TREATMENT OF NON-SOLID MAMMALIAN TUMORS WITH VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR ANTAGONISTS

BACKGROUND OF THE INVENTION

Cancer is the second leading cause of death next to heart attacks in the United States. There has been important progress in the development of new therapies in the treatment of this devastating disease. Much of the progress is due to a better understanding of cell proliferation in both normal cells and cancerous cells.

Normal cells proliferate by the highly controlled activation of growth factor receptors by their respective ligands. Examples of such receptors are the growth factor receptor tyrosine kinases.

Cancer cells also proliferate by the activation of growth factor receptors, but lose the careful control of normal proliferation. The loss of control may be caused by numerous factors, such as the overexpression of growth factors and/or receptors, and autonomous activation of biochemical pathways regulated by growth factors.

Some examples of receptors involved in tumorigenesis are the receptors for vascular endothelial growth factor (VEGFR), platelet-derived growth factor (PDGFR), insulin-like growth factor (IGFR), nerve growth factor (NGFR), and fibroblast growth factor (FGF).

During embryonic development, hematopoietic and early endothelial cells (angioblasts) originate from a common precursor cell known as hemangioblast. Given this common origin, several signaling pathways are shared by both hematopoietic and vascular cells. One such pathway is the VEGFR signaling pathway. VEGF receptors (VEGFR) include *FLT-1*, sequenced by Shibuya M. et al., Oncogene 5, 519-524 (1990) (VEGFR-1); *KDR*, described in PCT/US92/01300, filed February 20, 1992, and in Terman et al., Oncogene 6:1677-1683 (1991); and *FLK-1*, sequenced by

Matthews W. et al., Proc. Natl. Acad. Sci. USA, **88**:9026-9030 (1991) (KDR and FLK-1 are collectively referred to as VEGFR-2).

Unless otherwise stated or clearly inferred otherwise by context, this specification will follow the customary literature nomenclature of VEGF receptors. KDR will be referred to as the human form of VEGFR-2. FLK-1 will be referred to as the murine homolog of VEGFR2. FLT-1 is different from, but related to, the KDR/FLK-1 receptor.

VEGFR binds to several soluble factors including vascular endothelial growth factor (VEGF), which exerts proliferative and migratory effects on the endothelium. VEGFR-2 was thought to be exclusively expressed by endothelial cells. Recently, however, VEGFR2 has been shown to be present on a subset of multi-potent hematopoietic stem cells (1). Several studies have revealed that certain leukemic cells also expressed VEGFR-2 (2).

The two primary signaling tyrosine kinase receptors that mediate the various biological effects of VEGF are VEGFR-2 and VEGFR-1. Although the binding affinity of VEGFR-1 to VEGF is very high, with Kd values of 10-70pM (5), most studies have shown that VEGFR-2 is the critical receptor for transmitting cellular signals for the proliferation and differentiation of endothelial cells (6). VEGFR-1 appears to be more important for vascular remodeling. The relative significance of VEGF receptors in the regulation of vasculogenesis and angiogenesis has been established in studies in which the VEGFR-2 and VEGFR-1 genes were disrupted in murine embryonic stem cells by homologous recombination. Mice deficient in VEGFR-2 had drastic defects in vasculogenesis, angiogenesis, and hematopoiesis (7). In contrast, VEGFR-1 knockout mice developed abnormal vascular channels, suggesting a role for this receptor in the regulation of endothelial cell-cell or cell-matrix interactions (8).

Inhibition of angiogenesis through disruption of VEGFR-2 signaling results in inhibition of growth and metastasis of solid tumors. For example, neutralizing

monoclonal antibody (MoAb) to murine VEGFR-2 inhibited tumor growth and metastasis in murine models (9,10). Furthermore, glioblastoma growth was inhibited in mice dominant-negative for VEGFR-2 (11). Such inhibition of tumor growth is attributed to the inhibition of angiogenesis, effectively limiting the blood supply of the tumor.

Leukemias originate from hematopoietic stem cells at different stages of their maturation and differentiation. It is now well established that acute leukemias originate from immature hematopoietic stem cells that have the capacity to undergo self-renewal, whereas certain less aggressive leukemias such as chronic leukemias seem to originate from the more mature committed hematopoietic progenitor cells.

In any event, leukemic cells do not require an independent blood supply. Accordingly, leukemias, and other non-solid tumors are not suspectible to the prior art treatments described above.

Several studies have shown that VEGF is almost invariably expressed by all established leukemic cell lines as well as freshly isolated human leukemias, including the well studied HL-60 leukemic cell line (2,3). Using RT-PCR, several studies have shown that VEGFR-2 and VEGFR-1 are only expressed by certain human leukemias (2,3). However, none of these studies have shown whether expression of VEGF is associated with any parallel surface VEGFR-2/VEGFR-1 expression or functional response.

Current treatments of cancer traditionally include chemotherapy or radiation therapy. Such treatments are, however, not always effective, especially over long periods of time.

There is a need, therefore, for new methods of treating non-solid tumors. It is an object of this invention to provide such methods.

SUMMARY OF THE INVENTION

This and other objectives as will be apparent to those having ordinary skill in the art, have been achieved by providing a method of inhibiting the growth of non-solid tumors that are stimulated by a ligand of VEGFR in mammals. The method comprises treating the mammals with an effective amount of a VEGR antagonist.

In another embodiment, the method of the present invention comprises treating human patients with a combination of an effective amount of a VEGFR antagonist and a chemotherapeutic agent.

In yet another embodiment, the method of the present invention comprises treating human patients with a combination of an effective amount of a VEGFR antagonist and radiation.

BRIEF DESCRIPTION OF FIGURES

Figure 1. Human chloromas express FLT-1/VEGFR-1 and KDR/VEGFR-2. Sections from Human chloromas were stained by immunohistochemistry, as described in Methods. A: Control IgG, showing little non-specific staining (magnification: 400X); B: Factor VIII staining, showing specific blood vessel staining (magnification: 100X); C: KDR/VEGFR-2 staining, on leukemic cell areas (magnification: 400X); D: FLT-1/VEGFR-1 expression, also detected on a subset of leukemic cells (magnification: 400X). E and F: VEGF expression, showing specific leukemic cell (E) and, as a positive control, pericyte (white arrow, F) staining (magnification: 400X). These results are representative of 4 different samples.

Figure 2A: VEGFR-2 expression by leukemic cell lines. The cell lines HL-60 and HEL (as well as 5 primary leukemias) expressed KDR/VEGFR-2, as detected by RT-PCR. K562 cells were KDR negative. β-actin was used as an internal control.

Figure 2B: $VEGF_{165}$ induced dose-dependent VEGFR-2 phosphorylation on VEGFR-2-positive leukemic cell lines. Total cell lysates from HL-60 and HEL cells were immunoprecipitated with an anti-phosphotyrosine antibody and subsequently analyzed by western blotting using antibodies against VEGFR-1 (FLT-1) or VEGFR-2 (KDR), as described in Methods. Fibroblasts were used as negative controls, and showed no sign of VEGFR-1 or VEGFR-2 phosphorylation. Results shown are representative of 3 separate experiments.

Figure 3. $VEGF_{165}$ induced proliferation of a subset of leukemias, an effect mediated through KDR/VEGFR-2. $VEGF_{165}$ induced a significant (*, $p<0.003$) increase in proliferation of all KDR/VEGFR-2+ leukemias (2 cell lines and 2 primary leukemias are shown). This effect could be blocked by a neutralizing MoAb to KDR/VEGFR-2 (**, $p<0.04$), as described in Methods. The results shown are representative of 3 separate experiments.

Figure 4. A tyrosine kinase inhibitor with a high affinity for KDR/VEGFR-2 blocked $VEGF_{165}$ -induced leukemic cell proliferation. The tyrosine kinase inhibitor AG1433 significantly (*, $p<0.05$) blocked leukemic cell proliferation, confirming that, on leukemic cells, the mitogenic effects of $VEGF_{165}$ are mediated primarily through KDR/VEGFR-2. These results are representative of 2 separate experiments.

Figure 5. $VEGF_{165}$ induces MMP secretion by leukemic cells. A: zymographic analysis of leukemic cell supernatants, with or without $VEGF_{165}$ stimulation for 24 hrs. B: Quantification of the gelatinolytic activity detected on the culture supernatants. Incubation of leukemic cells with $VEGF_{165}$ for 24 hrs had a significant ($p<0.05$) effect on the level of MMPs released into the supernatant, as detected by zymography. The results shown are representative of 3 independent experiments.

Figure 6. $VEGF_{165}$ induces, in a dose-dependent manner (50 to 200ng/ml), leukemic cell migration through matrigel-coated transwells, a process mediated through KDR/VEGFR-

2 and FLT-1/VEGFR-1. Migration of HL-60 cells and 4 primary leukemias through matrigel-coated transwells, in response to 200ng/ml $VEGF_{165}$ is shown. This process requires MMP activity, as shown by the effects of a synthetic MMPi. MoAb to KDR/VEGFR-2 partially blocked leukemic cell migration, but incubation of the cells with both FLT-1/VEGFR-1 and KDR/VEGFR-2 MoAbs was more effective than either antibody alone (**, $p<0.05$). These results are representative of 3 independent experiments.

Figure 7. $VEGF_{165}$-induced HL-60 cell migration is blocked by AG1433. The synthetic protein kinase inhibitor AG1433 (used at 10μM) blocked $VEGF_{165}$-induced HL-60 cell migration (*, $p<0.04$). These results are representative of 2 different experiments and each condition was done in triplicate.

Figure 8. A. HL-60 injection into NOD-SCID mice induces high levels of human but not murine VEGF in mouse plasma. Mice were injected with $1x10^6$ HL-60 cells (i.v.) and the human (A) and murine VEGF (B) plasma levels were measure by ELISA at different time-points after injection. The results shown are representative of 2 separate experiments. B. Mouse survival (%) after HL-60 injection (iv). Mice were injected with $1x10^6$ cells i.v., and 3 days after leukemic cell injection treated (n=5) i.p. with 400μg IMC-1C11 (neutralizing MoAb to human VEGFR-2/KDR) or with PBS/BSA (n=8) 3 times a week. One mouse was sacrificed for histological analysis. The results shown are representative of 2 separate experiments.

Figure 9. Histology of liver (A, B) and spleen (C, D) from HL-60 injected mice. Untreated (control) mice had evidence of metastatic disease in the liver (white and red arrows, B) and spleen (D). On the contrary, mice treated with IMC-1C11 showed no evidence of leukemic cells in the liver (A) and the spleen had evidence of apoptosis (blue arrows, C) but normal histology.

Figure 10. On VEGFR-2 positive leukemias, VEGF may support leukemic cell growth through paracrine (increasing bone marrow endothelium cell mass) and autocrine

(directly stimulating leukemic cell proliferation) mechanisms. Antibodies against human VEGFR-2 (arrow) may block the autocrine loop induced by leukemia-derived human VEGF.

Figure 11. Nucleotide and Amino Acid Sequences of p1C11.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an improved method for treating non-solid tumors, particularly non-solid malignant tumors, in human patients.

Non-Solid Tumor Cells

Non-solid tumor cells include tumors that affect hematopoietic structures including components of the immune system. Some examples of non-solid tumors include leukemias, multiple myelomas and lymphomas. These tumor cells generally appear in the bone marrow and peripheral circulation.

The types of non-solid tumors that can be treated in accordance with the invention are any non-solid tumors that are stimulated by a ligand of VEGFR. The VEGFR family of receptors includes, for example, VEGFR-2, (KDR, flk-1) and VEGFR-1 (flt-1). Some examples of ligands that stimulate VEGFR include VEGF.

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.

The non-solid tumors may express VEGFR at normal levels or they may overexpress VEGFR at levels, for example, that are at least 10, 100 or 1000 times normal levels.

VEGFR antagonists

The non-solid tumors of the present invention are treated with a VEGFR antagonist. For the purposes of this specification, a VEGFR antagonist is any molecule that inhibits the stimulation of VEGFR by a VEGFR ligand. Such inhibition of stimulation inhibits the growth of cells that express VEGFR.

No particular mechanism of inhibition is implied as operating in the present invention. Nevertheless, VEGFR tyrosine kinases are generally activated by means of phosphorylation events. Therefore, the antagonists of the invention generally inhibit phosphorylation of VEGFR. Accordingly, phosphorylation assays are useful in predicting the antagonists useful in the present invention.

The growth of non-solid tumors is sufficiently inhibited in the patient to prevent or reduce the progression of the cancer (i.e. growth, invasiveness, metastasis, and/or recurrence of non-solid tumors). The VEGFR antagonist of the present invention can be cytostatic, i.e. inhibits the growth of the non-solid tumor. Preferably, the ERGR antagonist is cytolytic, i.e. destroys the tumor.

VEGFR antagonists include biological molecules or small molecules. Biological molecules include all lipids and polymers of monosaccharides, amino acids and nucleotides having a molecular weight greater than 450. Thus, biological molecules include, for example, oligosaccharides and polysaccharides; oligopeptides, polypeptides, peptides, and proteins; and oligonucleotides and polynucleotides. Oligonucleotides and polynucleotides include, for example, DNA and RNA.

Biological molecules further include derivatives of any of the molecules described above. For example, derivatives of biological molecules include lipid and glycosylation derivatives of oligopeptides, polypeptides, peptides and proteins.

Derivatives of biological molecules further include lipid derivatives of oligosaccharides and polysaccharides, e.g. lipopolysaccharides.

Most typically, biological molecules are antibodies, or functional equivalents of antibodies. Functional equivalents of antibodies have binding characteristics comparable to those of antibodies, and inhibit the growth of cells that express VEGFR. Such functional equivalents include, for example, chimerized, humanized and single chain antibodies as well as fragments thereof.

The functional equivalent of an antibody is preferably a chimerized or humanized antibody. A chimerized antibody comprises the variable region of a non-human antibody and the constant region of a human antibody. A humanized antibody comprises the hypervariable region (CDRs) of a non-human antibody. The variable region other than the hypervariable region, e.g. the framework variable region, and the constant region of a humanized antibody are those of a human antibody.

For the purposes of this application, suitable variable and hypervariable regions of non-human antibodies may be derived from antibodies produced by any non-human mammal in which monoclonal antibodies are made. Suitable examples of mammals other than humans include, for example, rabbits, rats, mice, horses, goats, or primates. Mice are preferred.

Functional equivalents further include fragments of antibodies that have binding characteristics that are the same as, or are comparable to, those of the whole antibody. Suitable fragments of the antibody include any fragment that comprises a sufficient portion of the hypervariable (i.e. complementarity determining) region to bind specifically, and with sufficient affinity, to VEGFR to inhibit growth of cells that express such receptors.

Such fragments may, for example, contain one or both Fab fragments or the $F(ab')_2$ fragment. Preferably the antibody fragments contain all six complementarity

determining regions of the whole antibody, although functional fragments containing fewer than all of such regions, such as three, four or five CDRs, are also included.

The preferred fragments are single chain antibodies, or Fv fragments. Single chain antibodies are polypeptides that comprise at least the variable region of the heavy chain of the antibody linked to the variable region of the light chain, with or without an interconnecting linker. Thus, Fv fragment comprises the entire antibody combining site. These chains may be produced in bacteria or in eukaryotic cells.

The antibodies and functional equivalents may be members of any class of immunoglobulins, such as: IgG, IgM, IgA, IgD, or IgE, and the subclasses thereof. The preferred antibodies are members of the IgG1 subclass. The functional equivalents may also be equivalents of combinations of any of the above classes and subclasses.

Antibodies may be made from the desired receptor by methods that are well known in the art. The receptors are either commercially available, or can be isolated by well known methods. For example, methods for isolating and purifying KDR are found in PCT/US92/01300. Methods for isolating and purifying flk-1 are found in Proc. Natl. Sci. 88:9026-30(1991). Methods for isolating and purifying flt-1 are found in Oncogene 5:519-24.

Methods for making monoclonal antibodies include the immunological method described by Kohler and Milstein in Nature 256, 495-497 (1975) and by Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al., Eds, Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985). The recombinant DNA method described by Huse et al. in Science 246, 1275-1281 (1989) is also suitable.

Briefly, in order to produce monoclonal antibodies, a host mammal is inoculated with a receptor or a fragment of a receptor, as described above, and then, optionally, boosted. In order to be useful, the receptor fragment must contain sufficient amino acid residues to define the epitope of the molecule being detected. If the fragment is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhold limpet hemocyanin and bovine serum albumin. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

Spleens are collected from the inoculated mammals a few days after the final boost. Cell suspensions from the spleens are fused with a tumor cell. The resulting hybridoma cells that express the antibodies are isolated, grown, and maintained in culture.

Antibodies that are essentially human may be produced in transgenic mammals, especially transgenic mice that are genetically modified to express human antibodies. Methods for making chimeric and humanized antibodies are also known in the art. For example, methods for making chimeric antibodies include those described in U.S. patents by Boss (Celltech) and by Cabilly (Genentech). See U.S. Patent Nos. 4,816,397 and 4,816,567, respectively. Methods for making humanized antibodies are described, for example, in Winter, U.S. Patent No. 5,225,539.

The preferred method for the humanization of antibodies is called CDR-grafting. In CDR-grafting, the regions of the mouse antibody that are directly involved in binding to antigen, the complementarity determining region or CDRs, are grafted into human variable regions to create "reshaped human" variable regions. These fully humanized variable regions are then joined to human constant regions to create complete "fully humanized" antibodies.

In order to create fully humanized antibodies that bind well to an antigen, it is advantageous to design the reshaped human variable regions carefully. The human variable regions into which the CDRs will be grafted should be carefully selected, and it is usually necessary to make a few amino acid changes at critical positions within the framework regions (FRs) of the human variable regions.

For example, the reshaped human variable regions may include up to ten amino acid changes in the FRs of the selected human light chain variable region, and as many as twelve amino acid changes in the FRs of the selected human heavy chain variable region. The DNA sequences coding for these reshaped human heavy and light chain variable region genes are joined to DNA sequences coding for the human heavy and light chain constant region genes, preferably γ1 and κ, respectively. The reshaped humanized antibody is then expressed in mammalian cells and its affinity for its target compared with that of the corresponding murine antibody and chimeric antibody.

Methods for selecting the residues of the humanized antibody to be substituted and for making the substitutions are well known in the art. See, for example, Co et al., Nature 351, 501-502 (1992); Queen et al., Proc. Natl. Acad. Sci. 86, 10029-1003 (1989) and Rodrigues et al., Int. J. Cancer, Supplement 7, 45-50 (1992). A method for humanizing and reshaping the 225 anti-EGFR monoclonal antibody described by Goldstein et al. in PCT application WO 96/40210. This method can be adapted to humanizing and reshaping antibodies against other growth factor receptor tyrosine kinases.

Methods for making single chain antibodies are also known in the art. Some suitable examples include those described by Wels et al. in European patent application 502 812 and Int. J. Cancer 60, 137-144 (1995). Single chain antibodies may also be prepared by screening phage display libraries. See below.

Other methods for producing the functional equivalents described above are disclosed in PCT Application WO 93/21319, European Patent Application 239 400,

PCT Application WO 89/09622, European Patent Application 338 745, U.S. Patent 5,658,570, U.S. Patent 5,693,780, and European Patent Application EP 332 424.

Preferred VEGFR antibodies are the chimerized, humanized, and single chain antibodies derived from a murine antibody for which the amino acid and nucleotide sequences of the heavy and light chain hypervariable regions are shown below. The chimerized antibody and single chain antibodies can be made in accordance with standard methods such as the methods described below. The humanized antibody can be prepared in accordance with the method described in example IV of PCT application WO 96/40210, which is incorporated herein by reference.

The sequences of the hypervariable (CDR) regions of the light and heavy chain are reproduced below. The nucleotide sequence is indicated below the amino acid sequence.

HEAVY CHAIN HYPERVARIABLE REGIONS (VH):

CDR1

SEQ ID NO: 1
Gly Phe Asn Ile Lys Asp Phe Tyr Met His

SEQ ID NO: 9
ggcttcaaca ttaaagactt ctatatgcac

CDR2

SEQ ID NO:2

Trp Ile Asp Pro Glu Asn Gly Asp Ser Asp Tyr Ala Pro Lys Phe Gln Gly

SEQ ID NO:10
tggattgatc ctgagaatgg tgattctgat tatgccccga agttccaggg c

CDR3

SEQ ID NO: 3

Tyr Tyr Gly Asp Tyr Glu Gly Tyr

SEQ ID NO: 11

tactatggtg actacgaagg ctac

LIGHT CHAIN HYPERVARIABLE REGIONS (VL):

CDR1

SEQ ID NO: 4

Ser Ala Ser Ser Ser Val Ser Tyr Met His

SEQ ID NO: 12

agtgccagct caagtgtaag ttacatgcac

CDR2

SEQ ID NO: 5

Ser Thr Ser Asn Leu Ala Ser

SEQ ID NO: 13

agcacatcca acctggcttc t

CDR3

SEQ ID NO: 6

Gln Gln Arg Ser Ser Tyr Pro Phe Thr

SEQ ID NO: 14

cagcaaagga gtagttaccc attcacg

The sequences of the entire light and heavy chain are reproduced below. The nucleotide sequence is indicated below the amino acid sequence.

Heavy Chain

SEQ ID NO:7

Gln Val Lys Leu Gln Gln Ser Gly Ala Glu Leu Val Gly Ser Gly Ala
Ser Val Lys Leu Ser Cys Thr Thr Ser Gly Phe Asn Ile Lys Asp Phe
Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile
Gly Trp Ile Asp Pro Glu Asn Gly Asp Ser Asp Tyr Ala Pro Lys Phe
Gln Gly Lys Ala Thr Met Thr Ala Asp Ser Ser Ser Asn Thr Ala Tyr
Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys
Asn Ala Tyr Tyr Gly Asp Tyr Glu Gly Tyr Trp Gly Gln Gly Thr Thr
Val Thr Val Ser Ser

SEQ ID NO: 15

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caggtcaagc tgcagcagtc tggggcagag cttgtggggt caggggcctc agtcaaattg 60
tcctgcacaa cttctggctt caacattaaa gacttctata tgcactgggt gaagcagagg 120
cctgaacagg gcctggagtg gattggatgg attgatcctg agaatggtga ttctgattat 180
gccccgaagt tccagggcaa ggccaccatg actgcagact catcctccaa cacagcctac 240
ctgcagctca gcagcctgac atctgaggac actgccgtct attactgtaa tgcatactat 300
ggtgactacg aaggctactg gggccaaggg accacggtca ccgtctcctc a          351
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Light Chain

SEQ ID NO: 8

Asp Ile Glu Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly

Glu Lys Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met

His Trp Phe Gln Gln Lys Pro Gly Thr Ser Pro Lys Leu Trp Ile Tyr

Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser

Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Arg Met Glu Ala Glu

Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Arg Ser Ser Tyr Pro Phe Thr

Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys

SEQ ID NO:16

gacatcgagc tcactcagtc tccagcaatc atgtctgcat ctccagggga gaaggtcacc 60
ataacctgca gtgccagctc aagtgtaagt tacatgcact ggttccagca gaagccaggc 120
acttctccca aactctggat ttatagcaca tccaacctgg cttctggagt ccctgctcgc 180
ttcagtggca gtggatctgg gacctcttac tctctcacaa tcagccgaat ggaggctgaa 240
gatgctgcca cttattactg ccagcaaagg agtagttacc cattcacgtt cggctcgggg 300
accaagctgg aaataaaa

Other examples of biological molecules useful as antagonists include soluble receptors. (Exp. Cell Res. 241:1, 161-170; Proc. Natl. Acad. Sci. 92:23, 10457-61)

In addition to the biological molecules discussed above, the antagonists useful in the present invention may also be small molecules. Any molecule that is not a biological molecule is considered in this specification to be a small molecule. Some examples of small molecules include organic compounds, organometallic compounds, salts of organic and organometallic compounds, saccharides, amino acids, and nucleotides. Small molecules further include molecules that would otherwise be considered biological molecules, except their molecular weight is not greater than 450. Thus, small molecules may be lipids, oligosaccharides, oligopeptides, and oligonucleotides, and their derivatives, having a molecular weight of 450 or less.

It is emphasized that small molecules can have any molecular weight. They are merely called small molecules because they typically have molecular weights less than 450. Small molecules include compounds that are found in nature as well as synthetic compounds. Preferably, the small molecules inhibit the growth of non-solid tumor cells that express VEGFR tyrosine kinase.

Some examples of small molecules useful as VEGFR molecules include the quinazolines, quinolines and cinnolines described by Hennequin et al. in J. Med. Chem. 42, 5369-5389 (1999). See also Annie et al., Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology 17, A41 (1998).

Administration of VEGFR antagonists

The present invention includes administering an effective amount of the VEGFR antagonist to human patients. Administering the VEGFR antagonists can be accomplished in a variety of ways including systemically by the parenteral and enteral routes. For example, VEGFR antagonists of the present invention can easily be administered intravenously (e.g., intravenous injection) which is a preferred route of delivery. Intravenous administration can be accomplished by contacting the VEGFR antagonists

with a suitable pharmaceutical carrier (vehicle) or excipient as understood by those skilled in the art.

The VEGFR antagonists of the present invention significantly inhibit the growth of non-solid tumor cells when administered to a human patient in an effective amount. As used herein, an effective amount is that amount effective to achieve the specified result of inhibiting the growth of the non-solid tumor.

Optimal doses of VEGFR antagonists can be determined by physicians based on a number of parameters including, for example, age, sex, weight, severity of the condition being treated, the antagonist being administered, and the route of administration. In general, a serum concentration of the antagonist that permits saturation of the target receptor is desirable. In the case of polypeptides and antibodies, for example, a concentration in excess of approximately 0.1 nM is normally sufficient. For example, a dose of 100 mg/m^2 of an antibody provides a serum concentration of approximately 20 nM for approximately eight days.

As a rough guideline, doses of antibodies may be given weekly in amounts of 10-300 mg/m^2. Equivalent doses of antibody fragments should be used at more frequent intervals in order to maintain a serum level in excess of the concentration that permits saturation of the receptors.

Combination Therapy

In one preferred embodiment the non-solid tumor can be treated with an effective amount of a combination of a VEGFR antagonist as described above, and chemotherapeutic agents, radiation or combinations thereof.

Examples of chemotherapeutic agents include alkylating agents, for example, nitrogen mustards, ethyleneimine compounds and alkyl sulphonates; antimetabolites, for example, folic acid, purine or pyrimidine antagonists; mitotic inhibitors, for example,

vinca alkaloids and derivatives of podophyllotoxin; cytotoxic antibiotics; and compounds that damage or interfere with DNA expression.

Particular examples of chemotherapeutic agents or chemotherapy include cisplatin, dacarbazine (DTIC), dactinomycin, mechlorethamine (nitrogen mustard), streptozocin, cyclophosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (adriamycin), daunorubicin, procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil, vinblastine, vincristine, bleomycin, paclitaxel (taxol), docetaxel (taxotere), aldesleukin, asparaginase, busulfan, carboplatin, cladribine, dacarbazine, floxuridine, fludarabine, hydroxyurea, ifosfamide, interferon alpha, leuprolide, megestrol, melphalan, mercaptopurine, plicamycin, mitotane, pegaspargase, pentostatin, pipobroman, plicamycin, streptozocin, tamoxifen, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil, taxol and combinations thereof.

Administering chemotherapeutic agents can be accomplished in a variety of ways including systemically by the parenteral and enteral routes, as described above.

In yet another embodiment the non-solid tumor can be treated with an effective amount of a VEGFR antagonist in combination with radiation. The source of radiation 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 with 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.

In the preferred embodiment, there is synergy when non-solid tumors in human patients are treated with the VEGFR antagonist and chemotherapeutic agents or radiation or combinations thereof. In other words, the inhibition of tumor growth by the VEGFR antagonist is enhanced when combined with chemotherapeutic agents or radiation or combinations thereof. Synergy may be shown, for example, by greater inhibition of non-solid tumor growth with combined treatment than would be expected from treatment with

either the VEGFR antagonist, chemotherapeutic agent or radiation alone. Preferably, synergy is demonstrated by remission of the cancer where remission is not expected from treatment with VEGFR antagonist, chemotherapeutic agent or radiation alone.

The VEGFR antagonist is administered before, during, or after commencing chemotherapeutic agent or radiation therapy, as well as any combination thereof, i.e. before and during, before and after, during and after, or before, during, and after commencing the chemotherapeutic agent and/or radiation therapy. For example when the VEGFR antagonist is an antibody, it is typically administered between 1 and 30 days, preferably between 3 and 20 days, more preferably between 5 and 12 days before commencing radiation therapy and/or chemotherapeutic agents.

The examples below demonstrate that certain subsets of leukemias not only produce VEGF but also express functional VEGFR-2 *in vivo* and *in vitro*, resulting in the generation of an autocrine loop that augments leukemic cell proliferation and migration. Approximately 50% of freshly isolated Acute Myeloblastic Leukemias (AML) examined expressed mRNA and protein for VEGFR-2. $VEGF_{165}$ induced phosphorylation of VEGFR-2 and increased proliferation of leukemic cells. $VEGF_{165}$ also induced the expression of metalloproteinase-9 (MMP-9) by leukemic cells and promoted their migration through reconstituted basement membrane.

Neutralizing MoAb to VEGFR-2 and a specific synthetic inhibitor of VEGFR-2 blocked $VEGF_{165}$-mediated proliferation of leukemic cells and VEGF-induced leukemic cell migration. Xenotransplantation of human leukemias into immunocompromised NOD-SCID mice resulted in significant elevation of human but not murine VEGF in plasma and death of inoculated mice within two weeks. Injection of a human specific neutralizing monoclonal antibody that selectively blocks signaling through human VEGFR-2, inhibited proliferation of xenotransplanted human leukemias and increased survival of the mice throughout the period of the experiment.

EXAMPLES

All chemicals and reagents were obtained from Sigma unless stated otherwise.

Example I. Inhibition of VEGFR

Collection of primary AML samples and isolation of mononuclear cells by Ficoll gradient

Peripheral blood samples from leukemic patients (diagnosed with acute leukemia) were collected by phlebotomy, diluted 1/2 in Hank's buffered saline (Gibco BRL), and overlaid on 5 mls of Lymphoprep (Ficoll, Accurate Chemical and Scientific Corporation, NY). Each sample was spun at 4000 rpm for 30 mins, and the mononuclear cell interphase was collected into a fresh tube and washed twice with Hank's for 5 mins at 2500 rpm. The resulting cell pellet was finally resuspended in RPMI/10% FCS.

Cell culture

The 3 AML cell lines used in this study, HL-60 (pro-myelomonocytic), HEL, (megakaryocytic) and K562 (erythroid) were cultured in RPMI with 10% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL). Prior to incubation with VEGF and/or antibodies/protein kinase inhibitor, cells were serum starved for 16 hrs to overnight in RPMI alone.

After collection and isolation of mononuclear cells from peripheral blood, primary leukemic cells were cultured overnight in RPMI/10% FCS, to eliminate possible contamination with monocytes/macrophages, which adhere readily to the tissue culture flasks. The cells remaining in suspension consist mainly of leukemic cell blasts. These leukemic cells were subsequently transferred to another flask and, prior to the addition of VEGF and/or anti-VEGFR antibodies, serum starved for 16 hrs to overnight, in serum free RPMI, as above.

For proliferation experiments, cells were cultured in 6 well plates (Corning), at a cell density of $1x10^5$ cells/well, in serum free RPMI. Cells were treated (10-50 ng/mL VEGF) or untreated (medium alone), and cultured in the presence or absence of 500ng to 1μg/mL of an

immunoneutralizing MoAb to KDR/VEGFR-2, IMC-1C11 (12) or a MoAb to FLT-1/VEGFR-1, clone 6.12 (both from ImClone Systems Incorporated). To confirm the mitogenic effects of VEGF on leukemic cell lines were mediated through VEGFR-2, a synthetic protein kinase inhibitor with a high affinity for VEGFR-2 (AG1433, IC_{50}: 9.3μM, Calbiochem, La Jolla, CA) was also used, at a concentration of 10μM, as recommended by the manufacturers. After 24, 48 and 96 hrs, viable cells (as determined by trypan blue exclusion) were counted in triplicate using a haemocytometer. Each experimental condition was done in triplicate and experiments with the leukemic cell lines were repeated 3 times.

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted using TRI-reagent, following the manufacturers instructions. cDNA was subsequently synthesized from total RNA using the Ready-to-go Kit (Amersham Pharmacia Biotech, Piscataway, NJ) and PCR was performed using a PCR thermal cycler (MWG Biotech, High Point, NC). The PCR program used to amplify KDR, FLT-1 and β-actin consisted of a pre-cycle of 5 mins at 94°C, 45 sec at 60°C and 45 sec at 72°C. Following this initial cycle, the reaction was continued for 35 cycles of 1 min at 94°C, 45 sec at 65°C and 2 mins at 72°C and concluded with 7 mins at 72°C. The primer sequences were as follows: β-actin forward primer: tcatgtttgagaccttcaa (SEQ ID NO: 17); β-actin reverse primer: gtctttgcggatgtccacg (SEQ ID NO: 18) (β-actin PCR product: 513 bp); VEGFR-2 forward primer: gtgaccaacatggagtcgtg (SEQ ID NO: 19); VEGFR-2 reverse primer: ccagagattccatgccactt (SEQ ID NO: 20) (VEGFR-2 PCR product: 660 bp); VEGFR-1 forward primer: atttgtgattttggccttgc (SEQ ID NO: 21); VEGFR-1 reverse primer: caggctcatgaacttgaaagc (SEQ ID NO: 22) (VEGFR-1 PCR product: 550 bp). Endothelial cell cDNA was used as positive control for all 3 sets of primers.

Protein extraction and western blotting

Phosphorylated VEGF receptors (VEGFR-1 and VEGFR-2) were detected by western blotting, after cell incubation with 20ng/ml $VEGF_{165}$ (R&D Systems, MN) for 10 min at 37°C. After this brief stimulation, total protein extracts from primary leukemic cells and cell lines were obtained by lysing the cells in cold RIPA buffer (50 mM Tris, 5 mM EDTA, 1% Triton X-114, 0.4% Sodium cacodylate and 150mM NaCl), in the presence of protease

inhibitors (1 mg/mL aprotinin, 10 mg/mL leupetin, 1 mM β-glycerophosphate, 1mM sodium orthovanadate and 1 mM PMSF). Embryonic fibroblasts (the MRC5 cell line) were used as negative control. After centrifugation to remove cell debris, supernatants (a total protein minimum of 500ng) were immunoprecipitated overnight at 4°C with protein-G agarose beads and an anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Ca) to precipitate phosphorylated proteins. These were resuspended in loading buffer and subjected to SDS-Page-acrylamide gel electrophoresis (7.5% gels) under reducing conditions (in the presence of β-mercaptoethanol). Proteins were subsequently blotted onto a nitrocellulose membrane following conventional protocols. Finally, blots were blocked in 1% BSA/PBS-1% Tween-20 for 1 hr at room temperature (RT) followed by incubation with primary and secondary antibodies. Rabbit Polyclonal anti-VEGFR-2 (Santa Cruz Biotechnology, Ca) and goat monoclonal anti-VEGFR-1 (R&D Systems, MN) antibodies were used at a concentration of 1μg/ml and secondary anti-rabbit IgG-HRP (for VEGFR-2) or anti-goat IgG-HRP (for VEGFR-1) were used at 1:6000. The ECL chemiluminescence detection system and ECL film (Amersham Pharmacia biotech, Piscataway, NJ) were used to visualize the presence of proteins on the nitrocellulose blots.

Gelatinolytic zymography

Supernatants from leukemic cell lines and primary cell cultures were collected after overnight incubation in serum-free medium, with or without $VEGF_{165}$, and their metalloproteinase activity was measured by gelatinolytic zymography, as previously described (13). Briefly, cell culture supernatants were treated with gelatin-agarose beads, to concentrate the gelatinases, and processed through SDS-Page-acrylamide gels containing 1% gelatin. The gels were subsequently incubated in 2.5% Triton X-100 for 1 hr at RT, rinsed in distilled water (DW) and placed in low-salt collagenase buffer (50 mM Tris- pH 7.6, 0.2 M NaCl, 5mM $CaCl_2$ and 0.2% v/v Brij-35) at 37°C for 18hrs. Bands of gelatinolytic activity were visualized after staining the gels with 10 mL of a 0.2% Coomassie blue solution and 190 mL destain (DW, Methanol and glacial acetic acid, 6:3:1) for 30-60 mins at RT. For each experiment, $1x10^6$ cells were plated into each well and experiments were done in triplicate. The Adobe Photoshop 4.0 software application and a Umax Astra scanner were used to scan the gels and the intensity of the gelatinolytic bands was assessed using NIH Image 1.58.

Migration experiments

Freshly isolated leukemic cells and cell lines were resuspended in serum-free RPMI and a stock of 10^6 cells/ml was prepared. A modified version of a previously described transwell migration technique (14) was used. Briefly, LC aliquots (100 μL) were added to 8 μm pore transwell inserts, coated with 25 μg of growth factor-depleted Matrigel (Beckton and Dickinson, San Jose, CA), and placed into the wells of a 24 well plate. The lower compartment contained serum free RPMI with or without 200 ng/ml $VEGF_{165}$ (R&D Systems, MN). For the purpose of blocking migration each condition was prepared in a separate aliquot and incubated with 1μg/mL of IMC-1C11, anti-VEGFR-1 (clone 6.12) neutralizing MoAb, 10μM AG1433 (Calbiochem, CA) or a broad range MMP inhibitor, 5,10-phenanthroline (1μM). The antibodies and the tyrosine kinase inhibitor used in these studies have been previously shown to block VEGF-induced endothelial cell proliferation and receptor phosphorylation (12,15,16). The migration was carried out at 37°C and 5% CO2 for 14-18 hrs. Migrated cells were collected from the lower compartment, spun down at 8000 rpm and counted using a haemocytometer. Only live cells, as determined by trypan blue exclusion, were considered in the quantification. Experiments were done in triplicate and results are shown as the number of cells migrated in response to VEGF.

Detection of KDR/VEGFR-2, FLT-1/VEGFR-1 and VEGF on ectopically implanted leukemias (chloromas) by immunohistochemistry.

Paraffin-embedded chloroma sections were immunohistochemically stained for VEGFR-1 and VEGFR-2, following conventional protocols. The antibodies used were: mouse MoAb to VEGFR-2, clone 6.64 (ImClone Systems Incorporated), used at 300 ng/ml; rabbit polyclonal antibody to VEGFR-1 (R&D Systems), used at 200ng/ml; vWF polyclonal antibody, used at 200ng/ml; VEGF polyclonal antibody (BioGenex, Ab No. 360p), used at 200ng/ml. Peroxidase-labelled secondary antibodies (against mouse and rabbit immunoglobulins) were used at a 1/6000 dilution. Sections were counterstained with Hematoxilin/Eosin, and observed under a light microscope.

In vivo experiments with HL-60 cells

Age and sex matched non-obese diabetic immunocompromised mice (NOD-SCID) were used in all experiments. HL-60 cells ($1x10^6$/mouse) were injected intravenously (i.v.) into 10 NOD-SCID mice and 3 days after injection the mice were divided into 2 groups of 5 mice. One group was treated intra-peritoneally with 400 μg of IMC-1C11 (12) 3 times a week while the control group was injected with PBS/1% BSA (diluent control for the antibody) for the duration of the experiment.

Quantification of VEGF levels in mouse plasma

Two ELISA kits (both from R&D systems), specific for human and murine VEGF, respectively, were used to determine VEGF concentrations in the plasma of mice injected with HL-60 cells. Plasma samples were collected at different time-points after leukemic cell injection and used without any further dilution. Each sample was assayed in triplicate and the measurements were done in two separate experiments. Both assays have a sensitivity limit of 7.5pg/ml and were developed according to the manufacturers instructions.

Human choloromas express VEGF, VEGFR-2 and VEGFR-1

Human leukemias are not only localized to the bone marrow and peripheral circulation but may also metastasize to tissues and form solid masses referred to as chloromas.

Immunohistochemical staining of human chloromas with antibodies specific for VEGFR-1 or VEGFR-2 revealed that, besides staining the endothelial lining of blood vessels, these receptors were also expressed by a subset of the leukemic cells (Fig 1C and D). The staining localized to the cell membrane, and positive cells appeared scattered throughout the sections (Fig 1C and D). Overall, there were more VEGFR-2-positive than VEGFR-1 positive areas in the sections analyzed. These receptors were primarily detected in different areas of the tumor, suggesting that the staining pattern of VEGFR-1 and VEGFR-2 may identify different cell populations. Furthermore, in the sections analyzed the leukemic cells also stained for VEGF (Fig 1, E and F), suggesting an autocine loop between VEGF and its receptors may contribute to the formation of chloromas.

Primary leukemias and leukemic cell lines produce VEGF and express VEGFR-2

Three leukemic cell lines and 10 primary acute leukemias (isolated from peripheral blood samples) were analyzed for the production of VEGF and expression of VEGFR-2 by ELISA and RT-PCR.

Of the leukemic cell lines analyzed, HL-60 and HEL expressed VEGFR-2 at the mRNA level, whereas K562 cells were negative (Fig 2A). RT-PCR analysis of primary leukemic samples showed that 5 out of 10 primary AML samples (50% total) expressed VEGFR-2. VEGFR-1 was also detected, by RT-PCR, on VEGFR-2-positive leukemias. Furthermore, all VEGFR-2 positive leukemic cell lines produced VEGF in vitro. The presence of functional receptors on VEGF-producing leukemic cells may thus create an autocrine loop supporting cell growth and survival.

$VEGF_{165}$ induces VEGFR-1 and VEGFR-2 phosphorylation on leukemic cells

$VEGF_{165}$ induced a dose-dependent increase in VEGFR-1 and VEGFR-2 phosphorylation on leukemic cell lines and primary leukemias, but not on fibroblasts or VEGFR negative leukemic cells (Fig 2B). In the absence of VEGF, leukemic cells had baseline VEGFR-1 and VEGFR-2 phosphorylation (Fig 2B), which may be due to the production of VEGF and expression of its receptors by the same cells.

The observation that $VEGF_{165}$ induced the phosphorylation of its receptors on a subset of leukemias suggested that it may also induce phenotypic changes on these cells. Therefore, the effect of $VEGF_{165}$ on leukemic cells was investigated further by looking at phenotypic changes such as increased proliferation, matrix-metalloproteinase (MMP) production and migration through reconstituted basement membrane. Anti-VEGFR MoAb and a specific protein kinase inhibitor, AG1433, were used, to investigate whether $VEGF_{165}$ induced its effects on leukemic cells through VEGFR-2.

VEGF induces leukemic cell proliferation, an effect mediated through VEGFR-2

$VEGF_{165}$ induced, in a dose-dependent manner, an increase in proliferation of VEGFR-2-

positive leukemias and leukemic cell lines (Fig 3). This effect could be blocked by incubating the cells with IMC-1C11, used at 1μg/ml (Fig 3, *, $p<0.05$). The mitogenic effects of $VEGF_{165}$ on leukemic cells appear to be mediated mainly through this receptor, as neutralizing MoAb to VEGFR-1 had no effect on $VEGF_{165}$-induced leukemic cell proliferation. Importantly, on leukemic cells that did not respond to $VEGF_{165}$, such as the K562 cell line and primary samples 6, 7, 8, 9 and 10, incubation with IMC-1C11 had no effect on VEGF-induced cell proliferation (Fig 3).

Experiments using the protein kinase inhibitor AG1433 confirmed that $VEGF_{165}$-induced leukemic cell proliferation mainly through VEGFR-2 (Fig 4). AG1433 significantly blocked $VEGF_{165}$-induced leukemic cell growth over a 72 hr period (**, $p<0.05$, Fig 4). As with neutralizing MoAb, incubation of K562 cells, VEGFR-2-negative leukemias or fibroblasts (not shown) with AG1433, in the presence or absence of $VEGF_{165}$, had no effect on cell proliferation (Fig 4).

These results show that $VEGF_{165}$ induces cell proliferation on a subset of leukemic cell lines and primary leukemias. This effect can be blocked by a MoAb to VEGFR-2 and by a synthetic protein kinase inhibitor with a high affinity for this receptor. This suggests that on leukemic cells, as reported for endothelial cells, $VEGF_{165}$ transduces its mitogenic effects through VEGFR-2.

VEGF induces MMP secretion/production by leukemic cells

$VEGF_{165}$ induces MMP production by smooth muscle cells, an effect that is usually correlated with the acquisition of a more invasive phenotype (17). We investigated whether $VEGF_{165}$ could induce a similar effect on leukemic cells.

Without stimulation, in serum-free conditions, the levels of MMP released into the supernatant by each sample were variable (Fig 5). It is suggested that MMP production by leukemic cells may identify a leukemic sub-type or sub-population with a more invasive phenotype. On the primary leukemias and/or cell lines investigated, the myelo-monocytic sub-types (shown in Fig 5: HL-60 cells, samples 2 and 3) have consistently shown a higher

level of basal MMP production and release. On MMP-producing leukemias, incubation with $VEGF_{165}$ significantly increased MMP-9 secretion by leukemic cells (Fig 5A and B) over an 18 hour period as determined by densitometry. On primary leukemias, MMP-9 was the main MMP released into the culture supernatants, with MMP-2 being absent in most cases (Fig 5). The levels of TIMP-1 produced by leukemic cells but showed only a minor variation after stimulation with $VEGF_{165}$ as determined by western blotting.

VEGF induces leukemic cell migration through matrigel-coated transwells, an effect mediated through VEGFR-1 and VEGFR-2

We investigated whether the $VEGF_{165}$-induced increase in MMP production by leukemic cells reflected the acquisition of a more invasive phenotype. This was demonstrated using a migration system in which transwell inserts were coated with a thin layer of matrigel, a model of invasion through the basement membrane. $VEGF_{165}$ induced leukemic cell migration of HL-60 cells and VEGFR-2+ primary leukemias in a dose-dependent manner (Fig 6). This process requires MMP production and activation, as the VEGF-induced cell migration was blocked by the use of a synthetic MMP inhibitor, 5,10-phenanthroline (Fig 6) and recombinant human TIMP-1.

The remaining cell lines and VEGFR-2 negative primary leukemias did not migrate in response to VEGF, even through bare (uncoated) transwells. This correlated with a decreased ability of these cells to secrete MMPs, but may also be due to a reduced level of VEGFR expression on these cells, namely VEGFR-1.

As shown above, the mitogenic effects of $VEGF_{165}$ on leukemic cells were mediated through VEGFR-2. However, in the migration system used in our studies, incubation of HL-60 cells with IMC-1C11 (at 1μg/ml) could only partially (40%) block $VEGF_{165}$-induced migration through matrigel (Fig 6). On the contrary, a MoAb to VEGFR-1, used at the same concentration, significantly blocked (60-70%) HL-60 cell migration (Fig 6 *, $p<0.05$), suggesting that on these cells $VEGF_{165}$ may induce MMP activation and cell migration by interacting with both receptors. Confirming this possibility, incubation of HL-60 cells with a combination of VEGFR-1 and VEGFR-2 MoAbs blocked $VEGF_{165}$-induced migration

more effectively (70-80%) than either antibody alone (Fig 6). Nevertheless, incubation of HL-60 cells with the VEGFR-2-specific tyrosine kinase inhibitor AG1433, significantly blocked $VEGF_{165}$-induced migration (70%) (Fig 7, *, $p<0.05$). Taken together, these results suggest that, on HL-60 cells, interaction of $VEGF_{165}$ with VEGFR-1 and VEGFR-2 may be necessary to induce cell migration and invasion, but the precise contribution of each receptor for this process requires further investigation.

On primary leukemias both anti-VEGFR-1 and VEGFR-2 MoAbs showed, overall, comparable migration-blocking effects (Fig 6). However, as for HL-60 cells, incubation of primary leukemias with a combination of anti-VEGFR-1 and VEGFR-2 MoAbs was more effective than either antibody alone, blocking $VEGF_{165}$-induced migration by 80% (Fig 6). Finally, the migration of leukemic cells in response to $VEGF_{165}$ could not be blocked by anti-MMP-2 antibodies in any of the cells tested, but was significantly reduced if cells were incubated with MMP-9-neutralizing antibodies, suggesting that MMP-9 may be the main proteinase involved in this migration process.

As mentioned above, the migration assay used in our studies requires active MMP secretion and activation, as well as cell chemotaxis in response to $VEGF_{165}$. Furthermore, the antibody and protein kinase inhibitor studies suggest that $VEGF_{165}$ induced signaling through VEGFR-1 and VEGFR-2 may be necessary for the cells to migrate and invade the basement membrane. This is not surprising, since VEGFR-1 has been shown to mediate monocyte migration (18) and also MMP production by smooth muscle cells (17).

Taken together, these results show that $VEGF_{165}$ induces a more invasive phenotype on a subset of leukemic cells, as determined by increased proliferation, MMP production and migration through the basement membrane. These effects are mediated primarily through VEGFR-2, although, particularly for migration and MMP activation, signaling through VEGFR-1 may also be required.

***HL-60 cells release VEGF* in vivo**

It is now well established that HL-60 cells release VEGF in picogram quantities *in vitro*.

Using NOD-SCID mice, we investigated whether these cells also released VEGF *in vivo*. As shown in figure 8A, human VEGF plasma levels increased significantly after leukemic cell injection, which correlated with an increase in circulating leukemic cells and a corresponding decrease in mouse survival (Fig 8B). Importantly, murine VEGF plasma levels remained very low (at or below the assay detection level, Fig 8A) throughout the experiment. These results support a role for autocrine VEGF in supporting leukemic cell growth and survival.

***Anti-human VEGFR-2/KDR moAb (IMC-1C11) blocks leukemic growth* in vivo**

In the absence of any treatment, mice injected with $1x10^6$ HL-60 cells (i.v.) survived only 14-18 days (n=8), highlighting the aggressive nature of these leukemic cells. Using IMC-1C11, we investigated whether leukemia-derived VEGF supported leukemic cell growth through an autocrine interaction with VEGFR-2. As shown in figure 8B, mice treated with IMC-1C11 survived throughout the period of the experiment (>80 days, Fig 8B, $p<0.005$). Importantly, it has recently been confirmed that the antibody used in these studies is specific for human VEGFR-2 (KDR) and does not cross react with murine flk-1 (Lu et al, *submitted*). Also, as mentioned above, there was an increase in human VEGF plasma levels in control (untreated) mice which correlated with a decrease in overall mouse survival (Fig 8A and B).

These *in vivo* results confirm the role of VEGF and VEGFR-2 in regulating leukemic cell growth and suggest that antibodies against VEGF receptors may represent a novel therapeutic approach for the treatment of a subset of leukemias.

Anti-human VEGFR-2/KDR moAb blocks the formation of liver and spleen metastasis

Histological analysis of liver and spleen, 14 days after start of treatment, from HL-60-injected PBS-treated mice revealed the presence of leukemic infiltrates (chloromas) in both organs (Fig 9B and D). Liver sections had several areas invaded by leukemic cells, with signs of active cellular proliferation (Fig 9B). Furthermore, the red pulp area of the spleen of these mice was largely replaced by leukemic cells (Fig 9D). On the contrary, mice treated with the neutralizing MoAb IMC-1C11, had normal liver and spleen histology (Fig 9A and C). In treated mice, there was no evidence of leukemic infiltrates in the liver (Fig 9A), and the spleen appeared normal, with evidence of apoptosis (Fig 9C). These results indicate that the

moAb to human VEGFR-2 blocked proliferation and invasiveness of HL-60 cells in inoculated mice.

Interpretation of Example I

In order to define the role of VEGF/VEGFR-2 autocrine loop in the regulation of leukemic cell in vivo, we have used a well-established xenotransplantation model where human leukemic cell line HL-60 are engrafted into immunodeficient mice (19-21). Injection of one million HL-60 cells into NOD-SCID mice results in the engraftment and formation of leukemic chloromas in the bone marrow, spleen, liver and peripheral circulation. We demonstrate that HL-60 cells produce human VEGF that could be detected at high levels in the plasma of HL-60 xenotransplanted mice, as determined by a human specific ELISA (Figure 8A). Rising levels of human VEGF were detected in plasma samples and increased over time as the HL-60 cells proliferated in the NOD-SCID mice. In contrast, plasma levels of murine VEGF produced were insignificant and did not increase during HL-60 proliferation (Figure 8A).

In the absence of any intervention the HL-60 xenografted NOD-SCID mice succumb to metastatic proliferation of chloromas within 14 days of inoculation. However, injection of MoAb specific to the VEGF binding domain of human VEGFR-2 resulted in the inhibition in proliferation of HL-60 human leukemia in NOD-SCID mice and their long-term survival throughout the experimental period (>80 days, Figure 8B). Histological examination of liver and spleen demonstrated that treatment with MoAb to VEGFR-2 effectively inhibits proliferation of leukemic infiltrates and Chloromas within the liver and spleen (Figure 9). These data strongly suggest that endogenously produced human VEGF by HL-60 promotes the growth of these leukemic cells through interaction with the human VEGFR-2 generating an autocrine loop, therefore confirming the in vitro data. Collectively, these data illustrate the plasticity of VEGFRs signaling within the vascular and hematopoietic lineages and their potential to support the proliferation certain subsets of VEGFR-2+ acute leukemias.

In humans, acute leukemias are classified based on progenitor cell type and differentiation state of the stem cell they originated from (M1 to M7). The majority of

leukemias are due to malignant transformation of the myeloid progenitors (M1, M2, M3, M4, M5), that are mostly localized to the bone marrow and the peripheral circulation. However, leukemic cells occasionally acquire the capacity to invade various tissues, setting up niches for the formation of large tumor masses referred to as chloromas. Based on our *in vivo* data, most of the chloromas tested to date express VEGFR-2 and VEGFR-1. Given that formation of chloromas, similar to vessel formation, requires sequential invasion, proliferation, and stabilization of the leukemic cells, it is not surprising that leukemic cells with the potential to form chloromas also express VEGFRs. For instance, HL-60 cells represent one leukemic model with the capacity to metastasize and to form a solid, vascularized tumor if implanted subcutaneously. Therefore, leukemias destined to form chloromas may utilize VEGFRs to upregulate the expression of MMPs and facilitate invasion into the tissues. Expression of VEGFR-2 may subsequently promote leukemic cell proliferation. In the absence of counter regulatory factors, the leukemias may continue to proliferate.

Based on our in vivo data, the growth of HL-60 cells xenotransplanted into NOD-SCID mice was inhibited by human specific neutralizing MoAb to VEGFR-2, suggesting that autocrine loops generated by VEGF/VEGFR-2, in addition to paracrine loops, mediate leukemic cell proliferation (Figure 10).

Example II. Producing Single Chain Antibodies

Cell lines and Proteins

Primary-cultured HUVEC was maintained in EBM-2 medium at 37°C, 5% CO2. Cells were used between passage 2-5 for all assays. $VEGF_{165}$ protein was expressed in baculovirus and purified. cDNA encoding the extracellular domain of *KDR* was isolated by RT-PCR from human fetal kidney mRNA and subcloned into the *Bgl* II and *BspE* I sites of the vector AP-Tag. In this plasmid the cDNA for *KDR* extracellular domain is fused in-frame with the cDNA for human placental AP. The plasmid was electroporated into NIH 3T3 cells together with the neomycin expression vector pSV-Neo and stable cell clones were selected with G418. The soluble fusion protein *KDR*-AP was purified from

cell culture supernatant by affinity chromatography using immobilized monoclonal antibodies to AP.

Mice immunization and construction of single chain antibody phage display library

Female BALB/C mice were given two intraperitoneal (i.p.) injections of 10 ug *KDR*-AP in 200 ul of RIBI Adjuvant System followed by one i.p. injection without RIBI adjuvant over a period of two months. The mice were also given a subcutaneous (s.c.) injection of 10 ug *KDR*-AP in 200 ul of RIBI at the time of the first immunization. The mice were boosted i.p. with 20 ug of *KDR*-AP three days before euthanasia. Spleens from donor mice were removed and the cells were isolated. RNA was extracted and mRNA was purified from total RNA of splenocytes. A scFv phage display library was constructed using the mRNA which was displayed on the surface of the filamentous phage M13.

In displaying the scFv on filamentous phage surface, antibody V_H and V_L domains are joined together by a 15 amino-acid-long linker $(GGGGS)^3$ (SEQ. ID No: 23) and fused to the N-terminal of phage protein III. A 15 amino-acid-long E tag, which is followed by an amber codon (TAG), was inserted between the C-terminal of V_L and the protein III for detection and other analytic purposes. The amber codon positioned between the E tag and the protein III enables the construct to make scFv in surface-displaying format when transformed into a suppressor host (such as TGI cells), and scFv in soluble form when transformed into a nonsupressor host (such as HB2151 cells).

The assembled scFv DNA was ligated into the pCANTAB 5E vector. The transformed TG1 cells were plated onto 2YTAG plates and incubated. The colonies were scraped into 10 ml of 2YT medium, mixed with 5 ml 50% glycerol and stored at -70°C as the library stock.

Biopanning

The library stock was grown to log phase, rescued with M13K07 helper phage and amplified overnight in 2YTAK medium (2YT containing 100 ug/ml of ampicillin and 50 ug/ml of kanamycin) at 30°C. The phage preparation was precipitated in 4% PEG/0.5M NaCl, resuspended in 3% fat-free milk/PBS containing 500 ug/ml of AP protein and

incubated at 37°C for 1 h to capture phage displaying anti-AP scFv and to block other nonspecific binding.

KDR-AP (10 ug/ml) coated Maxisorp Star tubes (Nunc, Denmark) were first blocked with 3% milk/PBS at 37°C for 1 h, and then incubated with the phage preparation at room temperature for 1 h. The tubes were washed 10 times with PBST followed by 10 times with PBS (PBS containing 0.1% Tween 20). The bound phage was eluted at room temperature for 10 min. with 1 ml of a freshly prepared solution of 100 mM triethylamine. The eluted phage were incubated with 10 ml of mid-log phase TG1 cells at 37°C for 30 min. stationary and 30 min. shaking. The infected TG1 cells were then plated onto 2YTAG plates and incubated overnight at 30°C.

Ninety-nine percent (185/186) of clones screened after the 3rd round of panning were found to be specific *KDR* binders. However, only 15 (8%) of these binders could block *KDR* binding to immobilized VEGF. DNA BstN I fingerprinting of these 15 clones indicated the presence of 2 different digestion patterns; whereas 21 randomly picked VEGF nonblockers yielded 4 different patterns. All the digestion patterns were also seen in clones identified after the 2nd round of panning. Representative clones of each digestion pattern were picked from clones recovered after the 2nd round of panning and subject to DNA sequencing. Out of 15 clones sequenced, 2 unique VEGF blockers and 3 nonblockers were identified. One scFv, p2A7, which neither binds to *KDR* nor blocks VEGF binding to *KDR*, was selected as a negative control for all studies.

Phage ELISA

Individual TG1 clones were grown at 37°C in 96 well plates and rescued with M13K07 helper phage as described above. The amplified phage preparation was blocked with 1/6 volume of 18% milk/PBS at RT for 1 h and added to Maxi-sorp 96-well microtiter plates (Nunc) coated with KDR-AP or AP (1 ug/ml x 100 ul). After incubation at room temperature for 1 h, the plates were washed 3 times with PBST and incubated with a rabbit anti-M13 phage Ab-HRP conjugate. The plates were washed 5 times, TMB peroxidase substrate added, and the OD at 450 nm read using a microplate reader and scFv antibodies were identified and sequenced.

Preparation of soluble scFv

Phage of individual clones were used to infect a nonsuppressor *E.coli* host HB2151 and the infectant selected on 2YTAG-N plates. Expression of scFv in HB2 151 cells was induced by culturing the cells in 2YTA medium containing 1 mM isopropyl-1-thio-B-D-galactopyranoside at 30°C. A periplasmic extract of the cells was prepared by resuspending the cell pellet in 25 mM Tris (pH 7.5) containing 20% (w/v) sucrose, 200 mM NaCl, 1 mM EDTA and 0.1 mM PMSF, followed by incubation at 4°C with gentle shaking for 1 h. After centrifugation at 15,000 rpm for 15 min., the soluble scFv was purified from the supernatant by affinity chromatography using the RPAS Purification Module (Pharmacia Biotech).

Example III. Assays

Quantitative KDR binding assay

Two assays were employed to examine quantitatively the binding of purified soluble scFv to *KDR*.

Four different clones, including the two VEGF blockers, p1C11 and plF12, one nonblocker, the dominant clone p2A6 and the nonbinder p2A7, were expressed in shaker flasks using a nonsuppressor host *E.coli* HB2151 cells. P1C11 has heavy chain and light chain sequences shown in Figure 11. The soluble scFv was purified from the periplasmic extracts of *E.coli* by anti-E-tag affinity chromatography. The yield of purified scFv of these clones ranged from 100 - 400 ug / liter culture.

In the direct binding assay, various amounts of soluble scFv were added to *KDR*-coated 96-well Maxi-sorp microtiter plates and incubated at room temerature for 1 h, after which the plates were washed 3 times with PBST. The plates were then incubated at room temerature for 1 h with 100 ul of mouse anti-E tag antibody followed by incubation with 100 pl of rabbit anti-mouse antibody-HRP conjugate. The plates were washed and developed following the procedure described above for the phage ELISA.

In another assay, i.e., the competitive VEGF blocking assay, various amounts of soluble scFv were mixed with a fixed amount of *KDR*-AP (50 ng) and incubated at room temerature for 1 h. The mixture were then transferred to 96-well microtiter plates coated with $VEGF_{165}$ (200 ng/well) and incubated at room temperature for an additional 2 h, after which the plates were washed 5 times and the substrate for AP was added to quantify the bound *KDR*-AP molecules. IC_{50}, i.e., the scFv concentration required for 50% inhibition of *KDR* binding to VEGF, was then calculated.

Clone plC11 and plF12, but not p2A6, also block *KDR* binding to immobilized VEGF. Clone p1C11, the dominant clone after each round of panning, showed the highest *KDR* binding capacity and the highest potency in blocking VEGF binding to *KDR* (Table 1). The antibody concentrations of clone p1C11 required for 50% of maximum binding to *KDR* and for 50% of inhibition of *KDR* binding to VEGF were 0.3 nM and 3 nM, respectively (See Table 1). FACS analysis demonstrated that plC11, plF12 and p2A6 were also able to bind to cell surface expressed receptor on HUVEC.

BIAcore analysis of the soluble scFv

The binding kinetics of soluble scFv to *KDR* were measured using BIAcore biosensor (Pharmacia Biosensor). *KDR*-AP fusion protein was immobilized onto a sensor chip and soluble scFv were injected at concentrations ranging from 62.5 nM to 1000 nM. Sensorgrams were obtained at each concentration and were evaluated using a program, BIA Evaluation 2.0, to determine the rate constant *kon* and *koff*. Kd was calculated from the ratio of rate constants *koff/kon*.

Table 1 shows the results of the surface plasmon resonance on a BIAcore instrument. The VEGF-blocking scFv, plC11 and plF12, bound to immobilized *KDR* with Kd of 2.1 and 5.9 nM, respectively. The non-blocking scFv, p2A6, bound to *KDR* with approximately a 6-fold weaker affinity (Kd, 11.2 nM) than the best binder p1C11, mainly due to a much faster dissociation rate. As anticipated, p2A7 did not bind to the immobilized *KDR* on the BIAcore.

Phosphorylation assay

Phosphorylation assays were performed with early passage HUVEC following a protocol described previously. Briefly, HUVEC were incubated in serum free EBM-2 base medium supplemented with 0.5% bovine serum albumin at room temperature for 10 min. in the presence or absence of scFv antibodies at 5 ug/ml, followed by stimulation with 20 ng/ml $VEGF_{165}$ at room temperature for an additional 15 min. The cells were lysed and the *KDR* receptor was immunoprecipitated from the cell lysates with Protein A Sepharose beads coupled to a rabbit anti-*KDR* polyclonal antibody (ImClone Systems Incorporated). The beads were washed, mixed with SDS loading buffer, and the supernatant subjected to Western blot analysis. To detect *KDR* phosphorylation, blots were probed with an anti-phosphotyrosine Mab, 4G10. For the MAP kinase activity assay, cell lysates were resolved with SDS-PAGE followed by Western blot analysis using a phospho-specific MAP kinase antibody. All signals were detected using ECL.

Results showed that VEGF-blocking scFv p1C11, but not the non-blocking scFv p-2A6, was able to inhibit *KDR* receptor phosphorylation stimulated by VEGF. Further, plC11 also effectively inhibited VEGF-stimulated activation of MAP kinases p44/p42. In contrast, neither plC11, nor IMC-2A6 inhibited FGF-stimulated activation of MAP kinases p44/p42.

Anti-mitogenic assay.

HUVEC (5 x 103 cells/well) were plated onto 96-well tissue culture plates (Wallach, Inc., Gaithersburg, MD) in 200 u1 of EBM-2 medium without VEGF, bFGF or EGF and incubated at 37°C for 72 h. Various amounts of antibodies were added to duplicate wells and pre-incubated at 37°C for 1 h, after which $VEGF_{165}$ was added to a final concentration of 16 ng/ml. After 18 h of incubation, 0.25 uCi of [3H]-TdR (Amersham) was added to each well and incubated for an additional 4 h. The cells were placed on ice, washed twice with serum-containing medium, followed by a 10 minute incubation at 4°C with 10% TCA. The cells were then washed once with water and solubilized in 25 ul of 2% SDS. Scintillation fluid (150 u1/well) was added and DNA incorporated radioactivity was determined on a scintillation counter (Wallach, Model 1450 Microbeta Scintillation Counter).

The ability of scFv antibodies to block VEGF-stimulated mitogenic activity on HUVEC is shown in Fig. 3. The VEGF-blocking scFv plC11 strongly inhibited VEGF induced DNA synthesis in HUVEC with an EC_{50}, i.e., the antibody concentration that inhibited 50% of VEGF-stimulated mitogenesis of HUVEC, of approximately 5 nM. The non-blocking scFv p2A6 showed no inhibitory effect on the mitogenic activity of VEGF. Neither p1C11 nor p2A6 inhibited bFGF-induced DNA synthesis in HUVEC.

Example IV. Producing Chimeric Antibodies

Cell lines and Proteins

Primary-cultured human umbilical vein endothelial cells (HUVEC) were maintained in EBM-2 medium at 37°C, 5% CO_2. Cells between passage 2-5 were used for all assays. $VEGF_{165}$ and *KDR*-alkaline phosphatase fusion proteins (*KDR*-AP) were expressed in baculovirus and NIH 3T3 cells, respectively, and purified following the procedures described above. The anti-*KDR* scFv plC11 and scFv p2A6, an antibody that binds to *KDR* but does not block *KDR*-VEGF interaction, were isolated from a phage display library constructed from a mouse immunized with *KDR* as described above. C225 is a chimeric IgG1 antibody directed against epidermal growth factor (EGF) receptor.

Cloning of the variable domains of scFv p1C11

The variable domains of the light (V_L) and the heavy (V_H) chains of plC11 were cloned from the scFv expression vector by PCR using primers 1 and 2, and primers 3 and 4, respectively. The leader peptide sequence for protein secretion in mammalian cells was then added to the 5' of the V_L and the V_H by PCR using primers 5 and 2, and primers 5 and 4, respectively.

Primer 1: 5' CTA GTA GCA ACT GCA ACT GGA GTA CAT TCA GAC ATC GAG CTC3'

Primer 2: 5' TCG ATC TAG AAG GAT CCA CTC ACG TTT TAT TTC CAG3'
*Bam*HI

Primer 3: 5' CTA GTA GCA ACT GCA ACT GGA GTA CAT TCA CAG GTC AAG CTG3'

Primer 4: 5' TCG AAG GAT CCA CTC ACC TGA GGA GAC GGT3'

*Bam*HI

Primer 5: 5' GGT CAA AAG CTT ATG GGA TGG TCA TGT ATC ATC CTT TTT

Hind III

CTA GTA GCA ACT3'

Construction of the expression vectors for the chimeric plCl1 IgG.

Separate vectors for expression of chimeric IgG light chain and heavy chains were constructed. The cloned V_L gene was digested with *Hind* III and *BamH* I and ligated into the vector pKN100 containing the human κ light chain constant region (C_L) to create the expression vector for the chimeric p1C11 light chain, IMC-1C11-L. The cloned V_H gene was digested with Hind III and BamH I and ligated into the vector pGID105 containing the human IgG1 (γ) heavy chain constant domain (C_H) to create the expression vector for the chimeric p1C11 heavy chain, IMC-1C11-H. Both constructs were examined by restriction enzyme digestion and verified by dideoxynucleotide sequencing.

As seen in Figure 4 both the V_H and the V_L domains are precisely fused on their 5' ends to a gene segment encoding a leader peptide sequence as marked. The V_H and the V_L domains are ligated via *Hind* III/*BamH* I sites into expression vector pG1D105, which contains a cDNA version of the human γ1 constant region gene, and pKN100, which contains a cDNA version of the human κ chain constant region gene, respectively. In each case, expression is under control of the HCMVi promoter and terminated by an artificial termination sequence. The light and the heavy chain complimentarily determining region (CDR) residues, defined according the hypervariable sequence definition of Kabat et al., are underlined and labeled CDR-H1 to H3 and CDR-L1 to L3, respectively.

IgG expression and purification.

COS cells were co-transfected with equal amounts of IMC-1C11-L and IMC-1C11-H plasmids for transient IgG expression. Subconfluent COS cells grown in DMEM / 10% FCS in 150 mm culture dishes were rinsed once with 20 ml of DMEM containing 40 mM Tris (pH 7.4), followed by incubation at 37°C for 4.5 h with 4 ml of DMEM / DEAE-Dextran / DNA mixture (DMEM containing 40 mM Tris, 0.4 mg/ml of DEAE-Dextran (Sigma), and 20 ug each of IMC-1C11-L and IMC-1C11 -H plasmids). The cells were incubated at 37°C for 1 h with 4 ml of DMEM / 2% FCS containing 100 nM of chloroquine (Sigma), followed by incubation with 1.5 ml of 20% glycerol / PBS at room temperature for 1 min. The cells were washed twice with DMEM / 5% FCS and incubated in 20 ml of the same medium at 37°C overnight. The cell culture medium was changed to serum-free DMEM / HEPES after the cells were washed twice with plain DMEM. The cell culture supernatant was collected at 48 h and 120 h after the transfection. The chimeric IgG was purified from the pooled supernatant by affinity chromatography using Protein G column following the protocol described by the manufacturer (Pharmacia Biotech). The IgG-containing fractions were pooled, buffer exchanged into PBS and concentrated using Centricon 10 concentrators (Amicon Corp., Beverly, MA). The purity of the IgG was analyzed by SDS-PAGE. The concentration of purified antibody was determined by ELISA using goat anti-human y chain specific antibody as the capture agent and HRP-conjugated goat anti-human k chain antibody as the detection agent. Standard curve was calibrated using a clinical grade antibody, C225.

After affinity purification by Protein G, a single protein band of ~150 kD was seen in SDS-PAGE. Western blot analysis using HRP-conjugated anti-human IgG1 Fc specific antibody confirmed the presence of human IgG Fc portion in the purified protein.

The results of the ELISA show that IMC-1C11 binds more efficiently to immobilized *KDR* than the parent scFv.

Example V. Assays and Analysis

FACS analysis.

Early passage HUVEC cells were grown in growth factor-depleted EBM-2 medium overnight to induce the expression of *KDR*. The cells were harvested and washed three times with PBS, incubated with IMC-plC11 IgG (5 ug/ml) for 1 h at 4°C, followed by incubation with a FITC labeled rabbit anti-human Fc antibody (Capper, Organon Teknika Corp., West Chester, PA) for an additional 60 min. The cells were washed and analyzed by a flow cytometer (Model EPICS®, Coulter Corp., Edison, NJ). As previously seen with the parent scFv p1C11, IMC-1C11 binds specifically to *KDR* expressed on early passage HUVEC.

Quantitative KDR binding assay.

Various amounts of antibodies were added to *KDR*-coated 96-well Maxi-sorp microtiter plates (Nunc. Danmark) and incubated at room temperature for 1 h, after which the plates were washed 3 times with PBS containing 0.1% Tween-20. The plates were then incubated at RT for 1 h with 100 ul of mouse anti-E tag antibody-HRP conjugate (Phannacia Biotech) for the scFv, or rabbit anti-human IgG Fc specific antibody-HRP conjugate (Cappel, Organon Teknika Corp.) for the chimeric IgG. The plates were washed 5 times, TMB peroxidase substrate (KPL, Gaithersburg, MD) added, and the OD at 450 nm read using a microplate reader (Molecular Device, Sunnyvale, CA). IMC-1C11 binds more efficiently to immobilized *KDR* receptor than the parent scFv.

BIA core analysis.

The binding kinetics of antibodies to *KDR* were measured using BIAcore biosensor (Pharmacia Biosensor). *KDR*-AP fusion protein was immobilized onto a sensor chip, and antibodies or VEGF were injected at concentrations ranging from 25 nM to 200 nM. Sensorgrams were obtained at each concentration and were evaluated using a program, BIA Evaluation 2.0, to determine the rate constants kon and koff . Kd was calculated as the ratio of rate constants *koff/kon.*

BIAcore analysis reveals that IMC-lC11 bind to *KDR* with higher affinity than the parent scFv (Table 2). The Kd of IMC-1C11 is 0.82 nM, compared to 2.1 nM for the

scFv. The increased affinity of IMC-1C11 is mainly due to a slower dissociation rate (koff) of the bivalent chimeric IgG. It is important to note that the affinity (Kd) of IMC-1C11 for binding to *KDR* is similar to that of the natural ligand VEGF for binding to *KDR*, which is 0.93 nM as determined in our BIAcore analysis (Table 2).

Competitive VEGF binding assay.

In the first assay, various amounts of antibodies were mixed with a fixed amount of *KDR*-AP (50 ng) and incubated at room temperature for 1 h. The mixtures were then transferred to 96-well microtiter plates coated with $VEGF_{165}$ (200 ng/well) and incubated at room temperature for an additional 2 h, after which the plates were washed 5 times and the substrate for AP (p-nitrophenyl phosphate, Sigma) was added to quantify the bound *KDR*-AP molecules. EC_{50}, i.e., the antibody concentration required for 50% inhibition of *KDR* binding to VEGF, was then calculated.

IMC-1C11 block *KDR* receptor from binding to immobilized VEGF in a dose-dependent manner. The chimeric antibody is more potent in blocking VEGF-*KDR* interaction with an IC_{50} (i.e., the antibody concentrations required to inhibit 50% of KDR trom binding to VEGF) of 0.8 nM, compared to that of 2.0 nM for the scFv. The control scFv p2A6 also binds *KDR* but does not block VEGF-*KDR* interaction.

In the second assay, various amounts of IMC-1C11 antibody or cold $VEGF_{165}$ protein were mixed with a fixed amount of 125I labeled $VEGF_{165}$ and added to 96-well microtiter plates coated with *KDR* receptor. The plates were incubated at room temperature for 2h, washed 5 times and the amounts of radiolabeled $VEGF_{165}$ that bound to immobilized *KDR* receptor were counted. Concentrations of IMC-1C11 and cold $VEGF_{165}$ required to block 50% of binding of the radiolabeled VEGF to immobilized *KDR* receptor were determined.

IMC-lC11 efficiently competes with ^{125}I labeled VEGF for binding to immobilized *KDR* receptor in a dose-dependent manner. As expected, C225, a chimeric antibody directed against EGF receptor does not bind to *KDR* receptor or block VEGF-*KDR* interaction.

Phosphorylation assay.

Subconfluent HUVEC cells were grown in growth factor depleted EBM-2 medium for 24 to 48h prior to experimentation. After pretreatment with 50 nM sodium orthovanadate for 30 min, the cells were incubated in the presence or absence of antibodies for 15 min, followed by stimulation with 20 ng/ml of $VEGF_{165}$, or 10 ng/ml of FGF at room temperature for an additional 15 min. The cells were then lysed in lysis buffer (50 nM Tris, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.25% sodium deoxycholate, 1 mM PMSF, 1 ug/ml leupeptin, 1 ug/ml pepstatin, 10 ug/ml aprotinin, pH 7.5) and the cell lysate used for both the *KDR* and MAP kinase phosphorylation assays. The *KDR* receptor was immunoprecipitated from the cell lysates with Protein A Sepharose beads (Santa Cruz Biotechnology, Inc., CA) coupled to an anti-*KDR* antibody, Mab 4.13 (ImClone Systems). Proteins were resolved with SDS-PAGE and subjected to Western blot analysis. To detect *KDR* phosphorylation, blots were probed with an antiphosphotyrosine Mab, PY20 (ICN Biomedicals, Inc. Aurora, OH). For the MAP kinase activity assay, cell lysates were resolved with SDS-PAGE followed by Western blot analysis using a phospho-specific MAP kinase antibody (New England BioLabs, Beverly, MA). All signals were detected using ECL (Amersham, Arlington Heights, IL). In both assays, the blots were reprobed with a polyclonal anti-*KDR* antibody (ImClone Systems) to assure that equal amount of protein was loaded in each lane of SDS-PAGE gels.

IMC-1C11 effectively inhibits VEGF-stimulated phosphorylation of *KDR* receptor and activation of p44/p42 MAP kinases. In contrast, C225 does not show any inhibition of VEGF-stimulated activation of *KDR* receptor and MAP kinases. Neither IMC-1C11, nor C225 alone has any effects on the activity of *KDR* receptor and p44/p42 MAP kinases. As previously seen with the scFv p1C11, IMC-1C11 does not inhibit FGF-stimulated activation of p44/p42 MAP kinases. Furthermore, neither scFv p2A6, nor the chimeric IgG form of p2A6 (c-p2A6), inhibits VEGF-stimulated activation of *KDR* receptor and MAP kinases.

Anti-mitogenic assay.

The effect of anti-*KDR* antibodies on VEGF-stimulated mitogenesis of human endothelial cells was determined with a [^{3}H]-TdR DNA incorporation assay using HUVEC. HUVEC (5 x 10^3 cells/well) were plated into 96-well tissue culture plates in 200 µl of EBM-2 medium without VEGF, bFGF or EGF and incubated at 37°C for 72 h. Various amounts of antibodies were added to duplicate wells and pre-incubated at 37°C for 1 hour, after which $VEGF_{165}$ was added to a final concentration of 16 ng/ml. After 18 hours of incubation, 0.25 µCi of [^{3}H]-TdR was added to each well and incubated for an additional 4 hours. DNA incorporated radioactivity was determined with a scintillation counter.

Both IMC-1C11 and scFv p1C11 effectively inhibit mitogenesis of HUVEC stimulated by VEGF. IMC-1C11 is a stronger inhibitor of VEGF-induced mitogenesis of HUVEC than the parent scFv. The antibody concentrations required to inhibit 50% of EGF-induced mitogenesis of HUVEC are 0.8 nM for IMC-1C11 and 6 nM for the scFv, respectively. As expected, scFv p2A6 does not show any inhibitory effect on VEGF-stimulated endothelial cell proliferation.

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Table 1: KDR - binding analysis of anti-KDR scFv antibodies

scFv clone	KDR binding[1] (ED_{50}, nM)	VEGF blocking[2] (IC_{50}, nM)	Binding kinetics[3]		
			kon (10^5 M^{-1} s^{-1})	*koff* (10^{-4} s^{-1})	K*d* (10^{-9} M)
p1C11	yes (0.3)	yes (3.0)	1.1	2.3	2.1
p1F12	yes (1.0)	yes (15)	0.24	1.4	5.9
p2A6	yes (5.0)	no (>300)	4.1	46.1	11.2
p2A7	no (NA)	no (>300)	NA	NA	NA

1. Determined by direct binding ELISA, numbers in the parenthesis represent the scFv concentrations that give 50% of maximum binding;
2. Determined by competitive VEGF blocking ELISA, numbers in the parenthesis represent the scFv concentrations required for 50% inhibition of KDR binding to immobilized VEGF; 3. Determined by BIAcore analysis. NA = not applicable.

Table 2. Binding kinetics of p1C11 scFv and IMC-1C11 to KDR receptor.*

Antibody	*kon* (10^5 M^{-1} s^{-1})	*koff* (10^{-4} s^{-1})	K*d* (10^{-9} M)
p1C11 scFv	1.11	2.27	2.1
IMC-1C11	0.63	0.52	0.82
VEGF	1.87	1.81	0.93

* All rates are determined by surface plasmon resonance using BIAcore system, and are mean of at least three separate determinations.

WHAT IS CLAIMED IS:

1. A method of inhibiting the growth of non-solid tumor cells that are stimulated by a ligand of vascular endothelial growth factor receptor (VEGFR) in mammals, the method comprising treating the mammals with an effective amount of a VEGFR antagonist.

2. A method according to claim 1 wherein the VEGFR is KDR.

3. A method according to claim 1 wherein the VEGFR is flk-1.

4. A method according to claim 1 wherein the VEGFR is flt-1.

5. A method according to claim 1 wherein the mammal is a human.

6. A method according to claim1 wherein the antagonist is a biological molecule.

7. A method according to claim 6 wherein the biological molecule is a monoclonal antibody specific for VEGFR or a fragment that comprises the hypervariable region thereof.

8. A method according to claim 7 wherein the antibody comprises at least one variable heavy-chain fragment comprising:

CDRH1, having the amino acid sequence shown in SEQ. ID. NO. 1;

CDRH2, having the amino acid sequence shown in SEQ. ID. NO. 2; and

CDRH3, having the amino acid sequence shown in SEQ. ID. NO. 3;

and at least one variable light-chain fragment comprising:

CDRL1, having the amino acid sequence shown in SEQ. ID. NO. 4;

CDRL2, having the amino acid sequence shown in SEQ. ID. NO. 5; and

CDRL3, having the amino acid sequence shown in SEQ. ID. NO. 6.

9. A method according to claim 7 wherein the antibody comprises:

at least one variable heavy-chain fragment having the amino acid sequence shown in SEQ. ID. NO. 7; and

at least one variable light-chain fragment having the amino acid sequence shown in SEQ. ID. NO. 8.

10. A method according to claim 7 wherein the monoclonal antibody is chimerized or humanized.

11. A method according to claim 1 wherein the antagonist is a small molecule.

12. A method according to claim 1 further comprising treating the non-solid tumor cells with radiation, chemotherapy or combinations thereof.

13. A method according to claim 1 wherein the tumor cells affect hematopoietic structures.

14. A method according to claim 13 wherein the tumor cells are bone marrow cells.

15. A method according to claim 14 wherein the tumor cells are leukemias.

16. A method according to claim 15 wherein the leukemias are acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), erythrocytic leukemia or monocytic leukemia.

17. A method according to claim 14 wherein the tumor cells are multiple myelomas.

18. A method according to claim 14 wherein the tumor cells are lymphoid cells.

19. A method according to claim 18 wherein the lymphoid cells are related to Hodgkin's disease or Non-Hodgkin's disease.

20. A method of inhibiting the growth of non-solid tumors that are stimulated by a ligand of vascular endothelial growth factor receptor (VEGFR) in mammals, the method comprising treating the mammals with an effective amount of a combination of a VEGFR antagonist and radiation.

21. A method according to claim 20 wherein the mammal is a human.

22. A method according to claim 20 wherein the antagonist is administered before radiation.

23. A method according to claim 20 wherein the antagonist is administered during radiation.

24. A method according to claim 20 wherein the antagonist is administered after the radiation.

25. A method according to claim 20 wherein the antagonist is administered before and during radiation.

26. A method according to claim 20 wherein the antagonist is administered during and after radiation.

27. A method according to claim 20 wherein the antagonist is administered before and after radiation.

28. A method according to claim 20 wherein the antagonist is administered before, during, and after radiation.

29. A method according to claim 20 wherein the source of the radiation is external to the mammal.

30. A method according to claim 20 wherein the source of radiation is internal to the mammal.

31. A method according to claim 20 wherein the antagonist is a monoclonal antibody.

32. A method according to claim 20 wherein the tumors affect hemopoietic structures.

33. A method of inhibiting the growth of nonsolid tumors that are stimulated by a ligand of vascular endothelial growth factor receptor (VEGFR) in mammals, comprising treating the mammals with an effective amount of an VEGFR antagonist and a chemotherapeutic agent.

34. A method according to claim 33 wherein the antagonist is administered before treatment with the chemotherapeutic agent.

35. A method according to claim 33 wherein the antagonist is administered during treatment with the chemotherapeutic agent.

36. A method according to claim 33 wherein the antagonist is administered after the treatment with the chemotherapeutic agent.

37. A method according to claim 33 wherein the antagonist is administered before and during treatment with the chemotherapeutic agent.

38. A method according to claim 33 wherein the antagonist is administered during and after treatment with the chemotherapeutic agent.

39. A method according to claim 33 wherein the antagonist is administered before and after treatment with the chemotherapeutic agent.

40. A method according to claim 33 wherein the antagonist is administered before, during, and after treatment with the chemotherapeutic agent.

41. A method according to claim 33 wherein the chemotherapeutic agent is selected from the group consisting of cisplatin, dacarbazine, dactinomycin, mechlorethamine, streptozocin, cyclophosphamide, carmustine, lomustine, doxorubicin, daunorubicin, procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil, vinblastine, vincristine, bleomycin, paclitaxel, docetaxel, aldesleukin, asparaginase, busulfan, carboplatin, cladribine, dacarbazine, floxuridine, fludarabine, hydroxyurea, ifosfamide, interferon alpha, leuprolide, megestrol, melphalan, mercaptopurine, plicamycin, mitotane, pegaspargase, pentostatin, pipobroman, plicamycin, streptozocin, tamoxifen, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil, taxol and combinations thereof.

42. A method according to claim 33 wherein the chemotherapeutic agent is selected from the group consisting of cisplatin, doxorubicin, taxol and combinations thereof.

43. A method according to claim 33 wherein the tumors cells affect hemopoietic structures.

44. A method according to claim 33 wherein the antagonist is a monoclonal antibody.