not expire between the time of each delivery, such that the anti-cancer agent and therapeutic agent-targeting agent composition would still be able to exert an advantageously combined effect on the tumor. In such instances, it is contemplated that one would contact the tumor with both agents within about 5 minutes to about one week of each other and, more preferably, within about 12-72 hours of each other, with a delay time of only about 12-48 hours being most preferred.

Exemplary anti-cancer agents that would be given prior to the therapeutic agent-targeting agent construct are agents that induce the expression of aminophospholipids within the tumor vasculature. For example, agents that stimulate localized calcium production and/or that induce apoptosis will generally result in increased PS expression, which can then be targeted using a subsequent anti-PS therapeutic agent-targeting agent construct. Therapeutic agent-targeting agent constructs would be first administered in other situations to cause tumor destruction, followed by, *e.g.*, anti-angiogenic therapies or therapies directed to targeting necrotic tumor cells.

The general use of combinations of substances in cancer treatment is well know. For example, U.S. Patent No. 5,710,134 (incorporated herein by reference) discloses components that induce necrosis in tumors in combination with non-toxic substances or "prodrugs". The enzymes set free by necrotic processes cleave the non-toxic "prodrug" into the toxic "drug", which leads to tumor cell death. Also, U.S. Patent No. 5,747,469 (incorporated herein by reference) discloses the combined use of viral vectors encoding p53 and DNA damaging agents. Any such similar approaches can be used with the present invention.

In some situations, it may even be desirable to extend the time period for treatment significantly, where several days (2, 3, 4, 5, 6 or 7), several weeks (1, 2, 3, 4, 5, 6, 7 or 8) or even several months (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. This would be advantageous in circumstances where one treatment was intended to substantially destroy the tumor, such as the therapeutic agent-targeting agent treatment, and another treatment was intended to prevent micrometastasis or tumor re-growth, such as the administration of an anti-angiogenic agent. The EN 7/44 antibody of Hagemeier *et al.* (1986) is not believed to be an effective anti-angiogenic agent, lacking binding to a surface accessible antigen, amongst other deficiencies.

It also is envisioned that more than one administration of either the therapeutic agent-targeting agent construct or the anti-cancer agent will be utilized. The therapeutic agent-targeting agent constructs and anti-cancer agents may be administered interchangeably, on alternate days or weeks; or a sequence of therapeutic agent-targeting agent treatment may be given, followed by a sequence of anti-cancer agent therapy. In any event, to achieve tumor regression using a combined therapy, all that is required is to deliver both agents in a combined amount effective to exert an anti-tumor effect, irrespective of the times for administration.

In terms of surgery, any surgical intervention may be practiced in combination with the present invention. In connection with radiotherapy, any mechanism for inducing DNA damage locally within tumor cells is contemplated, such as γ-irradiation, X-rays, UV-irradiation, microwaves and even electronic emissions and the like. The directed delivery of radioisotopes to tumor cells is also contemplated, and this may be used in connection with a targeting antibody or other targeting means.

Cytokine therapy also has proven to be an effective partner for combined therapeutic regimens. Various cytokines may be employed in such combined approaches. Examples of cytokines include IL-1α IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, G-CSF,

TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, IFN-γ. Cytokines are administered according to standard regimens, consistent with clinical indications such as the condition of the patient and relative toxicity of the cytokine. Uteroglobins may also be used to prevent or inhibit metastases (U.S. Patent No. 5,696,092; incorporated herein by reference).

J1. Chemotherapeutics

In certain embodiments, the therapeutic agent-targeting agent constructs of the present invention may be administered in combination with a chemotherapeutic agent. Chemotherapeutic drugs can kill proliferating tumor cells, enhancing the necrotic areas created by the overall treatment. The drugs can thus enhance the thrombotic action of the therapeutic agent-targeting agent constructs.

By inducing the formation of thrombi in tumor vessels, the therapeutic agent-targeting agent constructs can enhance the action of the chemotherapeutics by retaining or trapping the drugs within the tumor. The chemotherapeutics are thus retained within the tumor, while the rest of the drug is cleared from the body. Tumor cells are thus exposed to a higher concentration of drug for a longer period of time. This entrapment of drug within the tumor makes it possible to reduce the dose of drug, making the treatment safer as well as more effective.

Irrespective of the underlying mechanism(s), a variety of chemotherapeutic agents may be used in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated as exemplary include, *e.g.*, tamoxifen, taxol, vincristine, vinblastine, etoposide (VP-16), adriamycin, 5-fluorouracil (5FU), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), combretastatin(s) and derivatives and prodrugs thereof.

As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be generally around those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in

combination with other chemotherapeutics. By way of example only, agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered *via* injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Further useful agents include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for adriamycin, to 35-50 mg/m^2 for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of polynucleotide precursors may also be used. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU) are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Exemplary chemotherapeutic agents that are useful in connection with combined therapy are listed in **Table C**. Each of the agents listed therein are exemplary and by no means limiting. The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The physician responsible for administration will be able to determine the appropriate dose for the individual subject.

TABLE C

Chemotherapeutic Agents Useful in Neoplastic Disease

CLASS	TYPE OF AGENT	NONPROPRIETARY NAMES (OTHER NAMES)	DISEASE
Alkylating Agents	Nitrogen Mustards	Mechlorethamine (HN_2)	Hodgkin's disease, non-Hodgkin's lymphomas
		Cyclophosphamide Ifosfamide	Acute and chronic lymphocytic leukemias, Hodgkin's disease, non-Hodgkin's lymphomas, multiple myeloma, neuroblastoma, breast, ovary, lung, Wilms' tumor, cervix, testis, soft-tissue sarcomas
		Melphalan (L-sarcolysin)	Multiple myeloma, breast, ovary
		Chlorambucil	Chronic lymphocytic leukemia, primary macroglobulinemia, Hodgkin's disease, non-Hodgkin's lymphomas
	Ethylenimenes and Methylmelamines	Hexamethylmelamine	Ovary
		Thiotepa	Bladder, breast, ovary
	Alkyl Sulfonates	Busulfan	Chronic granulocytic leukemia
	Nitrosoureas	Carmustine (BCNU)	Hodgkin's disease, non-Hodgkin's lymphomas, primary brain tumors, multiple myeloma, malignant melanoma
		Lomustine (CCNU)	Hodgkin's disease, non-Hodgkin's lymphomas, primary brain tumors, small-cell lung
		Semustine (methyl-CCNU)	Primary brain tumors, stomach, colon
		Streptozocin (streptozotocin)	Malignant pancreatic insulinoma, malignant carcinoid
	Triazines	Dacarbazine (DTIC; dimethyltriazenoimidazolecarboxamide)	Malignant melanoma, Hodgkin's disease, soft-tissue sarcomas
Antimetabolites	Folic Acid Analogs	Methotrexate (amethopterin)	Acute lymphocytic leukemia, choriocarcinoma, mycosis fungoides, breast, head and neck, lung, osteogenic sarcoma
	Pyrimidine Analogs	Flouuracil (5-fluorouracil; 5-FU) Floxuridine (fluorodeoxyuridine; FUdR)	Breast, colon, stomach, pancreas, ovary, head and neck, urinary bladder, premalignant skin lesions (topical)

Antimetabolites, continued		Cytarabine (cytosine arabinoside)	Acute granulocytic and acute lymphocytic leukemias
	Purine Analogs and Related Inhibitors	Mercaptopurine (6-mercaptopurine; 6-MP)	Acute lymphocytic, acute granulocytic and chronic granulocytic leukemias
		Thioguanine (6-thioguanine; TG)	Acute granulocytic, acute lymphocytic and chronic granulocytic leukemias
		Pentostatin (2-deoxycoformycin)	Hairy cell leukemia, mycosis fungoides, chronic lymphocytic leukemia
Natural Products	Vinca Alkaloids	Vinblastine (VLB)	Hodgkin's disease, non-Hodgkin's lymphomas, breast, testis
		Vincristine	Acute lymphocytic leukemia, neuroblastoma, Wilms' tumor, rhabdomyosarcoma, Hodgkin's disease, non-Hodgkin's lymphomas, small-cell lung
	Epipodophyllotoxins	Etoposide Tertiposide	Testis, small-cell lung and other lung, breast, Hodgkin's disease, non-Hodgkin's lymphomas, acute granulocytic leukemia, Kaposi's sarcoma
	Antibiotics	Dactinomycin (actinomycin D)	Choriocarcinoma, Wilms' tumor, rhabdomyosarcoma, testis, Kaposi's sarcoma
		Daunorubicin (daunomycin; rubidomycin)	Acute granulocytic and acute lymphocytic leukemias
		Doxorubicin	Soft-tissue, osteogenic and other sarcomas; Hodgkin's disease, non-Hodgkin's lymphomas, acute leukemias, breast, genitourinary, thyroid, lung, stomach, neuroblastoma
		Bleomycin	Testis, head and neck, skin, esophagus, lung and genitourinary tract; Hodgkin's disease, non-Hodgkin's lymphomas
Natural Products, continued	Antibiotics, continued	Plicamycin (mithramycin)	Testis, malignant hypercalcemia
		Mitomycin (mitomycin C)	Stomach, cervix, colon, breast, pancreas, bladder, head and neck
	Enzymes	L-Asparaginase	Acute lymphocytic leukemia

	Biological Response Modifiers	Interferon alfa	Hairy cell leukemia., Kaposi's sarcoma, melanoma, carcinoid, renal cell, ovary, bladder, non-Hodgkin's lymphomas, mycosis fungoides, multiple myeloma, chronic granulocytic leukemia
Miscellaneous Agents	Platinum Coordination Complexes	Cisplatin (*cis*-DDP) Carboplatin	Testis, ovary, bladder, head and neck, lung, thyroid, cervix, endometrium, neuroblastoma, osteogenic sarcoma
	Anthracenedione	Mitoxantrone	Acute granulocytic leukemia, breast
	Substituted Urea	Hydroxyurea	Chronic granulocytic leukemia, polycythemia vera, essental thrombocytosis, malignant melanoma
	Methyl Hydrazine Derivative	Procarbazine (N-methylhydrazine, MIH)	Hodgkin's disease
	Adrenocortical Suppressant	Mitotane (*o,p'*-DDD)	Adrenal cortex
		Aminoglutethimide	Breast
Hormones and Antagonists	Adrenocorticosteroids	Prednisone (several other equivalent preparations available)	Acute and chronic lymphocytic leukemias, non-Hodgkin's lymphomas, Hodgkin's disease, breast
	Progestins	Hydroxyprogesterone caproate Medroxyprogesterone acetate Megestrol acetate	Endometrium, breast
	Estrogens	Diethylstilbestrol Ethinyl estradiol (other preparations available)	Breast, prostate
	Antiestrogen	Tamoxifen	Breast
	Androgens	Testosterone propionate Fluoxymesterone (other preparations available)	Breast
	Antiandrogen	Flutamide	Prostate
	Gonadotropin-releasing hormone analog	Leuprolide	Prostate

J2. Anti-Angiogenics

The term "angiogenesis" refers to the generation of new blood vessels, generally into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development and formation of the corpus luteum, endometrium and placenta. Uncontrolled (persistent and/or unregulated) angiogenesis is related to various disease states, and occurs during tumor growth and metastasis.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

As persistent, unregulated angiogenesis occurs during tumor development and metastasis, the treatment methods of this invention may be used in combination with any one or more "anti-angiogenic" therapies. Exemplary anti-angiogenic agents that are useful in connection with combined therapy are listed in **Table D**. Each of the agents listed therein is exemplary and by no means limiting.

TABLE D

Inhibitors and Negative Regulators of Angiogenesis

SUBSTANCES	REFERENCES
Angiostatin	O'Reilly *et al.*, 1994
Endostatin	O'Reilly *et al.*, 1997
16kDa prolactin fragment	Ferrara *et al.*, 1991; Clapp *et al.*, 1993; D'Angelo *et al.*, 1995; Lee *et al.*, 1998
Laminin peptides	Kleinman *et al.*, 1993; Yamamura *et al.*, 1993; Iwamoto *et al.*, 1996; Tryggvason, 1993
Fibronectin peptides	Grant *et al.*, 1998; Sheu *et al.*, 1997
Tissue metalloproteinase inhibitors (TIMP 1, 2, 3, 4)	Sang, 1998
Plasminogen activator inhibitors (PAI-1, -2)	Soff *et al.*, 1995
Tumor necrosis factor α (high dose, *in vitro*)	Frater-Schroder *et al.*, 1987
TGF-β1	RayChadhury and D'Amore, 1991; Tada *et al.*, 1994
Interferons (IFN-α, -β, γ)	Moore *et al.*, 1998; Lingen *et al.*, 1998
ELR- CXC Chemokines: IL-12; SDF-1; MIG; Platelet factor 4 (PF-4); IP-10	Moore *et al.*, 1998; Hiscox and Jiang, 1997; Coughlin *et al.*, 1998; Tanaka *et al.*, 1997
Thrombospondin (TSP)	Good *et al.*, 1990; Frazier, 1991; Bornstein, 1992; Tolsma *et al.*, 1993; Sheibani and Frazier, 1995; Volpert *et al.*, 1998
SPARC	Hasselaar and Sage, 1992; Lane *et al.*, 1992; Jendraschak and Sage, 1996
2-Methoxyoestradiol	Fotsis *et al.*, 1994
Proliferin-related protein	Jackson *et al.*, 1994
Suramin	Gagliardi *et al.*, 1992; Takano *et al.*, 1994; Waltenberger *et al.*, 1996; Gagliardi *et al.*, 1998; Manetti *et al.*, 1998
Thalidomide	D'Amato *et al.*, 1994; Kenyon *et al.*, 1997 Wells, 1998

Cortisone	Thorpe *et al.*, 1993 Folkman *et al.*, 1983 Sakamoto *et al.*, 1986
Linomide	Vukanovic *et al.*, 1993; Ziche *et al.*, 1998; Nagler *et al.*, 1998
Fumagillin (AGM-1470; TNP-470)	Sipos *et al.*, 1994; Yoshida *et al.*, 1998
Tamoxifen	Gagliardi and Collins, 1993; Linder and Borden, 1997; Haran *et al.*, 1994
Korean mistletoe extract (*Viscum album coloratum*)	Yoon *et al.*, 1995
Retinoids	Oikawa *et al.*, 1989; Lingen *et al.*, 1996; Majewski *et al.* 1996
CM101	Hellerqvist *et al.*, 1993; Quinn *et al.*, 1995; Wamil *et al.*, 1997; DeVore *et al.*, 1997
Dexamethasone	Hori *et al.*, 1996; Wolff *et al.*, 1997
Leukemia inhibitory factor (LIF)	Pepper *et al.*, 1995

A certain preferred component for use in inhibiting angiogenesis is a protein named "angiostatin". This component is disclosed in U.S. Patents 5,776,704; 5,639,725 and 5,733,876, each incorporated herein by reference. Angiostatin is a protein having a molecular weight of between about 38 kD and about 45 kD, as determined by reducing polyacrylamide gel electrophoresis, which contains approximately Kringle regions 1 through 4 of a plasminogen molecule. Angiostatin generally has an amino acid sequence substantially similar to that of a fragment of murine plasminogen beginning at amino acid number 98 of an intact murine plasminogen molecule.

The amino acid sequence of angiostatin varies slightly between species. For example, in human angiostatin, the amino acid sequence is substantially similar to the sequence of the above described murine plasminogen fragment, although an active human angiostatin sequence may start at either amino acid number 97 or 99 of an intact human plasminogen amino acid sequence. Further, human plasminogen may be used, as it has similar anti-angiogenic activity, as shown in a mouse tumor model.

Certain anti-angiogenic therapies have already been shown to cause tumor regressions, and angiostatin is one such agent. Endostatin, a 20 kDa COOH-terminal fragment of collagen XVIII, the bacterial polysaccharide CM101, and the antibody LM609 also have angiostatic activity. However, in light of their other properties, they are referred to as anti-vascular therapies or tumor vessel toxins, as they not only inhibit angiogenesis but also initiate the destruction of tumor vessels through mostly undefined mechanisms. Their combination with the present invention is clearly envisioned.

Angiostatin and endostatin have become the focus of intense study, as they are the first angiogenesis inhibitors that have demonstrated the ability to not only inhibit tumor growth but also cause tumor regressions in mice. There are multiple proteases that have been shown to produce angiostatin from plasminogen including elastase, macrophage metalloelastase (MME), matrilysin (MMP-7), and 92 kDa gelatinase B/type IV collagenase (MMP-9).

MME can produce angiostatin from plasminogen in tumors and granulocyte-macrophage colony-stimulating factor (GMCSF) upregulates the expression of MME by macrophages inducing the production of angiostatin. The role of MME in angiostatin generation is supported by the finding that MME is in fact expressed in clinical samples of hepatocellular carcinomas from patients. Another protease thought to be capable of producing angiostatin is stromelysin-1 (MMP-3). MMP-3 has been shown to produce angiostatin-like fragments from plasminogen *in vitro*.

The mechanism of action for angiostatin is currently unclear, it is hypothesized that it binds to an unidentified cell surface receptor on endothelial cells inducing endothelial cell to undergo programmed cell death or mitotic arrest. Endostatin appears to be an even more powerful anti-angiogenesis and anti-tumor agent although its biology is much less clear. Endostatin is effective at causing regressions in a number of tumor models in mice. Tumors do not develop resistance to endostatin and, after multiple cycles of treatment, tumors enter a dormant state during which they

do not increase in volume. In this dormant state, the percentage of tumor cells undergoing apoptosis was increased, yielding a population that essentially stays the same size. Endostatin is also thought to bind an unidentified endothelial cell surface receptor that mediates its effect.

CM101 is a bacterial polysaccharide that has been well characterized in its ability to induce neovascular inflammation in tumors. CM101 binds to and cross-links receptors expressed on dedifferentiated endothelium that stimulates the activation of the complement system. It also initiates a cytokine-driven inflammatory response that selectively targets the tumor. It is a uniquely antipathoangiogenic agent that downregulates the expression VEGF and its receptors. CM101 is currently in clinical trials as an anti-cancer drug, and can be used in combination herewith.

Thrombospondin (TSP-1) and platelet factor 4 (PF4) may also be used in combination with the present invention. These are both angiogenesis inhibitors that associate with heparin and are found in platelet α-granules. TSP-1 is a large 450kDa multi-domain glycoprotein that is constituent of the extracellular matrix. TSP-1 binds to many of the proteoglycan molecules found in the extracellular matrix including, HSPGs, fibronectin, laminin, and different types of collagen. TSP-1 inhibits endothelial cell migration and proliferation *in vitro* and angiogenesis *in vivo*. TSP-1 can also suppress the malignant phenotype and tumorigenesis of transformed endothelial cells. The tumor suppressor gene p53 has been shown to directly regulate the expression of TSP-1 such that, loss of p53 activity causes a dramatic reduction in TSP-1 production and a concomitant increase in tumor initiated angiogenesis.

PF4 is a 70aa protein that is member of the CXC ELR- family of chemokines that is able to potently inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. PF4 administered intratumorally or delivered by an adenoviral vector is able to cause an inhibition of tumor growth.

Interferons and metalloproteinase inhibitors are two other classes of naturally occurring angiogenic inhibitors that can be combined with the present invention. The anti-endothelial activity of the interferons has been known since the early 1980s, however, the mechanism of inhibition is still unclear. It is known that they can inhibit endothelial cell migration and that they do have some anti-angiogenic activity *in vivo* that is possibly mediated by an ability to inhibit the production of angiogenic promoters by tumor cells. Vascular tumors in particular are sensitive to interferon, for example, proliferating hemangiomas can be successfully treated with IFNα.

Tissue inhibitors of metalloproteinases (TIMPs) are a family of naturally occurring inhibitors of matrix metalloproteases (MMPs) that can also inhibit angiogenesis and can be used in combined treatment protocols with the present invention. MMPs play a key role in the angiogenic process as they degrade the matrix through which endothelial cells and fibroblasts migrate when extending or remodeling the vascular network. In fact, one member of the MMPs, MMP-2, has been shown to associate with activated endothelium through the integrin αvβ3 presumably for this purpose. If this interaction is disrupted by a fragment of MMP-2, then angiogenesis is downregulated and in tumors growth is inhibited.

There are a number of pharmacological agents that inhibit angiogenesis, any one or more of which may be used in combination with the present invention. These include AGM-1470/TNP-470, thalidomide, and carboxyamidotriazole (CAI). Fumagillin was found to be a potent inhibitor of angiogenesis in 1990, and since then the synthetic analogues of fumagillin, AGM-1470 and TNP-470 have been developed. Both of these drugs inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. TNP-470 has been studied extensively in human clinical trials with data suggesting that long-term administration is optimal.

Thalidomide was originally used as a sedative but was found to be a potent teratogen and was discontinued. In 1994 it was found that thalidomide is an

angiogenesis inhibitor. Thalidomide is currently in clinical trials as an anti-cancer agent as well as a treatment of vascular eye diseases.

CAI is a small molecular weight synthetic inhibitor of angiogenesis that acts as a calcium channel blocker that prevents actin reorganization, endothelial cell migration and spreading on collagen IV. CAI inhibits neovascularization at physiological attainable concentrations and is well tolerated orally by cancer patients. Clinical trials with CAI have yielded disease stabilization in 49 % of cancer patients having progressive disease before treatment.

Cortisone in the presence of heparin or heparin fragments was shown to inhibit tumor growth in mice by blocking endothelial cell proliferation. The mechanism involved in the additive inhibitory effect of the steroid and heparin is unclear although it is thought that the heparin may increase the uptake of the steroid by endothelial cells. The mixture has been shown to increase the dissolution of the basement membrane underneath newly formed capillaries and this is also a possible explanation for the additive angiostatic effect. Heparin-cortisol conjugates also have potent angiostatic and anti-tumor effects activity *in vivo*.

Further specific angiogenesis inhibitors, including, but not limited to, Anti-Invasive Factor, retinoic acids and paclitaxel (U.S. Patent No. 5,716,981; incorporated herein by reference); AGM-1470 (Ingber *et al.*, 1990; incorporated herein by reference); shark cartilage extract (U.S. Patent No. 5,618,925; incorporated herein by reference); anionic polyamide or polyurea oligomers (U.S. Patent No. 5,593,664; incorporated herein by reference); oxindole derivatives (U.S. Patent No. 5,576,330; incorporated herein by reference); estradiol derivatives (U.S. Patent No. 5,504,074; incorporated herein by reference); and thiazolopyrimidine derivatives (U.S. Patent No. 5,599,813; incorporated herein by reference) are also contemplated for use as anti-angiogenic compositions for the combined uses of the present invention.

Compositions comprising an antagonist of an $\alpha_v\beta_3$ integrin may also be used to inhibit angiogenesis in combination with the present invention. As disclosed in U.S. Patent No. 5,766,591 (incorporated herein by reference), RGD-containing polypeptides and salts thereof, including cyclic polypeptides, are suitable examples of $\alpha_v\beta_3$ integrin antagonists.

The antibody LM609 against the $\alpha_v\beta_3$ integrin also induces tumor regressions. Integrin $\alpha_v\beta_3$ antagonists, such as LM609, induce apoptosis of angiogenic endothelial cells leaving the quiescent blood vessels unaffected. LM609 or other $\alpha_v\beta_3$ antagonists may also work by inhibiting the interaction of $\alpha_v\beta_3$ and MMP-2, a proteolytic enzyme thought to play an important role in migration of endothelial cells and fibroblasts.

Apoptosis of the angiogenic endothelium in this case may have a cascade effect on the rest of the vascular network. Inhibiting the tumor vascular network from completely responding to the tumor's signal to expand may, in fact, initiate the partial or full collapse of the network resulting in tumor cell death and loss of tumor volume. It is possible that endostatin and angiostatin function in a similar fashion. The fact that LM609 does not affect quiescent vessels but is able to cause tumor regressions suggests strongly that not all blood vessels in a tumor need to be targeted for treatment in order to obtain an anti-tumor effect.

Non-targeted angiopoietins, such as angiopoietin-2, may also be used in combination with the present invention. As described above in the context of targeted delivery, the angiogenic effects of various regulators involve an autocrine loop connected with angiopoietin-2. The use of angiopoietin-2, angiopoietin-1, angiopoietin-3 and angiopoietin-4, is thus contemplated in conjunction with the present invention. Other methods of therapeutic intervention based upon altering signaling through the Tie2 receptor can also be used in combination herewith, such as using a soluble Tie2 receptor capable of blocking Tie2 activation (Lin *et al.*, 1998). Delivery of such a construct using recombinant adenoviral gene therapy has been shown to be effective in treating cancer and reducing metastases (Lin *et al.*, 1998).

J3. Apoptosis-Inducing Agents

Therapeutic agent-targeting agent treatment may also be combined with treatment methods that induce apoptosis in any cells within the tumor, including tumor cells and tumor vascular endothelial cells. Although many anti-cancer agents may have, as part of their mechanism of action, an apoptosis-inducing effect, certain agents have been discovered, designed or selected with this as a primary mechanism, as described below.

A number of oncogenes have been described that inhibit apoptosis, or programmed cell death. Exemplary oncogenes in this category include, but are not limited to, bcr-abl, bcl-2 (distinct from bcl-1, cyclin D1; GenBank accession numbers M14745, X06487; U.S. Patent No. 5,650,491; and 5,539,094; each incorporated herein by reference) and family members including Bcl-xl, Mcl-1, Bak, A1, A20. Overexpression of bcl-2 was first discovered in T cell lymphomas. bcl-2 functions as an oncogene by binding and inactivating Bax, a protein in the apoptotic pathway. Inhibition of bcl-2 function prevents inactivation of Bax, and allows the apoptotic pathway to proceed. Thus, inhibition of this class of oncogenes, *e.g.*, using antisense nucleotide sequences, is contemplated for use in the present invention in aspects wherein enhancement of apoptosis is desired (U.S. Patent No. 5,650,491; 5,539,094; and 5,583,034; each incorporated herein by reference).

Many forms of cancer have reports of mutations in tumor suppressor genes, such as p53. Inactivation of p53 results in a failure to promote apoptosis. With this failure, cancer cells progress in tumorigenesis, rather than become destined for cell death. Thus, provision of tumor suppressors is also contemplated for use in the present invention to stimulate cell death. Exemplary tumor suppressors include, but are not limited to, p53, Retinoblastoma gene (Rb), Wilm's tumor (WT1), bax alpha, interleukin-1b-converting enzyme and family, MEN-1 gene, neurofibromatosis, type 1 (NF1), cdk inhibitor p16, colorectal cancer gene (DCC), familial adenomatosis

polyposis gene (FAP), multiple tumor suppressor gene (MTS-1), BRCA1 and BRCA2.

Preferred for use are the p53 (U.S. Patent No. 5,747,469; 5,677,178; and 5,756,455; each incorporated herein by reference), Retinoblastoma, BRCA1 (U.S. Patent No. 5,750,400; 5,654,155; 5,710,001; 5,756,294; 5,709,999; 5,693,473; 5,753,441; 5,622,829; and 5,747,282; each incorporated herein by reference), MEN-1 (GenBank accession number U93236) and adenovirus E1A (U.S. Patent No. 5,776,743; incorporated herein by reference) genes.

Other compositions that may be used include genes encoding the tumor necrosis factor related apoptosis inducing ligand termed TRAIL, and the TRAIL polypeptide (U.S. Patent No. 5,763,223; incorporated herein by reference); the 24 kD apoptosis-associated protease of U.S. Patent No. 5,605,826 (incorporated herein by reference); Fas-associated factor 1, FAF1 (U.S. Patent No. 5,750,653; incorporated herein by reference). Also contemplated for use in these aspects of the present invention is the provision of interleukin-1β-converting enzyme and family members, which are also reported to stimulate apoptosis.

Compounds such as carbostyril derivatives (U.S. Patent No. 5,672,603; and 5,464,833; each incorporated herein by reference); branched apogenic peptides (U.S. Patent No. 5,591,717; incorporated herein by reference); phosphotyrosine inhibitors and non-hydrolyzable phosphotyrosine analogs (U.S. Patent No. 5,565,491; and 5,693,627; each incorporated herein by reference); agonists of RXR retinoid receptors (U.S. Patent No. 5,399,586; incorporated herein by reference); and even antioxidants (U.S. Patent No. 5,571,523; incorporated herein by reference) may also be used. Tyrosine kinase inhibitors, such as genistein, may also be linked to ligands that target a cell surface receptor (U.S. Patent No. 5,587,459; incorporated herein by reference).

J4. Immunotoxins and Coaguligands

The anti-aminophospholipid-conjugate based treatment methods of the invention may be used in combination with other immunotoxins and/or coaguligands in which the targeting portion thereof, *e.g.*, antibody or ligand, is directed to a relatively specific marker of the tumor cells, tumor vasculature or tumor stroma. In common with the chemotherapeutic and anti-angiogenic agents discussed above, the combined use of other targeted toxins or coagulants will generally result in additive, markedly greater than additive or even synergistic anti-tumor results.

Generally speaking, antibodies or ligands for use in these additional aspects of the invention will preferably recognize accessible tumor antigens that are preferentially, or specifically, expressed in the tumor site. The antibodies or ligands will also preferably exhibit properties of high affinity; and the antibodies, ligands or conjugates thereof, will not exert significant *in vivo* side effects against life-sustaining normal tissues, such as one or more tissues selected from heart, kidney, brain, liver, bone marrow, colon, breast, prostate, thyroid, gall bladder, lung, adrenals, muscle, nerve fibers, pancreas, skin, or other life-sustaining organ or tissue in the human body. The term "significant side effects", as used herein, refers to an antibody, ligand or antibody conjugate, that, when administered *in vivo*, will produce only negligible or clinically manageable side effects, such as those normally encountered during chemotherapy.

At least one binding region of these second anti-cancer agents employed in combination with the invention will be a component that is capable of delivering a toxin or coagulation factor to the tumor region, *i.e.*, capable of localizing within a tumor site. Such targeting agents may be directed against a component of a tumor cell, tumor vasculature or tumor stroma. The targeting agents will generally bind to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor vasculature or tumor stroma. However, once tumor vasculature and tumor cell destruction begins, internal components will be released, allowing additional targeting of virtually any tumor component.

Many tumor cell antigens have been described, any one which could be employed as a target in connection with the combined aspects of the present invention. Appropriate tumor cell antigens for additional immunotoxin and coaguligand targeting include those recognized by the antibodies B3 (U.S. Patent 5,242,813; incorporated herein by reference; ATCC HB 10573); KSI/4 (U.S. Patent 4,975,369; incorporated herein by reference; obtained from a cell comprising the vectors NRRL B-18356 and/or NRRL B-18357); 260F9 (ATCC HB 8488); and D612 (U.S. Patent 5,183,756; incorporated herein by reference; ATCC HB 9796). One may also consult the ATCC Catalogue of any subsequent year to identify other appropriate cell lines producing anti-tumor cell antibodies.

For tumor vasculature targeting, the targeting antibody or ligand will often bind to a marker expressed by, adsorbed to, induced on or otherwise localized to the intratumoral blood vessels of a vascularized tumor. Appropriate expressed target molecules include, for example, endoglin, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA (Liu *et al.*, 1997), a TIE, a ligand reactive with LAM-1, a VEGF/VPF receptor, an FGF receptor, $\alpha_v\beta_3$ integrin, pleiotropin and endosialin. Suitable adsorbed targets are those such as VEGF, FGF, TGFβ, HGF, PF4, PDGF, TIMP, a ligand that binds to a TIE and tumor-associated fibronectin isoforms. Antigens naturally and artificially inducible by cytokines and coagulants may also be targeted, such as ELAM-1, VCAM-1, ICAM-1, a ligand reactive with LAM-1, endoglin, and even MHC Class II (cytokine-inducible, *e.g.*, by IL-1, TNF-α, IFN-γ, IL-4 and/or TNF-β); and E-selectin, P-selectin, PDGF and ICAM-1 (coagulant-inducible *e.g.*, by thrombin, Factor IX/IXa, Factor X/Xa and/or plasmin).

The following patents and patent applications are specifically incorporated herein by reference for the purposes of even further supplementing the present teachings regarding the preparation and use of immunotoxins directed against expressed, adsorbed, induced or localized markers of tumor vasculature: U.S. Patents Nos. 5,855,866; 5,776,427; 5,863,538; 5,660,827; 5,855,866 and 5,877,289; and U.S.

Applications Serial No. 07/846,349; 08/295,868 (Patent No. 5,___,___; Issue Fee paid); 08/350,212 (Patent No. 5,___,___; Issue Fee paid); 08/457,869 (Notice of Allowance Received); 08/482,369 (U.S. Patent 5,___,___; Issue Fee paid); 08/487,427 (U.S. Patent 5,___,___; Issue Fee paid); and 08/479,727 (U.S. Patent 5,___,___; Issue Fee paid).

Suitable tumor stromal targets include components of the tumor extracellular matrix or stroma, or components those bound therein; including basement membrane markers, type IV collagen, laminin, heparan sulfate, proteoglycan, fibronectins, activated platelets, LIBS and tenascin. A preferred target for such uses is RIBS.

The following patents and patent applications are specifically incorporated herein by reference for the purposes of even further supplementing the present teachings regarding the preparation and use of tumor stromal targeting agents: U.S. Patent No. 5,877,289 and U.S. Applications Serial Nos. 08/482,369 (U.S. Patent 5,___,___; Issue Fee paid); 08/487,427 (U.S. Patent 5,___,___; Issue Fee paid); and 08/479,727 (U.S. Patent 5,___,___; Issue Fee paid).

The second anti-cancer therapeutics may be operatively attached to any of the cytotoxic or otherwise anti-cellular agents described herein for use in the anti-aminophospholipid immunotoxins. However, suitable anti-cellular agents also include radioisotopes. Toxin moieties will be preferred, such as ricin A chain and deglycosylated A chain (dgA) or even gelonin. Any one or more of the angiopoietins, or fusions thereof, may also be used as part of a second immunoconjugate for combined therapy.

The second, targeted agent for optional use with the invention may comprise a targeted component that is capable of promoting coagulation, *i.e.*, a coaguligand. Here, the targeting antibody or ligand may be directly or indirectly, *e.g.*, via another antibody, linked to any factor that directly or indirectly stimulates coagulation, including any of those described herein for use in the anti-aminophospholipid

coaguligands. Preferred coagulation factors for such uses are Tissue Factor (TF) and TF derivatives, such as truncated TF (tTF), dimeric and multimeric TF, and mutant TF deficient in the ability to activate Factor VII.

Effective doses of immunotoxins and coaguligands for combined use in the treatment of cancer will be between about 0.1 mg/kg and about 2 mg/kg, and preferably, of between about 0.8 mg/kg and about 1.2 mg/kg, when administered via the IV route at a frequency of about 1 time per week. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The physician responsible for administration will determine the appropriate dose for the individual subject.

* * *

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

VCAM-1 Expression on Tumor and Normal Blood Vessels

A. Materials and Methods

1. Materials

$Na^{125}I$ was obtained from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's tissue culture medium (DMEM) and Dulbecco PBS containing Ca^{2+} and Mg^{2+} were obtained from Gibco (Grand Island, NY). Fetal calf serum was

obtained from Hyclone (Logan, Utah). O-phenylenediamine, hydrogen peroxide, 3-aminopropyltriethoxy-silane and sterile, endotoxin-free saline (0.9% NaCl in 100 ml of water) were from Sigma (St. Louis, MO). SMPT was from Pierce (Rockford, IL). Proplex T containing factor VII (74 IU/ml), factor X and factor IX (17 IU/ml) was purchased from Baxter Diagnostics Inc. (McGraw Park, IL). Chromogenic substrate, S-2765, for measuring factor Xa proteolytic activity was obtained from Chromogenix (Franklin, OH). Purified factor Xa was purchased from American Diagnostica (Greenwich, CT). 96 and 48 flat bottom microtiter plates were obtained from Falcon (Becton Dickinson and Co., Lincoln Park, NJ). Sepharose-Protein G beads and S200 Superdex were purchased from Pharmacia (Piscataway, NJ). Recombinant murine IL-1α was purchased from R&D Systems (Minneapolis, MN).

2. Antibodies

The MK2.7 hybridoma, secreting a rat IgG1 antibody against murine VCAM-1, was obtained from the American Type Culture Collection (ATCC, Rockville, MD; ATCC CRL 1909). The characterization of this anti-VCAM-1 antibody has been reported by Miyake *et al.* (1991, incorporated herein by reference). The R187 hybridoma, secreting a rat IgG1 antibody against murine viral protein p30 gag, was also obtained from the ATCC, and was used as an isotype matched control for the anti-VCAM-1 antibody.

Mouse monoclonal antibody, 10H10, against human tissue factor was prepared as described in Morrissey *et al.* (1988), and in U.S. application Serial No. 08/482,369, each incorporated herein by reference.

MECA 32, a pan anti-mouse vascular endothelial cell antibody, was prepared as described by Leppink *et al.* (1989, incorporated herein by reference). MJ 7/18 rat IgG, reactive with murine endoglin, was prepared as described by Ge and Butcher (1994, incorporated herein by reference). The MECA 32 and MJ 7/18 antibodies served as positive controls for immunohistochemical studies.

Rabbit anti-rat and rat anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Dako (Carpinteria, CA).

3. Antibody Purification

Anti-VCAM-1 hybridoma, MK 2.7, and the irrelevant control hybridoma, R187, were grown in bioreactors (Heraeus, Inc., Germany) for 12 days. Supernatants were centrifuged, filtered through 0.22 μm filters and loaded onto Sepharose-Protein G columns. IgG was eluted with citric acid buffer, pH 3.5, dialyzed into PBS and stored thereafter at 4°C in the same buffer. Purity was estimated by SDS-PAGE and was routinely > 90%. Binding capacity of the purified anti-VCAM-1 antibody was assessed immunohistochemically on frozen sections of L540 tumor and by cell-based ELISA using IL-1α stimulated bEnd.3 cells, as described herein below.

4. Tumor-Bearing Mice and Immunohistochemistry

Male CB17 SCID mice (Charles River, Wilmington, MA) weighing approximately 25 g were injected with 1×10^7 L540 Hodgkin's lymphoma cells subcutaneously into the right flank. Tumors were allowed to grow to a size of 0.4-0.7 cm^3. Animals were anesthetized with metafane and their blood circulation was perfused with heparinized saline as described by Burrows *et al.* (1992, incorporated herein by reference). The tumor and major organs were removed and snap-frozen in liquid nitrogen.

Cryostat sections of the tissues were cut, incubated with the anti-VCAM-1 antibody and stained immunohistochemically to detect VCAM-1. Rat IgG was detected using rabbit anti-rat IgG conjugated to HRP followed by development with carbazole (Fries *et al.*, 1993).

B. Results

The blood vessels of the major organs and a tumor from mice bearing subcutaneous L540 human Hodgkin's tumors were examined immunohistochemically for VCAM-1 expression using an anti-VCAM-1 antibody. VCAM-1 expression on

tumor blood vessels was more peripheral than central. However, as demonstrated in Example VI and Example VII, the anti-VCAM-1 antibody and coaguligand were evidently binding to blood transporting vessels, as clearly shown by the ability of the coaguligand to arrest blood flow in all tumor regions and to cause destruction of the intratumoral region.

Overall, VCAM-1 expression was observed on 20-30% of total tumor blood vessels stained by the anti-endoglin antibody, MJ 7/18. VCAM-1 staining of the tumor vessels was largely observed on venules. VCAM-1 expression was similar in tumors up to 1500 mm^3, but larger tumors appeared to have reduced staining, with 5-10% of MJ 7/18 positive vessels being positive for VCAM-1.

Constitutive vascular expression of VCAM-1 was found in heart and lungs in both tumor-bearing and normal animals (Table 1). In the heart, strong staining was observed on venules and veins. Approximately 10% of MECA 32 positive vessels were positive for VCAM-1. Staining in lung endothelium was weak in comparison to heart and tumor, and was confined to a few large blood vessels. Strong stromal staining was observed in testis where VCAM-1 expression was strictly extravascular. Similar findings regarding constitutive VCAM-1 expression in rodent lung and testis were previously reported (Fries *et al.*, 1993).

TABLE 1

Expression of VCAM-1 on Endothelium in Tissues of L540 Tumor Bearing Mice and Localization of Anti-VCAM-1 Antibody

Tissue	VCAM-1 expression[a]	anti-VCAM-1 antibody localization[b]
Adrenal	-[c]	-
Brain Cerebellum	-	-
Brain Cortex	-	-
Heart	++	++
Kidney	-	-
Large Intestine	-	-
Liver	-	-
Lung	+	+
Pancreas	-	-
Small Intestine	-	-
Spleen	-	-
Testis	-[d]	-
L540 Hodgkin's tumor	+++	+++

[a] VCAM-1 was detected by anti-VCAM-1 antibody followed by anti-rat IgG-HRP.

[b] Localization of anti-VCAM-1 antibody *in vivo* was determined by injecting the antibody, exsanguinating the mice and staining tissues staining with anti-rat IgG-HRP.

[c] Intensity of staining was compared to pan-endothelial markers MJ 7/18 and MECA 32; - no staining; + weak; ++ moderate; +++ strong.

[d] No vascular expression was observed; however, extravascular stroma of testis was stained by anti-VCAM-1 antibody.

EXAMPLE II

Localization of Anti-VCAM-1 Antibody *In Vivo*

A. Methods

Male CB17 SCID mice (Charles River, Wilmington, MA) weighing approximately 25 g were injected with 1×10^7 L540 Hodgkin's lymphoma cells subcutaneously into the right flank. Tumors were allowed to grow to a size of 0.4-0.7 cm^3.

Mice were injected intravenously with 30 μg/25 g body weight of anti-VCAM-1 antibody, R187 antibody or corresponding coaguligands in 200 μl of saline. Two hours later, animals were anesthetized with metafane and their blood circulation was perfused with heparinized saline as described (Burrows *et al.*, 1992; incorporated herein by reference). The tumor and major organs were removed and snap-frozen in liquid nitrogen.

Cryostat sections of the tissues were cut and were stained immunohistochemically for the presence of rat IgG or TF. Rat IgG was detected using rabbit anti-rat IgG conjugated to HRP followed by development with carbazole (Fries *et al.*, 1993). Coaguligand was detected using the 10H10 antibody that recognizes human tissue factor, followed by HRP-labeled anti-mouse IgG. 10H10 antibody does not cross-react detectably with murine tissue factor (Morrissey *et al.*, 1988, incorporated herein by reference) or other murine proteins.

B. Results

Mice bearing subcutaneous L540 tumors were injected intravenously with anti-VCAM-1 antibody and, two hours later, the mice were exsanguinated. The tumor and normal organs were removed and frozen sections were prepared and examined immunohistochemically to determine the location of the antibody. Serial sections of the tissues were examined. Localized rat IgG was detected by HRP-labeled anti-rat Ig; and murine blood vessels were identified by pan-endothelial antibody, MECA 32.

Anti-VCAM-1 antibody was detected on endothelium of tumor, heart and lung (Table 1). The intensity and number of stained vessels was identical to that on serial sections of the same tissues stained directly with anti-VCAM-1 antibody (Table 1). Staining was specific as no staining of endothelium was observed in the tumor and organs of mice injected with a species isotype matched antibody of irrelevant specificity, R187. No localization of anti-VCAM-1 antibody was found in testis or any normal organ except heart and lung.

EXAMPLE III

Preparation of Anti-VCAM-1•tTF Coaguligand

An anti-VCAM-1•tTF conjugate or "coaguligand" was prepared as follows. Truncated tissue factor (tTF), with an additional added cysteine introduced at N-terminus (U.S. application Serial No. 08/482,369, incorporated herein by reference), was expressed in *E. coli* and purified as described by Stone *et al.* (1995, incorporated herein by reference). After purification, the sulfhydryl group of N' cysteine-tTF was protected by reaction with Ellman's reagent. The tTF derivative was stored in small volumes at -70°C.

To prepare the anti-VCAM-1 coaguligand, 5 ml of anti-VCAM-1 antibody IgG (2 mg/ml) in PBS were mixed with 36 µl of SMPT (10 mM) dissolved in dry DMF and incubated at room temperature for 1 h. The mixture was filtered through a column of Sephadex G25 equilibrated in PBS containing 1 mM EDTA. The fractions containing the SMPT-derivatized antibody were concentrated to 4 ml by ultrafiltration in an Amicon cell equipped with a 10,000 Da cut-off filter. Freshly thawed tTF derivative was incubated with 30 µl of DTT (10 mM) in H_20 for 10 min. at room temperature and was filtered through a column of Sephadex G25 equilibrated in PBS containing 1 mM EDTA. The eluted fractions containing reduced tTF were concentrated by ultrafiltration under nitrogen to a final volume of 3 ml.

The reduced tTF was mixed with the SMPT-derivatized antibody and the mixture was allowed to react for 24 h at room temperature. At the end of the

incubation, the reaction mixture was resolved by gel filtration on a column of Superdex S200 equilibrated in PBS. Fractions containing anti-VCAM-1•tTF having a M_r of 180,000 and corresponding to one molecule of antibody linked to one molecule of tTF were collected.

EXAMPLE IV

Binding of Anti-VCAM-1 Coaguligand to Activated Endothelial Cells

A. Methods

1. Iodination of 10H10 antibody

Anti-human tissue factor antibody, 10H10, was radiolabeled with ^{125}I using Chloramine T as described by Bocci (1964, incorporated herein by reference). The specific activity was approximately 10,000 cpm/μg, as calculated from protein determinations measured by a Bradford assay (Bradford, 1976).

2. Cells

L540 Hodgkin cells (L540 Cy), derived from a patient with end-stage disease, were prepared as described in Diehl *et al.* (1985, incorporated herein by reference), and were obtained from Prof. Volker Diehl (Klinik fur Innere Medizin der Universitaet, Köeln, Germany). bEnd.3 cells (murine brain endothelioma) were prepared as described in Bussolino *et al.* (1991) and Montesano *et al.* (1990), each incorporated herein by reference, and were obtained from Prof. Werner Risau (Max Planck Institute, Bäd Nauheim, Germany).

3. Tissue Culture

bEnd.3 cells and hybridomas were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 units/ml penicillin G and 2 μg/ml streptomycin. L540 cells were maintained in RPMI 1640 containing the same additives. All cells were subcultured once a week. bEnd.3 trypsinization was performed using 0.125% trypsin in PBS solution containing 0.2% EDTA. For binding studies, cells were seeded at a density of 5×10^4 cells/ml in 0.5 ml of medium

in 48 well plates and incubated for 48-96 h. Medium was refreshed 24 h before each study.

4. Binding of Coaguligand to Activated Endothelial Cells

Binding of the anti-VCAM-1 antibody and coaguligand to VCAM-1 on activated bEnd.3 cells was determined using a cell based ELISA, as described by Hahne (1993, incorporated herein by reference). bEnd.3 cells were incubated with 10 units/ml of IL-1α for 4 h at 37°C in 96-well microtiter plates. At the end of this incubation, medium was replaced by DPBS containing 2 mM Ca^{2+} and Mg^{2+} and 0.2% (w/v) gelatin as a carrier protein. The same buffer was used for dilution of antibodies and for washing of cell monolayers between steps.

Cells were incubated with 4 μg/ml of anti-VCAM-1•tTF conjugate, anti-VCAM•1 antibody or control reagents for 2 h, and were then washed and incubated for 1 h with rabbit anti-rat IgG-HRP conjugate (1:500 dilution). All steps were performed at room temperature. HRP activity was measured by adding O-phenylenediamine (0.5 mg/ml) and hydrogen peroxide (0.03% w/v) in citrate-phosphate buffer, pH 5.5. After 30 min., 100 μl of supernatant were transferred to 96 well plates, 100 μl of 0.18 M H_2SO_4 were added and the absorbance was measured at 492 nm. Each study was performed in duplicate and repeated at least twice.

5. Detection of Coaguligand Bound to Endothelial cells

Anti-VCAM-1 coaguligand and appropriate controls were incubated with IL-1α stimulated bEnd.3 cells in 96-well microtiter plates, as described above. Bound coaguligands were detected by identifying both the tissue factor component and the rat IgG component bound to bEnd.3 cells.

After removing the excess of unbound antibody, cells were incubated with 100 μl/well of ^{125}I-labeled 10H10 antibody (0.2 μg/ml) or ^{125}I-labeled rabbit anti-rat Ig (0.2 μg/ml) in binding buffer. After 2 h incubation at room temperature, cells were washed extensively and dissolved in 0.5 M of NaOH. The entire volume of 0.5 ml

was transferred to plastic tubes and counted in a γ counter. Each study was performed in duplicate and repeated at least twice.

B. Results

The ability of an anti-VCAM-1•tTF coaguligand to bind to IL-1α activated murine bEnd.3 cells was determined by measuring the binding of radioiodinated anti-TF antibody to coaguligand-treated cells *in vitro*. VCAM-1 expression by bEnd.3 cells is transiently inducible by IL-1α with a peak of VCAM-1 expression being obtained 4-6 h after addition of the cytokine (Hahne *et al.*, 1993). Strong binding of the coaguligand to activated bEnd.3 cells was observed (FIG. 1A).

At saturation, 8.7 fmoles of anti-TF antibody was bound to the cells, which is equivalent to 540,000 molecules of anti-TF antibody per cell. Binding of the coaguligand was specific; no detectable binding over background was observed with an isotype matched control coaguligand of irrelevant specificity. Binding of coaguligand to unstimulated cells was about half of that to activated cells and is probably attributable to constitutive VCAM-1 expression by cultured endothelioma cells.

In further studies, the anti-VCAM-1•tTF coaguligand was found to bind as strongly as unconjugated anti-VCAM-1 antibody to activated bEnd.3 cells, using detection by peroxidase-labeled anti rat IgG in the assay. This was done at both saturating and subsaturating concentrations. Thus, the conjugation procedure (Example III) did not diminish antibody's capacity to bind to VCAM-1 on intact endothelial monolayers.

EXAMPLE V

Factor X Activation by Endothelial Cell-Bound Coaguligand

A. Methods

The activity of the anti-VCAM-1•tTF coaguligand bound to activated bEnd.3 cells was determined indirectly by using a chromogenic assay to detect factor Xa (Schorer *et al.*, 1985; Nawroth *et al.*, 1985; each incorporated herein by reference). IL-1α-stimulated and unstimulated bEnd.3 cells were incubated with specific and control coaguligands in 96-well microtiter plates as described above. The cells were washed with PBS containing 2 mg/ml of BSA and were incubated with 150 µl/well of freshly prepared Proplex T solution diluted 1:20 in 50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mg/ml BSA (tissue culture grade, endotoxin-free) and 2.5 mM $CaCl_2$. After incubation for 60 min. at 37°C, 100 µl were withdrawn from each well, transferred to 96-well plates and mixed with 100 µl of the same buffer containing 12.5 mM EDTA (pH 8.1).

Chromogenic substrate S2765 for measuring factor Xa protcolytic activity was added in 50 µl, giving a final concentration of 300 µM. The breakdown of the substrate was determined by reading the absorbance at 405 nm over a 2 h period in a microplate reader (Molecular Devices, Palo Alto, CA).

Production of the chromogenic product was completely dependent on the presence of Proplex T and bEnd.3 cells preincubated with the specific coaguligand. Background hydrolysis of the substrate by Proplex T in the absence of cells was approximately 10% of the maximal value and was subtracted from each determination. Free coaguligands diluted in Proplex T solution were unable to generate factor Xa. The amount of Xa generated was calculated by reference to a standard curve constructed with known concentrations of purified factor Xa.

At the end of the study, cells were detached with trypsin-EDTA and counted. The results are expressed as the amount of factor Xa generated per 10^4 cells. Each study was performed in duplicate and was repeated at least 3 times.

B. Results

1. Factor X Activation

Anti-VCAM-1•tTF coaguligand bound to IL-1α-activated bEnd.3 cells was capable of specifically activating factor X. The rate of generation of factor Xa by anti-VCAM-1•tTF coated cells was 3.2 ng per 10^4 cells per hour, which is 7-10 fold higher than was observed with activated cells treated with a control coaguligand of irrelevant specificity or with tTF alone (FIG. 1B). Anti-VCAM-1•tTF in the absence of cells had undetectable factor X activating activity, confirming that cell binding is essential for coaguligand activity.

Anti-VCAM-1•tTF bound to unstimulated bEnd.3 cells activated factor X at a rate of 1.6 ng per 10^4 cells per hour. This rate is about half that observed with the IL-1α-stimulated cells, in accordance with the 50% lower amount of coaguligand that binds to unstimulated as compared with stimulated cells. Similar results to those shown in FIG. 1B were obtained in three separate studies.

2. Effect of Endothelial Cell Permeabilization

Permeabilization of bEnd.3 monolayers with saponin after treating them with anti-VCAM-1•tTF coaguligand increased the ability of the bound coaguligand to activate factor X by about 30-fold (Table 2). The rate of factor Xa generation by unstimulated cells treated with anti-VCAM-1•tTF increased from 1.6 to 49.2 ng per 10^4 cells per hour after permeabilization, while that of IL-1α stimulated cells increased from 3.2 to 98.8 ng per 10^4 cells per hour. The factor Xa generating activity of the permeabilized cells was due to the bound coaguligand rather than to endogenous TF since permeabilized untreated cells or cells treated with control coaguligand of irrelevant specificity had low factor Xa generating activity (2 ng per 10^4 cells per hour).

These results indicate that the coaguligand is able to function more efficiently in the environment of a permeabilized cell. Possibly, permeabilization exposes

negatively-charged phospholipids from within the cell that accelerate the formation of the coagulation-initiation complexes, or else prevents the inactivation of such complexes by TFPI.

TABLE 2

Generation of Factor Xa by Anti-VCAM-1•tTF Bound to Intact or Permeabilized bEnd.3 cells (ng per 10^4 cells per 60 min.)

Treatment[a]	Treatment[a]		Permeabilized cells[b]	
	Control	IL-1α	Control	IL-1α
Buffer	0.25[c]	0.43	0.45	2.0
tTF	0.26	0.42	0.39	2.1
Control IgG•tTF	0.26	0.43	0.41	2.1
Anti-VCAM-1•tTF	1.64	3.17	49.2	98.8

[a] IL-1α stimulated and unstimulated bEnd.3 cells were incubated with buffer alone or with 4 μg/ml of tTF, control IgG•tTF or anti-VCAM-1•tTF followed by 60 min. incubation with Proplex T solution at 37°C.

[b] Cells were treated with 0.2% saponin 5 min. before addition of Proplex T.

[c] Amount of factor Xa was determined as described above. Results are expressed as ng of factor Xa generated per 10^4 cells per 60 min. The arithmetic mean values from triplicate wells are shown. SE were less than 5 percent of the mean values.

EXAMPLE VI

Tumor Blood Vessel Thrombosis by Anti-VCAM-1 Coaguligand

A. Methods

SCID mice bearing L540 tumors (0.4-0.7 cm^3) were injected intravenously with 40 μg (total protein) of anti-VCAM-1•tTF or R187•tTF. This dose corresponds to 32 μg of antibody and 8 μg of tTF. Other animals received equivalent quantities of free antibody, free tTF or a mixture of both. Animals were anesthetized 4 or 24 h later and their blood circulations were perfused with heparinized saline. The tumor and

major organs were removed and were fixed in formalin and paraffin-embedded or snap-frozen for cryosectioning. Sections were cut through the center of the tissue or tumor. The number of thrombosed and non-thrombosed blood vessels in 5 cross-sections were counted. The percentage of thrombosed vessels was calculated.

B. Results

1. Thrombosis of Tumor Blood Vessels

This study shows that intravenous administration of the anti-VCAM-1•tTF coaguligand induces selective thrombosis of tumor blood vessels, as opposed to vessels in normal tissues, in tumor-bearing mice.

The anti-VCAM-1•tTF coaguligand was administered to mice bearing subcutaneous L540 tumors of 0.4 to 0.6 cm in diameter. Before coaguligand injection, tumors were healthy, having a uniform morphology lacking regions of necrosis. The tumors were well vascularized and had a complete absence of spontaneously thrombosed vessels or hemorrhages. Within four hours of coaguligand injection, 40-70% of blood vessels were thrombosed, despite the initial staining of only 20-30% of tumor blood vessels shown in Example I. The thrombosed vessels contained occlusive platelet aggregates, packed erythrocytes and fibrin. In several regions, the blood vessels had ruptured, spilling erythrocytes into the tumor interstitium.

By 24 h after coaguligand injection, the blood vessels were still occluded and extensive hemorrhage had spread throughout the tumor. Tumor cells had separated from one another, had pyknotic nuclei and were undergoing cytolysis. By 72 h, advanced necrosis was evident throughout the tumor. Necrosis was clearly present in the intratumoral region of the tumor, where VCAM-1 expression on the vessels was not originally prominent. The coaguligand binding was evidently effective to curtail blood flow in all tumor regions, resulting in widespread tumor destruction. Furthermore, it is likely that the initial coaguligand-induced thrombin deposition

results in increased induction of the VCAM-1 target antigen on central vessels, thus amplifying targeting and tumor destruction.

The thrombotic action of anti-VCAM-1•tTF on tumor vessels was antigen specific. None of the control reagents administered at equivalent quantities (tTF alone, anti-VCAM-1 antibody alone, tTF plus anti-VCAM-1 antibody or the control coaguligand of irrelevant specificity) caused thrombosis (Table 3).

TABLE 3

Anti-VCAM-1•tTF-Mediated Thrombosis in L540 Tumor Bearing Mice

Treatment[a]	Thrombosed Vessels (%)[b]		
	L540 Tumor	Heart and Lung	Other Organs
Saline	0-2	0	0
tTF	0-2	0	0
Anti-VCAM-1 Antibody	0-2	0	0
Anti-VCAM-1 Antibody + tTF	0-2	0	0
Control IgG•tTF	0-2	0	0
Anti-VCAM-1•tTF (< 0.3 cm3)[c]	0-10	0	0
Anti-VCAM-1•tTF (> 0.3 cm^3)	40-70	0	0

[a] L540 tumor-bearing mice were injected i.v. with one of the following reagents: saline; 8 μg of unconjugated tTF; 32 μg of unconjugated anti-VCAM-1 antibody; mixture of 8 μg of tTF and 32 μg of anti-VCAM-1 antibody; 40 μg of control IgG•tTF coaguligand; or 40 μg of anti-VCAM-1•tTF coaguligand. Animals were sacrificed 4 h after injection. Tissues were removed and fixed in formalin.

[b] Histological quantification was performed by counting numbers of thrombosed blood vessels in 5 cross sections of tissue. The number of thrombosed vessels is expressed as a percentage of total vessels. The range of results from three mice is given.

[c] L540 tumor bearing mice were divided into two groups (5-8 animals per group) having tumors smaller or larger than 0.3 cm^3.

2. Lack of Thrombosis of Normal Blood Vessels

In addition to the thrombosis of tumor blood vessels, this study also shows that intravenous administration of the anti-VCAM-1•tTF coaguligand does not induce thrombosis of blood vessels in normal organs.

Despite expression of VCAM-1 on vessels in the heart and lung of normal or L540 tumor-bearing mice (Table 1), thrombosis did not occur after anti-VCAM-1•tTF coaguligand administration. No signs of thrombosis, tissue damage or altered morphology were seen in 25 mice injected with 5 to 45 μg of coaguligand 4 or 24 h earlier. There was a normal histological appearance of the heart and lung from the same mouse that had major tumor thrombosis. All other major organs (brain, liver, kidney, spleen, pancreas, intestine, testis) also had unaltered morphology.

Frozen sections of organs and tumors from coaguligand-treated mice gave coincident staining patterns when developed with either the anti-TF antibody, 10H10, or an anti-rat IgG antibody and confirmed that the coaguligand had localized to vessels in the heart, lung and tumor. The intensity of staining was equal to that seen when coaguligand was applied directly to the sections at high concentrations followed by development with anti-TF or anti-rat IgG, indicating that saturation of binding had been attained *in vivo*.

These studies show that binding of coaguligand to VCAM-1 on normal vasculature in heart and lung is not sufficient to induce thrombosis, and that tumor vasculature provides additional factors to support coagulation.

EXAMPLE VII

In Vivo Tumor Destruction by Anti-VCAM-1 Coaguligand

A. Methods

Male CB17 SCID mice were injected subcutaneously with 1×10^7 L540 cells as described above. When the tumors had reached a volume of 0.4-0.6 cm^3, the mice were injected intravenously with either 20 μg of anti-VCAM-1•tTF, 16 μg anti-VCAM-1 antibody, 4 μg tTF, a mixture of 16 μg of anti-VCAM-1 antibody and 4 μg of tTF, 20 μg control IgG•tTF or saline. In some studies, the treatment was given 3 times, on days 0, 4 and 8. A minimum of 8 animals were treated in each group.

Animals were monitored daily for tumor measurements and body weight. Mice were sacrificed when tumors had reached a diameter of 2 cm^3, or earlier if tumors showed signs of necrosis or ulceration. Tumor volume was calculated according to the formula: $\pi/6 \times D \times d^2$, where D is the larger tumor diameter and d is the smaller diameter. Differences in tumor growth rates were tested for statistical significance using a non-parametric test (Mann-Whitney rank sum test) that makes no assumptions about tumor size being normally distributed (Gibbons, 1976).

B. Results

The anti-tumor activity of anti-VCAM-1•tTF coaguligand was determined in SCID mice bearing 0.3-0.4 cm^3 L540 tumors. The drug was administered i.v. 3 times at intervals of 4 days. The pooled results from 3 separate studies are presented in FIG. 2 and Table 4. Mean tumor volume of anti-VCAM-1•tTF treated mice was significantly reduced at 21 days of treatment ($P < 0.001$) in comparison to all other groups. Nine of a total of 15 mice treated with the specific coaguligand showed more than 50% reduction in tumor volume. This effect was specific since unconjugated tTF, control IgG coaguligand and mixture of free anti-VCAM-1 antibody and tTF did not affect tumor growth.

TABLE 4

Inhibition of Tumor Growth by Anti-VCAM-1•tTF Coaguligand

Treatment[a]	n	Mean tumor volume (mm^3)[b]		Tumor Growth Index[c]	P versus saline[d]
		Day 0	Day 21		
Saline	14	331 ± 61	2190 ± 210	6.91	-
TTF	13	341 ± 22	2015 ± 205	5.90	NS
Anti-VCAM-1	16	363 ± 24	1920 ± 272	5.28	NS
Anti-VCAM-1+tTF	13	349 ± 42	2069 ± 362	5.92	NS
Control IgG•tTF	8	324 ± 30	2324 ± 304	7.17	NS
Anti-VCAM-1•tTF	15	365 ± 28	1280 ± 130	3.50	< 0.001

[a] L540 tumor bearing mice were injected i.v. with one of the following reagents: saline; 8 μg of unconjugated tTF; 32 μg of unconjugated anti-VCAM-1 antibody; mixture of 8 μg of tTF and 32 μg of anti-VCAM-1 antibody; 40 μg of control IgG•tTF (R187) coaguligand; or 40 μg of anti-VCAM-1•tTF coaguligand. The treatment was repeated on day 4 and 7 after first injection.

[b] Mean tumor volume ± SD.

[c] The tumor growth index is the ratio of mean tumor volume on day 21 to mean tumor volume on day 0.

[d] Two tailed P values are for differences in tumor volume (day 21) for the treated groups versus the saline group as determined by the Mann-Whitney rank sum test.

EXAMPLE VIII

Phosphatidylserine Expression on Tumor Blood Vessels

A. Methods

1. Antibodies

Anti-phosphatidylserine (anti-PS) and anti-cardiolipin antibodies, both mouse monoclonal IgM antibodies, were produced as described by Rote (Rote *et al.*, 1993). Details of the characterization of the anti-PS and anti-cardiolipin antibodies were also reported by Rote *et al.* (1993, incorporated herein by reference).

2. **Detection of PS Expression on Vascular Endothelium**

L540 tumor-bearing mice were injected i.v. with 20 μg of either anti-PS or anti-cardiolipin mouse IgM antibodies. After 10 min., mice were anesthetized and their blood circulations were perfused with heparinized saline. Tumors and normal tissues were removed and snap-frozen. Serial sections of organs and tumors were stained with either HRP-labeled anti-mouse IgM for detection of anti-PS antibody or with anti-VCAM-1 antibody followed by HRP-labeled anti-rat Ig.

To preserve membrane phospholipids on frozen sections, the following protocol was developed. Animals were perfused with DPBS containing 2.5 mM Ca^{2+}. Tissues were mounted on 3-aminopropyltriethoxysilane-coated slides and were stained within 24 h. No organic solvents, formaldehyde or detergents were used for fixation or washing of the slides. Slides were re-hydrated by DPBS containing 2.5 mM Ca^{2+} and 0.2% gelatin. The same solution was also used to wash sections to remove the excess of reagents. Sections were incubated with HRP-labeled anti-mouse IgM for 3.5 h at room temperature to detect anti-PS IgM.

B. Results

To explain the lack of thrombotic effect of anti-VCAM-1•tTF on VCAM-1 positive vasculature in heart and lungs, the inventors developed a concept of differential PS localization between normal and tumor blood vessels. Specifically, they hypothesized that endothelial cells in normal tissues segregate PS to the inner surface of the plasma membrane phospholipid bilayer, where it is unable to participate in thrombotic reactions; whereas endothelial cells in tumors translocate PS to the external surface of the plasma membrane, where it can support the coagulation action of the coaguligand. PS expression on the cell surface allows coagulation because it enables the attachment of coagulation factors to the membrane and coordinates the assembly of coagulation initiation complexes (Ortel *et al.*, 1992).

The inventors' model of PS translocation to the surface of tumor blood vessel endothelial cells, as developed herein, is surprising in that PS expression does not

occur after, and does not inevitably trigger, cell death. PS expression at the tumor endothelial cell surface is thus sufficiently stable to allow PS to serve as a targetable entity for therapeutic intervention.

To confirm the hypothesis that tumor blood vessel endothelium expresses PS on the luminal surface of the plasma membrane, the inventors used immunohistochemistry to determine the distribution of anti-PS antibody after intravenous injection into L540 tumor bearing mice. Anti-PS antibody localized within 10 min. to the majority of tumor blood vessels, including vessels in the central region of the tumor that can lack VCAM-1. Vessels that were positive for VCAM-1 were also positive for PS. Thus, there is coincident expression of PS on VCAM-1-expressing vessels in tumors.

In the *in vivo* localization studies, none of the vessels in normal organs, including VCAM-1-positive vasculature of heart and lung, were stained, indicating that PS is absent from the external surface of the endothelial cells. In contrast, when sections of normal tissues and tumors were directly stained with anti-PS antibody *in vitro*, no differences were visible between normal and tumor, endothelial or other cell types, showing that PS is present within these cells but only becomes expressed on the surface of endothelial cells in tumors.

The specificity of PS detection was confirmed by two independent studies. First, a mouse IgM monoclonal antibody directed against a different negatively charged lipid, cardiolipin, did not home to tumor or any organs *in vivo*. Second, pretreatment of frozen sections with acetone abolished staining with anti-PS antibody, presumably because it extracted the lipids together with the bound anti-PS antibody.

EXAMPLE IX

Annexin V Blocks Coaguligand Activation of Factor X *In Vitro*

A. Methods

The ability of Annexin V to affect Factor Xa formation induced by coaguligand was determined by a chromogenic assay described above in Example V. IL-1α-stimulated bEnd.3 cells were incubated with anti-VCAM-•tTF and permeabilized by saponin. Annexin V was added at concentrations ranging from 0.1 to 10 μg/ml and cells were incubated for 30 min. before addition of diluted Proplex T. The amount of Factor Xa generated in the presence or absence of Annexin V was determined as described in Example V. Each treatment was performed in duplicate and repeated at least twice.

B. Results

The need for surface PS expression in coaguligand action is further indicated by the inventors' finding that annexin V, which binds to PS with high affinity, blocks the ability of anti-VCAM-1•tTF bound to bEnd.3 cells to generate factor Xa *in vitro*.

Annexin V added to permeabilized cells preincubated with anti-VCAM-1•tTF inhibited the formation of factor Xa in a dose-dependent manner (FIG. 3). In the absence of Annexin V, cell-bound coaguligand produced 95 ng of factor Xa per 10,000 cells per 60 min. The addition of increasing amounts of Annexin V (in the μg per ml range) inhibited factor Xa production. At 10 μg per ml, Annexin V inhibited factor Xa production by 58% (FIG. 3). No further inhibition was observed by increasing the concentration of Annexin V during the assay, indicating that annexin V saturated all available binding sites at 10 μg per ml.

EXAMPLE X

Annexin V Blocks Coaguligand Activity *In Vivo*

A. Methods

The ability of Annexin V to inhibit coaguligand-induced thrombosis *in vivo* was examined in L540 Hodgkin-bearing SCID mice. Tumors were grown in mice as described above in Example II. Two mice per group (tumor size 0.5 cm in diameter) were injected intravenously via the tail vein with one of the following reagents: a) saline; b) 100 μg of Annexin V; c) 40 μg of anti-VCAM-1•tTF; d) 100 μg of Annexin V followed 2 hours later by 40 μg of anti-VCAM-1•tTF.

Four hours after the last injection mice were anesthetized and perfused with heparinized saline. Tumors were removed, fixed with 4% formalin, paraffin-embedded and stained with hematoxylene-eosin. The number of thrombosed and non-thrombosed blood vessels were counted and the percentage of thrombosis was calculated.

B. Results

Annexin V also blocks the activity of the anti-VCAM-1•tTF coaguligand *in vivo*. Groups of tumor-bearing mice were treated with one of the control or test reagents, as described in the methods. Mice were given (a) saline; (b) 100 μg of Annexin V; (c) 40 μg of anti-VCAM-1•tTF coaguligand; or (d) 100 μg of Annexin V followed 2 hours later by 40 μg of anti-VCAM-1•tTF coaguligand. Identical results were obtained in both mice per group.

No spontaneous thrombosis, hemorrhages or necrosis were observed in tumors derived from saline-injected mice. Treatment with Annexin V alone did not alter tumor morphology.

In accordance with other data presented herein, 40 μg of anti-VCAM-1•tTF coaguligand caused thrombosis in 70% of total tumor blood vessels. The majority of

blood vessels were occluded with packed erythrocytes and clots, and tumor cells were separated from one another. Both coaguligand-induced anti-tumor effects, *i.e.*, intravascular thrombosis and changes in tumor cell morphology, were completely abolished by pre-treating the mice with Annexin V.

These findings confirm that the anti-tumor effects of coaguligands are mediated through the blockage of tumor vasculature. These data also demonstrate that PS is essential for coaguligand-induced thrombosis *in vivo*.

EXAMPLE XI

Externalized Phosphatidylserine is a Global Marker of Tumor Blood Vessels

A. Methods

PS exposure on tumor and normal vascular endothelium was examined in three animal tumor models: L540 Hodgkin lymphoma, NCI-H358 non-small cell lung carcinoma, and HT 29 colon adenocarcinoma (ATCC). To grow the tumors *in vivo*, 2×10^6 cells were injected into the right flank of SCID mice and allowed to reach 0.8-1.2 cm in diameter. Mice bearing large tumors (volume above 800 mm^3) were injected intravenously *via* the tail vein with 20 μg of either anti-PS or anti-cardiolipin antibodies. The anti-cardiolipin antibody served as a control for all studies since both antibodies are directed against negatively charged lipids and belong to the same class of immunoglobulins (mouse IgM).

One hour after injection, mice were anesthetized and their blood circulation was perfused with heparinized saline. Tumors and normal organs were removed and snap-frozen. Frozen sections were stained with anti-mouse IgM-peroxidase conjugate (Jackson Immunoresearch Labs) followed by development with carbazole.

B. Results

The anti-PS antibodies specifically homed to the vasculature of all three tumors (HT 29, L540 and NCI-H358) *in vivo*, as indicated by detection of the mouse IgM. The average percentages of vessels stained in the tumors were 80% for HT 29,

30% for L540 and 50% for NCI-H358. Vessels in all regions of the tumors were stained and there was staining both of small capillaries and larger vessels.

No vessel staining was observed with anti-PS antibodies in any normal tissues. In the kidney, tubules were stained both with anti-PS and anti-CL, and this likely relates to the secretion of IgMs by this organ (Table 5). Anti-cardiolipin antibodies were not detected in any tumors or normal tissues, except kidney.

These findings indicate that only tumor endothelium exposes PS to the outer site of the plasma membrane.

TABLE 5

Vessel Localization of Anti-PS and Anti-Cardiolipin Abs in Tumor-Bearing Mice*

Tissue	Anti-PS†	Anti-Cardiolipin†
L540 Cy tumor	++	-
H358 tumor	++	-
HT29 tumor	+++	-
Adrenal	-	-
Brain Cerebellum	-	-
Brain Cortex	-	-
Heart	-	-
Kidney	-‡	-‡
Large Intestine	-	-
Liver	-	-
Lung	-	-
Pancreas	-	-
Small Intestine	-	-
Spleen	-	-
Testes	-	-

*Biodistribution in normal organs of both anti-PS and anti-cardiolipin Abs was identical in all three tumor animal models.

†Anti-PS and anti-cardiolipin antibodies were detected on frozen sections using anti-mouse IgM-peroxidase conjugate. - no staining; + weak; ++ moderate; +++ strong, equivalent to pan endothelial marker Meca 32.

‡Tubular staining was observed in the kidneys of both and-PS and anti-CL recipients.

To estimate the time at which tumor vasculature loses the ability to segregate PS to the inner side of the membrane, the inventors examined anti-PS localization in L540 tumors ranging in volume from 140 to 1,600 mm^3. Mice were divided into 3

groups according to their tumor size: 140-300, 350-800 and 800-1,600 mm^3. Anti-PS Ab was not detected in three mice bearing small L540 tumors (up to 300 mm^3). Anti-PS Ab localized in 3 animals of 5 in the group of intermediate size L540 tumors and in all mice (4 out of 4) bearing large L540 tumors (Table 6). Percent of PS-positive blood vessels from total (identified by pan endothelial marker Meca 32) was 10-20% in the L540 intermediate group and 20-40% in the group of large L540 tumors (Table 6).

TABLE 6

PS Externalization Detected in Mid and Large Sized Tumors

Tumor Size (mm^3)	No. Positive Tumors/Total*	%PS-Positive Vessels/Total†
350-800	3/5	10-20
850-1,600	4/4	20-40

*Mice bearing L540 Cy tumors were divided into three groups according to tumor size. 20 μg of anti-PS antibodies were injected i.v. and allowed to circulate for 1 hour. Mouse antibodies were detected on frozen sections using anti-mouse IgM-peroxidase conjugate.

†Total number of blood vessels was determined using pan-endothelial Ab Meca 32. PS-positive and Meca-positive vessels were counted in 4 fields per cross section of tumor. Range of % PS-positive vessels within the same group is shown.

EXAMPLE XII

Anti-Tumor Effects of Unconjugated Anti-Phosphatidylserine Antibodies

A. Methods

The effects of anti-PS antibodies were examined in syngeneic and xenogeneic tumor models. For the syngeneic model, $1x10^7$ cells of murine colorectal carcinoma Colo 26 (obtained from Dr. Ian Hart, ICRF, London) were injected subcutaneously into the right flank of Balb/c mice. In the xenogeneic model, a human Hodgkin's

lymphoma L540 xenograft was established by injecting $1x10^7$ cells subcutaneously into the right flank of male CB17 SCID mice. Tumors were allowed to grow to a size of about 0.6-0.9 cm^3 before treatment.

Tumor-bearing mice (4 animals per group) were injected i.p. with 20 μg of naked anti-PS antibody (IgM), control mouse IgM or saline. Treatment was repeated 3 times with a 48 hour interval. Animals were monitored daily for tumor measurements and body weight. Tumor volume was calculated as described in Example VII. Mice were sacrificed when tumors had reached 2 cm^3, or earlier if tumors showed signs of necrosis or ulceration.

B. Results

The growth of both syngeneic and xenogeneic tumors was effectively inhibited by treatment with naked anti-PS antibodies (FIG. 4A and FIG. 4B). Anti-PS antibodies caused tumor vascular injury, accompanied by thrombosis, and tumor necrosis. The presence of clots and disintegration of tumor mass surrounding blocked blood vessels was evident.

Quantitatively, the naked anti-PS antibody treatment inhibited tumor growth by up to 60% of control tumor volume in mice bearing large Colo 26 (FIG. 4A) and L540 (FIG. 4B) tumors. No retardation of tumor growth was found in mice treated with saline or control IgM. No toxicity was observed in mice treated with anti-PS antibodies, with normal organs preserving unaltered morphology, indistinguishable from untreated or saline-treated mice.

Tumor regression started 24 hours after the first treatment and tumors continue to decline in size for the next 6 days. This was observed in both syngeneic and immunocompromised tumor models, indicating that the effect was mediated by immune status-independent mechanism(s). Moreover, the decline in tumor burden was associated with the increase of alertness and generally healthy appearance of the

animals, compared to control mice bearing tumors larger than 1500 mm^3. Tumor re-growth occurred 7-8 days after the first treatment.

The results obtained with anti-PS treatment of L540 tumors are further compelling for the following reasons. Notably, the tumor necrosis observed in L540 tumor treatment occurred despite the fact that the percentage of vessels that stained positive for PS in L540 tumors was less than in HT 29 and NCI-H358 tumors. This implies that even more rapid necrosis would likely result when treating other tumor types. Furthermore, L540 tumors are generally chosen as an experimental model because they provide clean histological sections and they are, in fact, known to be resistant to necrosis.

EXAMPLE XIII

Anti-Tumor Effects of Annexin Conjugates

The surprising finding that aminophospholipids are stable markers of tumor vasculature also means that antibody-therapeutic agent constructs can be used in cancer treatment. In addition to using antibodies as targeting agents, the inventors reasoned that annexins, and other aminophospholipid-binding proteins, could also be used to specifically deliver therapeutic agents to tumor vasculature. The following data shows the anti-tumor effects that result from the *in vivo* administration of annexin-TF constructs.

A. Methods

An annexin V-tTF conjugate was prepared and administered to nu/nu mice with solid tumors. The tumors were formed from human HT29 colorectal carcinoma cells that formed tumors of at least about 1.2 cm^3. The annexin V-tTF coaguligand (10 μg) was administered intravenously and allowed to circulate for 24 hours. Saline-treated mice were separately maintained as control animals. After the one day treatment period, the mice were sacrificed and exsanguinated and the tumors and major organs were harvested for analysis.

B. Results

The annexin V-tTF conjugate was found to induce specific tumor blood vessel coagulation in HT29 tumor bearing mice. Approximately 55% of the tumor blood vessels in the annexin V-tTF conjugate treated animals were thrombosed following a single injection. In contrast, there was minimal evidence of thrombosis in the tumor vasculature of the control animals.

EXAMPLE XIV

Phosphatidylserine Translocation in the Tumor Environment

The discovery of PS as an *in vivo* surface marker unique to tumor vascular endothelial cells prompted the inventors to further investigate the effect of a tumor environment on PS translocation and outer membrane expression. The present example shows that exposing endothelial cells *in vitro* to certain conditions that mimic those in a tumor duplicates the observed PS surfacc expression in intact, viable cells.

A. Methods

Mouse bEnd.3 endothelial cells were seeded at an initial density of 50,000 cells/well. Twenty-fours later cells were incubated with increasing concentrations of H_2O_2 (from 10 μM to 500 μM) for 1 hour at 37°C or left untreated. At the end of the incubation, cells were washed 3 times with PBS containing 0.2% gelatin and fixed with 0.25% glutaraldehyde. Identical wells were either stained with anti-PS IgM or trypsinized and evaluated for viability by the Trypan Blue exclusion test. For the anti-PS staining, after blocking with 2% gelatin for 10 min., cells were incubated with 2 μg/ml of anti-PS antibody, followed by detection with anti-mouse IgM-HRP conjugate.

Wells seeded with mouse bEnd.3 endothelial cells were also incubated with different effectors and compared to control, untreated wells after the same period of incubation at 37°C. The panel of effectors tested included TNF, LPS, bFGF, IL-1α, IL-1β and thrombin. After incubation, cells were washed and fixed and were again

either stained with anti-PS IgM or evaluated for viability using the Trypan Blue exclusion test, as described above.

B. Results

1. PS Induction by H_2O_2

Exposing endothelial cells to H_2O_2 at concentrations higher than 100 μM caused PS translocation in ~90% cells. However, this was accompanied by detachment of the cells from the substrate and cell viability decreasing to about 50-60%. The association of surface PS expression with decreasing cell viability is understandable, although it is still interesting to note that ~90% PS translocation is observed with only a 50-60% decrease in cell viability.

Using concentrations of H_2O_2 lower than 100 μM resulted in significant PS expression without any appreciable reduction in cell viability. For example, PS was detected at the cell surface of about 50% of cells in all H_2O_2 treated wells using H_2O_2 at concentrations as low as 20 μM. It is important to note that, under these low H_2O_2 concentrations, the cells remained firmly attached to the plastic and to each other, showed no morphological changes and had no signs of cytotoxicity. Detailed analyses revealed essentially 100% cell-cell contact, retention of proper cell shape and an intact cytoskeleton.

The 50% PS surface expression induced by low levels of H_2O_2 was thus observed in cell populations in which cell viability was identical to the control, untreated cells (*i.e.*, 95%). The PS expression associated with high H_2O_2 concentrations was accompanied by cell damage, and the PS-positive cells exposed to over 100 μM H_2O_2 were detached, floating and had disrupted cytoskeletons.

The maintenance of cell viability in the presence of low concentrations H_2O_2 is consistent with data from other laboratories. For example, Schorer *et al.* (1985) showed that human umbilical vein endothelial cells (HUVEC) treated with 15 μM

H_2O_2 averaged 90 to 95% viability (reported as 5% to 10% injury), whilst those exposed to 1500 μM H_2O_2 were only 0%-50% viable (50% to 100% injured).

The use of H_2O_2 to mimic the tumor environment *in vitro* is also appropriate in that the tumor environment is rich in inflammatory cells, such as macrophages, PMNs and granulocytes, which produce H_2O_2 and other reactive oxygen species. Although never before connected with stable tumor vascular markers, inflammatory cells are known to mediate endothelial cell injury by mechanisms involving reactive oxygen species that require the presence of H_2O_2 (Weiss *et al.*, 1981; Yamada *et al.*, 1981; Schorer *et al.*, 1985). In fact, studies have shown that stimulation of PMNs *in vitro* produces concentrations of H_2O_2 sufficient to cause sublethal endothelial cell injury without causing cell death (measured by chromium release assays) or cellular detachment; and that these H_2O_2 concentrations are attainable locally *in vivo* (Schorer *et al.*, 1985).

The present *in vitro* translocation data correlates with the earlier results showing that anti-PS antibodies localize specifically to tumor vascular endothelial cells *in vivo*, and do not bind to cells in normal tissues. The finding that *in vivo*-like concentrations of H_2O_2 induce PS translocation to the endothelial cell surface without disrupting cell integrity has important implications in addition to validating the original *in vivo* data and the inventors' therapeutic approaches.

Human, bovine and murine endothelial cells are all known to be PS-negative under normal conditions. Any previously documented PS expression has always been associated with cell damage and/or cell death. This is simply not the case in the present studies, where normal viability is maintained. This shows that PS translocation in tumor vascular endothelium is mediated by biochemical mechanisms unconnected to cell damage. This is believed to be the first demonstration of PS surface expression in morphologically intact endothelial cells and the first indication that PS expression can be disconnected from the apoptosis pathway(s). Returning to the operability of the present invention, these observations again confirm that PS is a

sustainable, rather than transient, marker of tumor blood vessels and a suitable candidate for therapeutic intervention.

2. PS Expression Does Not Correlate with Cell Activation

The relevance of this *in vitro* data to the tumor environment is also strengthened by the fact that other, general cell activators are without effect on PS translocation in endothelial cells. For example, the inventors tested TNF in similarly controlled studies and found it unable to induce PS surface expression, despite the expected increases in E-selectin and VCAM expression. Likewise, LPS, bFGF, IL-1α and IL-1β were all without effect on PS expression in appropriately controlled studies.

3. PS Induction by Thrombin

In contrast to the lack of effects of other cell activators, thrombin was observed to increase PS expression, although not to the same extent as H_2O_2. This data is also an integral part of the tumor-induction model of PS expression developed by the present inventors (thrombin-induced PS surface expression in normal tissues would also further coagulation as PS expression coordinates the assembly of coagulation initiation complexes (Ortel *et al.*, 1992)).

The tumor environment is known to be prothrombotic, such that tumor vasculature is predisposed to coagulation (U.S. Patent No. 5,877,289). As thrombin is a product of the coagulation cascade, it is present in tumor vasculature. In fact, the presence of thrombin induces VCAM expression, contributing to the inventors' ability to exploit VCAM as a targetable marker of tumor vasculature (U.S. Patent Nos. 5,855,866; 5,877,289). The present data showing that thrombin also induces PS expression is thus both relevant to targeting aminophospholipids with naked antibodies and therapeutic conjugates, and further explains the beneficial effects of the anti-VCAM coaguligand containing Tissue Factor (Example VII).

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

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1. A binding ligand comprising a targeting agent that binds to an aminophospholipid operatively attached to a therapeutic agent.

2. The binding ligand of claim 1, wherein said targeting agent comprises an anti-aminophospholipid antibody or antigen-binding fragment thereof.

3. The binding ligand of claim 2, wherein said targeting agent comprises an IgG or IgM anti-aminophospholipid antibody.

4. The binding ligand of claim 2, wherein said targeting agent comprises an scFv, Fv, Fab', Fab or $F(ab')_2$ antigen-binding region of an anti-aminophospholipid antibody.

5. The binding ligand of any preceding claim, wherein said targeting agent comprises a monoclonal anti-aminophospholipid antibody, or antigen-binding region thereof.

6. The binding ligand of claim 5, wherein said monoclonal anti-aminophospholipid antibody, or antigen-binding fragment thereof, is prepared by a preparative process comprising:

(a) preparing an anti-aminophospholipid antibody-producing cell; and

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(b) obtaining an anti-aminophospholipid monoclonal antibody from said antibody-producing cell.

7. The binding ligand of claim 6, wherein said anti-aminophospholipid antibody-producing cell is a human patient's cell, wherein said patient has a disease associated with the production of anti-aminophospholipid antibodies.

8. The binding ligand of claim 6, wherein said anti-aminophospholipid antibody-producing cell is obtained by *in vitro* stimulation of a mixed population of human peripheral blood lymphocytes with an immunogenically effective amount of an aminophospholipid sample.

9. The binding ligand of claim 6, wherein said anti-aminophospholipid antibody-producing cell is obtained by immunizing a non-human animal with an immunogenically effective amount of an aminophospholipid sample.

10. The binding ligand of claim 9, wherein said anti-aminophospholipid antibody-producing cell is obtained by immunizing a transgenic mouse that comprises a human antibody library with an immunogenically effective amount of an aminophospholipid sample.

11. The binding ligand of claim 6, wherein said preparative process comprises:

(a) fusing said anti-aminophospholipid antibody-producing cell with an immortal cell to prepare a hybridoma that produces an anti-aminophospholipid monoclonal antibody; and

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(b) obtaining said anti-aminophospholipid monoclonal antibody from said hybridoma.

12. The binding ligand of claim 6, wherein said preparative process comprises:

(a) immunizing a non-human animal with an immunogenically effective amount of an aminophospholipid sample;

(b) preparing a collection of antibody-producing hybridomas from the immunized animal;

(c) selecting from the collection a hybridoma that produces an anti-aminophospholipid antibody; and

(d) culturing the selected hybridoma to provide said anti-aminophospholipid monoclonal antibody.

13. The binding ligand of claim 12, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein said anti-aminophospholipid monoclonal antibody is a human monoclonal antibody.

14. The binding ligand of claim 6, wherein said preparative process comprises:

(a) obtaining the anti-aminophospholipid antibody-encoding nucleic acids from said anti-aminophospholipid antibody-producing cell; and

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(b) expressing said nucleic acids to obtain a recombinant anti-aminophospholipid monoclonal antibody.

15. The binding ligand of claim 6, wherein said preparative process comprises:

(a) immunizing an animal with an immunogenically effective amount of an aminophospholipid sample;

(b) preparing a combinatorial immunoglobulin phagemid library expressing RNA isolated from the spleen of the immunized animal;

(c) selecting from the phagemid library a clone that expresses an anti-aminophospholipid antibody; and

(d) expressing the anti-aminophospholipid antibody-encoding nucleic acids from said selected clone to provide a recombinant anti-aminophospholipid monoclonal antibody.

16. The binding ligand of claim 15, wherein the immunized animal is a transgenic mouse that comprises a human antibody library and wherein the recombinant anti-aminophospholipid monoclonal antibody is a recombinant human monoclonal antibody.

17. The binding ligand of any preceding claim, comprising a human, humanized or part-human chimeric anti-aminophospholipid antibody, or antigen-binding region thereof.

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18. The binding ligand of claim 1, wherein said targeting agent comprises an aminophospholipid binding protein or an aminophospholipid-binding fragment thereof.

19. The binding ligand of claim 18, wherein said targeting agent comprises a phosphatidylserine binding protein or a phosphatidylserine-binding fragment thereof.

20. The binding ligand of claim 19, wherein said targeting agent comprises an annexin or a phosphatidylserine-binding fragment thereof.

21. The binding ligand of claim 18, wherein said targeting agent comprises a phosphatidylethanolamine binding protein or a phosphatidylethanolamine-binding fragment thereof.

22. The binding ligand of claim 21, wherein said targeting agent comprises a kininogen or a phosphatidylethanolamine-binding fragment thereof.

23. The binding ligand of any preceding claim, wherein said targeting agent comprises two, three or multiple aminophospholipid binding sites.

24. The binding ligand of any preceding claim, wherein said targeting agent is a recombinant targeting agent.

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25. The binding ligand of any preceding claim, wherein said targeting agent binds to phosphatidylethanolamine.

26. The binding ligand of any preceding claim, wherein said targeting agent binds to phosphatidylserine.

27. The binding ligand of any preceding claim, wherein said targeting agent is attached to an anticellular or cytotoxic agent.

28. The binding ligand of claim 27, wherein said targeting agent is attached to a steroid, cytokine, antimetabolite, anthracycline, vinca alkaloid, antibiotic, alkylating agent, epipodophyllotoxin, DNA synthesis inhibitor, daunorubicin, doxorubicin or adriamycin.

29. The binding ligand of claim 27, wherein said targeting agent is attached to a plant-, fungus- or bacteria-derived toxin.

30. The binding ligand of claim 29, wherein said targeting agent is attached to an A chain toxin, bacterial endotoxin, lipid A moiety of bacterial endotoxin, ribosome inactivating protein, α-sarcin, gelonin, aspergillin, restrictocin, ribonuclease, diphtheria toxin or *Pseudomonas* exotoxin.

31. The binding ligand of claim 30, wherein said targeting agent is attached to ricin A chain or deglycosylated ricin A chain.

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32. The binding ligand of any preceding claim, wherein said targeting agent is attached to a coagulant.

33. The binding ligand of claim 32, wherein said targeting agent is attached to a human coagulation factor.

34. The binding ligand of claim 32, wherein said targeting agent is attached to a coagulation factor selected from the group consisting of Factor II/IIa, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, a vitamin K-dependent coagulation factor that lacks the Gla modification, Russell's viper venom Factor X activator, thromboxane A_2, thromboxane A_2 synthase and α2-antiplasmin.

35. The binding ligand of claim 32, wherein said targeting agent is attached to a Tissue Factor, dimeric Tissue Factor, trimeric Tissue Factor, polymeric Tissue Factor, mutant Tissue Factor or Tissue Factor derivative.

36. The binding ligand of claim 35, wherein said targeting agent is attached to a truncated Tissue Factor.

37. The binding ligand of any preceding claim, wherein said targeting agent is attached to a first and a second therapeutic agent.

38. The binding ligand of any preceding claim, wherein said targeting agent is attached to multiple therapeutic agents.



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39. The binding ligand of any preceding claim, wherein said targeting agent is directly attached to a therapeutic agent.

40. The binding ligand of claim 39, wherein said targeting agent is directly attached to a therapeutic agent by a direct covalent bond or via a chemical cross-linker.

41. The binding ligand of claim 39, wherein said targeting agent is directly attached to a therapeutic agent by expressing said binding ligand as a recombinant fusion protein.

42. The binding ligand of any one of claims 1 through 38, wherein said targeting agent is indirectly attached to a therapeutic agent via an antibody, or antigen binding region thereof, that binds to said therapeutic agent.

43. The binding ligand of claim 42, wherein said binding ligand is a bispecific antibody that comprises a first, targeting antibody, or antigen binding fragment thereof, operatively attached to a second antibody, or antigen binding fragment thereof, that binds to said therapeutic agent.

44. The binding ligand of claim 1, wherein said targeting agent is an anti-phosphatidylserine antibody, or antigen binding fragment thereof, that is directly or indirectly attached to truncated Tissue Factor.

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45. The binding ligand of any preceding claim, dispersed in a pharmaceutically acceptable formulation.

46. The binding ligand of any preceding claim, for use in delivering a therapeutic agent to an aminophospholipid expressed on the luminal surface of blood vessels of a vascularized tumor upon administration to an animal having a vascularized tumor.

47. The binding ligand of any preceding claim, for use in killing tumor vascular endothelial cells upon administration to an animal having a vascularized tumor.

48. The binding ligand of any preceding claim, for use in inducing coagulation in tumor vasculature upon administration to an animal having a vascularized tumor.

49. The binding ligand of any preceding claim, for use in destroying tumor vasculature upon administration to an animal having a vascularized tumor.

50. The binding ligand of any preceding claim, for use in treating cancer upon administration to an animal having a vascularized tumor.

51. Use of a binding ligand in accordance with any preceding claim in the manufacture of a medicament for treating cancer.

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52. A pharmaceutical composition comprising a biologically effective amount of a binding ligand in accordance with any one of claims 1 through 50.

53. The pharmaceutical composition of claim 52, wherein said composition comprises two binding ligands that each bind to a distinct aminophospholipid.

54. The pharmaceutical composition of claim 52 or 53, wherein said composition is formulated for intravenous administration.

54. The pharmaceutical composition of any one of claims 52 though 54, wherein said composition comprises a second anti-cancer agent.

55. The pharmaceutical composition of claim 54, wherein said second anti-cancer agent is a chemotherapeutic agent, radiotherapeutic agent, anti-angiogenic agent or apoptosis-inducing agent.

56. The pharmaceutical composition of claim 54, wherein said second anti-cancer agent is an antibody-therapeutic agent construct comprising a targeting antibody, or antigen binding fragment thereof, that binds to a surface-expressed, surface-accessible or surface-localized component of a tumor cell, tumor vasculature or tumor stroma; said targeting antibody or fragment thereof operatively linked to a therapeutic agent.

57. The pharmaceutical composition of claim 56, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to tumor cell or tumor stroma component.

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58. The pharmaceutical composition of claim 56, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed, surface-accessible, surface-localized, cytokine-inducible or coagulant-inducible component of intratumoral blood vessels of a vascularized tumor.

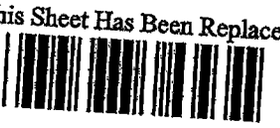
59. The pharmaceutical composition of claim 58, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-expressed component of intratumoral vasculature selected from the group consisting of an aminophospholipid, endoglin, a TGFβ receptor, E-selectin, P-selectin, VCAM-1, ICAM-1, PSMA, a VEGF/VPF receptor, an FGF receptor, a TIE, $\alpha_v\beta_3$ integrin, pleiotropin, endosialin and an MHC Class II protein.

60. The pharmaceutical composition of claim 58, wherein said second targeting antibody, or antigen-binding fragment thereof, binds to a surface-localized component of intratumoral vasculature selected from the group consisting of VEGF/VPF, FGF, TGFβ, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor/hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP.

61. The pharmaceutical composition of claim 56, wherein said second targeting antibody, or antigen-binding fragment thereof, is operatively linked to a cytotoxic agent.

62. The pharmaceutical composition of claim 56, wherein said second targeting antibody, or antigen-binding fragment thereof, is operatively linked to a coagulation

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factor or to an antibody, or antigen binding fragment thereof, that binds to a coagulation factor.

63. The pharmaceutical composition of claim 54, wherein said second anti-cancer agent is a naked antibody, or antigen-binding fragment thereof, that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor.

64. The pharmaceutical composition of any one of claims 52 though 63, wherein said composition is intended for human administration.

65. Use of a pharmaceutical composition in accordance with any one of claims 52 though 64 in the manufacture of a medicament for treating cancer.

66. A kit comprising a biologically effective combined amount of a binding ligand in accordance with any one of claims 1 through 50 and a second anti-cancer agent.

67. A kit comprising a biologically effective amount of a binding ligand in accordance with any one of claims 1 through 50 in combination with a diagnostically effective amount of a targeting agent-detectable agent construct that comprises a detectable agent operatively attached to a second targeting agent that binds to an aminophospholipid.

68. A kit comprising a pharmaceutical composition in accordance with any one of claims 52 though 64 in combination with a diagnostically effective amount of a

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targeting agent-detectable agent construct that comprises a detectable agent operatively attached to a second targeting agent that binds to an aminophospholipid.

69. The kit of claim 67 or 68, wherein said targeting agent-detectable agent construct comprises the X-ray detectable compound bismuth (III), gold (III), lanthanum (III) or lead (II).

70. The kit of claim 67 or 68, wherein said targeting agent-detectable agent construct comprises the detectable radioactive ion copper^{67}, gallium^{67}, gallium^{68}, indium^{111}, indium^{113}, iodine^{123}, iodine^{125}, iodine^{131}, mercury^{197}, mercury^{203}, rhenium^{186}, rhenium^{188}, rubidium^{97}, rubidium^{103}, technetium^{99m} or yttrium^{90}.

71. The kit of claim 67 or 68, wherein said targeting agent-detectable agent construct comprises the detectable nuclear magnetic spin-resonance isotope cobalt (II), copper (II), chromium (III), dysprosium (III), erbium (III), gadolinium (III), holmium (III), iron (II), iron (III), manganese (II), neodymium (III), nickel (II), samarium (III), terbium (III), vanadium (II) or ytterbium (III).

72. A method for treating an animal having a vascularized tumor, comprising administering to said animal a therapeutically effective amount of at least a first binding ligand that comprises at least a first therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid on the luminal surface of blood vessels of a vascularized tumor.

73. A method for imaging a vascularized tumor, comprising administering to an animal having a vascularized tumor a diagnostically effective amount of at least a first

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binding ligand that comprises a detectable agent operatively attached to a targeting agent that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor, and detecting the image of the tumor so formed.

74. A method for treating cancer, comprising:

(a) forming an image of a vascularized tumor by administering to an animal having a vascularized tumor a diagnostically effective amount of a targeting agent-detectable agent construct that comprises a detectable agent operatively attached to a targeting agent that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor, thereby forming a detectable image of the tumor vasculature; and

(b) subsequently administering to said animal a therapeutically effective amount of at least a first targeting agent-therapeutic agent construct comprising a therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid on the tumor blood vessel luminal surface and thereby destroys the tumor vasculature.

75. A method for treating cancer, comprising simultaneously or sequentially administering to an animal having a vascularized tumor a therapeutically effective combination of a targeting agent-therapeutic agent construct comprising a therapeutic agent operatively attached to a targeting agent that binds to an aminophospholipid on the luminal surface of blood vessels of the vascularized tumor and at least a second anti-cancer agent.

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