COMPOSITIONS TARGETING THE SOLUBLE EXTRACELLULAR DOMAIN OF E-CADHERIN AND RELATED METHODS FOR CANCER THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the priority date of U.S. Provisional Application No. 61/407,367, which was filed October 27, 2010. ~~The content of this earlier-filed provisional application is hereby incorporated by reference herein in its entirety.~~

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

This invention was made with government support awarded by the National Institutes of Health under Grant Nos. CA133910 and ES015832. The U.S. government has certain rights in this invention.

FIELD OF THE INVENTION

The compositions and methods of the present invention are related to targeting the extracellular domain of the cell-cell adhesion protein E-cadherin. The compositions include binding agents, such as antibodies, as well as antigenic fragments of the E-cadherin extracellular domain that can be used in therapeutic and prophylactic methods of treating cancer.

BACKGROUND

E-cadherin is an integral transmembrane glycoprotein that helps maintain epithelial cell-cell adhesion. Loss of E-cadherin function (full length) has been demonstrated to result in cellular de-differentiation, proliferation and increased invasiveness in cancers of the skin, lung, stomach, intestine and breast (Brouxhon *et al.*, *Cancer Res.* 67(16):7654-7664 (2007); Hirohashi, *Am. J. Pathol.* 153(2):333-339 (1998); Chen *et al.*, *Cancer Lett.* 201:97-106 (2003)). Moreover, loss of E-cadherin staining in biopsy specimens from breast cancer patients has been associated with a poor prognosis and short metastasis-free periods (Pederson *et al.*, *Brit. J. Cancer* 87:1281-1286 (2002)).

The full length protein is composed of an extracellular domain consisting of five subdomains designated EC1-EC5, a single transmembrane region, and a cytoplasmic domain (*see* Shiraishi *et al.*, *J. Immunol.* 175(2):1014-1021 (2005)). The EC1 subdomain, which is the most distant from the cell membrane surface, contains a histidine-alanine-valine (HAV) triplet

corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies of interest include primatized antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* apes, Old World monkeys, New World monkeys, prosimians) and human constant region sequences.

Various methods for generating monoclonal antibodies (mAbs) are well known in the art. See, *e.g.*, the methods described in U.S. Patent No. 4,196,265, ~~incorporated herein by reference~~. The most standard monoclonal antibody generation techniques generally begin along the same lines as those for preparing polyclonal antibodies (*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988)). Typically, a suitable animal can be immunized with a selected immunogen to stimulate antibody-producing cells. Rodents such as mice and rats are exemplary animals, although rabbits, sheep, frogs, and chickens can also be used. Mice can be particularly useful (*e.g.*, BALB/c mice are routinely used and generally give a higher percentage of stable fusions).

Following immunization, somatic cells with the potential for producing the desired antibodies, specifically B lymphocytes (B cells), can be selected for use in MAb generation and fusion 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 typically 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 can be used, as are known to those of skill in the art. For example, where the immunized animal is a mouse, one can 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 can use R210.RCY3, Y3-Ag 1.2.3, IR983F, 4B210 or one of the above listed mouse cell lines. U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6, all can be useful in connection with human cell fusions.

to form antibody fragments. The general technique for filamentous phage display is described (U.S. Patent No. 5,658,727). 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 a gene that encodes the member from the library. Additional methods for screening phagemid libraries are described (U.S. Patent Nos. 5,580,717; 5,427,908; 5,403,484; and 5,223,409).

One 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 (U.S. Patent No. 5,698,426, ~~incorporated herein by reference~~). 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.*, *Proc. Natl. Acad. Sci. USA* 88:4363 (1991); and Barbas *et al.*, *Proc. Natl. Acad. Sci. USA* 88:7978 (1991)). The surface expression library is screened for specific Fab fragments that bind neuraminidase molecules by standard affinity isolation procedures. The selected Fab fragments can be characterized by sequencing the nucleic acids encoding the polypeptides after amplification of the phage population.

One method for producing diverse libraries of antibodies and screening for desirable binding specificities is described (U.S. Patent Nos. 5,667,988 and 5,759,817). 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 degeneracies into CDR regions of immunoglobulin variable heavy and light chain variable domains, and display of mutagenized polypeptides on the surface of the phagemid. Thereafter, the display protein is screened for the ability to bind to a preselected antigen. A further variation of this method for producing diverse libraries of antibodies and screening for desirable binding specificities is described U.S. Patent No. 5,702,892, ~~incorporated herein by reference~~). In this method, only heavy chain sequences are employed, heavy chain sequences are

randomized at all nucleotide positions that encode either the CDRI or CDRIII hypervariable region, and the genetic variability in the CDRs can be generated independent of any biological process.

In addition to the combinatorial immunoglobulin phage expression libraries disclosed above, one molecular cloning approach is to prepare antibodies from transgenic mice containing human antibody libraries. Such techniques are described (U.S. Patent No. 5,545,807, ~~incorporated herein by reference~~). Such transgenic animals can be 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 method for producing human antibodies is described in U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661;016; and 5,770,429, wherein transgenic animals are described that are capable of switching from an isotype needed for B cell development to other isotypes.

The anti-sEcad immunoglobulins may be modified to reduce or abolish glycosylation. An immunoglobulin that lacks glycosylation may be an immunoglobulin that is not glycosylated at all; that is not fully glycosylated; or that is atypically glycosylated (*i.e.*, the glycosylation pattern for the mutant differs from the glycosylation pattern of the corresponding wild type immunoglobulin). The IgG polypeptides include one or more (*e.g.*, 1, 2, or 3 or more) mutations that attenuate glycosylation, *i.e.*, mutations that result in an IgG CH2 domain that lacks glycosylation, or is not fully glycosylated or is atypicially glycosylated. Mutations of the asparagine residue at amino acid 297 in human IgG1 is an example of such a mutation. The oligosaccharide structure can also be modified, for example, by eliminating the fusose moiety from the N-linked glycan.

Antibodies can also be modified to increase their stability and or solubility in vivo by conjugation to non-protein polymers, e.g, polyethylene glycol. Any PEGylation method can be used as long as the anti-sEcad antibody retains the ability to selectively bind the second, third, fourth or fifth subdomain of sEcad.

A wide variety of antibody/immunoglobulin frameworks or scaffolds can be employed so long as the resulting polypeptide includes at least one binding region that is specific for the target, *i.e.*, the second, third, fourth, or fifth subdomain of sEcad. Such frameworks or scaffolds include the five main idiotypes of human immunoglobulins, or fragments thereof (such as those disclosed elsewhere herein), and include immunoglobulins of other animal species, preferably

coding sequence for a given fragment of an antibody, a mutant antibody or fragment thereof, or a fusion protein or fragment thereof can be modified such that optimal expression in a particular organism is obtained, using appropriate codon bias tables for that organism.

Vectors containing nucleic acids such as those described herein also are provided. A "vector" is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Expression vectors include plasmid vectors, viral vectors, and the HSV amplicon particles as described in U.S. Application Publication No. 2006/0239970 ~~(which is hereby incorporated herein by reference)~~. Generally, a vector is capable of replication when associated with the proper control elements. Suitable vector backbones include, for example, those routinely used in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs, or PACs. The term "vector" includes cloning and expression vectors, as well as viral vectors and integrating vectors. An "expression vector" is a vector that includes a regulatory region. Suitable expression vectors include, without limitation, plasmids and viral vectors derived from, for example, bacteriophage, baculoviruses, and retroviruses. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, WI), Clontech (Palo Alto, CA), Stratagene (La Jolla, CA), and Invitrogen/Life Technologies (Carlsbad, CA).

The vectors provided herein also can include, for example, origins of replication, scaffold attachment regions (SARs), and/or markers. A marker gene can confer a selectable phenotype on a host cell. For example, a marker can confer biocide resistance, such as resistance to an antibiotic (*e.g.*, kanamycin, G418, bleomycin, or hygromycin). As noted above, an expression vector can include a tag sequence designed to facilitate manipulation or detection *(e.g.*, purification or localization) of the expressed polypeptide. Tag sequences, such as green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or Flag™ tag (Kodak, New Haven, CT) sequences typically are expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide, including at either the carboxyl or amino terminus.

The vector can also include a regulatory region. The term "regulatory region" refers to nucleotide sequences that influence transcription or translation initiation and rate, and stability and/or mobility of a transcription or translation product. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites,

destruction of cells. A non-cytotoxic agent may include an agent that can be activated to be cytotoxic. A non-cytotoxic agent may include a bead, liposome, matrix or particle (see, *e.g.*, U.S. Patent Publications 2003/0028071 and 2003/0032995 ~~which are incorporated by reference herein~~). Such agents may be conjugated, coupled, linked or associated with an antibody or other targeting agent disclosed herein.

Conventional cancer medicaments can be administered with the compositions disclosed herein. Useful medicaments include anti-angiogenic agents, *i.e.*, agents block the ability of tumors to stimulate new blood vessel growth necessary for their survival. Any anti-angiogenic agent known to those in the art can be used, including agents such as Bevacizumab (Avastin®, Genentech, Inc.) that block the function of vascular endothelial growth factor (VEGF). Other examples include, without limitation, Dalteparin (Fragmin®), Suramin ABT-510, Combretastatin A4 Phosphate, Lenalidomide, LY317615(Enzastaurin), Soy Isoflavone (Genistein; Soy Protein Isolate) AMG-706, Anti-VEGF antibody, AZD2171, Bay 43-9006 (Sorafenib tosylate), PI-88, PTK787/ZK 222584 (Vatalanib), SU11248 (Sunitinib malate), VEGF-Trap, XL184, ZD6474, Thalidomide, ATN-161, EMD 121974 (Cilenigtide) and Celecoxib (Celebrex®).

Other useful therapeutics include those agents that promote DNA-damage, *e.g.*, double stranded breaks in cellular DNA, in cancer cells. Any form of DNA-damaging agent know to those of skill in the art can be used. DNA damage can typically be produced by radiation therapy and/or chemotherapy. Examples of radiation therapy include, without limitation, external radiation therapy and internal radiation therapy (also called brachytherapy). Energy sources for external radiation therapy include x-rays, gamma rays and particle beams; energy sources used in internal radiation include radioactive iodine ($iodine^{125}$ or $iodine^{131}$), and from $strontium^{89}$, or radioisotopes of phosphorous, palladium, cesium, iridium, phosphate, or cobalt. Methods of administering radiation therapy are well known to those of ordinary skill in the art.

Examples of DNA-damaging chemotherapeutic agents include, without limitation, Busulfan (Myleran), Carboplatin (Paraplatin), Carmustine (BCNU), Chlorambucil (Leukeran), Cisplatin (Platinol), Cyclophosphamide (Cytoxan, Neosar), Dacarbazine (DTIC-Dome), Ifosfamide (Ifex), Lomustine (CCNU), Mechlorethamine (nitrogen mustard, Mustargen), Melphalan (Alkeran), and Procarbazine (Matulane).

Claims:

1. Use of an agent that specifically targets one or more of the second, third, fourth, or fifth subdomains (EC2, EC3, EC4 and EC5, respectively) of soluble E-cadherin (sEcad) but not the first subdomain (EC1) of sEcad, in the preparation of a pharmaceutical formulation for treating a patient who has cancer.

2. The use of claim 1, wherein the agent specifically targets EC4 and/or EC5.

3. The use of claim 2, wherein the agent specifically targets EC4.

4. The use of claim 2, wherein the agent specifically target EC5.

5. The use of any one of claims 1 to 4, wherein the agent is a protein scaffold.

6. The use of claim 5, wherein the protein scaffold is an antibody or a fragment thereof that specifically binds an epitope comprising amino acid residues in one or more of the EC2, EC3, EC4 or EC5 subdomains of sEcad but not in the EC1 subdomain of sEcad.

7. The use of claim 6, wherein the antibody is a humanized, chimeric, or human antibody.

8. The use of claim 6, wherein the antibody is a single chain antibody.

9. The use of claim 6, wherein the antibody is a monoclonal or polyclonal antibody.

10. The use of claim 6, wherein the antibody is of the immunoglobulin G (IgG) class or immunoglobulin M (IgM) class.

11. The use of any one of claims 1 to 10, wherein the agent is detectably labeled.

12. The use of any one of claims 1 to 11, wherein the agent kills malignant E-cadherin-expressing cells but does not kill non-malignant cells.

13. The use of claim 12, wherein the agent kills malignant E-cadherin-expressing cells by programmed cell death, growth arrest, anoikis, necrosis, or autophagy.

14. The use of any one of claims 1 to 13, wherein the agent is for administration in a pharmaceutical formulation that is free of cytotoxic amounts of any excipient.

15. The use of any one of claims 1 to 14, wherein the agent is delivered in a pharmaceutical formulation that:

(a) produces, upon administration to a patient, a serum level of the agent of about 1-10 mg/kg, or,

(b) produces, upon addition to a cell culture, a concentration of the agent of about 1-500 μg/mL of cell culture medium.

16. The use of claim 15, wherein the pharmaceutical agent produces a serum level of about 1-5 mg/kg in the patient or a concentration in the cell culture medium of about 1-400 μg/mL.

17. The use of any one of claims 1 to 16, wherein the agent is delivered in a pharmaceutical formulation by oral administration, intravenous administration, nasal or inhalation administration, intramuscular administration, intraperitoneal administration, transmucosal administration, or transdermal administration.

18. The use of any one of claims 1 to 17, further comprising the step of ~~providing~~ testing a biological sample from the patient and determining whether the sample includes an elevated level of sEcad or another predictive biomarker for cancer.

19. The use of claim 18, wherein the biological sample is a urine, saliva, cerebrospinal fluid, blood, or biopsy sample.

20. The use of claim 18, wherein the step is carried out before administering the agent and an elevated level of sEcad indicates that the patient is a good candidate for the treatment.

21. The use of claim 18, wherein the step is carried out at one or more times after administering the agent and a reduced level of sEcad indicates that the patient is responding well to the treatment.

22. The use of any one of claims 1 to 21, wherein the pharmaceutical formulation is formulated for administration in connection with~~further comprising the step of administering~~ a second cancer treatment.

23. The use of claim 22, wherein the second cancer treatment comprises administration of a chemotherapeutic agent, a radiation treatment, treatment with an antibody, or surgical intervention.

24. The use of any one of claims 1 to 23, wherein the cancer is within an epithelialized tissue.

25. The use of any one of claims 1 to 24, wherein the cancer is a cancer of the alimentary canal, central nervous system, breast, skin, reproductive system, lung, or urinary tract.

26. The use of claim 25, wherein the cancer of the alimentary canal is a cancer of the mouth, throat, esophagus, stomach, intestine, rectum or anus.

27. The use of claim 25, wherein the cancer of the skin is squamous cell carcinoma or melanoma.

28. The use of claim 25, wherein the cancer of the reproductive system is cervical cancer, uterine cancer, ovarian cancer, vulval or labial cancer, prostate cancer, testicular cancer, or cancer of the male genital tract.

29. Use of (a) an antigenic polypeptide that comprises an amino acid sequence from one or more of the EC2-EC5 subdomains of sEcad but excludes the EC1 subdomain, or an antigenically active fragment or other variant thereof or (b) an expression vector comprising a nucleic acid sequence encoding the antigenic polypeptide or the antigenically active fragment or other variant thereof, in the preparation of a pharmaceutical formulation for reducing the likelihood that a subject will develop cancer.

30. The use of claim 29, wherein the antigenic polypeptide comprises an amino acid sequence from EC4 and/or EC5.

31. The use of claim 29, wherein the antigenic polypeptide comprises an amino acid sequence from EC4.

32. The use of claim 29, wherein the antigenic polypeptide comprises an amino acid sequence from EC5.

33. The use of claim 29, wherein the antigenic polypeptide elicits the production of antibodies that specifically bind sEcad but do not bind E-cadherin expressed by non-malignant cells in the subject.

34. The use of claim 29, wherein the antigenic polypeptide or the expression vector is delivered in a pharmaceutical formulation by oral administration, intravenous administration, nasal or inhalation administration, intramuscular administration, intraperitoneal administration, transmucosal administration, or transdermal administration.

35. The use of any one of claims 29 to 34, wherein the cancer is within an epithelialized tissue.

36. The use of any one of claims 29 to 34, wherein the cancer is a cancer of the alimentary canal, central nervous system, breast, skin, reproductive system, lung, or urinary tract.

37. The use of claim 36, wherein the cancer of the alimentary canal is a cancer of the mouth, throat, esophagus, stomach, intestine, rectum or anus.

38. The use of claim 36, wherein the cancer of the skin is squamous cell carcinoma or melanoma.

39. The use of claim 36, wherein the cancer of the reproductive system is cervical cancer, uterine cancer, ovarian cancer, vulval or labial cancer, prostate cancer, testicular cancer, or cancer of the male genital tract.

40. A pharmaceutically acceptable composition comprising a therapeutically effective amount of (a) an antibody that specifically binds an epitope comprising amino acid residues in one or more of the EC2, EC3, EC4 or EC5 subdomains of sEcad but not in the EC1 subdomain of sEcad; (b) an antigenic polypeptide that comprises an amino acid sequence from one or more of the EC2-EC5 subdomains of sEcad but excludes the EC1 subdomain, or an antigenically active fragment or other variant thereof; or (c) an expression vector comprising a nucleic acid sequence encoding the antigenic polypeptide or the antigenically active fragment or other variant thereof.

41. A method for identifying an epitope in an sEcad, the method comprising:

(a) providing a polypeptide comprising sEcad or a fragment or other variant thereof;

(b) administering the polypeptide to an animal;

(c) isolating antibodies produced by the animal in response to the polypeptide; and

(d) exposing cancerous cells to the antibodies or to a monoclonal antibody generated therefrom, wherein the death of the cancerous cells indicates that the polypeptide comprises an epitope of sEcad that can be targeted or used as an agent in cancer treatment, prophylaxis, or imaging.

42. The method of claim 41, wherein the polypeptide comprises an amino acid sequence from one or more of the EC2, EC3, EC4, or EC5 subdomains of sEcad but excludes amino acid sequence from the EC1 subdomain of sEcad.

43. The method of claim 42, wherein the polypeptide comprises an amino acid sequence from one or more of EC4 or EC5.

44. The method of claim 43, wherein the polypeptide comprises an amino acid sequence confined to EC4.

45. The method of claim 43, wherein the polypeptide comprises an amino acid sequence confined to EC5.