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(71) Applicant (for all designated States except US): **MASSACHUSETTS GENERAL HOSPITAL** [US/US]; Building 149, 13th Street, Suite 5036, Charlestown, MA 02139 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **JAIN, Rakesh** [US/US]; Cambridge, MA (US). **KOZIN, Sergey** [RU/US]; Cambridge, MA (US). **BOUCHER, Yves** [US/US]; Cambridge, MA (US).

(74) Agents: **KAMAGE, Andrea, J.** et al.; Lerner, David, Littenberg, Krumholz & Mentlik LLP, 600 South Avenue West, Westfield, NJ 07090-1497 (US).

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(54) Title: METHOD OF TREATING RECURRENT TUMORS

(57) Abstract: The present invention provides a method of treating a recurring tumor in a mammal in need thereof comprising administering to the mammal a therapeutically effective amount of a vascular endothelial growth factor receptor (VEGFR) antagonist.



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METHOD OF TREATING RECURRENT TUMORS

CROSS-REFERENCE

5 This application claims the benefit of U.S. provisional patent application Ser. No. 60/898,098, filed January 30, 2007, the contents of which are incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

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The present invention is directed to methods of inhibiting or preventing the growth of tumors recurring after radiotherapy.

BACKGROUND OF THE INVENTION

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The treatment of tumor recurrences after radiotherapy is a challenging problem. Curative surgery in previously heavily irradiated areas is usually not feasible. Re-irradiation alone or in combination with chemotherapy is currently used in the clinic but the dose of repeat irradiation is significantly limited by the risk of serious normal tissue damage, which is greater than after the initial treatment. Re-irradiation at decreased doses is not typically effective and often used just palliatively. Although new, targeted therapies are occasionally used in treatment of the recurrences, these therapies are not always effective in reducing or inhibiting tumor growth.

20 Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein consisting of two 23 kD subunits with structural similarity to platelet derived growth factor (PDGF). Four different monomeric isoforms of VEGF exist, resulting from alternative splicing of mRNA. These include two membrane bound forms (VEGF₂₀₆ and VEGF₁₈₉) and two soluble forms (VEGF₁₆₅ and VEGF₁₂₁). In all human tissues except placenta, VEGF₁₆₅ is the most abundant isoform.

30 The biological response of VEGF is mediated through its high affinity receptors. VEGF receptors typically are class III receptor-type tyrosine kinases characterized by having several, typically 5 or 7, immunoglobulin-like loops in their amino-terminal extracellular receptor ligand-binding domains. The other two regions include a transmembrane region and a carboxy-terminal intracellular catalytic domain interrupted by an insertion of hydrophilic interkinase sequences of variable lengths, called the kinase

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insert domain. VEGF receptors include *fms*-like tyrosine kinase receptor (flt-1), sequenced by Shibuya et al., *Oncogene*, 5: 519-524 (1990) and kinase insert domain-containing receptor/fetal liver kinase (KDR/flk-1), described in WO 92/14248, filed February 20, 1992, and Terman et al., *Oncogene*, 6: 1677-1683 (1991), and sequenced by
5 Matthews et al., *Proc. Natl. Acad. Sci. USA*, 88: 9026-9030 (1991).

SUMMARY OF THE INVENTION

The present invention relates to methods of treating recurring tumors by
10 administering a therapeutically effective amount of a vascular endothelial growth factor receptor (VEGFR) antagonist to a patient in need thereof. In certain embodiments, the VEGFR antagonist is administered in combination with another agent or treatment such as chemotherapy, radiation, or other therapeutic agent.

In one aspect of the invention there are provided methods of treating recurring
15 tumors in a mammal, e.g., in a human, in need thereof by administering to the mammal a therapeutically effective amount of a vascular endothelial growth factor receptor (VEGFR) antagonist. In a particular embodiment, the recurring tumors are head and neck carcinomas or gliomas. In another particular embodiment, the recurring tumors are carcinomas, sarcomas, blastomas and gliomas. In another embodiment, the carcinomas,
20 sarcomas, blastomas and gliomas are epidermoid tumors, squamous tumors, head and neck tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, liver tumors, Kaposi's sarcoma, CNS neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas, cerebral metastases, melanomas, gastrointestinal
25 carcinomas, gastrointestinal sarcomas, renal carcinomas, renal sarcomas, rhabdomyosarcomas, glioblastomas, glioblastoma multiforme, leiomyosarcomas, squamous cell carcinomas, basal cell carcinomas, human malignant keratinocytes, leukemias, multiple myelomas, lymphomas, acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic
30 leukemia (CLL), erythrocytic leukemia, monocytic leukemia, Hodgkin's disease and Non-Hodgkin's disease.

In a further aspect of the invention, the VEGFR antagonist is an anti-VEGFR antibody. Preferably, the anti-VEGFR antibody binds to a VEGFR selected from the

group consisting of FLT-1, KDR and FLK-1. The anti-VEGFR antibody can be a monoclonal antibody, a fragment of an antibody, a derivative of an antibody, a chimerized antibody, a humanized antibody, a human antibody, or a single chain antibody. In a particular embodiment, the anti-VEGFR antibody is a single chain antibody that binds to VEGFR-2. In another particular embodiment, the anti-VEGFR antibody has the same specificity as rat IgG1 monoclonal antibody that is produced by hybridoma DC-101 (ATCC HB 11534). In another particular embodiment, the anti-VEGFR antibody has the same specificity as mouse monoclonal antibody Mab 6.12 produced by a hybridoma deposited as ATCC PTA-3344.

In a still further aspect of the invention, there are provided methods for treating recurring tumors in a mammal in need thereof by administering to the mammal a therapeutically effective amount of a VEGFR antagonist and one or more anti-neoplastic-agents. In one embodiment of the invention, the anti-neoplastic agent is a chemotherapeutic agent.

In another aspect of the invention, there are provided methods of treating recurring tumors in a mammal in need thereof by administering a therapeutically effective amount of a VEGFR antagonist and radiation.

A further aspect of the invention includes methods of treating recurring tumors in a mammal in need thereof by administering to the mammal a therapeutically effective amount of a VEGFR antagonist and an antibody that binds to EGFR.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs 1A and 1B demonstrate the therapeutic efficacy of DC101 treatment (on days 0, 3 and 6) in primary vs. recurrent 54A tumor xenografts after local irradiation at a dose of 20 Gy. FIG 1A is the average times necessary for tumors to double and triple their initial volumes ($\sim 260 \text{ mm}^3$, corresponding to $\sim 8 \text{ mm}$ in diameter), $n=19-23$ per group; FIG 1B is the individual values of time taken for a tumor to double its initial volume, two recurrent tumors were locally controlled (LC) for 120 days after DC101 therapy. Filled symbols are median values in groups.

FIGs 2A-2D demonstrate the dynamics of changes in vessel structure and function and interstitial fluid pressure (IFP) in primary and recurrent tumors during DC101 treatment. FIGs 2A and 2B are the density of lectin-perfused vessels and their coverage with α

SMA-stained pericytes. $n=6$ in all groups; FIGs 2C and 2D are serial measurements of DC101-induced changes in IFP in primary ($n=16$) and recurrent ($n=14$) tumors, and the kinetics of growth of these xenografts. Of note, the multiple IFP measurements had no significant effect on tumor size kinetics and eventually achieved DC101-induced growth delay for either tumor variant (not shown), as compared to the observations above without IFP evaluations. Asterisks indicate a significant difference ($p<0.05$) between primary and recurrent tumors for a particular time point. When all 3 pairs of values were analyzed collectively in *A* and *B*, the inter-model difference was highly significant ($p<0.01$) for both parameters.

FIGs 3A and 3B demonstrate the dynamics of tumor tissue necrosis (FIG. 3A) and tumor cell proliferation (FIG. 3B) during DC101 therapy, ($n=5-6$ per group). Asterisks indicate a significant difference ($p<0.05$) between primary and recurrent tumors for a particular time point.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of treating recurring tumors in a mammal. Recurring tumors are tumors that recur after radiation therapy and include both true relapses and new tumors within irradiated regions. Such tumors can be contrasted to primary, non-irradiated tumors. A treatment option, according to the present invention, is administering an effective amount of a VEGFR antagonist to a mammal suffering for a recurring tumor. Treatment includes (1) preventing a recurring tumor from developing; (2) inhibiting the growth of the recurring tumor, *e.g.* arresting its development; or (3) relieving the recurring tumor, *e.g.* causing regression of the symptoms of the recurring tumor. Inhibiting tumor growth includes slowing or stopping growth, as well as causing tumor regression. An effective amount for the treatment of a recurring tumor means an amount which, when administered to a patient suffering from a recurring tumor, is sufficient to effect treatment, as described above.

The recurring tumors that are treated according to the present invention can be any type of recurring tumor. The tumor can be solid or non-solid and can occur in virtually all parts of the body, including, for example, the breast, heart, lung, esophagus, small intestine, colon, rectum, stomach, spleen, kidney, bladder, head and neck, larynx, ovary, prostate, brain, pancreas, skin, bone, bone marrow, blood, thymus, uterus, testicles, cervix or liver.

Some examples of solid tumors that can be treated with the antagonists of the present invention include carcinomas, sarcomas, blastomas or gliomas. Some examples of such tumors include epidermoid tumors, squamous tumors, such as head and neck tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, including small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, and liver tumors. Other examples include Kaposi's sarcoma, CNS neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas and cerebral metastases, melanoma, gastrointestinal and renal carcinomas and sarcomas, rhabdomyosarcoma, glioblastoma, preferably glioblastoma multiforme, and leiomyosarcoma. Examples of vascularized skin cancers for which the antagonists of this invention are effective include squamous cell carcinoma, basal cell carcinoma and skin cancers that can be treated by suppressing the growth of malignant keratinocytes, such as human malignant keratinocytes. In a preferred embodiment, the recurring tumor is a head and neck carcinoma or glioma.

Some examples of non-solid tumors include leukemias, multiple myelomas and lymphomas. Some examples of leukemias include acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), erythrocytic leukemia or monocytic leukemia. Some examples of lymphomas include lymphomas associated with Hodgkin's disease and Non-Hodgkin's disease.

A VEGF receptor antagonist can be any antagonist that inhibits VEGF activity and includes an antibody, a ligand, a peptide, a DNA, a small molecule, or any other suitable antagonist. In order to be useful, the antibody, ligand, peptide, DNA, or small molecule must be sufficient to block binding to the receptor by one or both of the VEGF receptor ligands, VEGF and PlGF, and/or prevent activation of the VEGF receptor. By prevention of activation of the VEGF receptor is meant any decrease in the activation of the VEGF receptor. That is, the prevention of activation need not completely stop activation of the VEGF receptor. The VEGF receptor antagonist can bind to either the VEGF receptor or to the VEGF ligand to decrease activation of the VEGF receptor.

In one embodiment, the VEGF receptor antagonist binds specifically to an epitope on the extracellular domain of a VEGF receptor. The extracellular domain of a VEGF receptor is the ligand-binding domain. The ligand-binding domain may be found at either end of the receptor, but is normally found at the amino-terminal end. Some examples of VEGF receptors include the protein tyrosine kinase receptors referred to in the literature

as FLT-1, KDR and FLK-1. Unless otherwise stated or clearly suggested otherwise by context, this specification will follow the customary literature nomenclature of VEGF receptors. KDR will be referred to as the human form of a VEGF receptor having MW 180 kD (Terman et al., above). FLK-1 will be referred to as the murine homolog of KDR (Matthews et al., above). FLT-1 will be referred to as a form of VEGF receptor different from, but related to, the KDR/FLK-1 receptor. See Shibuya et al., above.

Other VEGF receptors include those that can be cross-link labeled with VEGF, or that can be co-immunoprecipitated with KDR. Some known forms of these VEGF receptors have molecular weights of approximately 170 KD, 150 KD, 130-135 KD, 120-125 KD and 85 KD. See, for example, Quinn et al. Proc. Nat'l. Acad. Sci 90, 7533-7537 (1993). Scher et al. J. Biol. Chem. 271, 5761-5767 (1996).

A VEGFR antagonist can also bind to a VEGF ligand.

In one embodiment of the present invention in which the VEGF receptor antagonist is an antibody, the antibody may be a monoclonal antibody, a fragment of an antibody, a derivative of an antibody, a chimerized antibody, a humanized antibody or a human antibody. The antibody may also be a single chain antibody (scFv) or a synthetic homolog of the antibody. As used herein, unless otherwise indicated or clear from the context, antibody domains, regions and fragments are accorded standard definitions as are well known in the art. See, e.g., Abbas et al., *Cellular and Molecular Immunology*, W.B. Saunders Company, Philadelphia, PA (1991).

The VEGF receptor antagonist antibodies of the subject invention are preferably monoclonal. The VEGF receptor antagonist may also be a fragment of an antibody. The fragment may be produced by cleaving a whole antibody, or by expressing DNA that encodes the fragment.

In one preferred embodiment, the VEGF receptor antagonist binds specifically to VEGFR-2. Particularly preferred are antigen-binding proteins that bind to the extracellular domain of VEGFR-2 and block binding by one or both of its ligands, VEGF and PlGF, and/or neutralize VEGF-induced or PlGF-induced activation of VEGFR-2. Thus, in a preferred embodiment, the VEGF receptor antagonist of the present invention comprises a scFv that binds to VEGFR-2 and blocks VEGF binding and/or PlGF binding to VEGFR-2. In another embodiment the monoclonal antibody is a rat IgG1 monoclonal antibody, specific for the murine VEGF receptor FLK-1, and produced by hybridoma

DC-101. Hybridoma cell line DC-101 was deposited Jan. 26, 1994 with the American Type Culture Collection designated ATCC HB 11534.

In other embodiments, the VEGF receptor antagonist binds specifically to VEGFR-1. Mab 6.12 binds to soluble and cell surface-expressed flt-1. ScFv 6.12
5 comprises the V_L and V_H domains of mouse monoclonal antibody Mab 6.12. A hybridoma cell line producing Mab 6.12, has been deposited as ATCC number PTA-3344.

The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent
10 Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC, which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a
15 license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

In a preferred embodiment, the VEGF receptor antagonist is an anti-VEGFR antibody that exhibits one or more of following properties:

1) The antibody binds to the external domain of VEGFR and inhibits ligand
20 binding. Inhibition can be determined, for example, by a direct binding assay using purified or membrane bound receptor. In this embodiment, the antibodies of the present invention or fragments thereof, preferably bind VEGFR at least as strongly as the natural ligands of VEGFR.

2) The antibodies neutralize VEGFR. Binding of a ligand to an external,
25 extracellular domain of VEGFR stimulates tyrosine kinase activity and receptor phosphorylation and/or the phosphorylation of other proteins involved in the various signaling pathways. Neutralization of VEGFR includes inhibition, diminution, inactivation and/or disruption of one or more of the activities normally associated with signal transduction. Neutralization can be determined *in vivo*, *ex vivo*, or *in vitro* using,
30 for example, tissues, cultured cell, or purified cellular components.

One measure of VEGFR neutralization is inhibition of the tyrosine kinase activity of the receptor. Tyrosine kinase inhibition can be determined using well-known methods; for example, by measuring the autophosphorylation level of recombinant kinase receptor,

and/or phosphorylation of natural or synthetic substrates. Thus, phosphorylation assays are useful in determining neutralizing antibodies in the context of the present invention. Phosphorylation can be detected, for example, using an antibody specific for phosphotyrosine in an ELISA assay or on a western blot. Some assays for tyrosine kinase activity are described in Panek et al., *J. Pharmacol. Exp. Thera.* 283: 1433-44 (1997) and Batley et al., *Life Sci.* 62:143-50 (1998). Antibodies of the invention cause a decrease in tyrosine phosphorylation of VEGFR of at least about 75%, preferably at least about 85%, and more preferably at least about 90% in cells that respond to ligand.

Another measure of VEGFR neutralization is inhibition of phosphorylation of downstream substrates of VEGFR. Accordingly, the level of phosphorylation of MEK and MAP can be measured. The decrease in phosphorylation is at least about 40%, and can be at least about 60%, or at least about 80%.

In addition, methods for detection of protein expression can be utilized to determine VEGFR neutralization, wherein the proteins being measured are regulated by VEGFR tyrosine kinase activity. These methods include immunohistochemistry (IHC) for detection of protein expression, fluorescence in situ hybridization (FISH) for detection of gene amplification, competitive radioligand binding assays, solid matrix blotting techniques, such as Northern and Southern blots, reverse transcriptase polymerase chain reaction (RT-PCR) and ELISA. See, e.g., Grandis et al., *Cancer*, 78:1284-92 (1996); Shimizu et al., *Japan J. Cancer Res.*, 85:567-71 (1994); Sauter et al., *Am. J. Pathol.*, 148:1047-53 (1996); Collins, *Glia* 15:289-96 (1995); Radinsky et al., *Clin. Cancer Res.* 1:19-31 (1995); Petrides et al., *Cancer Res.* 50:3934-39 (1990); Hoffmann et al., *Anticancer Res.* 17:4419-26 (1997); Wikstrand et al., *Cancer Res.* 55:3140-48 (1995).

Ex vivo assays can also be utilized to determine VEGFR neutralization. For example, receptor tyrosine kinase inhibition can be observed by mitogenic assays using cell lines stimulated with receptor ligand in the presence and absence of inhibitor. An example of such a mitogenic assay is a 3T3 cells mitogenic assay (3T3 (clone A31-714) cells from American Type Culture Collection (Manassas, VA)). Another method involves testing for inhibition of growth of VEGFR-expressing tumor cells or cells transfected to express VEGFR. Inhibition can also be observed using tumor models, for example, human tumor cells injected into a mouse.

The antibodies of the present invention are not limited by any particular mechanism of VEGFR neutralization. The anti-VEGFR antibodies of the present

invention can bind externally to VEGFR cell surface receptor, bind to VEGF, block binding of ligand and subsequent signal transduction mediated via the receptor-associated tyrosine kinase, and prevent phosphorylation of the VEGFR and other downstream proteins in the signal transduction cascade.

5 3) The antibodies down modulate VEGFR. The amount of VEGFR present on the surface of a cell depends on receptor protein production, internalization, and degradation. The amount of VEGFR present on the surface of a cell can be measured indirectly, by detecting internalization of the receptor or a molecule bound to the receptor. For example, receptor internalization can be measured by contacting cells that express
10 VEGFR with a labeled antibody. Membrane-bound antibody is then stripped, collected and counted. Internalized antibody is determined by lysing the cells and detecting label in the lysates.

Another way is to directly measure the amount of the receptor present on the cell following treatment with an anti-VEGFR antibody or other substance, for example, by
15 fluorescence-activated cell-sorting analysis of cells stained for surface expression of VEGFR. Stained cells are incubated at 37°C and fluorescence intensity measured over time. As a control, part of the stained population can be incubated at 4°C (conditions under which receptor internalization is halted).

Another measure of down-modulation is reduction of the total receptor protein
20 present in a cell, and reflects degradation of internal receptors. Accordingly, treatment of cells (particularly cancer cells) with antibodies of the invention results in a reduction in total cellular VEGFR. In a preferred embodiment, the reduction is at least about 70%, more preferably at least about 80%, and even more preferably at least about 90%.

For treatment of human subjects, antibodies according to the invention are
25 preferably human. Alternatively, the antibodies can be from non-human primates or other mammals, or be humanized or chimeric antibodies.

Antibody fragments according to the invention can be produced by cleaving a whole antibody, or by expressing DNA that encodes the fragment. Fragments of antibodies may be prepared by methods described by Lamoyi et al., *J. Immunol. Methods*,
30 56: 235-243 (1983) and by Parham, *J. Immunol.* 131: 2895-2902 (1983). Such fragments may contain one or both Fab fragments or the F(ab')₂ fragment. Such fragments may also contain single-chain fragment variable region antibodies, i.e. scFv, dibodies, or other antibody fragments. Methods of producing such functional equivalents are disclosed in

PCT Application WO 93/21319, European Patent Application No. EP 239400; PCT Application WO 89/09622; European Patent Application EP 338745; and European Patent Application EP 332424.

Preferred host cells for transformation of vectors and expression of the antibodies
5 of the present invention are mammalian cells, e.g., COS-7 cells, Chinese hamster ovary (CHO) cells, and cell lines of lymphoid origin such as lymphoma, myeloma (e.g. NS0), or hybridoma cells. Other eukaryotic hosts, such as yeasts, can be alternatively used.

The transformed host cells are cultured by methods known in the art in a liquid medium containing assimilable sources of carbon (carbohydrates such as glucose or
10 lactose), nitrogen (amino acids, peptides, proteins or their degradation products such as peptones, ammonium salts or the like), and inorganic salts (sulfates, phosphates and/or carbonates of sodium, potassium, magnesium and calcium). The medium furthermore contains, for example, growth-promoting substances, such as trace elements, for example iron, zinc, manganese and the like.

15 High affinity anti-VEGFR antibodies according to the present invention can be isolated from a phage display library constructed from human heavy chain and light chain variable region genes. For example, a variable domain of the invention can be obtained from a peripheral blood lymphocyte that contains a rearranged variable region gene. Alternatively, variable domain portions, such as CDR and FW regions, can be obtained
20 from different sources and recombined. Further, portions of the variable domains (e.g., FW regions) can be synthetic consensus sequences.

Antibodies and antibody fragments of the present invention can be obtained, for example, from naturally occurring antibodies, or Fab or scFv phage display libraries. It is understood that, to make a single domain antibody from an antibody comprising a V_H and
25 a V_L domain, certain amino acid substitutions outside the CDRs can be desired to enhance binding, expression or solubility. For example, it can be desirable to modify amino acid residues that would otherwise be buried in the V_H - V_L interface.

Further, antibodies and antibody fragments of the invention can be obtained by standard hybridoma technology (Harlow & Lane, ed., Antibodies: A Laboratory Manual,
30 Cold Spring Harbor, 211-213 (1998), which is incorporated by reference herein) using transgenic mice (e.g., KM mice from Medarex, San Jose, Calif.) that produce human immunoglobulin gamma heavy and kappa light chains. In a preferred embodiment, a substantial portion of the human antibody producing genome is inserted into the genome

of the mouse, and is rendered deficient in the production of endogenous murine antibodies. Such mice may be immunized subcutaneously (s.c.) with PDGFR α (usually in complete Freund's adjuvant) with boosts as needed. Immunization methods are well known in the art.

5 Antibodies that can be used according to the invention include complete immunoglobulins, antigen binding fragments of immunoglobulins, as well as antigen binding proteins that comprise antigen binding domains of immunoglobulins. Antigen binding fragments of immunoglobulins include, for example, Fab, Fab', and F(ab')₂. Other antibody formats have been developed which retain binding specificity, but have
10 other characteristics that may be desirable, including for example, bispecificity, multivalence (more than two binding sites), compact size (e.g., binding domains alone).

 Single chain antibodies lack some or all of the constant domains of the whole antibodies from which they are derived. Therefore, they can overcome some of the problems associated with the use of whole antibodies. For example, single-chain
15 antibodies tend to be free of certain undesired interactions between heavy-chain constant regions and other biological molecules. Additionally, single-chain antibodies are considerably smaller than whole antibodies and can have greater permeability than whole antibodies, allowing single-chain antibodies to localize and bind to target antigen-binding sites more efficiently. Furthermore, the relatively small size of single-chain antibodies
20 makes them less likely to provoke an unwanted immune response in a recipient than whole antibodies.

 Multiple single chain antibodies, each single chain having one V_H and one V_L domain covalently linked by a first peptide linker, can be covalently linked by at least one or more peptide linker to form a multivalent single chain antibodies, which can be
25 monospecific or multispecific. Each chain of a multivalent single chain antibody includes a variable light chain fragment and a variable heavy chain fragment, and is linked by a peptide linker to at least one other chain. The peptide linker is composed of at least fifteen amino acid residues. The maximum number of amino acid residues is about one hundred.

30 Two single chain antibodies can be combined to form a diabody, also known as a bivalent dimer. Diabodies have two chains and two binding sites, and can be monospecific or bispecific. Each chain of the diabody includes a V_H domain connected to a V_L domain. The domains are connected with linkers that are short enough to prevent

pairing between domains on the same chain, thus driving the pairing between complementary domains on different chains to recreate the two antigen-binding sites.

Three single chain antibodies can be combined to form triabodies, also known as trivalent trimers. Triabodies are constructed with the amino acid terminus of a V_L or V_H domain directly fused to the carboxyl terminus of a V_L or V_H domain, i.e., without any linker sequence. The triabody has three Fv heads with the polypeptides arranged in a cyclic, head-to-tail fashion. A possible conformation of the triabody is planar with the three binding sites located in a plane at an angle of 120 degrees from one another. Triabodies can be monospecific, bispecific or trispecific.

Thus, antibodies of the invention and fragments thereof include, but are not limited to, naturally occurring antibodies, bivalent fragments such as $(Fab')_2$, monovalent fragments such as Fab, single chain antibodies, single chain Fv (scFv), single domain antibodies, multivalent single chain antibodies, diabodies, triabodies, and the like that bind specifically with antigens.

According to certain embodiments of the present invention, a VEGFR antagonist is administered in combination with one or more anti-neoplastic agents. Any suitable anti-neoplastic agent can be used, such as a chemotherapeutic agent, radiation, or combinations thereof. The anti-neoplastic agent can be an alkylating agent or an anti-metabolite. Examples of alkylating agents include, but are not limited to, cisplatin, cyclophosphamide, melphalan, and dacarbazine. Examples of anti-metabolites include, but are not limited to doxorubicin, daunorubicin, paclitaxel, and gemcitabine.

In a preferred embodiment, the anti-neoplastic agent is a chemotherapeutic agent. Preferred chemotherapeutic agents include amifostine (ethyol), cisplatin, dacarbazine (DTIC), dactinomycin, mechlorethamine (nitrogen mustard), streptozocin, cyclophosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (adriamycin), doxorubicin lipo (doxil), gemcitabine (gemzar), daunorubicin, daunorubicin lipo (daunoxome), procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil, vinblastine, vincristine, bleomycin, paclitaxel (taxol), docetaxel (taxotere), aldesleukin, asparaginase, busulfan, carboplatin, cladribine, camptothecin, CPT-11, 10-hydroxy-7-ethyl-camptothecin (SN38), dacarbazine, floxuridine, fludarabine, hydroxyurea, ifosfamide, idarubicin, mesna, interferon alpha, interferon beta, irinotecan, mitoxantrone, topotecan, leuprolide, megestrol, melphalan, mercaptopurine, plicamycin, mitotane, pegaspargase, pentostatin, pipobroman, plicamycin, streptozocin, tamoxifen,

teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil and combinations thereof. In a more preferred embodiment, the chemotherapeutic agent is irinotecan.

In other embodiments, a VEGFR antagonist is administered in combination with radiation. The source of radiation, used in combination with a VEGF receptor antagonist, can be either external or internal to the patient being treated. When the source is external to the patient, the therapy is known as external beam radiation therapy (EBRT). When the source of radiation is internal to the patient, the treatment is called brachytherapy (BT). The radiation is administered in accordance with well known standard techniques using standard equipment manufactured for this purpose, such as AECL Theratron and Varian Clinac. The dose of radiation depends on numerous factors as is well known in the art. Such factors include the organ being treated, the healthy organs in the path of the radiation that might inadvertently be adversely affected, the tolerance of the patient for radiation therapy, and the area of the body in need of treatment. The dose will typically be between 1 and 100 Gy, and more particularly between 2 and 80 Gy. Some doses that have been reported include 35 Gy to the spinal cord, 15 Gy to the kidneys, 20 Gy to the liver, and 65-80 Gy to the prostate. It should be emphasized, however, that the invention is not limited to any particular dose. The dose will be determined by the treating physician in accordance with the particular factors in a given situation, including the factors mentioned above.

The distance between the source of the external radiation and the point of entry into the patient may be any distance that represents an acceptable balance between killing target cells and minimizing side effects. Typically, the source of the external radiation is between 70 and 100 cm from the point of entry into the patient.

Brachytherapy is generally carried out by placing the source of radiation in the patient. Typically, the source of radiation is placed approximately 0-3 cm from the tissue being treated. Known techniques include interstitial, intercavitary, and surface brachytherapy. The radioactive seeds can be implanted permanently or temporarily. Some typical radioactive atoms that have been used in permanent implants include iodine-125 and radon. Some typical radioactive atoms that have been used in temporary implants include radium, cesium-137, and iridium-192. Some additional radioactive atoms that have been used in brachytherapy include americium-241 and gold-198.

The dose of radiation for brachytherapy can be the same as that mentioned above for external beam radiation therapy. In addition to the factors mentioned above for determining the dose of external beam radiation therapy, the nature of the radioactive atom used is also taken into account in determining the dose of brachytherapy.

5 In certain other embodiments of the present invention, a VEGFR antagonist is administered in combination with an endothelial growth factor receptor (EGFR) antagonist. An EGFR antagonist, according to the present invention can be an extracellular antagonist or an intracellular antagonist and more than one antagonist may be employed. Extracellular antagonists include, but are not limited to, proteins or other
10 biological molecules that bind to EGFR. In certain embodiments of the invention, an extracellular antagonist binds to the extracellular domain of EGFR and inhibits binding of EGFR to one or more of its ligands and/or neutralizes ligand-induced activation of EGFR. Ligands for EGFR include EGF, TGF- α , amphiregulin, heparin-binding EGFR (HB-EGF) and betacellulin. Extracellular EGFR antagonists can also include substances that inhibit
15 EGFR dimerization with other EGFR receptor subunits (*i.e.* EGFR homodimers) or heterodimerization with other growth factor receptors (*e.g.*, HER2).

In a preferred embodiment, the EGFR antagonist is an antibody that binds to EGFR and blocks ligand binding. One example of an EGFR antibody is cetuximab (IMC-C225) (GenBank Accession No. 1NQLA), which is a chimeric (human/mouse) IgG
20 monoclonal antibody. *See e.g.*, U.S. Patent No. 4,943,533 (Mendelsohn *et al.*); U.S. Patent No. 6,217,866 (Schlessinger *et al.*); U.S. Application Nos. 08/973,065 (Goldstein *et al.*) and 09/635,974 (Teufel); WO 99/60023 (Waksal *et al.*) and WO 00/69459, all of which are incorporated by reference herein. Cetuximab specifically binds to EGFR and blocks binding of a ligand, such as EGF. Cetuximab Fab contains the Fab fragment of
25 Cetuximab, *i.e.*, the heavy and light chain variable region sequences of murine antibody M225 (U.S. App. Ser. No. 2004/0006212, incorporated herein by reference) with human IgG1 C_H1 heavy and kappa light chain constant domains. (Cetuximab includes all three IgG1 heavy chain constant domains.) The CDR regions of the heavy chain of cetuximab have the following sequences: a CDR1 region with a sequence of N Y G V H (SEQ ID
30 NO: 1), a CDR2 region with a sequence of V I W S G G N T D Y N T P F T S (SEQ ID NO: 2), and a CDR3 region with a sequence of A L T Y Y D Y E F A Y (SEQ ID NO: 3). The CDR regions of the light chain of Cetuximab have the following sequences: a CDR1 region with a sequence of R A S Q S I G T N I H (SEQ ID NO: 4), a CDR2 region with a

sequence of Y A S E S I S (SEQ ID NO: 5), and a CDR3 region with a sequence of Q Q N N N W P T T (SEQ ID NO: 6).

Another example of an EGFR antibody is ABX-EGF, which is a fully human IgG₂ monoclonal antibody specific for EGFR. ABX-EGF binds EGFR with high specificity, blocking binding of EGFR to both of its ligands, EGF and TGF- α . *See e.g.*, Figlin *et al.*, Abstract 1102 presented at the 37th Annual Meeting of ASCO, San Francisco, CA, 12-15 May 2001, which is incorporated by reference herein. The sequence and characterization of ABX-EGF, which was formerly known as clone E7.6.3, is disclosed in U.S. Patent No. 6,235,883 (Abgenix, Inc.) at col. 28, line 62 through col. 29, line 36 and in Fig. 29-34, which is incorporated by reference herein. (*See also* Yang *et al.*, *Critical Rev. Oncol./Hematol.*, 38(1): 17-23, 2001, which is incorporated by reference herein.)

Another examples of an EGFR antibody is HERCEPTIN® (trastuzumab), which is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity in a cell-based assay (K_d of 5 nM) to the extracellular domain of the human EGFR2 protein, HER2. The antibody is an IgG₁ kappa that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2. *See, e.g.*, International Patent Publication No. WO 01/89566 (Mass), which is incorporated by reference herein.

Other EGFR antibodies include EMD 72000 (Merck KGaA), which is a humanized version of the murine anti-EGFR monoclonal antibody EMD 55900; h-R3 (TheraCIM), which is a humanized anti-EGFR monoclonal antibody; Y10, which is a murine monoclonal antibody and was raised against a murine homologue of the human EGFRvIII mutation; and MDX-447 (Medarex). *See* U.S. Patent Nos. 5,558,864 (Bendig *et al.*), 5,884,093 (Kettleborough *et al.*), and 5,891,996 (Mateo de Acosta del Rio *et al.*), all of which are incorporated by reference herein.

Intracellular EGFR antagonists can be biological molecules, but are usually small molecules, such as synthetic kinase inhibitors that act directly on the cytoplasmic domain of EGFR to inhibit EGFR-mediated signal transduction. One example of a small molecule EGFR antagonist is IRESSA™ (ZD1939), which is a quinoxaline derivative that functions as an ATP-mimetic to inhibit EGFR. *See* U.S. Patent No. 5,616,582 (Zeneca Limited); WO 96/33980 (Zeneca Limited) at p. 4; *see also*, Rowinsky *et al.*, Abstract 5 presented at the 37th Annual Meeting of ASCO, San Francisco, CA, 12-15 May 2001; Anido *et al.*, Abstract 1712 presented at the 37th Annual Meeting of ASCO,

San Francisco, CA, 12-15 May 2001. Another example of a small molecule EGFR antagonist is TARCEVA® (OSI-774), which is a 4-(substitutedphenylamino)quinoxaline derivative [6,7-Bis(2-methoxy-ethoxy)-quinazolin-4-yl]- (3-ethynyl-phenyl)amine hydrochloride] EGFR inhibitor. See WO 96/30347 (Pfizer Inc.) at, for example, page 2,
5 line 12 through page 4, line 34 and page 19, lines 14-17. See also Moyer *et al.*, *Cancer Res.*, 57: 4838-48 (1997); Pollack *et al.*, *J. Pharmacol.*, 291: 739-48 (1999).

TARCEVA® may function by inhibiting phosphorylation of EGFR and its downstream PI3/Akt and MAP (mitogen activated protein) kinase signal transduction pathways resulting in p27-mediated cell-cycle arrest. See Hidalgo *et al.*, Abstract 281 presented at
10 the 37th Annual Meeting of ASCO, San Francisco, CA, 12-15 May 2001.

Other small molecules are also reported to inhibit EGFR, many of which are thought to be specific to the tyrosine kinase domain of an EGFR. Some examples of such small molecule EGFR antagonists are described in WO 91/116051, WO 96/30347, WO 96/33980, WO 97/27199 (Zeneca Limited). WO 97/30034 (Zeneca Limited), WO
15 97/42187 (Zeneca Limited), WO 97/49688 (Pfizer Inc.), WO 98/33798 (Warner Lambert Company), WO 00/18761 (American Cyanamid Company), and WO 00/31048 (Warner Lambert Company). Examples of specific small molecule EGFR antagonists include C1-1033 (Pfizer), which is a quinoxaline (N-[4-(3-chloro-4-fluoro-phenylamino)-7-(3-morpholin-4-yl-propoxy)-quinazolin-6-yl]-acrylamide) inhibitor of tyrosine kinases,
20 particularly EGFR and is described in WO 00/31048 at page 8, lines 22-6; PKI166 (Novartis), which is a pyrrolopyrimidine inhibitor of EGFR and is described in WO 97/27199 at pages 10-12; GW2016 (GlaxoSmithKline), which is an inhibitor of EGFR and HER2; EKB569 (Wyeth), which is reported to inhibit the growth of tumor cells that overexpress EGFR or HER2 *in vitro* and *in vivo*; AG-1478 (Tryphostin), which is a
25 quinazoline small molecule that inhibits signaling from both EGFR and erbB-2; AG-1478 (Sugen), which is a bisubstrate inhibitor that also inhibits protein kinase CK2; PD 153035 (Parke-Davis) which is reported to inhibit EGFR kinase activity and tumor growth, induce apoptosis in cells in culture, and enhance the cytotoxicity of cytotoxic chemotherapeutic agents; SPM-924 (Schwarz Pharma), which is a tyrosine kinase
30 inhibitor targeted for treatment of prostate cancer; CP-546,989 (OSI Pharmaceuticals), which is reportedly an inhibitor of angiogenesis for treatment of solid tumors; ADL-681, which is a EGFR kinase inhibitor targeted for treatment of cancer; PD 158780, which is a pyridopyrimidine that is reported to inhibit the tumor growth rate of A4431 xenografts in

mice; CP-358,774, which is a quinazoline that is reported to inhibit autophosphorylation in HN5 xenografts in mice; ZD1839, which is a quinazoline that is reported to have antitumor activity in mouse xenograft models including vulvar, NSCLC, prostrate, ovarian, and colorectal cancers; CGP 59326A, which is a pyrrolopyrimidine that is reported to inhibit growth of EGFR-positive xenografts in mice; PD 165557 (Pfizer); CGP54211 and CGP53353 (Novartis), which are dianilnophthalimides. Naturally derived EGFR tyrosine kinase inhibitors include genistein, herbimycin A, quercetin, and erbstatin.

Further small molecules reported to inhibit EGFR and that are therefore within the scope of the present invention are tricyclic compounds such as the compounds described in U.S. Patent No. 5,679,683; quinazoline derivatives such as the derivatives described in U.S. Patent No. 5,616,582; and indole compounds such as the compounds described in U.S. Patent No. 5,196,446.

It will be appreciated that useful small molecule to be used in the invention are inhibitors of EGFR, but need not be completely specific for EGFR.

In a combination therapy, the VEGFR antagonist is administered before, during, or after commencing therapy with another agent, as well as any combination thereof, i.e., before and during, before and after, during and after, or before, during and after commencing the anti-neoplastic agent therapy. For example, an VEGFR antibody can be administered between 1 and 30 days, preferably 3 and 20 days, more preferably between 5 and 12 days before commencing radiation therapy. In a preferred embodiment of the invention, chemotherapy is administered concurrently with or, more preferably, subsequent to antibody therapy.

In the present invention, any suitable method or route can be used to administer antagonists of the invention, and optionally, to co-administer anti-neoplastic agents and/or antagonists of other receptors. The anti-neoplastic agent regimens utilized according to the invention, include any regimen believed to be optimally suitable for the treatment of the patient's tumor. Different tumors can require use of specific anti-tumor antibodies and specific anti-neoplastic agents, which will be determined on a patient to patient basis. Routes of administration include, for example, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration. The dose of antagonist administered depends on numerous factors, including, for example, the type of antagonists, the type and severity of the tumor being treated and the route of administration of the antagonists.

It should be emphasized, however, that the present invention is not limited to any particular method or route of administration.

One of skill in the art would understand that dosages and frequency of treatment depend on the tolerance of the individual patient and on the pharmacological and pharmacokinetic properties of blocking or inhibitory agent used. Ideally, one wishes to achieve saturable pharmacokinetics for the agent used. A loading dose for an anti-VEGFR antibody can range, for example, from about 10 to about 1000 mg/m², preferably from about 200 to about 400 mg/m². This can be followed by several additional daily or weekly dosages ranging, for example, from about 200 to about 400 mg/m². The patient is monitored for side effects and the treatment is stopped when such side effects are severe.

One of skill in the art would also know how to monitor the progress of the treatment in order to determine an effective dose. For example, one such way is to monitor MRI, CT, or other brain scans.

EXAMPLES

Example 1

I. MATERIALS AND METHODS

a. Tumor implantation and irradiation

Human lung tumor 54A was implanted subcutaneously into the calf area of the right hindleg of athymic NCr/Sed nude (nu/nu) mice. When tumors grew to 6 mm in diameter, they were locally γ -irradiated with a single dose of 20 or 30 Gy (at a dose rate of ~ 5 Gy/min).

b. DC101 and collagenase treatments and evaluation of tumor response to therapy

Both primary (untreated) and recurrent (after irradiation) tumor xenografts were treated with the monoclonal antibody DC101 or collagenase starting when a tumor reached 8 mm in mean diameter. Three intraperitoneal injections of DC101 (ImClone Systems, Inc., NY), 27 mg/kg each, were given with a 3-day interval. DC101 specifically blocks the signaling of mouse VEGFR2/Flk-1 and does not cross-react with human VEGFR2/KDR or mouse and human VEGFR1. Collagenase (Clostridiopeptidase A, Sigma) was injected intratumorally (50 μ g/ml in PBS, 2 injections of 20 μ l each per tumor) 3 times with a 2-day interval. Tumor size was measured 3 times a week. The time

taken for a tumor to double or triple its initial volume V_0 (from ~ 260 to $520/780 \text{ mm}^3$) was determined.

c. Tumor histology and immunohistochemistry

5 To stain for perfused vessels, 0.1 ml of biotinylated tomato lectin (Vector Laboratories, Burlingame, CA) was injected intravenously and 5 minutes later the mouse was sacrificed. The excised tumors were formalin-fixed, embedded in paraffin and 5 μm sections were cut. To localize the lectin, the sections were stained with a streptavidin-conjugated fluorochrome (Alexa 647, Molecular Probes Inc., Eugene, OR). Then these
10 sections were also stained for $\alpha\alpha$ -smooth muscle actin (αSMA ; Cy-3-conjugated mouse monoclonal antibody, clone 1A4, Sigma), to highlight perivascular cells. TUNEL staining for apoptosis and Ki67 for tumor cell proliferation were performed. To determine collagen content in tumors, sections were stained with Masson trichrome. Images were captured using either Olympus confocal or bright-field microscopes and then processed
15 with Adobe Photoshop 7.0 software (Adobe Systems, Inc.) and/or the NIH Image 1.63 software. A macro within the latter software was used to calculate the co-localization of αSMA -positive cells with perfused vessels.

d. Expression of angiogenesis-related molecules in tumors

20 Primary and recurrent tumors of 8 mm in size ($n = 6$) were snap frozen in liquid nitrogen and stored at -80°C . Pooled tumor aliquots were first used for analysis of mRNA expression while the remaining powder was used for protein analysis. Total RNA was extracted with RNeasy Mini Kit (QIAGEN Inc., Valencia, CA). To screen for relative gene expression, we used cDNA gene chips containing 96 human or mouse genes
25 involved in angiogenesis and vessel maturation (GEArray Q Series, SuperArray, Bethesda, MD) and quantified chemoluminescent spots on membranes by densitometry (FluoroChem 800 system, Alpha Innotech, San Leandro, CA). The relative abundance of a transcript was normalized to β -actin. Protein extraction from the stored powder was performed using NET buffer and total protein concentrations were determined in
30 individual sample by the Standard Lowry Method (Bio-Rad DC protein assay, Bio-Rad Hercules, CA). Human and mouse VEGF, and human PlGF solid-phase ELISAs (the Quantikine Immunoassays, R&D Systems, Inc., Minneapolis, MN) were then performed.

e. **Interstitial fluid pressure (IFP) measurements**

IFP was measured in tumors with the wick-in-needle technique prior to and 2 and 8 days after the initiation of DC101 treatment, each time in two tumor regions. Before IFP measurements mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.m.) and placed on a heating pad to maintain normal body temperature.

f. **Statistical analysis**

Differences between the means in groups were evaluated by *t*-tests, paired or for two independent samples, and considered significant for $p < 0.05$. The comparison of a collection of several groups for one tumor type with a collection of the corresponding groups for another tumor type was carried out using *t*-test for linear contrasts in one-way ANOVA. Unless indicated otherwise, all error values represent the standard error of the mean.

15 **II. RESULTS AND DISCUSSION**

a. **Recurrent tumors are more sensitive to DC101 than primary tumors**

Following a minor increase in size over the first days after irradiation at 6 mm, apparent tumor dimensions remained almost unchanged after 20 Gy, and slowly decreased by 1-2 mm after 30 Gy. Regardless of the dose, when the tumors relapsed, they reached 8 mm in diameter (the point of DC101 treatment initiation) at a stabilized growth rate. The mean time ($\pm$ SD) between tumor irradiation and re-growth to 8 mm was 20.3 $\pm$ 4.2 and 35.9 $\pm$ 6.1 days following 20 and 30 Gy, respectively. Primary (non-irradiated) tumors grew from 6 to 8 mm typically in 4-5 days.

The stabilized growth of recurrent tumors was slower versus their non-irradiated counterparts. **Fig. 1A** shows that after 20 Gy, the time needed to double and triple tumor volume V_0 increased 1.6 and 1.8-fold, respectively. The tumor growth results obtained are similar to the findings in other experimental studies and consistent with the classical tumor bed effect, which usually reaches saturation at ~ 20 Gy.

During DC101 therapy, the growth was modestly inhibited in primary tumors while it was completely arrested in most recurrences, with an occasional decrease in size. After DC101 treatment, primary tumors immediately started to grow at a rate indistinguishable from untreated controls. In contrast, most recurrent tumors remained dormant for a while after the last antibody injection, although eventually they re-grew at

approximately the same rate as untreated relapses. All growth retardations occurred under the level of 1.5-2 Vo and therefore the minimum volume at which we determined the complete therapeutic effects of DC101 was 2Vo (**Fig. 1A**). The tumor growth delay induced by this antibody (the difference between DC101-treated and untreated groups) was significantly longer in relapsed than primary tumors at both 2Vo ($p<0.001$) and 3Vo ($p<0.01$). The similar slopes of the curves between these two size levels demonstrate that DC101 had only a temporary effect on the growth rate of both tumor variants. Because primary and recurrent xenografts grew at different rates, we calculated the mean specific tumor growth delay induced by DC101 (normalized to tumor doubling times of the corresponding controls), which was 40 % greater in radiation recurrences. The individual variability within groups was larger among relapsed tumors, especially in the case of DC101 treatment (increased inter-tumor variabilities of certain parameters were also noticed for tumors implanted in pre-irradiated sites. **Fig. 1B** shows the individual tumor volume doubling times for all the groups and demonstrates that in the 19 recurrent tumors treated with DC101, 3 tumors were relatively resistant and 6 tumors were especially sensitive (including 2 tumors that were locally controlled for 120 days).

For both DC101-treated (n=8) and untreated (n=9) tumors, the results of the 30 Gy tumor irradiation were virtually identical to those obtained with the 20 Gy dose.

Therefore in the other experiments we only used the 20 Gy dose for tumor irradiation.

Collectively these results indicate that DC101 induced a greater growth retardation in recurrent than primary 54A tumor xenografts. We found this enhanced anti-tumor effect of DC101 on radiation recurrences after a relatively short period of antibody administration. Therefore, greater benefits may be expected from a longer administration of anti-angiogenic agents.

b. The decrease in functional vascular density in recurrent tumors is associated with less mature vessels and a reduction in PlGF concentration

Table 1 shows that the percentage of necrotic area was significantly increased in tumor recurrences, while the fractions of proliferating and apoptotic tumor cells were not significantly different. There was also a strong trend for a lower functional vessel density in recurrent than primary tumors.

Although the perfused vessel density was approximately proportional to the viable tumor parenchyma in both tumor types, the fraction of perfused vessels covered by perivascular cells decreased by 44% in recurrences (Table 1).

To determine if differences in the vasculature of primary and recurrent tumors were related to the expression of angiogenesis-related genes we used human and mouse-specific angiogenic gene arrays. Except for human PlGF expression, which was down-regulated by 2.7 fold in relapsed xenografts, the expression of other genes either did not change or the difference was not more than 2 fold. In agreement with the gene-array results, there was no significant difference in human and mouse VEGF protein levels between primary and recurrent tumors while human PlGF was 1.64 fold lower in recurrences (Table 1). The decreased PlGF levels could affect the density and maturation of blood vessels and the slower growth rate of radiation recurrences. It is known that PlGF, a specific ligand of VEGFR1, can potentiate the angiogenic effects of VEGF, enhance the survival of tumor-associated endothelial cells and monocytes/macrophages, and stimulate the recruitment of perivascular cells and vessel stabilization.

c. Collagenase accelerates the growth of recurrent tumors

The quantitative evaluation of the Masson trichrome staining revealed a 3.3-fold higher fraction of tissue occupied by the collagen stain in recurrent than primary tumors (Table 1).

Based on *in vitro* studies showing that collagen inhibits the growth of tumor cells and spheroids [26, 27], we assumed that the increased collagen accumulation in recurrent tumors inhibited their growth. To test this hypothesis, we injected bacterial collagenase intratumorally to degrade collagen fibers. Collagenase did not change the growth of primary tumors (time taken for tumors to grow to 3Vo was 6.9 ± 0.4 and 7.0 ± 0.5 days after PBS and collagenase treatment, respectively) but partially restored the growth rate of recurrent tumors (the corresponding values were 16.3 ± 1.0 and 12.9 ± 0.7 days, $p < 0.02$). Thus, radiation-induced accumulation of collagen may affect the growth rate of recurrent tumors independent of a change in tumor cell proliferation (Table 1).

d. DC101 reduces the tumor IFP in both primary and recurrent tumors

Interstitial hypertension in solid tumors is regulated by vascular permeability, and the organization and composition of the extracellular matrix could also play a role. Based

on differences in the vasculature and collagen content of primary and recurrent tumors, we could expect differences in IFP. However, the mean IFP of primary and recurrent tumors was similar, which is consistent with the absence of difference in VEGF levels between the two tumor variants (Table 1). The IFP of primary and recurrences was
5 decreased to the same extent on day 2 and 8 after the initiation of DC101 treatment (**Fig. 2C**).

DC101 selectively reduces the tumor cell proliferation in recurrent tumors but does not affect the vessel density or perivascular cell coverage in both tumor variants. In both tumor types the DC101-induced growth inhibition was not associated with
10 changes in the density of perfused vessels (Fig. 2A) or the fraction of vessels covered by α SMA-positive perivascular cells (Fig. 2B). The vasculature of 54A primary tumors and recurrences (even with less vessels covered by perivascular cells) was more resistant to DC101 than the vasculature of other tumor types treated with similar or higher-doses of DC101. Although there was no change in vascular density it is quite possible that DC101
15 affected angiogenesis preferentially in recurrent tumors. DC101 almost immediately arrested the growth of relapses (**Fig. 2D**), suggesting that angiogenesis was almost completely inhibited. In contrast, primary tumors continued to grow with no change in vascular density.

The effects of DC101 on tumor growth was also related to secondary effects on
20 tumor cells (this antibody is highly specific for mouse VEGFR2). DC101 increased necrosis in both primary and recurrent tumors (**Fig. 3A**) while neoplastic cell apoptosis was not significantly changed in either model (not shown). Importantly, in recurrences, DC101 decreased tumor cell proliferation by 55 % within 2 days of treatment initiation, whereas there was no similar change in primary tumors (**Fig. 3B**). Thus, neoplastic cells
25 in both tumor variants responded to therapy but in recurrences they were more sensitive to the anti-proliferative stimuli triggered by the VEGFR2 blockade.

III. CONCLUSION

Several characteristics of experimental recurrences following irradiation were
30 uncovered that distinguish them from primary tumors of the same origin and size. Based on the fraction of vessels covered by perivascular cells, vessels of recurrences are less mature which may be due to lower PlGF levels. While the slower growth rate of recurrent tumors has been associated with a reduction in vessel density, the elevated

collagen content in tumor relapses could also inhibit their growth. Our results also show that relapsed tumors are more susceptible to anti-VEGFR2 therapy, which suggests possible benefits to using anti-angiogenic agents even as a palliative monotherapy for patients with in-field recurrences.

5

Table 1. Comparison of molecular, structural and functional characteristics of 8 mm primary vs. recurrent 54A tumors

Parameter	Primary tumors	Recurrent tumors
Density of perfused vessels (1/mm ²)	11.8±1.7 (10)	7.8±0.9* (10)
Fraction of perfused vessels covered with pericytes	0.31±0.04 (6)	0.17±0.03** (6)
IFP (mmHg)	11.2±0.5 (28)	11.8±0.5 (24)
Necrosis (%)	9.8±1.6 (6)	30.1±7.4** (6)
Fraction of collagen staining (%)	1.10±0.17 (5)	3.41±0.63*** (5)
Tumor cell proliferation (%)	24.9±3.3 (6)	19.8±3.4 (6)
Tumor cell apoptosis (apoptotic cells/mm ²)	107±25.3 (6)	142±37.6 (5)
Human VEGF (pg/mg total protein)	423±39 (12)	389±59 (12)
Mouse VEGF	18.1±2.4	19.5±2.9

(pg/mg total protein)	(12)	(12)
Human PlGF	105±7	64±7****
(pg/mg total protein)	(12)	(12)

Data are mean±SE from number of tumors indicated parenthetically.

Significant differences between tumor types: * $p=0.06$, ** $p<0.05$, *** $p<0.01$,

**** $p<0.001$.

What is claimed is:

1. A method of treating a recurring tumor in a mammal in need thereof
5 comprising:

administering to the mammal a therapeutically effective amount of a vascular endothelial growth factor receptor (VEGFR) antagonist.

2. The method of claim 1, wherein the tumor is a head and neck carcinoma or a
10 glioma.

3. The method of claim 1, wherein the tumor is selected from the group consisting of carcinomas, sarcomas, blastomas and gliomas.

- 15 4. The method of claim 1, wherein the tumor is selected from the group consisting of epidermoid tumors, squamous tumors, head and neck tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, liver tumors, Kaposi's sarcoma, CNS neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas,
20 cerebral metastases, melanomas, gastrointestinal carcinomas, gastrointestinal sarcomas, renal carcinomas, renal sarcomas, rhabdomyosarcomas, glioblastomas, glioblastoma multiforme, leiomyosarcomas, squamous cell carcinomas, basal cell carcinomas, human malignant keratinocytes, leukemias, multiple myelomas, lymphomas, acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), acute lymphocytic leukemia
25 (ALL), chronic lymphocytic leukemia (CLL), erythrocytic leukemia, monocytic leukemia, Hodgkin's disease and Non-Hodgkin's disease.

5. The method of claim 1, wherein the antagonist is an anti-VEGFR antibody.

6. The method of claim 5, wherein the anti-VEGFR antibody binds to a VEGFR
5 selected from the group consisting of FLT-1, KDR and FLK-1.

7. The method of claim 5, wherein the anti-VEGFR antibody is selected from the
group consisting of a monoclonal antibody, a fragment of an antibody, a derivative of an
antibody, a chimerized antibody, a humanized antibody, and a single chain antibody.
10

8. The method of claim 5, wherein the anti-VEGFR antibody is a single chain
antibody that binds to VEGFR-2.

9. The method of claim 5, wherein the anti-VEGFR antibody has the same
15 specificity as rat IgG1 monoclonal antibody that is produced by hybridoma DC-101
(ATCC HB 11534).

10. The method of claim 5, wherein the anti-VEGFR antibody has the same
specificity as mouse monoclonal antibody Mab 6.12 produced by a hybridoma deposited
20 as ATCC PTA-3344.

11. The method of claim 1, further comprising administering one or more anti-
neoplastic-agents.

12. The method of claim 1, wherein the anti-neoplastic agent is a
25 chemotherapeutic agent.

13. The method of claim 1, further comprising treating the mammal with
radiation.
30

14. The method of claim 1, further comprising administering an antibody that
binds to EGFR.

15. The method of claim 1, wherein the mammal is a human.

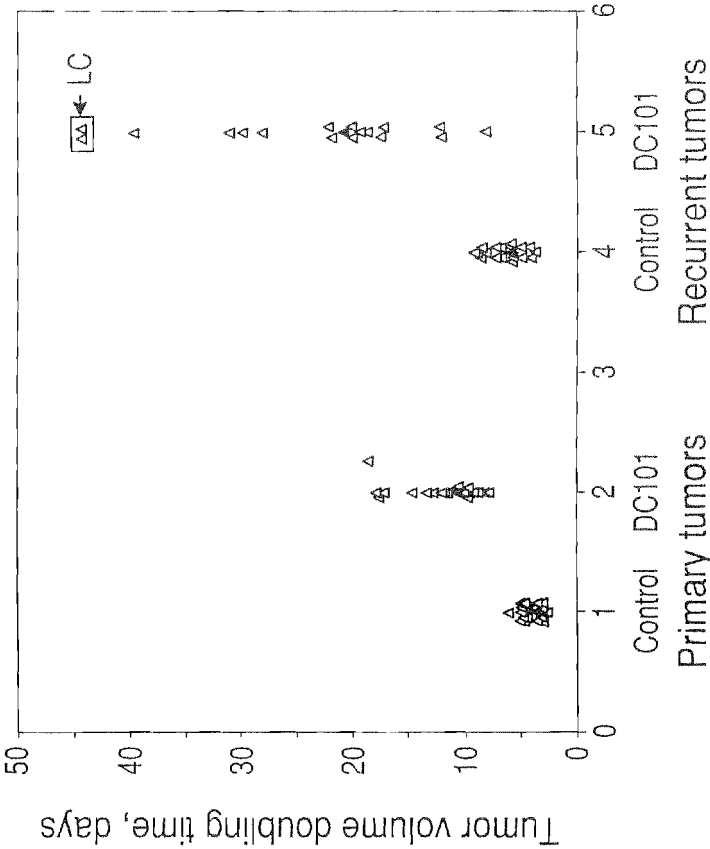


FIG. 1B

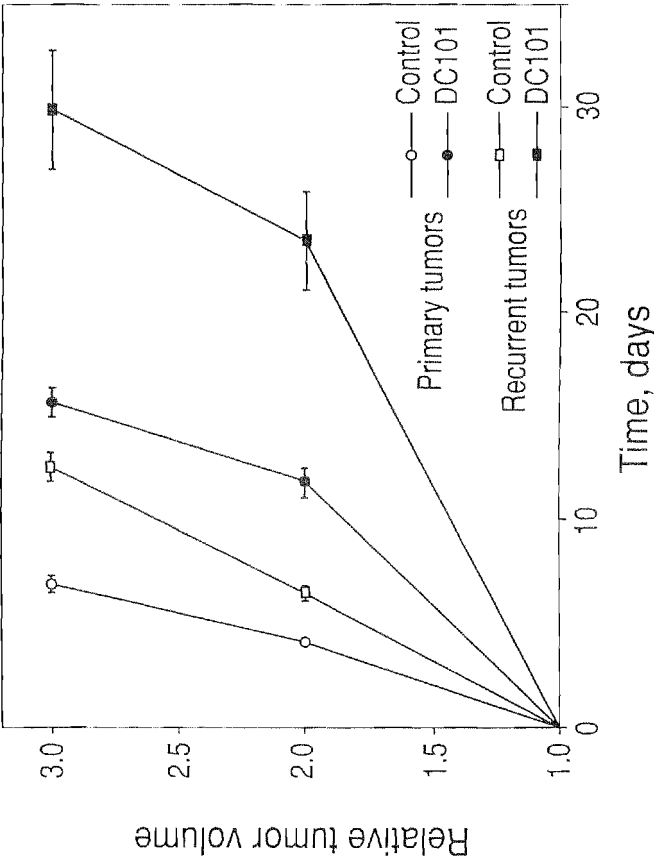
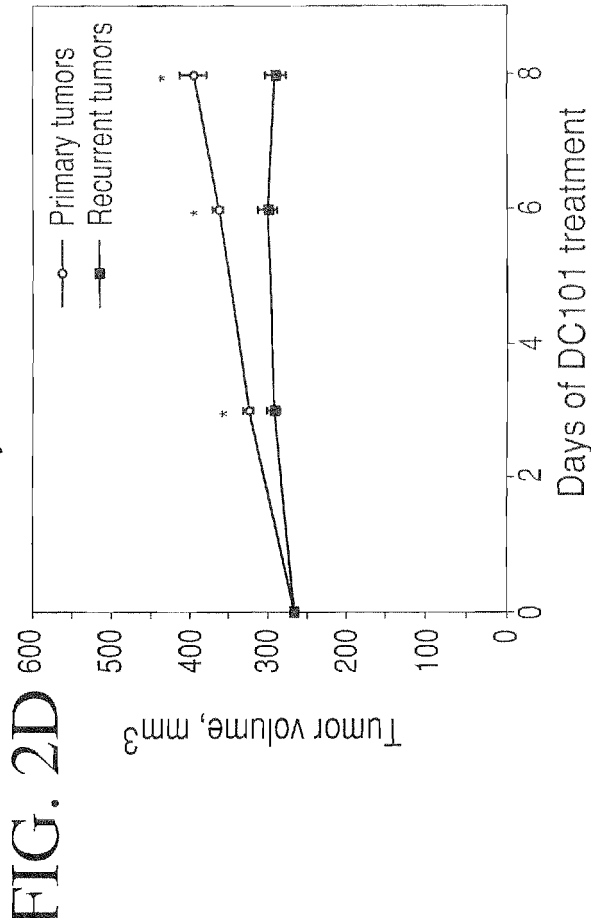
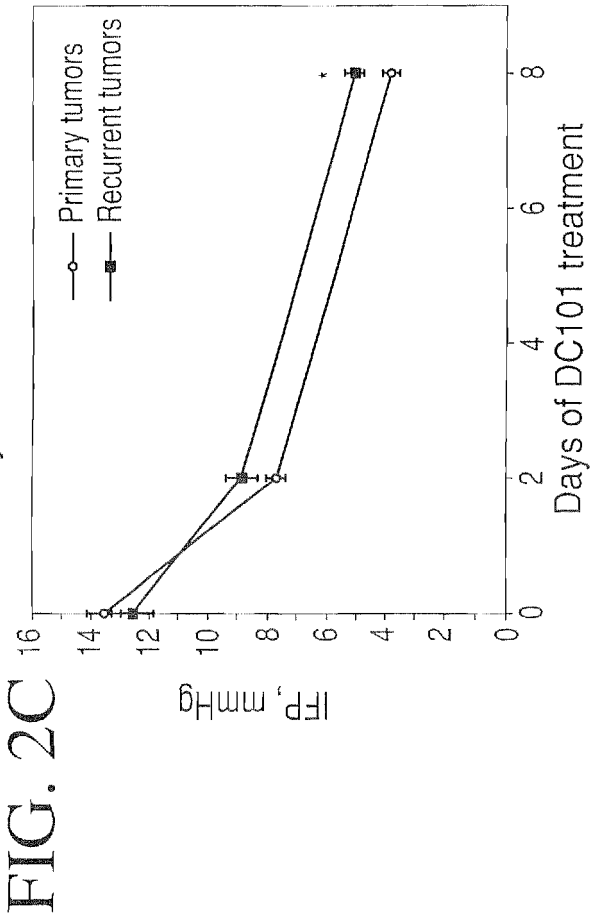
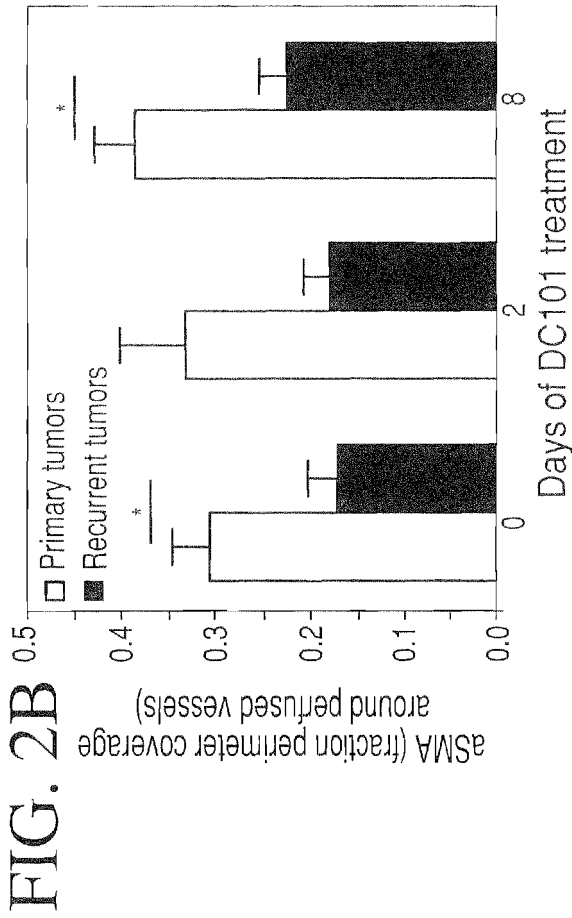
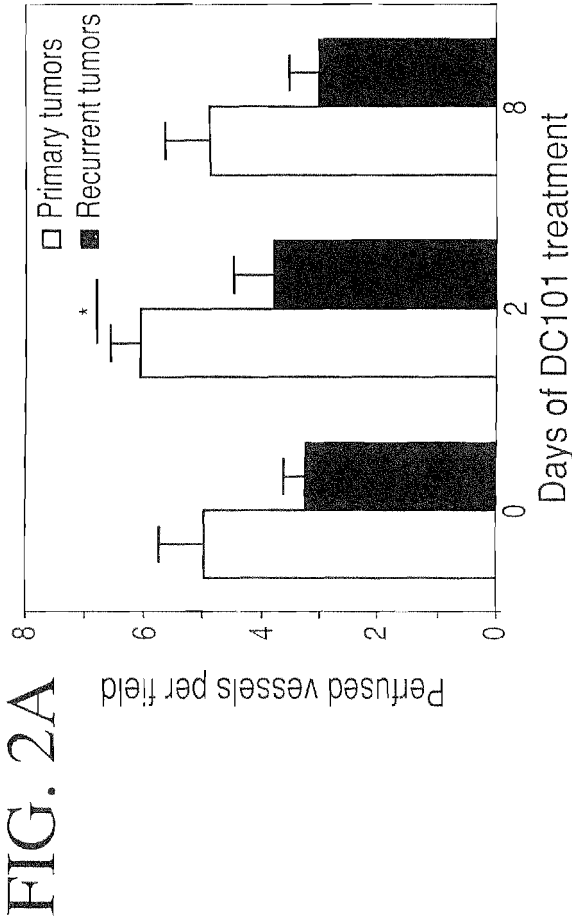


FIG. 1A



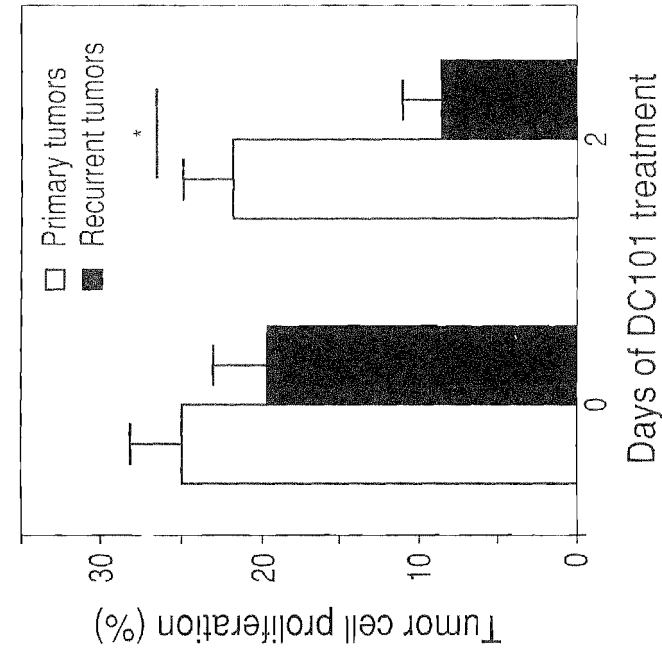


FIG. 3B

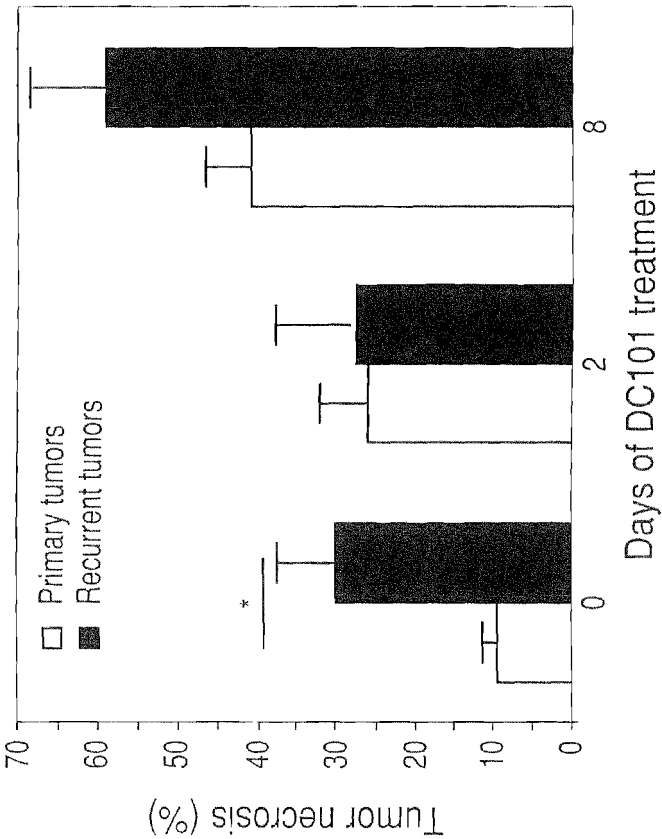


FIG. 3A