PROTEASE-REGULATED ANTIBODIES

[01] This application claims the benefit of Serial No. 61/617,837 filed on March 30, 2012, which is incorporated herein by reference in its entirety.

[02] All documents cited in this disclosure are incorporated herein by reference in their entireties.

[03] This application incorporates by reference a 64 kb text file created on March 26, 2012 and named "0297301274sequencelisting.txt," which is the sequence listing for this application.

TECHNICAL FIELD

[04] The technical field is treatment of hemophilia and other coagulopathies.

BRIEF DESCRIPTION OF THE DRAWINGS

[05] **FIG. 1**. Illustration of an embodiment of a protease-regulated antibody. VH, heavy chain variable region; VL, light chain variable region; CH, heavy chain constant region; CL, light chain constant region.

[06] **FIG. 2**. Western blot of two protease-regulated anti-TFPI Fab fragments ("Fab-1" and "Fab-2"), with and without thrombin digestion.

[07] **FIG. 3**. Graph showing tissue factor pathway inhibitor (TFPI) binding of Fab-1 and Fab-2, with and without thrombin digestion, assayed by ELISA.

[08] **FIG. 4**. Graph showing BIACORE™ measurement of TFPI binding of Fab-1 (Fab1) and Fab-2 (Fab2) with and without thrombin digestion.

[09] **FIG. 5**. SDS-PAGE of purified IgG antibody expressed in HEK293 6E cells.

[10] **FIG. 6**. Western Blot of parental IgG ("Parent IgG;" top) and IgG2-linker1 (bottom). Lane 1, digestion with thrombin (1 unit); lane 2, no protease (control); lane 3, digestion with bovine plasmin; lane 4, digestion with enterokinase (0.02 μg); lane 5, digestion with bovine Factor Xa (1 μg); lane 6, digestion with matriptase (MTSP) (0.5

μg); lane 7, digestion with urokinase (uPA) (0.25 μg); lane 8, digestion with human rhinovirus 3C protease (HRV 3C) (2 units).

[11] **FIG. 7.** Graph showing TFPI binding of IgG with and without thrombin digestion, assayed by ELISA.

[12] **FIG. 8**. Graph showing BIACORE™ measurement of TFPI binding of parental IgG and IgG-linker1, with and without thrombin digestion.

[13] **FIG. 9.** Western blot , showing protease digestion of IgG-Linker1 and WT antibody with human proteases.

DETAILED DESCRIPTION

[14] This disclosure provides protease-regulated antibodies which specifically bind to tissue factor pathway inhibitor (TFPI). The antibodies are useful for treating bleeding disorders such as hemophilia. In some embodiments, protease-regulated anti-TFPI antibodies can be cleaved by thrombin and/or plasmin. By initially inhibiting TFPI, such protease-regulated anti-TFPI antibodies promote the generation of thrombin and/or plasmin, which in turn cleaves the antibodies and removes or significantly reduces their binding activity to TFPI. This negative feedback allows the antibodies to promote coagulation within a safe therapeutic window.

1. Protease-regulated anti-TFPI antibodies

[15] Protease-regulated antibodies disclosed herein specifically bind to TFPI; *i.e.*, they bind to TFPI with an affinity that is higher (*e.g.*, at least two-fold higher) than their binding affinity for an irrelevant antigen (*e.g.*, BSA, casein). The term "tissue factor pathway inhibitor" or "TFPI" as used herein refers to any variant, isoform and species homolog of human TFPI that is naturally expressed by cells.

[16] In some embodiments, protease-regulated antibodies bind to TFPI with an affinity of at least about 10^5 M^{-1} to about 10^{12} M^{-1} (*e.g.*, 10^5 M^{-1}, $10^{5.5}$ M^{-1}, 10^6 M^{-1}, $10^{6.5}$ M^{-1}, 10^7 M^{-1}, $10^{7.5}$ M^{-1}, 10^8 M^{-1}, $10^{8.5}$ M^{-1}, 10^9 M^{-1}, $10^{9.5}$ M^{-1}, 10^{10} M^{-1}, $10^{10.5}$ M^{-1}, 10^{11} M^{-1}, $10^{11.5}$ M^{-1}, 10^{12} M^{-1}). The affinity (K_d) of antibody binding to an antigen can be assayed using any method known in the art including, for example, immunoassays

such as enzyme-linked immununospecific assay (ELISA), Bimolecular Interaction Analysis (BIA) (*e.g.*, Sjolander & Urbaniczky; Anal. Chem. 63:2338-2345, 1991; Szabo, et al., Curr. Opin. Struct. Biol. 5:699-705, 1995), and fluorescence-activated cell sorting (FACS) for quantification of antibody binding to cells that express an antigen. BIA is a technology for analyzing biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIACORE^{TM}). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

[17] A protease-regulated anti-TFPI antibody can be constructed using a substantially full-length immunoglobulin molecule (*e.g.*, IgG1, IgG2a, IgG2b, IgG3, IgG4, IgM, IgD, IgE, IgA), an antigen binding fragment thereof, such as a Fab or F(ab′)$_2$, or a construct containing an antigen binding site, such as a scFv, Fv, or diabody, which is capable of specific binding to TFPI. The term "antibody" also includes other protein scaffolds that are able to orient antibody complementarity-determining region (CDR) inserts into the same active binding conformation as that found in natural antibodies such that the binding to TFPI observed with these chimeric proteins is maintained relative to the TFPI binding activity of the natural antibody from which the CDRs were derived.

[18] An "isolated antibody" as used herein is an antibody which is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that binds to TFPI is substantially free of antibodies that bind antigens other than TFPI). An isolated antibody that binds to an epitope, isoform, or variant of human TFPI may, however, have cross-reactivity to other related antigens, *e.g.*, from other species (*e.g.*, TFPI species homologs). An isolated antibody can be substantially free of other cellular material and/or chemicals.

[19] The protease-regulated antibodies disclosed herein are engineered to comprise a protease cleavage site recognized by one or more proteases. As used herein, "protease cleavage site" refers to an amino acid sequence that is recognized and cleaved by a protease. In some embodiments, the protease cleavage site is positioned between its variable and constant regions. In some embodiments, protease-regulated anti-TFPI antibodies include one or more protease cleavage sites that can be cleaved by thrombin, plasmin, and/or Factor Xa . In some embodiments, the amino acid sequence between the variable and constant regions of a protease-regulated anti-TFPI antibody

comprises a polypeptide linker in addition to the protease cleavage site (as illustrated, for example, in **FIG. 1**). The linker can be a single amino acid or a polypeptide sequence (*e.g.*, up to 100 amino acids). For example, the linker can be GGGGS (SEQ ID NO:149). Other useful linkers include those shown in SEQ ID NOS:151-176. In other embodiments, no linker is present, and the cleavage site itself is inserted between the variable and constant regions.

[20] At least two optimal cleavage sites for thrombin have been determined: (1) X_1-X_2-P-R-X_3-X_4 (SEQ ID NO:147), where X_1 and X_2 are hydrophobic amino acids and X_3 and X_4 are nonacidic amino acids; and (2) GRG. Thrombin specifically cleaves after the arginine residue. Plasmin can also cleave the two aforementioned cleavage sites, however with less specificity as compared to thrombin. Other useful thrombin cleavage sites are provided as SEQ ID NOS:1-60. Other useful plasmin cleavages sites are provided as SEQ ID NOS:12, 47, 48, 53, and 61-130. In some embodiments, the cleavage site is LVPRGS (SEQ ID NO:137).

[21] In some embodiments, a Factor Xa cleavage site, such as I-(E or D)-G-R (SEQ ID NO:148), is used. Other useful Factor Xa cleavage sites are provided as SEQ ID NOS:29, 59, and 61-69. Other thrombin and FXa cleavage sites or sequences can be found from previous publication authored by Bianchini [Bianchini EP et al 2002 JBC]. One protease-regulated antibody may comprise more than one protease cleavage sites.

[22] In addition to cleavage site, a second binding site of protease, so-called exosite, can be introduced into a protease-regulated TFPI antibody to make the cleavage more efficient. The exosite of thrombin can be from the native exosite of protease substrates or inibitor, such as PAR1, fibrinogen and hirudin. The exosite can also be a derivative of native exosite.

[23] Protease-regulated TFPI antibodies can be produced synthetically or recombinantly. A number of technologies are available to produce antibodies. For example, phage-antibody technology can be used to generate antibodies (Knappik *et al., J. Mol. Biol. 296*:57-86, 2000). Another approach for obtaining antibodies is to screen a DNA library from B cells as described in WO 91/17271 and WO 92/01047. In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab

fragments. Phage displaying antibodies are selected by affinity enrichment for binding to a selected protein. Antibodies can also be produced using trioma methodology (*e.g.*, Oestberg *et al., Hybridoma 2*:361-367, 1983; U.S. Patent 4,634,664; U.S. Patent 4,634,666).

[24] Antibodies can also be purified from any cell that expresses the antibodies, including host cells that have been transfected with antibody-encoding expression constructs. The host cells can be cultured under conditions whereby the antibodies are expressed. Purified antibody can be separated from other cellular components that can associate with the antibody in the cell, such as certain proteins, carbohydrates, or lipids, using methods well known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis. A preparation of purified antibodies can contain more than one type of antibody.

[25] Alternatively, protease-regulated anti-TFPI antibodies can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (*e.g.*, Merrifield, J. *Am. Chem. Soc. 85*:2149-2154, 1963; Roberge *et al., Science 269*:202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Optionally, fragments of antibodies can be separately synthesized and combined using chemical methods to produce a full-length molecule.

[26] A protease-regulated anti-TFPI antibody can also be constructed in a "single chain Fv (scFv) format," in which a protease cleavage site is inserted in or around a peptide linker between the variable region of light chain and the variable region of heavy chain of a scFv. As the peptide linker is necessary to hold together the two variable regions of a scFv for antigen binding, cleavage of the peptide linker or flanking region allows a protease of interest to inactivate or to down-regulate the binding of scFv to its antigen. The amino acid sequence SEQ ID NO:179 (which can be encoded by SEQ ID NO:180) is an example of a protease-regulated anti-TFPI antibodies in scFv format.

[27] In some embodiments, protease-regulated TFPI antibodies are constructed in "IgG format," having two binding sites, and can comprise one, two, three, or four protease cleavage sites on the heavy chain, light chain, or both. In each case, a protease cleavage site can be flanked on either or both sides by a linker. Further, in each case, the cleavage sites can be the same or different. "IgG-linker1" (Example 5) and "IgG-linker2" are examples of protease-regulated anti-TFPI antibodies in IgG format:

	light chain		heavy chain	
	amino acid	DNA	amino acid	DNA
IgG-linker1	SEQ ID NO:139	SEQ ID NO:141	SEQ ID NO:140	SEQ ID NO:142
IgG-linker2	SEQ ID NO:143	SEQ ID NO:145	SEQ ID NO:144	SEQ ID NO:146

2. Negative Feedback

[28] Protease-regulated antibodies can include a protease cleavage site of a protease upregulated or generated as a result of the function of the antibody. Such protease can then cleave the protease-regulated antibody thereby providing a negative feedback loop which can be useful in preventing excess activity of the protease-regulated antibody.

[29] For example, protease-regulated anti-TFPI antibodies which can be cleaved by thrombin and/or plasmin can exhibit such a negative feedback effect. Such protease-regulated anti-TFPI antibodies promote the generation of thrombin and/or plasmin. Thrombin and plasmin will in turn cleave the protease-regulated anti-TFPI antibodies and remove or significantly reduce their binding activity to TFPI. This negative feedback allows the antibodies to promote coagulation within a safe therapeutic window.

3. Polynucleotides

[30] This disclosure also provides polynucleotides encoding protease-regulated antibodies. These polynucleotides can be used, for example, to produce quantities of the antibodies for therapeutic use.

[31] Antibody-encoding cDNA molecules can be made with standard molecular biology techniques, using mRNA as a template. Thereafter, cDNA molecules can be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook, et al., (Molecular Cloning: A Laboratory Manual, (Second Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.; 1989) Vol. 1-3). An amplification technique, such as PCR, can be used to obtain additional copies of the polynucleotides. Alternatively, synthetic chemistry techniques can be used to synthesize polynucleotides encoding protease-regulated anti-TFPI antibodies.

[32] To express a polynucleotide encoding an antibody, the polynucleotide can be inserted into an expression vector that contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods that are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding antibodies and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described, for example, in Sambrook, et al. (1989) and in Ausubel, et al., (Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1995).

[33] A variety of expression vector/host systems can be utilized to contain and express sequences encoding antibodies. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV); or bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems.

[34] The control elements or regulatory sequences are those non-translated regions of the vector—enhancers, promoters, 5' and 3' untranslated regions—which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in strength and specificity. Depending on the vector system and host, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (*e.g.*, heat shock, RUBISCO, and storage protein genes) or from plant viruses (*e.g.*, viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses can be used. If it is necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding an antibody, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

[35] General texts describing additional useful molecular biological techniques, including the preparation of antibodies, are Berger and Kimmel (Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press, Inc.); Sambrook, et al., (Molecular Cloning: A Laboratory Manual, (Second Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.; 1989) Vol. 1-3); Current Protocols in Molecular Biology, (F. M. Ausabel et al. [Eds.], Current Protocols, a joint venture between Green Publishing Associates, Inc. and John Wiley & Sons, Inc. (supplemented through 2000)); Harlow et al., (Monoclonal Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988), Paul [Ed.]); Fundamental Immunology, (Lippincott Williams & Wilkins (1998)); and Harlow, et al. (Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1998)).

4. Pharmaceutical Compositions

[36] A protease-regulated anti-TFPI antibody can be provided in a pharmaceutical composition comprising a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier preferably is non-pyrogenic. A pharmaceutical composition comprising a protease-regulated anti-TFPI antibody can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not

limited to, saline, buffered saline, dextrose, and water. A variety of aqueous carriers can be employed, *e.g.*, 0.4% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These solutions can be sterilized by conventional, well known sterilization techniques (*e.g.*, filtration). The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of protease-regulated anti-TFPI antibody in a pharmaceutical composition can vary widely, *i.e.*, from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected. See U.S. Pat. No. 5,851,525. If desired, more than one different protease-regulated anti-TFPI antibody can be included in a pharmaceutical composition.

[37] In addition to the active ingredients, pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries that facilitate processing of the compositions into preparations which can be used pharmaceutically. Pharmaceutical compositions can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means.

[38] After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

5. Methods

[39] Pharmaceutical compositions comprising one or more protease-regulated anti-TFPI antibodies can be administered to a patient alone, or in combination with other agents, drugs or coagulation factors, to treat hemophilia or other clotting disorders. A "therapeutically effective dose" of protease-regulated anti-TFPI antibody refers to that amount of protease-regulated anti-TFPI antibody that will promote coagulation or reduce bleeding time. The determination of a therapeutically effective dose is well within the capability of those skilled in the art.

[40] A therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually rats, mice, rabbits, dogs, or pigs. An animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

[41] Therapeutic efficacy and toxicity, *e.g.*, ED_{50} (the dose therapeutically effective in 50% of the population) and LD_{50} (the dose lethal to 50% of the population) of a protease-regulated anti-TFPI antibody can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}.

[42] Pharmaceutical compositions that exhibit large therapeutic indices are preferred. Data obtained from cell culture assays and animal studies are used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

[43] The exact dosage will be determined by the practitioner, in light of factors related to the patient who requires treatment. Dosage and administration are adjusted to provide sufficient levels of the protease-regulated TFPI antibody or to maintain the desired effect. Factors that can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and

frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

[44] In some embodiments, therapeutically effective *in vivo* dosages of a protease-regulated anti-TFPI antibody are in the range of about 5 μg to about 100 mg/kg, about 1 mg to about 50 mg/kg, about 10 mg to about 50 mg/kg of patient body weight.

[45] The mode of administration of a pharmaceutical composition comprising a protease-regulated anti-TFPI antibody can be any suitable route which delivers the antibody to the host (*e.g.*, subcutaneous, intramuscular, intravenous, or intranasal administration).

[46] In some embodiments, a protease-regulated anti-TFPI antibody is administered without other therapeutic agents. In some embodiments, a protease-regulated anti-TFPI antibody is administered in combination with other agents, such as drugs or coagulation factors, to enhance initial production of thrombin while ensuring that the thrombin level stays below the range that may cause thrombosis in some people with coagulopathy. The administration of the protease-regulated anti-TFPI antibody can be before, after, or at substantially the same time as the administration of other agents.

[47] Nothing in this specification should be considered as limiting the scope of this disclosure. All examples presented are representative and non-limiting. The above-described embodiments can be modified or varied, as appreciated by those skilled in the art in light of the above teachings. It is therefore to be understood that, within the scope of the claims and their equivalents, the embodiments disclosed herein can be practiced otherwise than as specifically described.

EXAMPLE 1

Construction of protease-regulated anti-TFPI Fab fragments

[48] Two protease-regulated anti-TFPI Fabs, "Fab-1" and "Fab-2," were based on the anti-TFPI antibody sequences shown in SEQ ID NO:177 (heavy chain) and SEQ ID NO:178 (light chain). Both Fabs have the thrombin/plasmin protease cleavage site, LVPRGS (SEQ ID NO:137) inserted C-terminal to both variable domains. The

cleavage site in Fab-1 is flanked by a $(Gly)_4Ser$ linker. Fab-2 contains the six amino acid cleavage site alone, no linker is present. Thrombin and plasmin will cleave C-terminal to the Arg (R) residue of LVPRGS (SEQ ID NO:137). DNA encoding the Fabs was synthesized by GenScript with optimized codons for bacterial expression. The amino acid and DNA sequences for the Fabs are identified in the table below.

Fab-1 variable and constant light amino acid sequence	SEQ ID NO:131
Fab-1 variable and constant heavy amino acid sequence	SEQ ID NO:133
Fab-1 DNA Sequence	SEQ ID NO:134
Fab-2 variable and constant light amino acid sequence	SEQ ID NO:135
Fab-2 variable and constant heavy amino acid sequence	SEQ ID NO:136
Fab-2 DNA Sequence	SEQ ID NO:138

[49] The Fab coding regions were digested with the restriction enzymes *Bsa*I and *Hind*III (New England Biolabs). The DNA fragments were purified using an agarose gel and subcloned into pBADmycHisA (Invitrogen). The cloned DNA was ligated and transformed using standard techniques. Positive clones were confirmed by DNA sequencing and used for BL21 *E.coli* expression.

EXAMPLE 2

Western Blot of thrombin-cleaved Fabs

[50] Approximately 2.5 μg of the crudely purified Fab-1 and Fab-2 were digested with 0, 2 or 10 units of thrombin (Novagen) for 1hr at 37 ˚C. Digests were run on a 4-15% CRITEREON™ TGX™ gel (Bio-Rad). Protein were transferred to a nitrocellulose membrane and probed with an anti-human Fab antibody (Southern Biotech).

[51] The Western blot of Fab-1 and Fab-2 cleavage is shown in FIG. 2. All samples were reduced. Bands at approximately 12 kDA were observed for both Fabs when treated with thrombin. These bands were not present in the samples without thrombin digestion, indicating that the small size protein was the product of thrombin cleavage.

EXAMPLE 3

TFPI ELISA of thrombin digested Fab-1 and Fab-2

[52] Approximately 2.5 μg of partially purified Fab-1 and Fab-2 were digested with 2 units of biotinylated thrombin (Novagen) at 23 ˚C overnight. Streptavidin sepharose beads (100 μL) were added to the digestion to deplete the thrombin. The digested Fab samples were applied to a column that captures the sepharose bead/thrombin complex. The eluate contained the digested Fabs.

[53] A MAXISORP® 96-well plate (Nunc) was coated with 1 μg/mL of TFPI in PBS overnight at 4 ˚C. The plate was blocked for 1hr at room temperature (RT) in 5% non-fat dry milk PBS/0.5% TWEEN-20® (PBS-T). Serial two-fold dilutions of undigested and digested Fab-1 and Fab-2 were added to the wells (100 μL/well) and incubated for 1hr at RT. The plates were washed five times with PBS-T. A secondary HRP-conjugated anti-Fab antibody was added (100 μL of a 1:10,000 dilution) for detection with an AMPLEX® Red (Invitrogen) solution. As shown in **FIG. 3,** thrombin digestion significantly reduced the signal of TFPI binding.

EXAMPLE 4

BIACORE™ measurements of thrombin-digested Fab-1 and Fab-2

[54] Approximately 2.5 μg of crudely purified Fab-1 and Fab-2 were digested with 2 units of biotinylated thrombin (Novagen) at 23 ˚C overnight. Streptavidin sepharose beads (100 μL) were added to the digestion to deplete the thrombin. The digested Fab samples were applied to a column that captures the sepharose bead/thrombin complex. The eluate contained the digested Fabs.

[55] For BIACORE™ analysis, human TFPI (American Diagnostica) was immobilized using amine coupling at targeted level of 100 relative units (RU), and the antibodies were injected in the mobile phase. HBS-p was used as the running buffer. A reference channel was prepared using blank immobilization where the surface was activated and then inactivated without any immobilized protein. A manual run was performed to measure elevated RU of the Fabs and a buffer control.

[56] As shown in **FIG. 4**, The parental Fab without cleavable linker generated 24.6 RU, comparable to undigested Fab-1 (25.6 RU) and Fab2 (27.9 RU). After thrombin cleavage, the binding signal of Fab-1 and Fab-2 was reduced by more than 50%, indicating that cleaved Fab-1 and Fab-2 not only lost the constant domain, but also lost binding activity to TFPI.

EXAMPLE 5

IgG expression and purification

[57] A protease-regulated anti-TFPI immunoglobulin molecules, "IgG-linker1," was constructed based on parental anti-TFPI antibody sequences shown in SEQ ID NO:177 (heavy chain) and SEQ ID NO:178 (light chain). To facilitate molecular cloning, a *Blp*I site was introduced in the heavy chain coding sequence. Compared to the position of protease cleavable linker in Fab-1, the introduction of BlpI site shifts the linker position of IgG-linker1 two amino acids to the constant region.

[58] HEK293 6E cells were transfected with constructed IgG expression vectors, and the culture supernatant containing the IgG antibodies was harvested. The antibodies were purified using an affinity column of MABSELECT SURE™ followed by SUPERDEX™ 200 chromatography. The purified IgG-linker1 on SDS-PAGE is shown **FIG. 5**.

EXAMPLE 6

Western Blot of parental IgG and IgG-linker1

[59] Purified parental IgG and IgG-linker1 (0.5 μg) were digested with thrombin, bovine plasmin, bovine Factor Xa, matriptase (MTSP), urokinase (uPA), or human rhinovirus 3C protease (HRV 3C). The antibodies were incubated with the proteases for 1hr at 37°C. Digests were run on a 4-20% CRITEREON™ TGX™ gel (Bio-Rad). Protein was transferred to a nitrocellulose membrane and probed with an anti-human IgG heavy and light chain antibody (Pierce). Western blots of IgG2 and IgG2-linker1 are shown in **FIG. 6**. All samples were reduced.

[60] Intact IgG produced two bands under reducing conditions. The two bands correspond to the heavy chain (50 kD) and the light chain (25 kD). Digested antibody IgG-linker1 showed a shift in molecular weight vs. undigested antibody. The following proteases were used to digests the antibodies: thrombin, plasmin, bovine Factor Xa, MTSP, and uPA. The digested IgG-linker1 antibody showed a shift in the molecular weight of the heavy chain from 50 kD to 37 kD. This size shift correlates with the loss of the VH domain from the heavy chain. There was also a molecular weight shift of the 25 kD light chain to a faint band around 16 kDa, which indicates cleavage of the VL domain from the light chain. The proteases did not cleave parental IgG, indicating that molecular weight loss was a result of the protease digestion due to the cleavage site engineered into the antibody.

EXAMPLE 7

TFPI-binding ELISA of thrombin digested parental IgG and IgG-linker1

[61] One microgram of the full length antibodies, parental IgG and IgG-linker1, were digested with 1 unit of biotinylated thrombin (Novagen) for 1 hr at 37°C. Then 50 µL of streptavidin sepharose beads were added to the digestion to deplete the thrombin. The digested IgG samples were applied to a column that captures the sepharose bead/thrombin complex. The eluate contains the digested IgGs.

[62] A MAXISORP® 96-well plate (Nunc) was coated with 1 µg/mL of TFPI in PBS over night at 4 C°. The plate was blocked for 1hr at room temperature (RT) in 5% non-fat dry milk PBS/0.5% TWEEN® 20 (PBS-T). Serial two-fold dilutions of undigested and digested parental IgG and IgG-linker1 were added to the wells (100 µL/well) and incubated for 1hr at RT. The plate was washed five times in PBS-T. A secondary HRP conjugated anti-Fab- antibody was added (100 µL of a 1:10,000 dilution) for detection with an AMPLEX RED ® (Invitrogen) solution.

[63] The results of the TFPI binding ELISA are shown in FIG. 7. Loss of TFPI binding by thrombin digested IgG Linker-1 is shown. The TFPI binding of undigested IgG-linker1 was similar to the TFPI binding of the undigested and thrombin-digested parental IgG.

EXAMPLE 8

BIACORE™ analysis of parental IgG and IgG-linker1

[64] A CM4 sensor chip was immobilized with a low density of human TFPI using an amine coupling kit (GE HealthCare). Kinetic assays of parental IgG and IgG-linker1 were conducted using different concentration of the antibodies, followed by regeneration with pH 1.5 glycine buffer. The parental IgG and IgG- linker1 antibodies at a concentration of 1 μg were digested with 1 unit of biotinylated thrombin (Novagen) for 1 hr at 37C°. The antibodies with or without digestion were injected to a BIACORE™ system for TFPI-binding analysis. The following figure shows the signal generated from injection 45 μl of 6.25 μg/ml antibodies or control samples.

[65] In kinetics assay, parental IgG and IgG2-linker1 have association rates (ka) of $1.536x10^6$ /Ms and 1.902×10^6/Ms, respectively. The two antibodies did not have measurable dissociation in 30 minutes. This indicates that the insertion of linker1 did not significantly change binding activity of the antibody.

[66] The effect of thrombin cleavage on the antibodies is shown in **FIG. 8**. Thrombin digestion slightly decreased the parental IgG binding on TFPI, whereas thrombin digestion reduced IgG2-linker1 binding on TFPI from 20 RU to 5 RU, a 75% decrease.

EXAMPLE 9

Western Blot of protease/coagulation factor treated IgG-Linker1 and Parental IgG

[67] The following human proteases/coagulation factors were used to digest 80nM of IgG-Linker 1 and the WT antibody: thrombin (0.1μM), plasmin (0.1μM), Factor VIIa (0.01μM), Factor IXa (0.089μM), Factor Xa (13μM), Factor XIa (0.031μM), Factor XIIIa (0.03μM). The treated material was run on a 4-15% CRITEREON™ TGX gel (Bio-Rad). Proteins were transferred to a nitrocellulose membrane and probed with an anti-human IgG antibody (Pierce).

[68] Human thrombin, plasmin and FactorXa digested IgG-Linker1, thrombin digesting most efficiently (**FIG. 9**) as shown by the appearance of a 37kDa band. The proteases

did not cleave the parental IgG, indicating that molecular weight loss was a result of the protease digestion due to the cleavage site engineered into the antibody.

CLAIMS

1. A protease-regulated antibody which specifically binds to tissue factor pathway inhibitor (TFPI), comprising a variable domain, a constant domain, and an amino acid sequence joining the variable domain to the constant domain, wherein the amino acid sequence comprises a protease cleavage site.

2. The protease-regulated antibody of claim 1, wherein the protease cleavage site is a thrombin cleavage site.

3. The protease-regulated antibody of claim 1, wherein the protease cleavage site is a plasmin cleavage site.

4. The protease-regulated antibody of claim 1, wherein the protease cleavage site is a Factor Xa cleavage site.

5. The protease-regulated antibody of claim 1 which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:131, amino acids 1-239 of SEQ ID NO:133, SEQ ID NO:135, amino acids 1-229 of SEQ ID NO:136, SEQ ID NO:139, SEQ ID NO:140, SEQ ID NO:143, and SEQ ID NO:144.

6. The protease-regulated antibody of any of claims 1-5, wherein the amino acid sequence further comprises an amino acid linker.

7. The protease-regulated antibody of any of claims 1-6 which is a full-length antibody.

8. The protease-regulated antibody of any of claims 1-6 which is an antibody fragment.

9. A pharmaceutical composition comprising the protease-regulated antibody of any of claims 1-8 and a pharmaceutically acceptable vehicle.

10. An isolated nucleic acid molecule encoding the protease-regulated antibody of any of claims 1-8.

11. A method of treating a coagulopathy, comprising administering to a patient in need thereof a therapeutically effective amount of the protease-regulated antibody of any of claims 1-8.

12. The method of claim 11, wherein the coagulopathy is hemophilia.

13. A method of modulating procoagulant activity of an anti-TFPI antibody comprising adding a protease cleavage site into the anti-TFPI antibody.

14. The method of claim 13, wherein the protease cleave site is a thrombin cleavage site.

15. The method of claim 13, wherein the protease cleavage site is a plasmin cleavage site.

16. The method of claim 13, wherein the protease cleavage site is a Factor Xa cleavage site.

17. A method of promoting generation of thrombin or plasmin, comprising contacting TFPI with a protease-regulated antibody of any of claims 1-8.