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Benævnelse: FREMGANGSMÅDE TIL BEHANDLING AF CANCER, DER OMFATTER EN VEGF-B-ANTAGONIST

Fremdragne publikationer:
WO-A1-00/64261
SCOTNEY P D ET AL: "Human vascular endothelial growth factor B: characterization of recombinant isoforms and generation of neutralizing monoclonal antibodies."
LEONARD P ET AL: "Crystal Structure of Vascular Endothelial Growth Factor-B in Complex with a Neutralising Antibody Fab Fragment"
UNDERINER TL ET AL: "DEVELOPMENT OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR (VEGF) KINASE INHIBITORS AS ANTI-ANGIOGENIC AGENTS IN CANCER THERAPY"
DESCRIPTION

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of cancer therapy and prophylaxis. More particularly, the present invention provides growth factor antagonists which inhibit the growth of cancers including tumors and pre-cancerous tissue. Even more particularly, the present invention is directed to antagonists of vascular endothelial growth factor-B and their use to inhibit the growth of cancer including tumor tissue and pre-cancerous tissue.

DESCRIPTION OF THE PRIOR ART

[0002] Bibliographic details of the publications referred to in this specification are also collected at the end of the description.

[0003] Reference to any prior art is not, and should not be taken as an acknowledgement or any form of suggestion that this prior art forms part of the common general knowledge in any country.


[0005] In addition to normal physiological processes, the pathological growth of tumors is also known to be dependent on the degree of new blood vessel formation in the tumor bed (Carmeliet et al., 2000 supra; Folkman, Nature Medicine 1:27-31, 1995; Hanahan & Folkman, Cell 86:353-364, 1996). VEGF-A mRNA is upregulated in many human tumors and VEGF-A appears to be an important angiogenic factor frequently utilized by tumors to switch on blood
vessel growth (Dvorak et al., Semin Perinatol 24:75-78, 2000; Ferrara & Alitalo, 1999 supra; Yancopoulos, 2000 supra; Benjamin & Keshet, Proc Natl Acad Sci USA 94:8761-8766, 1997; Ferrara and Davis-Smyth, Endocr. Rev 18:4-25, 1997). VEGF-A also increases vascular permeability, and this is thought to be important for tumor invasion and metastasis (Dvorak et al., Curr Top Microbiol Immunol 237:97-132, 1999). As a result there has been a significant effort towards the development of agents that target angiogenic factors such as VEGF-A in order to inhibit tumor growth (Ferrara et al., 2003 supra). One such agent is bevacizumab, a humanized mouse monoclonal antibody that binds to, and inhibits the activity of, VEGF-A. Bevacizumab (Avastin) has recently been approved by the FDA for the treatment of colorectal cancer.

[0006] VEGF-A is now recognized as the founding member of a family of structurally related molecules. The 'VEGF family' comprises six members including prototype VEGF-A, placenta growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E (Eriksson & Alitalo, Curr Top Microbiol Immunol 237:41-57, 1999). The biological functions of the VEGF family are mediated by the differential activation of at least three structurally homologous tyrosine kinase receptors, VEGFR-1/Flt-1, VEGFR-2/Flik-1/KDR and VEGFR-3/Fit-4. VEGF-A, VEGF-B and PLGF also bind to the non-tyrosine kinase receptors neuropilin-1 and -2, Soker et al., Cell 92:735-45, 1998; Neufeld et al., Trends Cardiovasc Med. 12:13-19, 2002). According to their receptor binding patterns, the VEGF family can be divided into three subgroups: (1) VEGF-A, which binds to VEGFR-1 and VEGFR-2; (2) PLGF and VEGF-B, which bind only to VEGFR-1 and; (3) VEGF-C and VEGF-D, which interact with both VEGFR-2 and VEGFR-3 (Ferrara & Alitalo, 1999 supra; Ferrara et al., 2003 supra).

[0007] As noted above, VEGF-A is the most thoroughly characterized member of the VEGF family and an accumulation of evidence has led to the conclusion that VEGFR-2 is the major mediator of VEGF-A associated biological activities such as endothelial cell proliferation, migration and survival, angiogenesis and vascular permeability (Ferrara et al., 2003 supra). In addition to VEGFR-2, VEGF-C and -D also bind to, and activate, VEGFR-3. VEGFR-3 is expressed primarily on lymphatic endothelial cells and VEGF-C and -D are thought to be key regulators of lymphatic angiogenesis [or lymphangiogenesis] (Makinen et al., Nature Medicine 7:199-205, 2001; Skobe et al., Nature Medicine 7:192-8, 2001; Stacker et al., Nature Medicine 7:186-91, 2001). In contrast to VEGF-A, -C and -D and the downstream effects of signaling through VEGFR-2 or -3, the precise role of VEGF-B and signaling through VEGFR-1 remains poorly understood.

[0008] VEGFR-1 is expressed on a variety of cell types (Clauss et al., J. Biol. Chem. 271:17629-17634, 1996; Wang & Keiser, Circ. Res. 83:832-840, 1998; Niida et al., J. Exp. Med. 190:293-298, 1999) and expression, at least on endothelial cells, is upregulated by hypoxia and a HIF-1α dependent mechanism (Gerber et al., J. Biol. Chem. 272:23659-23667, 1997). However, only weak autophosphorylation of VEGFR-1 is observed in response to VEGF-A, and VEGF-A binding to VEGFR-1 appears not to activate the downstream signals required for key endothelial cell responses such as proliferation and survival (de Vries et al., Science 255:989-991, 1992; Waltenberger et al., J. Biol. Chem. 269:26988-26995, 1994; Keyt
et al., J. Biol. Chem. 271:5638-5646, 1996; Rahimi et al., J. Biol. Chem. 275:16986-16992, 2000). The observation that the VEGF-1 specific ligand, PLGF, enhanced the activity of VEGF-A on endothelial cells suggested that VEGF-R-1 might function as a decoy receptor i.e. PLGF displaced VEGF-A from VEGF-R-1 making it available to bind to, and signal through VEGF-R-2 (Park et al., J Biol Chem. 269:25646-25654, 1994). In vivo data from genetically modified mice further suggested a non-signaling decoy role for VEGF-R-1. VEGF-R-1<sup>−/−</sup> mice died in utero between days 8.5 and 9.5 and although endothelial cells developed, they failed to organize into vascular channels (Fong et al., Development 126:3015-3025, 1999). Lethality was attributed to excessive angioblast proliferation and this in turn, was attributed to enhanced VEGF-A action (Fong et al., 1999 supra). The observation that mice expressing VEGF-R-1 lacking the kinase domain were healthy and showed no overt defect in vascular development provided further support for the decoy hypothesis, as the truncated receptor could still bind VEGF-A, but not transmit intracellular signals (Hiratsuka et al., Proc. Natl. Acad. Sci. USA 4:9349-9354, 1998).

[0009] Analysis of VEGF-B<sup>−/−</sup> mice has also failed to resolve the confusion surrounding the precise physiological (and pathological) role of VEGF-R-1 specific ligands and VEGF-R-1 signaling. In contrast to VEGF-A<sup>−/−</sup> mice, VEGF-B<sup>−/−</sup> mice display no overt defects in vascular development and are healthy and fertile (Bellomo et al., Circ. Res. 86:E29-E35, 2000). In one report the hearts of VEGF-B<sup>−/−</sup> mice were reduced in size and the response to coronary occlusion and myocardial recovery from ischemia were compromised (Bellomo et al 2000, supra). Although heart morphology appeared normal, the authors concluded that VEGF-B is essential for the establishment of a fully functional coronary vasculature. In contrast, a second report describing VEGF-B<sup>−/−</sup> mice reported only a minor atrial conduction defect (Aase et al., Circulation 104:358-364, 2001).

[0010] As a result of the confusion surrounding the role for VEGF-R-1 and VEGF-R-1-specific ligands in the regulation of blood vessel formation, the potential of VEGF-B as a therapeutic target for inhibition of tumor growth and metastasis is unclear. Although VEGF-B has been shown, along with other factors, to be expressed in a variety of tumors (Salven et al, Am J Pathol, 153:103-108, 1998), evidence of upregulation is limited (Li et al., Growth Factors 19:49-59, 2001) and there have been no reports of the efficacy of VEGF-B specific antagonists in xenograft or other relevant animal models. In fact, the potential of VEGF-B (and PLGF) is further confused by the disclosure by Cao et al in International PCT Publication No. WO03/62788, which suggests that increasing VEGF-B expression inhibits VEGF-A induced angiogenesis, and thus represents a potential approach to the treatment of diseases caused by VEGF-A activity and VEGF-A induced angiogenesis.


[0012] In accordance with the present invention, it has been surprisingly determined that
antagonists of VEGF-B are useful in reducing the growth and development of cancer including tumor tissue.

SUMMARY OF THE INVENTION

[0013] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprised" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0014] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

[0015] A method of inhibiting the growth of cancer is disclosed herein including tumor tissue and pre-cancerous tissue using an antagonist of VEGF-B. Compositions are also described comprising one or more VEGF-B antagonists alone or in combination with other anti-cancer agents or other angiogenesis-inhibiting agents.

[0016] An antagonist described herein may be an antibody which inhibits interaction between VEGF-B and VEGFR-1, an antisense compound which reduces VEGF-B expression, or an interfering nucleic acid which reduces VEGF-B expression.

[0017] In particular, the invention provides an isolated anti-VEGF-B antibody which competes for binding with a second anti-VEGF-B antibody and which isolated antibody binds to human VEGF-B with a K_D value of 1 x 10^{-7} M or less, is cross-reactive for human VEGF-B and murine VEGF-B and inhibits human VEGF-B induced signaling through VEGFR-1, wherein the second antibody comprises a light chain variable region having the amino acid sequence shown in SEQ ID NO:3 and a heavy chain variable region having the amino acid sequence shown in SEQ ID NO:4.

[0018] The preferred antibodies bind to VEGF-B and interfere with VEGF-B interaction with its receptor. The antibody and other antagonists disclosed herein are proposed for use in treating certain conditions mediated in whole or in part, or directly or indirectly, by VEGF-B. Thus methods are disclosed herein for treating these conditions in a subject comprising administering a VEGF-B antagonist.

[0019] In the case of antibodies or other antagonists directed towards human VEGF-B, some level of cross-reactivity with other mammalian forms of VEGF-B may be desirable in certain circumstances, such as for example, for the purpose of testing antibodies or other antagonists in animal models for their effect on a particular disease and for conducting toxicology studies in
a manner where VEGF-B/VEGFR-1 receptor signaling in the test animal is affected by the test antibody or other antagonists. Species where cross-reactivity of an antibody or other antagonists to human VEGF-B may be desirable include mouse, dog and monkey. The antibodies of the present invention exhibit cross-reactivity to human VEGF-B and murine VEGF-B.

[0020] The antibodies of the present invention bind with the receptor binding domain (RBD) of VEGF-B and inhibit VEGF-B induced signaling through VEGFR-1. A particularly preferred group of antibodies of the present invention are those that bind with the human VEGF-B RBD and which also bind, interact, or otherwise associate to the murine VEGF-B RBD and inhibit VEGF-B induced signaling through the VEGFR-1.

[0021] Preferably, the antibodies are monoclonal antibodies ("mAbs") or antigen-binding fragments thereof. Even more preferably, the antibodies are humanized antibodies including deimmunized or chimeric antibodies or human antibodies suitable for administration to humans. Humanized antibodies, prepared, for example, from murine monoclonal antibodies and human monoclonal antibodies which are prepared, for example, using transgenic mice or by phage display are particularly preferred.

[0022] Antibodies described herein include the murine monoclonal antibodies 1C6, 2F5, 2H10 and 4E12, and humanized, deimmunized or chimeric forms of mAbs 1C6, 2F5, 2H10 and 4E12.

[0023] Methods of modulating VEGF-B-mediated diseases or conditions by the administration of VEGF-B antagonists are disclosed herein, in particular antibodies of the present invention. Conditions to be treated in accordance with the present invention include cancers and tumors, and in particular solid tumors. Pre-cancerous conditions and dispersed tumors, such as for example, myelomas and lymphomas may also be treated.

[0024] Accordingly, the present invention also provides a composition comprising an isolated antibody of the invention described above. The invention also provides the antibody and composition of the invention described above for use in medicine. In a particular embodiment, the invention provides the antibody and composition of the invention described above for use in treating cancer in a mammal.

[0025] A summary of sequence identifiers used throughout the subject specification is provided in Table 1.

TABLE 1

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<tr>
<th>SEQUENCE ID NO:</th>
<th>DESCRIPTION</th>
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<td>2</td>
<td>Amino acid sequence of the murine VEGF-B&lt;sub&gt;10-108&lt;/sub&gt;</td>
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<td>DESCRIPTION</td>
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<td>Nucleotide sequence of 2H10 CDR-L1 optimised for expression in <em>E. coli</em></td>
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<td>Nucleotide sequence of 2H10 CDR-H2 optimised for expression in <em>E. coli</em></td>
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<tr>
<td>41</td>
<td>Nucleotide sequence of 2H10 CDR-H3</td>
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[0026] A list of abbreviations used in the subject specification are shown in Table 2.
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>Vascular Endothelial Growth Factor-B</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>VEGF-B Receptor</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placenta Growth Factor</td>
</tr>
<tr>
<td>Mabs</td>
<td>Monoclonal antibodies</td>
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<td>1C6</td>
<td>Murine monoclonal antibody to VEGF-B</td>
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<tr>
<td>2F5</td>
<td>Murine monoclonal antibody to VEGF-B</td>
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<tr>
<td>2H10</td>
<td>Murine monoclonal antibody to VEGF-B</td>
</tr>
<tr>
<td>4E12</td>
<td>Murine monoclonal antibody to VEGF-B</td>
</tr>
<tr>
<td>RBD</td>
<td>Receptor Binding Domain</td>
</tr>
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<td>CDR</td>
<td>Complementarily Determining Regions</td>
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<td>Murine VEGF-B</td>
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<tr>
<td>hVEGF-B</td>
<td>Human VEGF-B</td>
</tr>
<tr>
<td>VEGF-B_{10-108}</td>
<td>Truncated form of VEGF-B comprising amino acids 10 through 108</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription-Polymerase Chain Reaction</td>
</tr>
</tbody>
</table>

**BRIEF DESCRIPTION OF THE FIGURES**

[0027]

**Figure 1** shows the heavy and light chain variable region amino acid sequences of anti-VEGF-B mAb 2H10. Amino acid numbering is according to Kabat et al., (Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991). CDR regions (underlined) are defined according to the sequence definition of Kabat et al. (*supra*), except for CDR-H1, which is the combined sequence and structural definition of Chothia and Lesk, *J. Mol. Biol.* 196:901-917, 1987.

**Figure 2** shows the heavy and light chain variable region amino acid sequences of anti-VEGF-B mAb 4E12. Amino acid numbering is according to Kabat et al., (*supra*). CDR regions (underlined) are defined according to the sequence definition of Kabat et al. (*supra*), except for CDR-H1, which is the combined sequence and structural definition of Chothia and Lesk (*supra*).

**Figure 3** shows the heavy and light chain variable region amino acid sequences of anti-VEGF-B mAb 2F5. Amino acid numbering is according to Kabat et al., (*supra*). CDR regions (underlined) are defined according to the sequence definition of Kabat et al. (*supra*), except for
CDR-H1, which is the combined sequence and structural definition of Chothia and Lesk (supra).

**Figure 4** shows the Human Variable Light Chain Germline (PK9/JK4) amino acid sequence derived from the EmBL database sequences X93622 and J00242 (human unrearranged germline antibody light chain regions PK9 and JK4 respectively) and the Human Variable Heavy Chain Germline (DP75/JH4a) amino acid sequence derived from the EmBL database sequences HSIGDP75 and J00256 (human unrearranged germline antibody heavy chain regions DP75 and JH4a respectively). The CDR regions are underlined.

**Figure 5** shows the Human Variable Light Chain Germline (PK9/JK4) amino acid sequence and the Human Variable Heavy Chain Germline (DP75/JH4a) amino acid sequence following CDR-grafting with the CDR's of the murine anti-VEGF-B monoclonal antibody 2H10. The CDR regions are underlined. The amino acid at position 74 in the heavy chain sequence (Kabat numbering H73) was backmutated from Thr to Lys as described in Example 10.

**Figure 6** shows the nucleic acid sequences for the 2H10 CDR regions optimised for expression in *E. coli*.

**Figure 7** shows the heavy and light chain variable region amino acid sequences of anti-VEGF-B mAb 1C6. Amino acid numbering is according to Kabat et al., (supra). CDR regions (underlined) are defined according to the sequence definition of Kabat et al. (supra), except for CDR-H1, which is the combined sequence and structural definition of Chothia and Lesk (supra).

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

[0028] Before describing the present invention in detail, it is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations of components, manufacturing methods, dosage or diagnostic regimes, or the like. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0029] The singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "an antibody" includes a single antibody, as well as two or more antibodies; reference to "a VEGF-B" includes a single VEGF-B as well as two or more VEGF-B molecules; reference to "an antagonist" includes a single antagonist as well as two or more antagonists; and so forth.

[0030] In describing and claiming the present invention, the following terminology is used in accordance with the definitions set forth below.
[0031] The terms "antagonist", "compound", "active agent", "pharmacologically active agent", "medicament" and "active" are used interchangeably herein to refer to a VEGF-B antagonist that induces a desired pharmacological and/or physiological effect. The terms also encompass pharmaceutically acceptable and pharmacologically active forms of those active agents specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the terms "antagonist", "compound", "active agent", "pharmacologically active agent", "medicament" and "active" are used, then it is to be understood that this includes the active agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs, etc.

[0032] Reference to an "antagonist", "compound", "active agent", "pharmacologically active agent", "medicament" and "active" may include combinations of two or more of such components, such as for example, two or more antibodies. A "combination" also includes multi-part combinations such as a two-part composition where the agents are provided separately and given or dispensed separately or admixed together prior to dispensation.

[0033] For example, a multi-part pharmaceutical pack may have two or more antibodies maintained separately or an anti-VEGF-B antibody and an anti-cancer agent or an angiogenesis-inhibiting agent.

[0034] The terms "effective amount" and "therapeutically effective amount" of an agent as used herein mean a sufficient amount of the VEGF-B antagonist to provide the desired therapeutic or physiological effect or outcome including inhibiting angiogenesis and/or inhibiting growth of cancer including tumor tissue. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount". The exact amount of agent required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation. The ability of a VEGF-B antagonist, preferably an antibody, to inhibit cancer can be evaluated in an animal model system predictive of efficacy in human tumors. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

[0035] By "pharmacologically acceptable" carrier and/or diluent is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, agents used for adjusting tonicity, buffers, chelating agents, and absorption delaying agents and the like.

[0036] Similarly, a "pharmacologically acceptable" salt, ester, amide, prodrug or derivative of a
compound as described herein is a salt, ester, amide, prodrug or derivative that is not biologically or otherwise undesirable.

[0037] The terms "treating" and "treatment" as used herein refer to reduction in severity and/or frequency of symptoms of cancer, elimination of symptoms and/or underlying cause of cancer, prevention of the occurrence of symptoms of cancer and/or their underlying cause and improvement or remediation or amelioration of damage following a cancer.

[0038] The terms "cancer" and "tumor" may be used interchangeably and include pre-cancerous conditions.

[0039] "Treating" a subject may involve prevention of cancer growth or other adverse physiological event in a susceptible subject as well as treatment of a clinically symptomatic subject by ameliorating the symptoms of cancer.

[0040] A "subject" as used herein refers to an animal, preferably a mammal and more preferably a human who can benefit from the pharmaceutical formulations and methods of the present invention and their use in medicine. There is no limitation on the type of animal that could benefit from the presently described pharmaceutical formulations and their use in medicine. A subject regardless of whether a human or non-human animal may be referred to as an individual, patient, animal, host or recipient as well as subject. The compounds of the present invention have applications in human medicine and veterinary medicine.

[0041] Preferred animals are humans or laboratory test animals.

[0042] Examples of laboratory test animals include mice, rats, rabbits, guinea pigs, hamsters, cats and dogs.


[0044] The terms "polynucleotide", "nucleic acid" or "nucleic acid molecule" refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

[0045] The terms "polynucleotide sequence", "nucleic acid sequence" or "nucleotide sequence" refer to a series of nucleotide bases (also referred to as "nucleotides") in a nucleic
acid, such as DNA or RNA, and means any chain of two or more nucleotides.

[0046] The terms "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in production of the product.

[0047] The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

[0048] The term "amplification" of nucleotide sequence as used herein may denote the use of the polymerase chain reaction (PCR) to increase the concentration of a particular nucleotide sequence within a mixture of nucleotide sequence sequences. Saiki, et al., Science 239: 487, 1988 provide a description of PCR. Nucleic acids disclosed herein include those which encode an anti-VEGF-B antibody, an anti-VEGF-B antibody heavy or light chain, an anti-VEGF-B antibody heavy or light chain variable region, an anti-VEGF-B antibody heavy or light chain constant region, an anti-VEGF-B antibody CDR (CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2 or CDR-H3), a murine-human chimeric antibody as described herein, a CDR-Fab as described herein or humanized antibodies as described herein which can be amplified by PCR.

[0049] The term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, preferably no more than 100 nucleotides, that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labelled for example, by incorporation of 32P-nucleotides, 3H-nucleotides, 14C-nucleotides, 35S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. A labelled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. Oligonucleotides (one or both of which may be labelled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

[0050] The sequence of any nucleic acid (for example, a nucleic acid encoding an VEGF-B gene or a nucleic acid encoding an anti-VEGF-B antibody or a fragment or portion thereof) may be sequenced by any method known in the art such as by chemical sequencing or enzymatic sequencing. "Chemical sequencing" of DNA may be done by the method of Maxam and Gilbert, (Proc. Natl. Acad. Sci. USA 74(2): 560-564, 1977), in which DNA is randomly cleaved using individual base-specific reactions. "Enzymatic sequencing" of DNA may be done by the method of Sanger (Sanger, et al., Proc. Natl, Acad. Sci. USA 74(12): 5463 5467, 1977).

[0051] Nucleic acids described herein may be flanked by natural regulatory (expression
control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5’- and 3’-non-coding regions, and the like.

[0052] A "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell and initiating transcription of a coding sequence. A promoter sequence is generally bounded at its 3’ terminus by the transcription initiation site and extends upstream in the 5’ direction to include the minimum number of bases or elements necessary to initiate transcription at any level. A transcription initiation site as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase may be found within the promoter sequence. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid described herein. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Pat. Nos. 5,385,839 and 5,168,062) and the SV40 early promoter region (Benoist, et al., Nature 290:304-310, 1981).

[0053] A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be trans-RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

[0054] The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to be converted into a product; for example, producing a protein by activating the cellular functions involved in transcription and translation of a nucleotide sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as RNA (such as mRNA) or a protein (such as the anti-VEGF-B antibody). The expression product itself may also be said to be "expressed" by the cell.

[0055] The terms "vector", "cloning vector" and "expression vector" mean the vehicle (such as a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

[0056] The term "transfection" or "transformation" means the introduction of a nucleic acid into a cell. These terms may refer to the introduction of a nucleic acid encoding an anti-VEGF-B antibody or fragment thereof into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

[0057] The term "host cell" means any cell of any organism that is selected, modified,
transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression or replication, by the cell, of a gene, a DNA or RNA sequence, a protein or an enzyme.

[0058] The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include E. coli host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

[0059] The present invention relates generally to antibodies that inhibit the growth of cancers including tumorous tissue as well as pre-cancerous tissue by inhibiting the signalling function of VEGF-B. This may occur by inhibiting VEGF-B activity or inhibiting VEGF-B interaction with a receptor. Accordingly, a method for the treatment of cancer and compositions and agents useful for same are disclosed herein. The present invention provides antibodies which antagonize VEGF-B/VEGFR-1 interaction.

[0060] Other VEGF-B antagonists disclosed herein include an antisense compound which reduces VEGF-B expression, or an interfering nucleic acid which reduces VEGF-B expression. An interfering nucleic acid molecule includes synthetic or DNA-derived RNAi.

[0061] VEGF-B antagonists may be prepared by a range of methods. For example, nucleic acid-based compounds may be prepared using the general approaches taught in US Patent Application 20040102389 and include, enzymatic nucleic acid molecules (ribozymes such as Inozyme, G-cleaver, amberzyme, zinzyme), DNAzymes, 2-5A antisense chimeras, triplex forming nucleic acid, decoy nucleic acids, aptamers, allozymes, antisense nucleic acids containing RNA cleaving chemical groups (Cook et al., U.S. Pat. No. 5,359,051), small interfering nucleic acid (siRNA, Beigelman et al., U.S. S No. 60/409,293).

[0062] Antisense compounds which reduce VEGF-B expression are described in Zhang and Doble, International PCT Publication No. WO03/105754. Interfering nucleic acid molecules, such as short interfering nucleic acid (siRNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against VEGF-B and/or which reduce VEGF-B expression may be prepared using the general approach described in McSwiggen et al., International PCT Publication No. WO 03/070910.

[0063] The preferred antagonists disclosed herein are directed towards human VEGF-B. However, cross-reactive VEGF-B antagonists, i.e. that antagonize human and other mammalian forms of VEGF-B may be desirable.

[0064] The antibodies of the invention preferably are monoclonal antibodies. The antibodies are in isolated, homogenous or fully or partially purified form.

[0065] Most preferably, the antibodies are humanized or chimeric or are human antibodies
suitable for administration to humans. These include humanized antibodies prepared, for example, from murine monoclonal antibodies, and human monoclonal antibodies which may be prepared, for example, using transgenic mice as described below, or by phage display. A "humanized" antibody includes a deimmunized antibody.

[0066] Reference to "VEGF-B" includes all naturally occurring isoforms of VEGF-B, including VEGF-B₁₆₇ and VEGF-B₁₈₆ (Olofsson et al., J Biol Chem. 271:19310-19317, 1996), naturally occurring processed forms of VEGF-B, together with any genetically engineered forms based on the sequence of the naturally occurring VEGF-B proteins. VEGF-B is a member of the VEGF family of growth factors, and like all members of this family incorporates a VEGF homology domain (Olofsson et al., Curr Opin in Biotech. Vol 10:528-535, 1999). The homology domain is characterised by a structural motif referred to as a cystine-knot, and it is this part of the protein that retains peptides sequences principally responsible for binding with VEGFR-1. For human and murine VEGF-B, the VEGF homology domain is contained within a genetically engineered form that spans amino acids 10-108 of the mature forms of both VEGF-B₁₆₇ and VEGF-B₁₈₆. The VEGF homology domain of VEGF-B can also be referred to as the receptor binding domain (RBD). Engineered VEGF-B₁₀₋₁₀₈ binds to the ligand binding domain of VEGFR-1, and shows activity in a chimeric receptor assay designed to demonstrate signalling through VEGFR-1 (Scotney et al., Clin Exp Pharmacol Physiol (Australia), 29(11): 1024-9, 2002).

[0067] VEGF-B molecules disclosed herein include human VEGF-B₁₆₇ (NCBI Accession Number AAB06274; protein sequence includes the signal sequence), human VEGF-B₁₈₆ (NCBI Accession Number AAC50721; protein sequence includes the signal sequence), murine VEGF-B₁₆₇ (NCBI Accession Number AAB06273; protein sequence includes the signal sequence), murine VEGF-B₁₈₆ (NCBI Accession Number AAC52823; protein sequence includes the signal sequence) and engineered forms thereof referred to as human VEGF-B₁₀₋₁₀₈ (SEQ ID NO:1) and murine VEGF-B₁₀₋₁₀₈ (SEQ ID NO:2).

[0068] The sequence of human VEGF-B₁₀₋₁₀₈ corresponds to the sequence of amino acids from amino acid 31 (H) to amino acid 129 (K) inclusive of the human VEGF-B₁₆₇ protein sequence shown in NCBI Accession Number AAB06274, and to the sequence of amino acids from amino acid 31 (H) to amino acid 129 (K) inclusive of the human VEGF-B₁₈₆ protein sequence shown in NCBI Accession Number AAC50721.

[0069] The sequence of murine VEGF-B₁₀₋₁₀₈ corresponds to the sequence of amino acids from amino acid 31 (H) to amino acid 129 (K) inclusive of the murine VEGF-B₁₆₇ protein sequence shown in NCBI Accession Number AAB06273 and to the sequence of amino acids from amino acid 31 (H) to amino acid 129 (K) inclusive of the murine VEGF-B₁₈₆ protein sequence shown in NCBI Accession Number AAC52823.

[0070] Examples of antibodies disclosed herein include those that bind to VEGF-B and block
the signaling of VEGF-B through VEGFR-1, thereby inhibiting a VEGF-B-induced biological activity. Such antibodies may be raised against a VEGF-B polypeptide or an antigenic part thereof or an immunogenic part, such as for example, VEGF-B$_{167}$ or peptides containing the receptor binding domain (RBD) such as VEGF-B$_{10-108}$. Antibodies that have the ability to block the signaling of VEGF-B through VEGFR-1 may be selected using cell based or biochemical assays, such as, but not limited to those assays described herein. Preferably, antibodies are raised against a human VEGF-B polypeptide or immunogenic parts thereof. Preferably, antibodies which block human VEGF-B signaling are selected.

[0071] Antibodies of the present invention bind to human VEGF-B and inhibit human VEGF-B induced signaling through VEGFR-1.

[0072] Antibodies that bind to human VEGF-B$_{10-108}$ (SEQ ID NO:1) and inhibit VEGF-B induced signaling through VEGFR-1 and that bind to the human VEGF-B RBD and inhibit VEGF-B induced signaling through VEGFR-1 are disclosed herein.

[0073] In the case of antibodies directed towards human VEGF-B, some level of cross-reactivity with other mammalian forms of VEGF-B may be desirable in certain circumstances, such as for example, for the purpose of testing antibodies in animal models for their effect on a particular disease and for conducting toxicology studies in a manner where VEGF-B/VEGFR-1 signaling in the test animal is affected by the test antibody. Species where cross-reactivity of an antibody to human VEGF-B may be desirable include mouse, rat, dogs and monkey. The antibodies of the present invention exhibit cross-reactivity to human VEGF-B and murine VEGF-B ("mVEGF-B"). Cross-reactivity means that an antibody binds with high affinity to human VEGF-B and VEGF-B from the other species, and inhibits interaction of VEGF-B with VEGFR-1. Cross-reactivity for other VEGF-B antagonists, such as antisense or interfering RNAs, means that they may reduce expression of human VEGF-B and VEGF-B from the other species.

[0074] Preferred antibodies of the present invention and for use in medicine according to the present invention are antibodies that are cross-reactive with VEGF-B from two or more species and inhibit VEGF-B induced signaling through VEGFR-1.

[0075] Antibodies of the present invention bind to human VEGF-B and to murine VEGF-B and inhibit VEGF-B induced signaling through VEGFR-1.

[0076] Antibodies are disclosed herein that bind to human VEGF- B$_{10-108}$ (SEQ ID NO:1) and to murine VEGF- B$_{10-108}$ (SEQ ID NO:2) and inhibit VEGF-B induced signaling through VEGFR-1.

[0077] Antibodies are disclosed herein that bind to the human VEGF-B RBD and to the murine VEGF-B RBD and inhibit VEGF-B induced signaling through VEGFR-1 are also disclosed herein.
[0078] Preferably, the antibodies are monoclonal antibodies.

[0079] Most preferably, the antibodies are human or humanized including deimmunized monoclonal antibodies suitable for use in human therapeutics.

[0080] Antibodies that are human or humanized monoclonal antibodies that bind to VEGF-B and block the signaling of VEGF-B through VEGFR-1 are disclosed herein.

[0081] Antibodies that are human or humanized monoclonal antibodies that bind to human VEGF-B and inhibit human VEGF-B induced signaling through VEGFR-1 are disclosed herein.

[0082] Antibodies that are human or humanized monoclonal antibodies that bind to human VEGF-B_{10-108} (SEQ ID NO:1) and inhibit VEGF-B induced signaling through VEGFR-1 are disclosed herein.

[0083] Antibodies that are human or humanized monoclonal antibodies that bind to the human VEGF-B RBD and inhibit VEGF-B induced signaling through VEGFR-1 are disclosed herein.

[0084] Antibodies that are human or humanized monoclonal antibodies that are cross-reactive with VEGF-B from two or more species and inhibit VEGF-B induced signaling through VEGFR-1 are disclosed herein.

[0085] Antibodies that are human or humanized monoclonal antibodies that bind to human VEGF-B and to murine VEGF-B and inhibit VEGF-B signaling through VEGFR-1 are disclosed herein.

[0086] Antibodies that are human or humanized monoclonal antibodies that bind to human VEGF-B_{10-108} (SEQ ID NO:1) and which also bind to murine VEGF-B_{10-108} (SEQ ID NO:2) and inhibit VEGF-B signaling through VEGFR-1 are disclosed herein.

[0087] Antibodies that are human or humanized monoclonal antibodies that bind to the hVEGF-B RBD and which also bind to the mVEGF-B RBD and inhibit VEGF-B signaling through VEGFR-1 are also disclosed herein.

[0088] The present invention provides an isolated anti-VEGF-B antibody which competes for binding with a second anti-VEGF-B antibody and which isolated antibody binds to human VEGF-B with a $K_D$ value of $1 \times 10^{-7}$M or less, is cross-reactive for human VEGF-B and murine VEGF-B and inhibits human VEGF-B induced signaling through VEGFR-1, wherein the second antibody comprises a light chain variable region having the amino acid sequence shown in SEQ ID NO:3 and a heavy chain variable region having the amino acid sequence shown in SEQ ID NO:4. Antigen-binding fragments of the competing antibodies, and human or humanized forms of the competing antibodies or their antigen-binding fragments are also provided.
[0089] Examples of antibodies that compete with the monoclonal antibody 2H10 for binding to VEGF-B include 1C6 and 2F5.

[0090] Reference to an "antibody" or "antibodies" includes reference to all the various forms of antibodies, including but not limited to: full antibodies (e.g. having an intact Fc region), including, for example, monoclonal antibodies; antigen-binding fragments of antibodies including, for example, Fv, Fab, Fab' and F(ab')2 fragments; humanized antibodies; human antibodies (e.g., produced in transgenic animals or through phage display); and immunoglobulin-derived polypeptides produced through genetic engineering techniques. Unless otherwise specified, the terms "antibody" or "antibodies" and as used herein encompasses both full antibodies and antigen-binding fragments thereof.

[0091] Unless stated otherwise, specificity in respect of an antibody disclosed herein is intended to mean that the antibody binds substantially only to its target antigen with no appreciable binding to unrelated proteins. However, it is possible that an antibody will be designed or selected to bind to two or more related proteins. A related protein includes different splice variants or fragments of the same protein or homologous proteins from different species. Such antibodies are still considered to have specificity for those proteins and are encompassed by the present invention. The term "substantially" means in this context that there is no detectable binding to a non-target antigen above basal, i.e. non-specific, levels.

[0092] Antibodies which bind VEGF-B are referred to as anti-VEGF-B antibodies.


[0094] One method for producing an antibody of the present invention comprises immunizing a non-human animal, such as a mouse or a transgenic mouse, with a VEGF-B polypeptide, or immunogenic parts thereof, such as, for example, a peptide containing the RBD, whereby antibodies directed against the VEGF-B polypeptide or immunogenic parts are generated in said animal. Various means of increasing the antigenicity of a particular immunogen, such as administering adjuvants or conjugated antigens, comprising the antigen against which an antibody response is desired and another component, are well known to those in the art and may be utilized. Immunizations typically involve an initial immunization followed by a series of booster immunizations. Animals may be bled and the serum assayed for antibody titer. Animals may be boosted until the titer plateaus. Conjugates may be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[0095] Both polyclonal and monoclonal antibodies can be produced by this method. The
methods for obtaining both types of antibodies are well known in the art. Polyclonal antibodies are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of a VEGF-B polypeptide, or immunogenic parts thereof, collecting serum from the animal and isolating VEGF-B specific antibodies by any of the known immunoabsorbent techniques. Antibodies produced by this technique are generally less favoured, because of the potential for heterogeneity of the product.

[0096] The use of monoclonal antibodies is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. Monoclonal antibodies may be produced by conventional procedures.

[0097] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., Nature 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using for example, the techniques described in Clackson et al., Nature 352:624-628, 1991 and Marks et al., J. Mol Biol. 222:581-597, 1991.

[0098] A method is disclosed herein for producing a hybridoma cell line which comprises immunizing a non-human animal, such as a mouse or a transgenic mouse, with a VEGF-B polypeptide, such as, for example, human VEGF-B_{167}, or immunogenic parts thereof, such as, for example, a peptide containing the RBD, such as VEGF-B_{10-108} (SEQ ID NO:1) and (SEQ ID NO:2); harvesting spleen cells from the immunized animal; fusing the harvested spleen cells to a myeloma cell line to generate hybridoma cells; and identifying a hybridoma cell line that produces a monoclonal antibody that binds a VEGF-B polypeptide.

[0099] Such hybridoma cell lines and the anti-VEGF-B monoclonal antibodies produced by them are disclosed herein. Monoclonal antibodies secreted by the hybridoma cell lines are purified by conventional techniques. Hybridomas or the monoclonal antibodies produced by them may be screened further to identify monoclonal antibodies with particularly desirable properties, such as the ability to inhibit VEGF-B-signaling through VEGFR-1.

[0100] The VEGF-B polypeptide or immunogenic part thereof that may be used to immunize animals in the initial stages of the production of the antibodies of the present invention may be from any mammalian source. Preferably, the VEGF-B polypeptide or immunogenic part thereof
is human VEGF-B and includes the RBD region.

[0101] Antigen-binding fragments of antibodies of the present invention may be produced by conventional techniques. Examples of such fragments include, but are not limited to, Fab, Fab', F(ab')₂ and Fv fragments, including single chain Fv fragments (termed scFv or scFv). Antibody fragments and derivatives produced by genetic engineering techniques, such as disulphide stabilized Fv fragments (dsFv), single chain variable region domain (Abs) molecules, minibodies and diabodies are also contemplated for use in accordance with the present invention.

[0102] Such fragments and derivatives of monoclonal antibodies directed against VEGF-B may be prepared and screened for desired properties, by known techniques, including the assays described herein. The assays described herein provide the means to identify fragments and derivatives of the antibodies of the present invention that bind to VEGF-B, as well as identify those fragments and derivatives that also retain the activity of inhibiting signaling by VEGF-B through VEGFR-1. Certain of the techniques involve isolating DNA encoding a polypeptide chain (or a portion thereof) of a mAb of interest, and manipulating the DNA through recombinant DNA technology. The DNA may be fused to another DNA of interest, or altered (e.g. by mutagenesis or other conventional techniques) to add, delete, or substitute one or more amino acid residues, for example.

[0103] DNA encoding antibody polypeptides (e.g. heavy or light chain, variable region only or full length) may be isolated from B-cells of mice that have been immunized with VEGF-B. The DNA may be isolated using conventional procedures.

[0104] The present invention contemplates any slight modifications of the amino acid or nucleotide sequences which correspond to the antibodies, antigen-binding fragments or humanized forms of the invention. In particular, the present invention contemplates sequence conservative variants of the nucleic acids which encode the antibodies or antigen-binding fragments of the invention. "Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. (Figure 6) Function-conservative variants of the antibodies of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein have been changed without altering the overall conformation and function of the protein, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and methionine; acidic amino acids which may be interchangeable include aspartic acid and glutamic acid and basic amino acids which may be interchangeable include histidine, lysine and arginine.
The present invention includes anti-VEGF-B antibodies and fragments thereof which are encoded by nucleic acids as described herein as well as nucleic acids which hybridize thereto. Preferably, the nucleic acids hybridize under low stringency conditions, more preferably under moderate stringency conditions and most preferably under high stringency conditions. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, et al., supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions may be 55°C, 5 times SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5 times SSC and 0.5% SDS. Typical moderate stringency hybridization conditions are similar to the low stringency conditions except the hybridization is carried out in 40% formamide, with 5 or 6 times SSC. High stringency hybridization conditions are similar to low stringency conditions except the hybridization conditions are carried out in 50% formamide, 5 or 6 times SSC and, optionally, at a higher temperature (e.g., 57 to 68°C). In general, SSC is 0.15M NaCl and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for calculating the melting temperature have been derived (see Sambrook, et al. supra). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, et al. supra).

Also described herein are nucleic acids comprising nucleotide sequences and polypeptides comprising amino acid sequences which are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical to the nucleotide and amino acid sequences described herein when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. Polypeptides comprising amino acid sequences which are at least about 70% similar, preferably at least about 80% similar, more preferably at least about 90% similar and most preferably at least about 95% similar (e.g., 95%, 96%, 97%, 98%, 99%, 100%) to the amino acid sequences described herein when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences, are also described herein.

Sequence identity refers to exact matches between the nucleotides or amino acids of two sequences which are being compared. Sequence similarity refers to both exact matches between the amino acids of two polypeptides which are being compared in addition to matches between non-identical, biochemically related amino acids. Biochemically related amino acids
which share similar properties and may be interchangeable are discussed above.


[0109] The anti-VEGF-B antibody molecules of the present invention may also be produced recombinantly (for example, in an E.coli expression system). There are several methods by which to produce recombinant antibodies which are known in the art. One example of a method for recombinant production of antibodies is disclosed in US. Patent No: 4,816,567. Transformation can be by any known method for introducing polynucleotides into a host cell. Methods for introduction of heterologous polynucleotides into mammalian cells are well known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, biolistic injection and direct microinjection of the DNA into nuclei. In addition, nucleic acid molecules may be introduced into mammalian cells by viral vectors. Methods of transforming cells are well known in the art. See, for example, U.S. Pat. Nos. 4,399,216; 4,912,040; 4,740,461 and 4,959,455.

[0110] In one aspect, the present invention provides a method for the production of the antibodies of the present invention, said method comprising: (a) culturing the isolated host cells of the invention described below for a period of time sufficient to allow for expression of the antibody; and (b) purifying the expressed antibody.

[0111] Vectors available for cloning and expression in host cell lines are well known in the art, and include but are not limited to vectors for cloning and expression in mammalian cell lines, vectors for cloning and expression in bacterial cell lines and vectors for cloning and expression insect cell lines. The antibodies can be recovered using standard protein purification methods.

[0112] In another aspect, the present invention provides antibodies comprising CDRs having the sequences shown in SEQ ID NOs:21 to 26, or SEQ ID NOs:45 to 50 or sequences with at least 95% identity thereto or antibodies comprising the variable light chain and the variable heavy chain sequences shown in SEQ ID NOs:19 and 20, or SEQ ID NOs:43 and 44 or sequences with at least 95% identity thereto.

[0113] In a further aspect, the present invention provides an expression vector comprising a nucleic acid molecule encoding the light chain and the heavy chain of the isolated antibody of the invention comprising CDRs having the sequences shown in SEQ ID NOs:21 to 26, or SEQ ID NOs: 45 to 50 or sequences with at least 95% identity thereto, wherein said expression vector is capable of expressing said nucleic acids in a prokaryotic or eukaryotic host cell.

[0114] In still a further aspect, the present invention provide an expression vector comprising a nucleic acid molecule encoding the antibody of the invention comprising the variable light chain sequences and the variable heavy chain sequences shown in SEQ ID NOs: 19 and 20,
or SEQ ID NOs:43 and 44.

[0115] Vectors comprising nucleic acid sequences encoding antibodies comprising the variable light chain sequences and the variable heavy chain sequences shown in SEQ ID NOs:29 and 30 are disclosed herein.

[0116] In still a further aspect, the present invention provides an isolated prokaryotic or eukaryotic host cell comprising an expression vector of the present invention.

[0117] Mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, 3T3 cells, and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as Sf9. cells, amphibian cells, bacterial cells, plant cells and fungal cells. When recombinant expression vectors encoding the heavy chain or antigen-binding portion thereof, the light chain and/or antigen-binding portion thereof are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown.

[0118] Antibodies can be recovered from the culture medium using standard protein purification methods. Further, expression of antibodies of the invention (or other moieties therefrom) from production cell lines can be enhanced using a number of known techniques. For example, the glutamine synthetase gene expression system (the GS system) is a common approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with European Patent Nos. 0 216 846, 0 256 055, and 0 323 997 and European Patent Application No. 89303964.4.

[0119] It is likely that antibodies expressed by different cell lines or in transgenic animals will have different glycosylation from each other. However, all antibodies encoded by the nucleic acid molecules provided herein, or comprising the amino acid sequences provided herein may find utility in the therapeutic uses disclosed herein, regardless of the glycosylation of the antibodies.

[0120] Phage display is another example of a known technique whereby derivatives of antibodies may be prepared. In one approach, polypeptides that are components of an antibody of interest are expressed in any suitable recombinant expression system, and the expressed polypeptides are allowed to assemble to form antibody molecules.

[0121] Single chain antibodies may be formed by linking heavy and light chain variable region
(Fv region) fragments via an amino acid bridge (short peptide linker), resulting in a single polypeptide chain. Such single-chain Fvs (scFvs) have been prepared by fusing DNA encoding a peptide linker between DNAs encoding the two variable region polypeptides (VL and VH). The resulting antibody fragments can form dimers or trimers, depending on the length of a flexible linker between the two variable domains (Kortt et al., Protein Engineering 10: 423, 1997). Techniques developed for the production of single chain antibodies include those described in U.S. Patent No. 4,946,778; Bird (Science 242: 423, 1988), Huston et al. (Proc. Natl. Acad. Sci. USA 85: 5879, 1988) and Ward et al. (Nature 334: 544, 1989). Single chain antibodies derived from antibodies provided herein are encompassed by the present invention.

[0122] In one embodiment, the present invention provides antibody fragments or chimeric, recombinant or synthetic forms of the antibodies of the present invention that bind to VEGF-B from two or more animal species, and inhibit signaling by VEGF-B through VEGFR-1.

[0123] Techniques are known for deriving an antibody of a different subclass or isotype from an antibody of interest, i.e., subclass switching. Thus, IgG1 or IgG4 monoclonal antibodies may be derived from an IgM monoclonal antibody, for example, and vice versa. Such techniques allow the preparation of new antibodies that possess the antigen-binding properties of a given antibody (the parent antibody), but also exhibit biological properties associated with an antibody isotype or subclass different from that of the parent antibody. Recombinant DNA techniques may be employed. Cloned DNA encoding particular antibody polypeptides may be employed in such procedures, e.g. DNA encoding the constant region of an antibody of the desired isotype.

[0124] The monoclonal production process described above may be used in animals, for example mice, to produce monoclonal antibodies. Conventional antibodies derived from such animals, for example murine antibodies, are known to be generally unsuitable for administration to humans as they may cause an immune response. Therefore, such antibodies may need to be modified in order to provide antibodies suitable for administration to humans. Processes for preparing chimeric and/or humanized antibodies are well known in the art and are described in further detail below.

[0125] The monoclonal antibodies herein specifically include "chimeric" antibodies in which the variable domain of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a non-human species (e.g., murine), while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from humans, 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).

[0126] "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from the non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which the complementarity determining regions (CDRs) of the recipient are replaced by the
corresponding CDRs from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired properties, for example specificity, and affinity. In some instances, framework region residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the complementarity determining regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework region residues are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature 321:522-525, 1986; Reichmann et al., Nature 332:323-329, 1988; Presta, Curr. Op. Struct. Biol. 2:593-596, 1992; Liu et al., Proc. Natl. Acad. Sci. USA 84: 3439, 1987; Larrick et al., Bio/Technology 7: 934, 1989; and Winter and Harris, TIPS 14: 139, 1993.

[0127] The complementarity determining regions (CDRs) of a given antibody may be readily identified, for example using the system described by Kabat et al. in Sequences of Proteins of Immunological Interest, 5th Ed., US Dept. of Health and Human Services, PHS, NIH, NIH Publication No. 91-3242, 1991).

[0128] For example, the murine monoclonal antibodies 1C6, 2F5, 2H10 and 4E12 can be humanized to reduce the immunogenicity of the antibodies in a target host. Murine monoclonal antibodies 1C6, 2F5, 2H10 and 4E12 have antagonistic effect against VEGF-B and inhibit signaling through VEGFR-1. However, the potential immunogenicity of the mAbs 1C6, 2F5, 2H10 and 4E12 in other hosts, and in particular humans, makes the use of mAbs 1C6, 2F5, 2H10 and 4E12 unsuitable as therapeutic agents in these hosts.

[0129] In a particular embodiment, antibodies of the present invention comprise within the variable region of their light chain, at least one of the CDRs found in the light chain of mAb 2F5. The CDRs of mAb 2F5 are disclosed in Figure 3 and in SEQ ID NOs:21-26. Thus, among the antibodies contemplated by the present invention are those that comprise from one to all three of the CDR sequences from the light chain variable region of mAb 2F5. Further, among the antibodies contemplated by the present invention are those that comprise from one to all three of the CDR sequences from the heavy chain variable region of mAb 2F5. In a preferred embodiment, antibodies of the present invention comprise one, two, three, four, five or all six CDR sequences from the heavy and light chain variable regions of mAb 2F5.

[0130] In a particular embodiment, antibodies of the present invention comprise within the variable region of their light chain, at least one of the CDRs found in the light chain of mAb 1C6. The CDRs of mAb 1C6 are disclosed in Figure 7 and in SEQ ID NOs:45-50. Thus, among the antibodies contemplated by the present invention are those that comprise from one to all three of the CDR sequences from the light chain variable region of mAb 1C6. Further, among the antibodies contemplated by the present invention are those that comprise from one to all
three of the CDR sequences from the heavy chain variable region of mAb 1C6. In a preferred embodiment, antibodies of the present invention comprise one, two, three, four, five or all six CDR sequences from the heavy and light chain variable regions of mAb 1C6.

[0131] Procedures for generating human antibodies in non-human animals have been developed and are well known to those skilled in the art. For example, transgenic mice into which genetic material encoding one or more human immunoglobulin chains has been introduced may be used to produce the antibodies of the present invention. Antibodies produced in the animals incorporate human immunoglobulin polypeptide chains encoded by the human genetic material introduced into the animal. Examples of techniques for production and use of such transgenic animals are described in U.S. Patent Nos. 5,814,318, 5,569,825, and 5,545,806.

[0132] Another method for generating human antibodies is phage display. Phage display techniques for generating human antibodies are well known to those skilled in the art, and include the methods used by companies such as Cambridge Antibody Technology and MorphoSys and which are described in International Patent Publication Nos. WO 92/01047, WO 92/20791, WO 93/06213 and WO 93/11236.

[0133] Antibodies of the present invention may be employed in vitro or in vivo to inhibit a biological activity that results from VEGF-B signaling. Among other uses for the antibodies of the present invention are assays (either in vitro or in vivo) to detect the presence of VEGF-B polypeptides and immunoaffinity chromatography to purify VEGF-B polypeptides.

[0134] Therefore, in one embodiment, antibodies of the present invention may be used in therapeutic applications to treat diseases or conditions caused or exacerbated (directly or indirectly) by the signaling of VEGF-B through the VEGF-B receptor. A therapeutic application involves in vivo administration of an antibody of the present invention to a mammal in an amount effective to inhibit signaling by VEGF-B through VEGFR-1. Preferably, the antibodies are human or humanized monoclonal antibodies of the present invention.

[0135] The antibodies may be used to treat diseases or conditions induced by VEGF-B including but not limited to cancer, in particular tumors, more particularly solid tumors.

[0136] Antibodies disclosed herein include the murine monoclonal antibodies 1C6, 2H10, 4E12 and 2F5, and humanized forms of mAbs 1C6, 2F5, 2H10 and 4E12.

[0137] Antibodies disclosed herein also include, but are not limited to monoclonal antibodies directed against amino acid residues 10 to 108 of the human VEGF-B (SEQ ID NO: 1) and/or amino acid residues 10 to 108 of the murine VEGF-B (SEQ ID NO:2) and which bind to VEGF-B from two or more animal species.

[0138] Particular monoclonal antibodies disclosed herein include the mAbs 1C6, 2H10, 4E12 and 2F5; mAbs that bind to the same epitope as the mAbs 1C6, 2H10, 4E12 and 2F5; a mAb
that competes with the mAbs 1C6, 2H10, 4E12 or 2F5 for binding to human VEGF-B; a mAb that competes with the mAbs 1C6, 2H10, 4E12 or 2F5 for binding to human VEGF-B_{10-108}; a mAb that possesses a biological activity of the mAbs 1C6, 2H10, 4E12 or 2F5; and an antigen-binding fragment of any of the foregoing antibodies that inhibits binding of VEGF-B or VEGF-B_{10-108} to VEGFR-1. Antibodies in accordance with these disclosures include 1C6, 2H10, 4E12 and 2F5 and humanized forms thereof as discussed in the Examples.

[0139] An antibody disclosed herein may have a binding affinity for human VEGF-B or VEGF-B_{10-108} that is substantially equivalent to the binding affinity of a mAb selected from 1C6, 2H10, 4E12 and 2F5 for human VEGF-B or VEGF-B_{10-108}. mAbs of any isotype (including but not limited to IgG4, IgG1), derived from mAbs 1C6, 2H10, 4E12 and 2F5 are also disclosed herein. Hybridoma cell lines that produce any such monoclonal antibodies also are disclosed herein.

[0140] Antibody affinities may be determined using a biosensor-based approach as described in the examples section. Antibodies of the invention bind human VEGF-B with a K_D value of 1 x 10^{-7}M or less; preferably 1 x 10^{-6}M or less; more preferably 1 x 10^{-9}M or less; and most preferably 5 x 10^{-10}M or less as determined by surface plasmon resonance. One example of a biological activity of a mAb selected from 1C6, 2H10, 4E12 and 2F5 is its ability to bind to VEGF-B and inhibit signaling by VEGF-B through VEGFR-1. A mAb disclosed herein may possess biological activity or VEGF-B blocking activity substantially equivalent to that of a mAb selected from 1C6, 2H10, 4E12 and 2F5. Such activity may be measured in any suitable assay (e.g. as measured in the VEGFR-1/EpoR/BaF3 proliferation assay described herein or in a reporter gene assay such as that described by Scotney et al. (supra)).

[0141] In one assay, Ba/F3 cells are transfected with a chimeric receptor incorporating the intracellular domain of the erythropoietin receptor (EpoR) and the extracellular domain of VEGFR-1. When the engineered Ba/F3 cells are in the presence of an appropriate amount of VEGF-B, proliferation of the cells, as measured using an MTS dye reduction assay occurs. This is referred to as the VEGFR-1/EpoR/BaF3 proliferation assay. Anti-VEGF-B antibodies that inhibit binding of VEGF-B to VEGFR-1 inhibit VEGF-B induced cell proliferation in this assay.

[0142] Anti-VEGF-B antibodies that antagonize VEGF-B signaling through VEGFR-1 will inhibit VEGF-B-mediated activation of the reporter molecule in a reporter gene based assay such as that described by Scotney et al., supra.

[0143] Those skilled in the art appreciate that the cell based assays described herein, for example described above and in the Examples, may be utilised as a basis for screening for modulators of VEGF-B/receptor interaction. While such methods are well known to those skilled in the art, a brief description of the method is provided herein. The method involves subjecting appropriately engineered cells to a signal producing amount of VEGF-B under conditions where, in the absence of any antagonism of VEGF-B/ receptor binding, a signal, for example proliferation or reporter luciferase expression, may be detected. The same
experimental procedure is then conducted in the presence of one or more test compounds and the level of signal detected compared with that detected in the absence of a test compound. Test compounds which alter the level of signal detected compared with that detected in the absence of a test compound are then selected for further study. Test compounds may include phage display libraries of antibody variable domains and the like, or panels of monoclonal antibodies against VEGF-B may be screened across the assay.

[0144] Treatment of VEGF-B associated diseases and disorders may be by the administration of pharmaceutical compositions comprising a VEGF-B antagonist, preferably antibodies of the present invention, and one or more pharmaceutically acceptable carriers and/or diluents.

[0145] Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, agents used for adjusting tonicity, buffers, chelating agents, and absorption delaying agents and the like. The use of such media and agents is well known in the art. Except insofar as any conventional media or agent is incompatible with the active form, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[0146] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dilution medium comprising, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of surfactants. The preventions of the action of microorganisms can be brought about by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include agents to adjust tonicity, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. The compositions may also include buffers and chelating agents.

[0147] Sterile injectable solutions are prepared by incorporating the active form in the required amount in the appropriate solvent and optionally other active ingredients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum drying and the freeze-drying technique which yield a powder of active form plus any additionally desired ingredient.

[0148] The amount of active form in such therapeutically useful compositions is such that a suitable dosage will be obtained.
The compositions of the present invention are useful in modifying VEGF-B mediated conditions including but not limited to tumors, in particular solid tumors, and any other VEGF-B mediated diseases or conditions. Pre-cancerous conditions and dispersed tumors, such as for example, myelomas and lymphomas may also be treated.

The human and humanized antibodies of the present invention and in particular humanized forms of the mAbs 1C6 and 2F5 are useful in the treatment of such conditions. Any adverse condition resulting from VEGF-B interaction with VEGF-B receptor may be treated by the administration of the antibodies of the present invention such as humanized forms of the mAbs selected from 1C6 and 2F5.

The type of tumors contemplated for treatment using the antibody and compositions of the present invention include without being limited to breast tumors, colorectal tumors, adenocarcinomas, mesothelioma, bladder tumors, prostate tumors, germ cell tumor, hepatoma/cholangio, carcinoma, neuroendocrine tumors, pituitary neoplasm, small round cell tumor, squamous cell cancer, melanoma, atypical fibroxanthoma, seminomas, nonseminomas, stromal leydig cell tumors, sertoli cell tumors, skin tumors, kidney tumors, testicular tumors, brain tumors, ovarian tumors, stomach tumors, oral tumors, bladder tumors, bone tumors, cervical tumors, esophageal tumors, laryngeal tumors, liver tumors, lung tumors, vaginal tumors and Wilm’s tumor.

Dosage regimens may be adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the VEGF-B antagonists, such as an antibody of the present invention, employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a composition of the invention may be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be by injection, preferably proximal to the site of the target (e.g., tumor). If desired, the effective daily dose of a pharmaceutical composition may be administered as two, three, four, five, six or more subdoses administered separately at appropriate intervals throughout the day.

For therapeutic applications, the VEGF-B antagonists, such as anti-VEGF-B antibodies of the present invention, are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that
may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The VEGF-B antagonists, such as the anti-VEGF-B antibodies, also are suitably administered by intra tumoral, peritumoral, intrallesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

[0155] For the prevention or treatment of VEGF-B mediated diseases or conditions, the appropriate dosage of VEGF-B antagonists, such as an anti-VEGF-B antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antagonist is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antagonist, and the discretion of the attending physician. The antagonist may be suitably administered to the patient at one time or over a series of treatments.

[0156] Depending on the type and severity of the disease, about 1 μg/kg to about 50 mg/kg (e.g., 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1 μg/kg to about 20 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, the treatment is repeated until a desired suppression of disease symptoms occurs. The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

[0157] Preferably, the subject is a human. However, veterinary applications are also contemplated for livestock animals as well as companion animals. In such cases it would be necessary to prepare an appropriate antibody designed to avoid an immunogenic response to the antibody by the mammal.

[0158] Thus a method is disclosed herein for treating a VEGF-B mediated disease or condition in a human subject, said method comprising administering to said subject a therapeutically effective amount of a VEGF-B antagonist for a time and under conditions sufficient to produce a desired suppression of the disease or condition.

[0159] Also disclosed herein is the use of a VEGF-B antagonist in the manufacture of a medicament in the treatment or prophylaxis of cancer including tumor tissue and precancerous tissue in a subject.

[0160] Preferably the VEGF-B antagonist for use in the above method is an antibody. Preferably the antibody is a monoclonal antibody,

[0161] Preferred antibodies for use in the above method are those which bind human VEGF-B with a $K_D$ value of $1 \times 10^{-7}$M or less; preferably $1 \times 10^{-8}$M or less; even more preferably $1 \times 10^{-9}$M or less.
M or less; and most preferably $5 \times 10^{-9}$M or less.

[0162] Therefore, a method is disclosed herein for ameliorating the effects of VEGF-B mediated cancer in a human subject, said method comprising administering to said subject an effective amount of a humanized form of a mAb selected from 1C6, 2H10, 4E12 and 2F5 or an antibody with equivalent VEGF-B blocking activity for a time and under conditions sufficient to reduce the growth or spread of the cancer.

[0163] Also disclosed herein is the use of a humanized form of a mAb selected from 1C6, 2H10, 4E12 and 2F5 or antibody with equivalent VEGF-B blocking activity in the manufacture of a medicament for the treatment or prophylaxis of cancer in a subject.

EXAMPLE 1

Preparation of VEGF proteins

General

[0164] Human and murine VEGF-B proteins and VEGF-A proteins were produced according to the general methodology previously described for human VEGF-B B\textsubscript{10-108} (Scotney \textit{et al.}, 2002, \textit{supra}, Scrofani \textit{et al}, 2000, Protein Science, 9:2018-2025). This is described below in more detail for the production of murine VEGF-B B\textsubscript{10-108}. Proteins produced using this methodology initially have an additional 16 amino acids at the N-terminus that incorporate a 6xHis tag and a Genenase I cleavage site between the tag and the start of the actual VEGF-B amino acid sequence. Where desired, these additional amino acids are removed by enzymatic cleavage using the general procedure outlined below. The presence or absence of the tag and cleavage site on a protein is indicated as follows: 6xHis.hVEGF-B\textsubscript{10-108} indicates a protein containing amino acids 10-108 of the mature human VEGF-B with the N-terminal tag and cleavage site. hVEGF-B\textsubscript{10-108} indicates the same protein without the N-terminal tag and cleavage site. Other proteins are represented using the same approach.

Production of murine 6xHis. VEGF-B

[0165] Following generally the procedure of Scotney \textit{et al.} (\textit{supra}), murine VEGF-B\textsubscript{10-108} with a 6xHis tag and a Genenase I cleavage (6xHis.mVEGF-B\textsubscript{10-108}) was produced as a recombinant protein by bacterial fermentation following transformation with 6xHis.mVEGF-B\textsubscript{10-108}pET-15b (pET-15b vector from Novagen USA Cat # 70755-3) into the E. coli BL21-Codon-
Plus-[DE3]-RP strain (Stratagene, USA). To determine expression levels, samples were collected pre- and 1, 2 and 3 hrs post-induction with IPTG and assessed by SDS-PAGE and Western blot using an anti-6xHis mAb (murine mAb, QIAGEN USA). Bound mAb was visualized by autoradiography using HRP-conjugated secondary reagent (sheep anti-mouse antibody, Chemicon USA) together with a luminescence substrate (PerkinElmer USA).

[0166] Following cell lysis 6xHis.mVEGF-B$_{10-108}$ was purified from E. coli inclusion bodies by metal affinity chromatography (nickel affinity, Amersham) under denaturing and reducing conditions. Fractions were eluted from the column using 6M urea, 10mM Tris, 100mM phosphate [pH of 4.5] and were analyzed using SDS-PAGE. Western blot analysis was also performed on the eluted fractions using an anti-6xHis mAb. Bound mAb was visualized by autoradiography using a HRP-conjugated secondary reagent together with a luminescence substrate (PerkinElmer USA).

[0167] Purified 6xHis.mVEGF-B from nickel affinity was diluted to 100 µg/ml in 6 M guanidine hydrochloride (gd), 100 mM Tris (pH 8.5), reduced by the addition of DTT to 20 mM and dialyzed against 1M gd, 100 mM Tris (pH 8.5), 5 mM cysteine and 1 mM cystine for 2 days. Refolded 6xHis.mVEGF-B was then dialyzed against 0.1 M acetic acid prior to purification by reverse phase HPLC (RP-HPLC, Zorbax 300 SB-C8 column 250x21 mm i.d.). The fractions containing predominantly dimeric material (as assessed by non-reducing SDS-PAGE) were pooled for assaying. Western blot analysis (SDS-PAGE under reducing conditions) was performed on the RP-HPLC fractions using an anti-6xHis mAb. Bound mAb was visualized by autoradiography using a HRP-conjugated secondary reagent together with a luminescence substrate. Note that when VEGF-B is refolded correctly the N-terminus of the protein is concealed and the anti-6xHis mAb does not bind to the protein.

[0168] Analysis of the activity of purified, refolded 6xHis.mVEGF-B$_{10-108}$ was determined using the VEGFR-1/EpoR/BaF3 cell proliferation assay as described herein and demonstrated a dose dependant proliferation of the VEGFR-1/EpoR/BaF3 cells in response to recombinant 6xHis.mVEGF-B$_{10-108}$, and the positive control proteins hVEGF-A$_{11-109}$, hVEGF-B$_{10-108}$ and 6xHis.hVEGF-B$_{10-108}$.

Enzymatic Cleavage Procedure

[0169] Note that where indicated the N-terminal 6xHis tag was removed from human VEGF-A and/or VEGF-B preparations by enzymatic cleavage as follows. Briefly, 5mg of lyophilised tagged protein was resuspended in a minimum volume of 1 mM acetic acid

[0170] (500 µl) and 100X volume (50 ml) of Genenase I digest solution (5 µg/ml Genenase I, 100 mM Tris-HCl, 5 mM CaCl2, 200 mM NaCl, 0.02% Tween-20) added. The material was incubated for 24 hrs at 21°C with gentle mixing before being dialysed into 0.1 M acetic acid to stop the reaction. The digested material was then purified by reverse phase chromatography
and stored as lyophilised aliquots as previously described (Scotney et al., supra).

**EXAMPLE 2**

*Human VEGF-B specific mouse monoclonal antibodies antagonise the activity of VEGF-B in cell-based and biochemical assays*

[0171] Monoclonal antibodies were raised against recombinant human (h) VEGF-B isoforms using standard procedures (see Harlow and Lane, Antibodies - A Laboratory Manual, Cold Spring Harbour Laboratory, Chapter 6, 1988). Briefly, BALB/c or C57BL/6 mice were immunised via the intraperitoneal (i.p) route with approximately 10-30 µg of hVEGF-B (one of either hVEGF-B\textsubscript{167}, hVEGF-B\textsubscript{10-108} or hVEGF-B\textsubscript{186} with or without an N-terminal 6xHis tag) emulsified in complete Freunds Adjuvant (CFA) for the first immunisation and incomplete Freunds Adjuvant for subsequent immunisations (at least 2, and no less than 3-4 weeks apart). Three days prior to fusion and at least 3-4 weeks after the final i.p. immunisation mice were boosted with approximately 10 µg of hVEGF-B in phosphate buffered saline (PBS) via the intravenous (i.v.) route. Following fusion of spleen cells to the Sp2/O-Ag14 myeloma fusion partner and subsequent HAT (hypoxanthine, aminopterin and thymidine) selection, hybridomas secreting hVEGF-B specific mAbs were identified by ELISA and, after expansion, cloned by limit dilution on at least two occasions.

**Cell based assay**

[0172] Monoclonal Abs were assessed for antagonist activity using a novel VEGFR-1/EpoR/BaF3 proliferation assay. This assay is based on engineered BaF3 (DSMZ, Cat No. ACC 300) cells expressing a chimeric receptor incorporating the intracellular domain of the human erythropoietin receptor (EpoR) and the extracellular domain of VEGFR-1. VEGFR-1 ligands such as VEGF-A and VEGF-B trigger dimerisation of the chimeric receptor - the subsequent phosphorylation of the cytoplasmic domain of the EpoR leads to the activation of downstream signal transduction molecules and proliferation of the BaF3 cells. This approach has been used to develop proliferation-based biological assays for a number of cytokines and growth factors (for examples see Murayama et al., J. Biol. Chem., 269, 5976-5980, 1994; Fukada et al., Immunity 5, 449-460, 1996; Stacker et al., J. Biol. Chem., 274, 34884-34892, 1999). The engineered BaF3 cells are maintained in DMEM (Invitrogen, USA) supplemented with 10% FCS, Zeocin (250 µg/ml, Invitrogen, USA), hVEGF-A\textsubscript{165} (50 ng/ml) penicillin (50 units/ml, Invitrogen, USA), streptomycin (50 µg/ml, Invitrogen, USA) and GlutaMAX-I (2 mM, Invitrogen, USA).

[0173] For analysis of mouse mAb antagonist activity washed VEGFR-1/EpoR/BaF3 cells (5x10\textsuperscript{4} cells/well, flat bottom 96 well microtitre plates) were pre-incubated with titrating test
mAb for 1 hr prior to the addition of the indicated concentration of hVEGF-A_{165}, hVEGF-B_{167} or hVEGF-B_{10-108} for 72 hrs at 37°C with 10% CO₂ in air. After 72 hrs, proliferation was assessed using an MTS dye reduction assay (Mosmann, T., J. Immunol Methods, 65, 55-63, 1983). The results demonstrated that mAbs 2H10, 4E12, 2F5 and 1C6 were able to antagonise the activity of hVEGF-B_{167} (VB167) and hVEGF-B_{10-108} (VB108) but not hVEGF-A_{165}(VA165).

**Biochemical assay**

[0174] The ability of monoclonal antibodies to block binding of VEGF-B to a ligand-binding fragment of VEGFR-1 was measured in a novel competition ELISA. A recombinant fragment of VEGFR-1, corresponding to the second Ig-like domain (D2-VEGFR-1, see Scotney et al., supra; Weisemann et al., Cell 91, 695-704, 1997), was used to coat microtitre plates. A fixed, subsaturating concentration of recombinant hVEGF-B_{10-108} (5 nM), which had been pre-incubated with serial dilutions of test mAb, was then incubated in these plates. After 1 hr, plates were washed and successively treated with rabbit antihuman VEGF-B_{167} serum mouse anti-rabbit-Ig horse radish peroxidase conjugate, and TMB substrate. The ELISA signal, measured colourimetrically at 450nm after acid quenching, was proportional to the amount of VEGF-B_{10-108} captured by plate-bound D2-VEGFR-1. Dose-dependent inhibition of binding by mAb was indicative of competition between mAb and D2-VEGFR-1 for binding to VEGF-B_{10-108}. The results of the assay revealed that mAbs 2H10, 1C6 and 2F5, but not 4E12, can antagonise binding of VEGF-B_{10-108} to D2-VEGFR-1. The data suggest that antagonist mAb 4E12 binds to an area of VEGF-B not involved in the direct interaction with D2-VEGFR-1.

**EXAMPLE 3**

*Kinetics analysis of human VEGF-B specific antagonist mouse monoclonal antibodies*

**Kinetic analysis**

[0175] A Biosensor-based approach was used for kinetic analysis of mAb binding to target VEGF-B using a BIACore™ 2000 surface plasmon resonance instrument (Biacore AB, Uppsala, Sweden). A biosensor chip was activated for covalent coupling of VEGF-B using N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier’s (Biacore AB, Uppsala, Sweden) instructions. A solution of 5μg/ml VEGF-B_{10-108} in 20 mM sodium acetate, pH 4.8 was prepared and an aliquot (35 μl) was injected at a flow rate of 2 μl/min to achieve approximately 500-600 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.
[0176] For kinetics measurements, two-fold serial dilutions of test antibody starting at 125nM to 0.98nM were injected in Biacore™ buffer (20mM HEPES, pH 7.8, 0.15M NaCl, 3.4mM EDTA, 0.005% Tween-20) with 1mg/ml bovine serum albumin (BSA) at 25°C at a flow rate of 15μl/min. Simultaneous analysis of association (k_a) and dissociation (k_d) rate constants using BiAevaluation™ V3.02 software (Biacore AB, Uppsala, Sweden) was used to determine the equilibrium binding constant (K_D). Binding by each test mAb was assessed in duplicate and a summary of the data generated to date is presented in Table 3.

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**EXAMPLE 4**

*Monoclonal antibodies raised against human VEGF-B cross react with, and inhibit the activity of, mouse VEGF-B*

[0177] Monoclonal antibodies raised against hVEGF-B were tested for cross-reactivity with mVEGF-B using a standard ELISA format. mAbs were incubated with plate bound hVEGF-B_{10-100} or 6xHis.mVEGF-B_{10-100} at a concentration of 1.25 μg/ml and bound mAb visualized using a HRP-conjugated secondary reagent and TMB substrate. Results demonstrate that only mAb 4E12 did not interact with both human and murine VEGF-B.

[0178] Cross-reactivity was further verified by Western blot analysis. 500 ng of hVEGF_{10-108} and 6xHis.mVEGF-B_{10-108} were run on a non-reducing SDS-PAGE and transferred to PVDF membrane using standard procedures. Blots were probed with the test mAbs (2F5, 2H10 or 4E12) or a negative control mAb (6A9) at a concentration of 10μg/ml. The bound mAbs were visualized by autoradiography using HRP-conjugated secondary reagent together with a luminescence substrate. mAbs 2F5 and 2H10 bound both human and murine VEGF-B_{10-108}, while mAb 4E12 bound only hVEGF-B.

[0179] mAbs 2H10, 1C6, 2F5 and 4E12 were also tested for their ability to inhibit the proliferative activity of 1 nM hVEGF-A, 50 nM 6xHis.hVEGF-B_{10-108} and 50 nM 6xHis.mVEGF-B_{10-108} using the VEGFR-1/EpoR/BaF3 proliferation assay as previously described. mAbs
2H10, 1C6 and 2F5 inhibited activity of both human and murine 6xHis.VEGF-B_{10-108}, while mAb 4E12 was active only against the human form of VEGF-B_{10-108}.

EXAMPLE 5

Detection of VEGF-B in Human Tumor Cell Line Conditioned Medium

[0180] The human tumor cell lines, 2008 (ovarian), A431 (epidermoid carcinoma), H460 (non-small cell lung), HT-29 (colon), MDA-MB-231 (breast) and PC-3 (prostate) were obtained from the Peter MacCallum Cancer Centre. The cell lines were routinely passaged as adherent cells in RPMI media (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), penicillin (50 units/ml, Invitrogen, USA) and streptomycin (50 μg/ml, Invitrogen, USA) and GlutaMAX (Invitrogen, USA) at 37°C with 5% CO₂ in air. For analysis of VEGF-B production cell lines were diluted approximately 1:20 from a confluent culture into 100 mm petridishes containing 8.0 ml of media with or without heparin (100 μg/ml, Sigma, USA). Culture supernatants were recovered by centrifugation after 14 days and total VEGF-B protein level (VEGF-B_{167} plus VEGF-B_{186} isoforms) assessed a standard sandwich ELISA assay. The sandwich ELISA was based on microtitre plate bound anti-VEGF-B mAb (VEGF-B_{167} and VEGF-B_{186} reactive), with captured VEGF-B detected using a rabbit anti-VEGF-B polyclonal antibody and, subsequently, a HRP-conjugated anti-rabbit Ig reagent (Chemicon). Test VEGF-B was quantitated using a standard curve established with recombinant VEGF-B protein.

[0181] All human tumor cell lines assessed were demonstrated to express VEGF-B. The VEGF-B_{167} isoform retains a C-terminal heparin binding domain and, as a result, a proportion of expressed protein is known to remain cell-associated. Heparin was included in the culture media in order to release this material from the cell surface and in some cases this resulted in slightly higher levels of VEGF-B detected in the cell supernatant.

EXAMPLE 6

Human Tumor Cell Line HT-29 Mouse Xenograft Study

[0182] Novel therapeutics agents that potentially limit the growth of human tumors can be assessed for efficacy in vivo using well characterised mouse xenograft models. As described above in Example 5 a number of human tumor cell lines, all of which grow as solid tumors when grafted into immunodeficient mice, express VEGF-B protein.

[0183] Human colon carcinoma HT-29 tumor cells growing exponentially in vitro were
harvested, washed and injected (5x10⁶/mouse) into the subcutaneous tissue of the right hind-limb of an anesthetized athymic nude mice. Treatment was commenced when tumors reached a size of approximately 100mm³ (approximately 2 weeks) at which point mice were divided into treatment groups of 10 animals. For analysis of anti-VEGF-B mAb 2H10, one group of 10 mice was treated twice weekly with an intraperitoneal (ip) injection of approximately 400 µg of test 2H10 in a volume of 200 µl, while a second group of mice was treated with the identical amount of an isotype matched control mAb (mAb C44, colchicine specific, ATCC Cat No. CRL-1943). Treatment was continued for a period of 5-7 weeks and the width and length of tumors was determined at regular intervals (for example every 4-5 days) using electronic calipers. Tumor volume (mm³) was calculated according to the formula: 0.5 x length x width².

[0184] By day 38 post initiation of treatment, there was a 41% reduction in tumor volume in mice treated with the anti-VEGF-B mAb 2H10 when compared with tumors in the mice treated with the isotype matched control mAb. This compares favourably with the anti-tumor activity observed for a mAb directed against VEGF-A using human colon carcinoma HT-29 tumor cells in a similar tumor xenograft model (Asano, M et al, Jpn J Cancer Research, 90(1): 3-100, 1999).

EXAMPLE 7

**Cloning of mAb 2H10 variable region genes for the generation of a therapeutic humanized mab**

[0185] Messenger RNA was prepared from hybridoma cells producing 2H10 mAb, and the variable region sequences were reverse transcribed and amplified by RT-PCR. Partially degenerate PCR primers based on the N-terminal amino acid sequence and the antibody isotype were used to amplify the light chain variable region. The heavy chain variable region was amplified using a forward primer designed to anneal to sequence encoding the N-terminal leader peptide, and a reverse primer based on the antibody isotype (Coloma et al. 1991, Larrick et al. 1989). The subsequent PCR products were sequenced to reveal the amino acid sequence of the variable regions of 2H10. The amino acid sequences of the variable regions of antagonist mAbs 4E12 and 2F5 were determined using an identical strategy (see Table 1; Figures 8-10)

EXAMPLE 8

*Human Tumor Cell Line DU145 Mouse Xenograft Study*
[0186] As noted in Example 6 novel therapeutics agents that potentially limit the growth of human tumors can be assessed for efficacy in vivo using well characterised mouse xenograft models.

[0187] Human prostate carcinoma DU145 tumor cells growing exponentially in vitro were harvested, washed and injected (2x10^6 in 1:1 PBS:Matrigel/mouse) into the subcutaneous tissue of the right flank of an anesthetized SCID mice. Treatment was commenced when tumors reached a size of approximately 100mm^3 at which point mice were divided into treatment groups of 10 animals. For analysis of anti-VEGF-B mAb 2H10, one group of 10 mice was treated twice weekly with an intraperitoneal (ip) injection of approximately 400 μg of test 2H10 in a volume of 200 μl, while a second group of mice was treated with the identical amount of an isotype matched control mAb (mAb C44, colchicine specific, ATCC Cat No. CRL-1943). Treatment was continued for a period of 4-5 weeks and the width and length of tumors was determined at regular intervals (for example every 4-5 days) using electronic calipers. Tumor volume (mm^3) was calculated according to the formula: 0.5 x length x width^2.

[0188] By day 33 post initiation of treatment, there was a significant reduction in tumor volume in mice treated with the anti-VEGF-B mAb 2H10 when compared with tumors in the mice treated with the isotype matched control mAb. The level of reduction in tumor growth was similar to that observed for the HT-29 tumor experiments described above.

EXAMPLE 9

Human Tumor Cell Line A431 Mouse Xenograft Study

[0189] As noted in Example 6 novel therapeutics agents that potentially limit the growth of human tumors can be assessed for efficacy in vivo using well characterised mouse xenograft models.

[0190] Human squamous cell carcinoma A431 tumor cells growing exponentially in vitro were harvested, washed and injected (3x10^6 in 1:1 PBS:Matrigel/mouse) into the subcutaneous tissue of the right flank of an anesthetized athymic nude mice. Treatment was commenced when tumors reached a size of approximately 100mm^3 at which point mice were divided into treatment groups of 10 animals. For dose-response analysis of anti-VEGF-B mAb 2H10, groups of 10 mice were treated twice weekly with an intraperitoneal (ip) injection of approximately 1000 μg, 400 μg, 40 μg or 4 μg of test 2H10 in a volume of 200 μl, while a fifth group of mice was treated twice weekly with an ip injection of 1000 μg of an isotype matched control mAb (mAb C44, colchicine specific, ATCC Cat No. CRL-1943). Treatment was continued for a period of 17 days and the width and length of tumors was determined at regular intervals (for example every 2-3 days) using electronic calipers. Tumor volume (mm^3)
was calculated according to the formula: $0.5 \times \text{length} \times \text{width}^2$.

[0191] By day 17 post initiation of treatment, there was a significant reduction in tumor volume in mice treated with the anti-VEGF-B mAb 2H10, with the extent of reduction proportional to the dose of 2H10 administered (i.e. level of reduction of tumor growth: 1000 μg dose > 400 μg dose > 40 μg dose > 4 μg dose). When compared with the 4 μg / dose group, the level of reduction in tumor growth observed with 1000 μg / dose was approximately 40-45%. Compared with the 4 μg dose of 2H10, control mAb C44 at 1000 μg / dose appeared to cause some non-specific reduction in tumor growth.

EXAMPLE 10

*Humanisation of Anti-VEGF-B mAb 2H10*

*Generation of CDR-grafted Fabs and mouse-human chimeric Fabs*

[0192] The VBASE database (database of human antibody genes http://vbase.mrc-cpe.cam.ac.uk/) was used to identify human germline variable region sequences closely related to the 2H10 variable region sequences described in Example 7 and shown in Figure 1. The EmBL database sequences X93622 and J00242 (human unarranged germline antibody light chain regions PK9 and JK4 respectively) and HSIGDP75 and J00256 (human unarranged germline antibody heavy chain regions DP75 and JH4a respectively) (Figure 4) were selected for subsequent CDR grafting.

[0193] Nucleic acid sequences encoding 2H10 CDR-grafted human germline Fab (CDR-Fab) were prepared by replacing the nucleotides encoding the CDRs of the human germline variable region sequences with the nucleotide sequences for the 2H10 CDRs (Figure 5). The variable region sequences were then fused to nucleotide sequences encoding the corresponding human constant domains of the light and heavy chains to give cDNAs encoding the CDR-Fab. The CDR-Fab nucleotide sequences were modified to optimise the codons for expression in *E. coli* and then inserted into a single *E. coli* expression vector to generate a dicistronic construct to express soluble functional CDR-Fabs. For a general description of the expression of antibody fragments in *E.coli* refer to Corisdeo S. and Wang B. (2003), Functional expression and display of an antibody Fab fragment in Escherichia coli: study of vector design and culture conditions. Protein Expression and Purification 34: 270-279.

[0194] Figure 6 shows the nucleic acid sequences for the 2H10 CDR regions optimised for expression in *E.coli*.

[0195] A mouse-human chimeric Fab, (consisting of the murine 2H10 heavy and light chain
variable regions fused to the corresponding human constant domains), and a soluble 2H10 murine Fab protein, (prepared by the proteolytic digestion of the full length murine 2H10 antibody using the papain digestion protocol described in "Antibody Production: Essential Techniques" Peter J. Delves, 1997, John Wiley & Sons, Chichester, UK) were also prepared for VEGF-B binding studies. The mouse-human chimeric Fab and the 2H10 murine Fab had similar binding affinity for VEGF-B and were used to assess whether the CDR-Fab required framework optimization to optimize the presentation of the murine CDRs by the human framework and improve VEGF-B binding affinity.

Comparison of the binding affinities of the chimeric and CDR-grafted Fabs

[0196] The binding affinity of the CDR-Fab for VEGF-B may be compared to the VEGF-B binding affinity of the mouse-human chimeric Fab and the 2H10 murine Fab in a number of ways, such as in competition-based binding assays (for example, as phage displayed Fabs in an ELISA format) or as purified soluble protein by BIacore analysis.

[0197] The binding affinity of the CDR-Fab for immobilised human VEGF-B was compared with the 2H10 murine Fab by measuring surface plasmon resonance (SPR) using a BIACore instrument. The association rate constant (k_on), the dissociation rate constants (k_off), and the equilibrium binding constants (K_D) were calculated for the CDR-Fab and the 2H10 murine Fab using data from injections of each Fab at several different concentrations. The CDR-Fab was found to have a faster dissociation rate than the 2H10 murine Fab, resulting in a weaker binding affinity for VEGF-B (Table 4). Optimisation of key framework residues was therefore undertaken to improve the binding affinity of the CDR-Fab.

[0198] The variable region sequences of the CDR-Fab and the mouse-human chimeric Fab were aligned to identify framework residues within the CDR-Fab which may require optimization (Foot J. and Winter G. (1992) Antibody Framework Residues Affecting the Conformation of the Hypervariable Loops. J. Mol. Biol. 224: 487-499). Six key framework residues were identified, these being L71, H67, H69, H71, H73 and H75. A panel of twelve CDR-Fab framework variants were generated by individual and combined changes of the key framework residues back to the original murine sequence (six single backmutations and pairs of H67, H69, H71 and H73).

[0199] The twelve CDR-Fab framework variants were expressed in E. coli and their dissociation rates were compared to the dissociation rates for the mouse-human chimeric Fab and the CDR-Fab by BIACore. CDR- Fab variants with the H73 backmutation, either alone or in combination with one of H67, H69 OR H71, were found to have similar dissociation rates to the mouse-human chimeric Fab (Table 5).

[0200] The binding affinities of the four H73 CDR-Fab framework variants (H73, H67/73, H69/73 AND H71/73) were then compared with the murine-human chimeric Fab in a
competition ELISA. These five Fabs were tested in solution for their ability to bind to plate bound human VEGF-B (1 ug/ml) in the presence of titrating amounts of VEGF-B in solution, and the IC50s were determined (the soluble VEGF-B concentration at which 50% binding to immobilised VEGF-B is displaced). The IC50 for each CDR-Fab variant and the murine-human chimeric Fab was used to rank the VEGF-B binding affinity. The CDR-Fab variant with the single H73 backmutation was found to have the highest VEGF-B binding affinity, similar to that of the murine-human chimeric Fab (Table 5).

The equilibrium binding constant ($K_D$) of the H73 CDR- Fab framework variant for immobilised human VEGF-B was then determined by BIAcore and found to be within 3-fold of the equilibrium binding constant for the 2H10 murine Fab (337 pM compared with 115 pM respectively).

Table 4

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Table 5

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* measured by competition ELISA, where relative binding affinity is equal to IC<sub>50</sub> CDR-Fab variant/ IC<sub>50</sub> murine-human chimera.

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1 5 10

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Tyr Phe
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ala Ala Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Cys Gln Gln Ser Tyr Ser Thr Pro Pro
85 90 95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Gly Tyr

Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met

Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe

Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ile Ser Thr Ala Tyr

Met Glu Leu Ser Arg Leu Arg Ser Asp Thr Ala Val Tyr Tyr Cys

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Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly

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Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Gly Lys Thr Leu Pro Pro

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys

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35 40 45
Gly His Ile Asn Pro Gly Asn Gly Thr Asn Tyr Asn Glu Lys Phe
50 55 60
Lys Arg Arg Val Thr Met Thr Arg Asp Lys Ser Ile Ser Thr Ala Tyr
65 70 75 80
Met Glu Leu Ser Arg Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
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Ala Arg Ser Tyr Ser Asn Tyr Val Arg Ala Met Asp Tyr Trp Gly Gln
100 105 110
Gly Thr Leu Val Val Thr Val Ser Ser
115 120

Mus musculus

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Escherichia coli

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tacatcaaa cattacacta 21

Mus musculus
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 20         25   30
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 35         40   45
Tyr Trp Ala Ser Thr Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
 50         55   60
Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Asn Val Gln Ser
 65         70   75   80
Glu Asp Leu Ala Asp Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Leu Thr
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Phe Gly Ala Gly Ala Thr Lys Leu Glu Leu
100        105
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20 25 30
Tyr Ile His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Glu Trp Ile
35 40 45
 Ala Trp Phe Tyr Pro Gly Asn Val Asn Thr Asn Tyr Asn Glu Lys Phe
50 55 60
Lys Asp Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ala Ala Tyr
65 70 75 80
Leu Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95
Thr Arg Ser Pro Tyr Tyr Gly Tyr Val Phe Asp Phe Trp Gly Gln Gly
100 105 110

Thr Thr Leu Thr Val Ser Ser
115

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  1  5

<210> 48
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  1  5  10

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  1  5  10  15

Asp

<210> 50
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<213> Mus musculus

<400> 50
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  1  5  10

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav


2. Isoleret antistof ifølge krav 1, hvor det isolerede antistof er et monoklonalt antistof.

3. Isoleret antistof ifølge krav 2, hvor det isolerede antistof er humant eller humaniseret.

4. Isoleret antistof ifølge krav 1, der omfatter en letkæde med CDR’er, som har aminosyresekvensen vist i SEQ ID NO: 21 til 23 eller en aminosyresekvens, der er mindst 95 % identisk med SEQ ID NO: 21 eller 22 eller 23, og en tungkæde med CDR’er, som har aminosyresekvensen vist i SEQ ID NO: 24 til 26 eller en aminosyresekvens, der er mindst 95 % identisk med SEQ ID NO: 24 eller 25 eller 26.

5. Isoleret antistof ifølge krav 1, der omfatter en letkæde med CDR’er, som har aminosyresekvensen vist i SEQ ID NO: 45 til 47 eller en aminosyresekvens, der er mindst 95 % identisk med SEQ ID NO: 45 eller 46 eller 47, og en tungkæde med CDR’er, som har aminosyresekvensen vist i SEQ ID NO: 48 til 50 eller en aminosyresekvens, der er mindst 95 % identisk med SEQ ID NO: 48 eller 49 eller 50.

6. Isoleret antistof ifølge et hvilket som helst af kravene 1 til 5, der er et antigenbindende fragment.

7. Isoleret antistof ifølge krav 6, hvor det antigenbindende fragment er udvalgt blandt et Fv-, Fab- eller F(ab')₂-fragment,
et enkeltkædet Fv-fragment (sFv eller scFv), et
disulfidstabiliseret Fv-fragment (dsFv) og minibodies og
diabodies.

8. Præparat, der omfatter et isoleret antistof ifølge et
hvilket som helst af kravene 1 til 7.

9. Ekspressionsvektor, der omfatter et nukleinsyremolekyle,
som koder for letkæden og tungkæden af det isolerede antistof
ifølge et hvilket som helst af kravene 1 til 5, hvor
ekspressionsvektoren er i stand til at udtrykke nukleinsyrerne
i en prokaryot eller eukaryot værtscelle.

10. Isoleret prokaryot eller eukaryot værtscelle, der
omfatter en ekspressionsvektor ifølge krav 9.

11. Fremgangsmåde til produktion af et isoleret anti-VEGF-B-
antistof ifølge et hvilket som helst af kravene 1 til 5,
hvilken fremgangsmåde omfatter: (a) dyrkning af de isolerede
værtscelle ifølge krav 10 i et tidsrum, der er tilstrækkeligt
til muliggørelse af ekspression af antistoffet; og (b)
oprensning af det udtrykte antistof.

12. Isoleret antistof ifølge et hvilket som helst af kravene
1 til 7 eller præparat ifølge krav 8 til anvendelse i medicin.

13. Isoleret antistof ifølge et hvilket som helst af kravene
1 til 7 eller præparat ifølge krav 8 til anvendelse til
behandling af cancer hos et pattedyr.

14. Isoleret antistof til anvendelse eller sammensætning til
anvendelse ifølge krav 13, hvor canceren er en tumor, en
præcancerøs tilstand, et myelom eller et lymfom, fortrinsvis
hvor tumoren er udvalgt blandt brysttumorer, kolorektale
tumorer, adenokarcinomer, mesoteliom, blæretumorer,
prostatatumorer, germcelletumor, hepatom/cholangio, karcinom,
neuroendocrine tumorer, hypofyse-neoplasma, lille rund celle-
tumor, pladecellecancer, melanom, atypisk fibroxantom,
seminomer, nonseminomer, stromale leydig celle-tumorer, sertoli celle-tumorer, hudtumorer, nyretumorer, testikeltumorer, hjernetumorer, ovarietumorer, mavetumorer, orale tumorer, blæretumorer, knogletumorer, cervikale tumorer, øsofageale tumorer, laryngeale tumorer, levertumorer, lungetumorer, vaginale tumorer og Wilm's tumor.

15. Isoleret antistof til anvendelse eller præparat til anvendelse ifølge krav 13 til 14, hvor canceren er en fast tumor.
DRAWINGS

2H10 light chain variable region

10  20  30  40
EIQMTQTTSSLSASLGDRVTISCRASQDISNFLWYQQKP
50  60  70  80
DGTVKLLIYYTSTLHSGVPSRFSGSGTDYSLTISNLEQ
90  100
EDIATYFCQQGKTLPPFGGGTKLEIK

2H10 heavy chain variable region

10  20  30  40
QVQLQPGTELVKFGASVKLSCKASGYTFTGFIMHWVKQR
50  a  60  70  80
PGQGLEWIGHINPGGQNYNEKFKRAMATLVKSSSTAYM
abc  90  100abc  110
QLSSLTSED SAVYYCAR SYNV RAMDYWGQQGTSVTSS

Figure 1
4E12 light chain variable region

10  20  30  40  
DIVMTQSQKFMSVSVTCKASQNVNVNLAYQQQK

50  60  70  80  
GQSPRPLTSASSKCSGVPDRFTGSGFTGTDFTLTISNVQS

90  100  
EDLAEYFCQQYHSFPLTFGAGAKLDK

4E12 heavy chain variable region

10  20  30  40  
QVPQQFAGELVKFGASVKSCKASGDTITNSWICWVTQR

50  a  60  70  80  
PQGLEWIGDIFPSGHTNYEKEFKNRATLVDTSSSTAYM

abc  90  100  110  
LSSLTSDSAYVYCVIENYAVFAYWCGTLTVSAA
2F5 light chain variable region

10  20  30  40
DIVMTQSHKFMSTSGDRTVSITCKASODVGTAVAWYQQKPR
50  60  70  80
GQSPKLLIYWASTHRHTGVPDRFTGSGTDTFLTLISNVQS
90  100
EDLADYFCQOYSSLTFAGATKLEIK

2F5 heavy chain variable region

10  20  30  40
QVQLQQSGPELVKGTSVRISSCKAGKYTPTFYIHWKQR
50  a  60  70  80
PGQGLEWIGWFPGNVNTNNEKLGKATLTDKSSSAYL
abc  90  100ab  110
QLNSLTSEDASAVYFCTRSPYGYFVFDYWGQGGTLTVSS

Figure 3
Human Variable Light Chain: Germline PK9/JK4

DIQMTQSPSSLSASVGDRVTITCRASQSISYPLNQYQKPGKAPKLILYAASSLQGVPS
RFSGSGSTDFTLTISSLQPEDFATYYCCQYSPTPTFGGTKVEIK

Human Variable Heavy Chain: Germline DP75/JH4a

QVQLVQSGAEVKPGASVVKVSCKASGYTFTGYYMHWVRQAPGQGLEWWMGINPNSGCTNY
AQRKFGQRTIEMTRDS1STAYMELSRLRSDTAVYYCAR3YSNIVYEDYWGQGTVSS

Figure 4
2H10 CDR-Grafted Human Variable Light Chain: Germline PK9/JK4

DIQMTQSPSLSASVGRVTITCRASQDISNFLNLWYQQKFGKAPKLILYYTSLHSGVPS
RFSGSGSGYDFTLTISSLQPEDFATYYCQQCGKLPTFGGGTKEIK

2H10 CDR-Grafted Human Variable Heavy Chain: Germline DP75/JH4a

QVQLVQSGAEVKPGASVKSCKASGTYTFTGFWIHVRQAPGQGLEWMGHINPGNNGGTY
NEKFKRRVTVTTRDKSIAYMLSRLRSDDTAVVYYCARSYSNYVRAMDYWQGTLVTSS

Figure 5
2H10 V<sub>L</sub> Chain CDR Regions

**CDR-L1**
AGGGCAAGTCAGGACATTAGCAATTTTTAAAC  (murine)
CGTGCAGGCAGGATATAGCAACTTTCTGAAC  (E.coli)

**CDR-L2**
TACACATCAAACATTACACTCA  (murine)
TATACCAGCACCCCTGATAGC  (E.coli)

**CDR-L3**
CAACAGGGTTAAAACGCTTCTCCACCG  (murine)
CAGCAGGGCATAAACCTGCGCCGACC  (E.coli)

2H10 V<sub>H</sub> Chain CDR Regions

**CDR-H1**
GGCTACACTTTTCACTGGCTTCTGGATAACAC  (murine)
GGCTATACCTTTACCGGCTTTTGGATTCAT  (E.coli)

**CDR-H2**
CATATTAACTCTGGCAATCGGGACTAATCATAATGAGAAGTTCAAGAGA  (murine)
CATATTAAACCCGGCAACGGCGGTACCAAACATTAACGAAAAATTTAACGT  (E.coli)

**CDR-H3**
TCTTNTAGTAACCTGCGGCTATGGACTAC  (murine)
AGCTATAGCRAACTATGTCGCTGGATGGATTAT  (E.coli)

Figure 6
1C6 light chain variable region

10  20  30  40
DIVMTQSHKFMSTSVGDRVSITCKASQDVGSAVAVYWQQKP

50  60  70  80
GQSPKLLLIVASTRHTGVPDRFTGSGLTNTFTISNVQDS

90  100
EDLADYFCQOYSSLSLTFGAGATKLELK

1C6 heavy chain variable region

10  20  30  40
QVQLQQSGPELVKFGASVRISCKASGYTFTFFYIHWWKQR

50  a  60  70  80
PCQGLEWIAWFYPGNVTNYKNEKFKDKATLTADKSAAAYL

abc  90  100ab  110
QLNLQTDSEAVYFCRSPYGYVFDFWQGQSTTLTVSS

Figure 7