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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A01N 37/18, 43/04, A61K 38/00, 31/70, C12Q 1/68, C12P 21/06, 19/34, 21/08, C07H 21/02, 21/04, C07K 1/00, 2/00, 4/00, 7/00, 14/00, 16/00, 17/00

A1

(11) International Publication Number: **WO 00/64261**

(43) International Publication Date: 2 November 2000 (02.11.00)

(21) International Application Number: PCT/US00/11096

(22) International Filing Date: 26 April 2000 (26.04.00)

(30) Priority Data:
60/130,935 26 April 1999 (26.04.99) US

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(81) Designated States: AU, CA, CN, FI, JP, KR, NZ, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR–B (VEGF–B) IN DEVELOPING BONES AND USES THEREOF

(57) Abstract

This invention relates to the expression of VEGF–B in developing bones. Methods are provided for stimulating angiogenesis of a bone, for stimulating the healing of bone fractures or breaks, for enhancing the acceptance and/or securing of an implant to a bone and for the treatment or alleviation of the inflammation of the epiphysis of a long bone as well as the use of a pharmaceutical composition which comprises a polypeptide having an amino acid sequence of at least 85 % sequence identity with the sequence of SEQ ID NO: 2 or 4, or fragment thereof having the biological activity of VEGF–B to stimulate angiogenesis of a bone.

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ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR-B (VEGF-B) IN DEVELOPING BONES AND USES THEREOF

This invention relates generally to methods utilizing the growth factor VEGF-B, and specifically to methods utilizing VEGF-B to affect the growth of bones.

BACKGROUND OF THE INVENTION

The two major components of the mammalian vascular system are the endothelial and smooth muscle cells. The endothelial cells form the lining of the inner surface of all blood vessels and lymphatic vessels in the mammal. The formation of new blood vessels can occur by two different processes, vasculogenesis or angiogenesis (for review *see* Risau, W., *Nature* **386:** 671-674, 1997). Vasculogenesis is characterized by the *in situ* differentiation of endothelial cell precursors to mature endothelial cells and association of these cells to form vessels, such as occurs in the formation of the primary vascular plexus in the early embryo. In contrast, angiogenesis, the formation of blood vessels by growth and branching of pre-existing vessels, is important in later embryogenesis and is responsible for the blood vessel growth which occurs in the adult. Angiogenesis is a physiologically complex process involving proliferation of endothelial cells, degradation of extracellular matrix, branching of vessels and subsequent cell adhesion events. In the adult, angiogenesis is tightly controlled and limited under normal circumstances to the female reproductive system. However angiogenesis can be switched on in response to tissue damage. Importantly solid tumors are able to induce angiogenesis in surrounding tissue, thus sustaining tumor growth and facilitating the formation of metastases (Folkman, J., *Nature Med.* **1**: 27-31, 1995). The molecular mechanisms underlying the complex angiogenic processes are far from being understood.

Angiogenesis is also involved in a number of pathologic conditions, where it plays a role or is involved directly in different sequelae of the disease. Some examples include neovascularization associated with various liver diseases, neovascular sequelae of diabetes, neovascular sequelae to hypertension, neovascularization in post-trauma, neovascularization due to head trauma, neovascularization in chronic liver infection (e.g. chronic hepatitis), neovascularization due to heat or cold trauma, dysfunction related to excess of hormone, creation of hemangiomas and restenosis following angioplasty. Accordingly, inhibition of angiogenesis is useful in preventing, treating or alleviating these pathological conditions and to reduce blood supply to solid tumors.

Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGFα), and hepatocyte growth factor (HGF). See for example Folkman *et al.*, *J. Biol. Chem.*, **267:** 10931-10934, 1992 for a review.

It has been suggested that a particular family of endothelial cell-specific growth factors, the vascular endothelial growth factors (VEGFs), and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF/VEGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs). The PDGF/VEGF family of growth factors belongs to the cystine-knot

superfamily of growth factors, which also includes the neurotrophins and transforming growth factor-ß.

Eight different proteins have been identified in the PDGF/VEGF family, namely two PDGFs (A and B), VEGF and five members that are closely related to VEGF. The five members closely related to VEGF are: VEGF-B, described in International Patent Application PCT/US96/02957 (WO 96/26736) and in U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki; VEGF-C or VEGF2, described in Joukov *et al.*, *EMBO J.*, **15:** 290-298, 1996, Lee *et al.*, *Proc. Natl. Acad. Sci. USA*, **93:** 1988-1992, 1996, and U.S. Patents 5,932,540 and 5,935,540 by Human Genome Sciences, Inc; VEGF-D, described in International Patent Application No. PCT/US97/14696 (WO 98/07832), and Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 548-553, 1998; the placenta growth factor (PlGF), described in Maglione *et al.*, Proc. Natl. Acad. Sci. USA, **88:** 9267-9271, 1991; and VEGF3, described in International Patent Application No. PCT/US95/07283 (WO 96/39421) by Human Genome Sciences, Inc. Each VEGF family member has between 30% and 45% amino acid sequence identity with VEGF. The VEGF family members share a VEGF homology domain which contains the six cysteine residues which form the cystine-knot motif. Functional characteristics of the VEGF family include varying degrees of mitogenicity for endothelial cells, induction of vascular permeability and angiogenic and lymphangiogenic properties.

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has been isolated from several sources. Alterative mRNA splicing of a single VEGF gene gives rise to five isoforms of VEGF. VEGF shows highly specific mitogenic activity for endothelial cells. VEGF has important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis and in angiogenesis during

adult life (Carmeliet *et al.*, *Nature*, **380:** 435-439, 1996; Ferrara *et al.*, *Nature*, **380:** 439-442, 1996; reviewed in Ferrara and Davis-Smyth, *Endocrine Rev.*, **18:** 4-25, 1997). The significance of the role played by VEGF has been demonstrated in studies showing that inactivation of a single VEGF allele results in embryonic lethality due to failed development of the vasculature (Carmeliet *et al.*, *Nature*, **380:** 435-439, 1996; Ferrara *et al.*, *Nature*, **380:** 439-442, 1996). The isolation and properties of VEGF have been reviewed; see Ferrara *et al.*, *J. Cellular Biochem.*, **47:** 211-218, 1991 and Connolly, *J. Cellular Biochem.*, **47:** 219-223, 1991.

In addition VEGF has strong chemoattractant activity towards monocytes, can induce the plasminogen activator and the plasminogen activator inhibitor in endothelial cells, and can also induce microvascular permeability. Because of the latter activity, it is sometimes referred to as vascular permeability factor (VPF). VEGF is also chemotactic for certain hematopoetic cells. Recent literature indicates that VEGF blocks maturation of dendritic cells and thereby reduces the effectiveness of the immune response to tumors (many tumors secrete VEGF) (Gabrilovich *et al.*, *Blood* **92**: 4150-4166, 1998; Gabrilovich *et al.*, *Clinical Cancer Research* **5**: 2963-2970, 1999).

VEGF-B has similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique by screening for cellular proteins which might interact with cellular retinoic acid-binding

protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02957 (WO 96/26736), in U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki and in Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, **93:** 2576-2581, 1996. The entire disclosure of the International Patent Application PCT/US96/02957 (WO 96/26736), of U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki are incorporated herein by reference.

VEGF-C was isolated from conditioned media of the PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase VEGFR-3 (Flt4), using cells transfected to express VEGFR-3. VEGF-C was purified using affinity chromatography with recombinant VEGFR-3, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov *et al.*, *EMBO J.*, **15:** 290-298, 1996.

VEGF-D was isolated from a human breast cDNA library, commercially available from Clontech, by screening with an expressed sequence tag obtained from a human cDNA library designated "Soares Breast 3NbHBst" as a hybridization probe (Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 548-553, 1998). Its isolation and characteristics are described in detail in International Patent Application No. PCT/US97/14696 (WO98/07832).

The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. VEGF-D is strongly expressed in heart, lung and skeletal muscle. Intermediate levels of VEGF-D are expressed in spleen, ovary, small intestine and colon, and a lower expression occurs in kidney, pancreas, thymus, prostate and testis. No VEGF-D mRNA

was detected in RNA from brain, placenta, liver or peripheral blood leukocytes.

PlGF was isolated from a term placenta cDNA library. Its isolation and characteristics are described in detail in Maglione *et al.*, *Proc. Natl. Acad. Sci. USA*, **88:** 9267-9271, 1991. Presently its biological function is not well understood.

VEGF3 was isolated from a cDNA library derived from colon tissue. VEGF3 is stated to have about 36% identity and 66% similarity to VEGF. The method of isolation of the gene encoding VEGF3 is unclear and no characterization of the biological activity is disclosed.

Similarity between two proteins is determined by comparing the amino acid sequence and conserved amino acid substitutions of one of the proteins to the sequence of the second protein, whereas identity is determined without including the conserved amino acid substitutions.

As noted above, the PDGF/VEGF family members act primarily by binding to receptor tyrosine kinases. In general, receptor tyrosine kinases are glycoproteins, which consist of an extracellular domain capable of binding a specific growth factor(s), a transmembrane domain, which is usually an alpha-helical portion of the protein, a juxtamembrane domain, which is where the receptor may be regulated by, e.g., protein phosphorylation, a tyrosine kinase domain, which is the enzymatic component of the receptor and a carboxy-terminal tail, which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

Five endothelial cell-specific receptor tyrosine kinases have been identified, namely VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), Tie and Tek/Tie-2. These receptors differ in their specificity and affinity. All of

these have the intrinsic tyrosine kinase activity which is necessary for signal transduction.

The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and PlGF. VEGF-C has been shown to be the ligand for VEGFR-3, and it also activates VEGFR-2 (Joukov *et al.*, *The EMBO Journal*, **15:** 290-298, 1996). VEGF-D binds to both VEGFR-2 and VEGFR-3 (Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 548-553, 1998). A ligand for Tek/Tie-2 has been described in International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc. The ligand for Tie has not yet been identified.

Recently, a novel 130-135 kDa VEGF isoform specific receptor has been purified and cloned (Soker *et al.*, *Cell*, **92:** 735-745, 1998). The VEGF receptor was found to specifically bind the $VEGF_{165}$ isoform via the exon 7 encoded sequence, which shows weak affinity for heparin (Soker *et al.*, *Cell*, **92:** 735-745, 1998). Surprisingly, the receptor was shown to be identical to human neuropilin-1 (NP-1), a receptor involved in early stage neuromorphogenesis. PlGF-2 also appears to interact with NP-1 (Migdal *et al.*, *J. Biol. Chem.*, **273:** 22272-22278, 1998).

VEGFR-1, VEGFR-2 and VEGFR-3 are expressed differently by endothelial cells. Generally, both VEGFR-1 and VEGFR-2 are expressed in blood vessel endothelia (Oelrichs *et al.*, *Oncogene*, **8:** 11-18, 1992; Kaipainen *et al.*, *J. Exp. Med.*, **178:** 2077-2088, 1993; Dumont *et al.*, *Dev. Dyn.*, **203:** 80-92, 1995; Fong *et al.*, *Dev. Dyn.*, **207:** 1-10, 1996) and VEGFR-3 is mostly expressed in the lymphatic endothelium of adult tissues (Kaipainen *et al.*, *Proc. Natl. Acad. Sci. USA*, **9:** 3566-3570, 1995). VEGFR-3 is also expressed in the blood vasculature surrounding tumors.

Although VEGFR-1 is mainly expressed in endothelial cells during development, it can also be found in hematopoetic precursor cells during early stages of embryogenesis (Fong *et al.*, *Nature*, **376:** 66-70, 1995). In adults, monocytes and macrophages also express this receptor (Barleon *et al.*, *Blood*, **87:** 3336-3343, 1995). In embryos, VEGFR-1 is expressed by most, if not all, vessels (Breier *et al.*, *Dev. Dyn.*, **204:** 228-239, 1995; Fong *et al.*, *Dev. Dyn.*, **207:** 1-10, 1996). Thus, VEGFR-1 is expressed in all tissues expressing VEGF-B suggesting that VEGF-B is a physiological ligand for VEGFR-1.

The receptor VEGFR-3 is widely expressed on endothelial cells during early embryonic development but as embryogenesis proceeds becomes restricted to venous endothelium and then to the lymphatic endothelium (Kaipainen *et al.*, *Cancer Res.*, **54:** 6571-6577, 1994; Kaipainen *et al.*, *Proc. Natl. Acad. Sci. USA*, **92:** 3566-3570, 1995). VEGFR-3 is expressed on lymphatic endothelial cells in adult tissues. This receptor is essential for vascular development during embryogenesis.

The essential, specific role in vasculogenesis, angiogenesis and/or lymphangiogenesis of VEGFR-1, VEGFR-2, VEGFR-3, Tie and Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos. Disruption of the VEGFR genes results in aberrant development of the vasculature leading to embryonic lethality around midgestation. Analysis of embryos carrying a completely inactivated VEGFR-1 gene suggests that this receptor is required for functional organization of the endothelium (Fong *et al.*, *Nature*, **376:** 66-70, 1995). However, deletion of the intracellular tyrosine kinase domain of VEGFR-1 generates viable mice with a normal vasculature (Hiratsuka *et al.*, *Proc. Natl. Acad. Sci. USA*, **95:** 9349-9354, 1998). The reasons underlying these differences remain to be explained but

suggest that receptor signalling via the tyrosine kinase is not required for the proper function of VEGFR-1. Analysis of homozygous mice with inactivated alleles of VEGFR-2 suggests that this receptor is required for endothelial cell proliferation, hematopoesis and vasculogenesis (Shalaby *et al.*, *Nature*, **376:** 62-66, 1995; Shalaby *et al.*, *Cell*, **89:** 981-990, 1997). Targeted inactivation of both copies of the VEGFR-3 gene in mice resulted in defective blood vessel formation characterized by abnormally organized large vessels with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at post-coital day 9.5 (Dumont *et al.*, *Science*, **282:** 946-949, 1998). On the basis of these findings it has been proposed that VEGFR-3 is required for the maturation of primary vascular networks into larger blood vessels. However, the role of VEGFR-3 in the development of the lymphatic vasculature could not be studied in these mice because the embryos died before the lymphatic system emerged. Nevertheless it is assumed that VEGFR-3 plays a role in development of the lymphatic vasculature and lymphangiogenesis given its specific expression in lymphatic endothelial cells during embryogenesis and adult life. This is supported by the finding that ectopic expression of VEGF-C, a ligand for VEGFR-3, in the skin of transgenic mice, resulted in lymphatic endothelial cell proliferation and vessel enlargement in the dermis. Furthermore this suggests that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov *et al.*, *EMBO J.*, **15:** 290-298, 1996).

Of the eight known VEGFs, VEGF-B is most closely related to VEGF (Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, **93:** 2576-2581). VEGF-B has similar angiogenic and other properties to those of VEGF, but is distributed and expressed

in tissues differently from VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

VEGF-B is expressed in several tissues and most abundantly in the heart, brain, skeletal muscle and kidney and occurs as two splice isoforms of 167 and 186 amino acids, respectively (Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, **93**: 2576-2581; Olofsson *et al.*, *J. Biol. Chem.*, 1996 **271**: 19310-19317). Because VEGF-B binds VEGFR-1, it may have a role in the regulation of blood vessel physiology. Previous studies on the localization of VEGF-B during embryonic development have been fragmentary with only limited general insights into the relationship between VEGF-B expressing cells and the vasculature of the developing embryos (Lagercrantz *et al.*, *Biochem. Biophys. Res. Commun.*, 1996, **220**: 147-152; Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, **93**: 2576-2581; Lagercrantz *et al.*, *Biochim. Biophys. Acta.*, 1998. **1398**: 157-163).

SUMMARY OF THE INVENTION

The invention generally provides methods utilizing the growth factor VEGF-B, and specifically to methods utilizing VEGF-B to affect the growth of bones.

In a first aspect of the invention, a method is provided for stimulating vascularization of a bone by angiogenesis. This method comprises the step of administering an effective angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4 (VEGF-B isoforms: hVEGF-B_{167} and hVEGF-B_{186}, respectively) or a fragment thereof having the biological activity of VEGF-B. As used herein, the term "VEGF-B" collectively refers to a polypeptide having an amino acid sequence of at least 85% sequence identity with the

sequence of SEQ ID NO:2 or 4, or fragments thereof as herein defined, and to a polynucleotide sequence which can code for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B. This nucleotide sequence/polynucleotides can be naked and/or in a vector or liposome.

The angiogenesis stimulating amount of VEGF-B or a fragment thereof can be provide by the use of an expression vector comprising a nucleotide sequence which codes for VEGF-B (see SEQ ID NO:1 or 3) or a fragment thereof having the biological activity of VEGF-B, or a polynucleotide coding for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4 (see, for example, SEQ ID NO:1 or 3), or a fragment thereof having the biological activity of VEGF-B.

This method can be used for bones which include, but are not limited to, long bones and vertebrae.

The abilities referred to herein as "biological activities of VEGF-B" and can be readily tested by methods known in the art. Preferably the polypeptide or the encoded polypeptide from a polynucleotide has the ability to stimulate one or more of endothelial cell proliferation, differentiation, migration, survival or vascular permeability including, but not limited to, proliferation, differentiation, migration, survival or vascular permeability of vascular endothelial cells and/or lymphatic endothelial cells. VEGF-B can also have antagonistic effects on cells which have hitherto not been described, but are included in the biological activities of VEGF-B.

Polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain the biological activity of VEGF-B are clearly to be understood to

be within the scope of the invention. Persons skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymatic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid analog may retain the required aspects of the biological activity of VEGF-B. Such compounds can readily be made and tested by methods known in the art, and are also within the scope of the invention.

In addition, possible additional isoforms of the VEGF-B polypeptide which result from alternative splicing, as are known to occur with VEGF, and naturally-occurring allelic variants of the nucleic acid sequence encoding VEGF-B are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do not result in any substantial functional alteration of the encoded polypeptide.

Preferred vectors of the invention are expression vectors in which a nucleic acid according to the invention is operatively connected to one or more appropriate promoters and/or other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing VEGF-B, or a fragment thereof having the biological activity of VEGF-B. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenoviral- or retroviral-based vectors or liposomes. A variety of such vectors is known in the art.

Optionally VEGF-B, a fragment thereof having the biological activity of VEGF-B may be administered together

with, or in conjunction with, one or more of VEGF, VEGF-C, VEGF-D, VEGF-F, PlGF, PDGF-A, PDGF-B, FGF and/or heparin.

In a second aspect of the invention, a method is provided for stimulating the healing of bone fractures or breaks. This method comprises the step of administering an angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B, or a polynucleotide coding for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

In a third aspect of the invention, a method is provided for enhancing the acceptance and/or securing of an implant to a bone. This method comprises the steps of applying an implant to a bone; and administering an effective angiogenesis stimulating amount of VEGF-B, or a fragment or analog thereof having the biological activity of VEGF-B, a polynucleotide coding for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

In a fourth aspect of the invention, there is provided a method of treatment or alleviation of the inflammation of an epiphysis of a long bone. This method comprises the step of administering an effective inflammation inhibiting amount of an antagonist to a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

The antagonist may be any agent that prevents the action of VEGF-B, either by preventing the binding of VEGF-B to its corresponding receptor on the target cell, or by preventing

activation of the receptor. Suitable antagonists include, but are not limited to, antibodies directed against VEGF-B; competitive or non-competitive inhibitors of binding of VEGF-B to its corresponding receptor; and anti-sense nucleotide sequences as described below.

Where VEGF-B or a VEGF-B antagonist is to be used for therapeutic purposes, the dose and route of application will depend upon the nature of the patient and condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include oral, subcutaneous, intramuscular, intraperitoneal or intravenous injection, parenteral, topical application, implants *etc.* Topical application of VEGF-B may be used in a manner analogous to VEGF. Where used to enhance angiogenesis, an effective amount of the truncated active form of VEGF-B is administered to an organism in need thereof in a dose between about 0.1 and 1000 μg/kg body weight.

The VEGF-B or a VEGF-B antagonist may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of VEGF-B or a VEGF-B antagonist, and a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant. Examples of such a carrier or adjuvant include, but are not limited to, saline, buffered saline, Ringer's solution, mineral oil, talc, corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, elixirs, syrups, wafers, ointments or other conventional forms. The formulation to suit the mode of administration. Compositions

which comprise VEGF-B may optionally further comprise one or more of VEGF, PDGF-A, PDGF-B, VEGF-C, VEGF-D, PlGF and/or heparin. Compositions comprising VEGF-B will contain from about 0.1% to 90% by weight of the active compound(s), and most generally from about 10% to 30%.

For intramuscular preparations, a sterile formulation, preferably a suitable soluble salt form of the truncated active form of VEGF-B, such as hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as pyrogen-free water (distilled), physiological saline or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

The preferred anti-sense nucleotide sequence is complementary to at least a part of a DNA sequence which encodes a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof that has the biological activity of VEGF-B. In addition the anti-sense nucleotide sequence can be to the promoter region of the VEGF-B gene or other non-coding region of the gene which may be used to inhibit, or at least mitigate, VEGF-B expression.

A further aspect of the invention involves the use of a pharmaceutical composition which comprises an effective angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B, and a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant or diluent to stimulate angiogenesis of a bone.

It is also known that VEGF-B forms both homodimers and heterodimers with VEGF. Thus, the invention includes both homodimers of VEGF-B polypeptide and heterodimers of VEGF-B and VEGF, VEGF-C, VEGF-D, PlGF, PDGF-A or PDGF-B.

It will be clearly understood that for the purposes of this specification the word "comprising" means "included but not limited to". The corresponding meaning applies to the word "comprises".

Other objects, advantages and novel features of the present invention will become apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows analyses of mouse VEGF-B_{186} and mVEGF-$B_{exon1-5}$ from baculovirus-infected Sf9 insect cells;

Figure 2A-H shows immunohistochemical localization of VEGF-B in the developing heart and large vessels; and

Figure 3A-H shows immunohistochemical localization of VEGF-B in developing muscle, developing long bone, cartilage and glandular tissues.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A role of VEGF-B in the vascularization of developing bones is suggested based on the pattern of expression of this growth factor. Thus, VEGF-B can be used to stimulate vascularization of a bone by angiogenesis which can be used to stimulate the healing process of a damaged bone or enhance the acceptance and/or securing of an implant to a bone.

Expression of VEGF-B in baculovirus infected insect cells

Recombinant mVEGF-B_{186} and mVEGF-$B_{exon1-5}$ were tagged at the amino terminus with 6X Histidine residues and then expressed in baculovirus-infected *Sf9* cells. The baculovirus expression vector pACGP67A was a gift from Dr. Johanna Veijola. Full length mVEGF-B_{186} cDNA (SEQ ID NO:5) was amplified by polymerase chain reaction (PCR) using two primers to allow direct cloning into the expression vector and including the appropriate modifications of the expressed protein; sense 5'-ATC GAG ATC TTC ATC ACC ATC ACC ATC ACG GAG ATG ACG ATG ACA AAC CTG TGT CCC AGT TT-3' (SEQ ID NO:6) (including a Bgl II site for in frame cloning and a 6X Histidine tail and enterokinase site, but excluding the endogenous signal sequence) antisense 5'-AGC TAG ATC TCT AAG CCC CGC CCT TG-3' (SEQ ID NO:7)(including a Bgl II site for cloning). A truncated mVEGF-B protein, encoded by sequences corresponding to exons 1-5 (Olofsson *et al.*, *J. Biol. Chem.*, 1996 **271**: 19310-19317, Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, 1998 **95**: 11709-11714), was similarly generated with same sense primer and the antisense primer 5'-TAG CAG ATC TGT ACT ACC TGT CTG GCT TCA CAG A-3' (SEQ ID NO:8) (including a Bgl II site for cloning and C-terminus truncated after exon 5). Recombinant baculovirus was generated in Sf9 cells (obtained from ATCC) and reamplified and the titer of the virus stocks was determined as recommended by the supplier (Pharmingen Baculovirus Expression Vector System: Procedures and Methods Manual). The Sf-9 cells were maintained in medium (Nordcell TNM-FH 1x without L-glutamine) containing 10% fetal bovine serum (Gibco BRL).

For production of recombinant proteins, the Sf9 cells were adapted in four steps to serum free medium (Sf 900 II SMF, Gibco BRL), and the cells were infected with a multiplicity of infection of 7 at a density of 2 X 10^6

cells/ml. Medium containing recombinant protein was harvested 4 days post infection and incubated with Ni-NTA-Agarose (Qiagen) overnight at +4°C (5 μl of packed gel per ml of medium). After washing with 50 mM sodium phosphate buffer pH 8, containing 300 mM NaCl, the proteins were eluted with the same buffer containing 150 mM imidazole (for full length mVEGF-B_{186}) or containing 200 mM imidazole (for mVEGF-$B_{exon1-5}$).

The eluted proteins were analyzed on 12.5% acrylamide gels under reducing and nonreducing conditions. For immunoblotting analyses, the proteins were electrotransferred onto Hybond filters for 45 min. The recombinant proteins on the blots were visualized using an antipeptide Ig against the N-terminus (Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996 **93**: 2576-2581.)(Figure 1A) or using a rabbit antiserum raised against a glutathionine-S-transferase fusion protein containing the unique C-terminal amino acid residues of mVEGF-B_{186} (Figure 1C).

Antibodies to mVEGF-B

The highly purified mVEGF-B_{186} was used to immunize rabbits. 25 μg of protein was emulsified in 500 μl of Freunds Complete Adjuvant and injected intradermally. The rabbits were boostered at biweekly intervals with the same amounts of protein emulsified in Freunds Incomplete Adjuvant. Affinity purified Ig to mVEGF-B_{186} was obtained by passing immune sera over a Sepharose 4B column (Pharmacia Biotech) containing immobilized mVEGF-B_{186}. Following extensive washing, the bound Igs were eluted in 300 mM sodium citrate buffer pH 3.0 containing 500 mM NaCl. Eluted Igs were dialyzed against PBS and concentrated.

Figure 1A shows the immunoblot analysis under non-reducing conditions (-) and reducing (+) conditions using the antipeptide Ig against the N-terminus. Immunoblot analysis

of the eluates under non-reducing conditions revealed that both proteins are disulfide-linked homodimers migrating with apparent molecular weights of 48-55,000 and 34,000, respectively, and significant amounts of both proteins appeared as high molecular weight smears. Under reducing conditions, the 186 isoform migrated as 30-32 kDa species while the truncated protein, mVEGF-$B_{exon1-5}$, appeared as a homogenous 21 kDa species. The immunoglobulins raised to mVEGF-B_{186} also react with mVEGF-$B_{exon1-5}$ and mVEGF-B_{167}. The molecular weight standards are indicated to the left in the Figure.

Figure 1B shows the SDS-PAGE analysis of highly purified recombinant mVEGF-B_{186} analyzed under non-reducing (-) and reducing (+) conditions. The separated proteins were visualized by staining with Coomassie Brilliant Blue.

To check the specificity of the affinity-purified Igs, human VEGF (R&D Systems), mVEGF-$B_{exon1-5}$, and human VEGF-C (Joukov *et al.*, *EMBO J.*, 1996 **15**: 290-298) were subjected to immunoblot analysis, as described above, using 300 ng of protein/lane. Figure 1C shows that the affinity-purified Igs were specific only for VEGF-B. Thus, the Igs to VEGF-B recognize the common N-terminal domain of the various mVEGF-B isoforms generated either by alternative splicing of mRNA or by proteolytic processing of mVEGF-B_{186}, and show no cross-reactivity with several other VEGF members.

Immunohistochemical localization of VEGF-B in mouse embryos

The spatial and temporal patterns of expression of the VEGF-B protein in mouse embryos were determined by immunohistochemistry using standard procedures and employing affinity purified Ig fractions on tissue sections of E10.5 to E17.5 embryos.

The embryos were fixed in 4% paraformaldehyde overnight

at 4°C and processed for cryosectioning. 14μm cryosections were used for the stainings. After sectioning, the slides were air dried for 1 to 3 hours followed by a 10 minute post fixation with 4% paraformaldehyde. After washing 3 x 5 minutes with phosphate buffered saline (PBS) containing 0.3% Triton X-100 (PBS-T) the slides were incubated in 0.3% H_2O_2 in PBS-T for 30 minutes to quench the endogenous peroxidase activity. This was followed by washing 2 x 5 minutes with PBS-T and 2 x 5 minutes in PBS. Blocking of non-specific binding was done using 3% bovine serum albumin (BSA) in PBS for 30 minutes. The slides were incubated with the affinity purified antibody against mVEGF-B at a concentration of 0.5 μg/ml overnight in 4°C. After washing, the slides were incubated with the secondary Ig (goat anti-rabbit HRP, Vector Laboratories) at a dilution of 1:200 for 1 hour. After washing, the slides were incubated with the AB complex (Vector Laboratories) for 1 hour and washed with Tris pH 7.4. Either 3,3'-diaminobenzidine tetrahydrochloride (DAB from SIGMA) or 3-amino-9-ethyl carbazole (AEC from Vector Laboratories) were used for color development. The reaction was quenched by washing in Tris-HCl buffer. As negative controls, the anti mVEGF-B Ig was either preabsorbed with a 10X molar excess of $mVEGFB_{186}$, tissue sections were incubated with only the secondary Ig or preimmune rabbit IgG was used as the primary antibody.

In Figures 2A and 2B, the control experiments verified that the staining procedure specifically detected VEGF-B. Blocking experiments using primary Ig pre-treated with an excess of purified $mVEGF\text{-}B_{186}$ gave no specific staining nor did pre-immune IgG fractions or only the secondary antibodies.

Expression in the heart and large vessels

Expression of VEGF-B in the developing heart was observed. Figure 2B and 2C shows that the immunostaining was confined to the cardiac myocytes of the atria and the ventricles while the developing atrioventricular septal region, formed largely from the endocardial cushion tissue, was negative. Intense staining for VEGF-B was seen in the outflow tract proximal to the heart while more distally, the outflow tract lacked detectable expression. Figure 2B and 2D shows that no staining of the endothelial cells was seen in the outflow tract, in the endocardium or epicardium. Figure 2D also shows that, at higher magnification, the cardiac myocytes showed an intracellular staining with a preferential perinuclear localization. The extracellular matrix surrounding the cardiac myocytes also showed some immunoreactivity.

In the E10.5 embryo, vascular smooth muscle cells showed no expression of VEGF-B, but around E12.5, these cells became positive, with staining around several of the larger vessels, e.g. the dorsal aorta, the carotid artery and the subclavian artery. Figure 2G shows that at E14.5, VEGF-B also appeared in the vascular smooth muscle cells of the intervertebral and lung vessels and in the arteries of the kidney at E15.5. By E17.5, staining was seen also in the arterial smooth muscle cells of brown fat and intestine. As seen in Figure 2E-G, no expression was seen in the endothelial cells of any vessels. Figure 2E shows that in the aorta, VEGF-B staining of the smooth muscle cell layer was weak, with scattered positive cells. Figure 2H shows that VEGF-B was not expressed in the tonic smooth muscle cells of the aortic arch.

Expression in the developing muscles

As seen in Figure 3A-B, 3E and 3G, in E10.5, the myotomal component of the somites was stained for VEGF-B and this expression continued in later stages of the developing skeletal muscle cells. VEGF-B was detected in myoblasts of the intersegmental muscles in the developing vertebral column in E12.5 embryos. At the later stages, E15.5 and 17.5, all skeletal muscles were positive, including the muscles of the extremities, the vibrissae etc. Expression of VEGF-B in the muscle cells of the tongue and extrinsic ocular muscles was first detected around E12. Subcutaneous muscles started to express VEGF-B at E14.5 and the muscles associated with the hair follicles at E15.5. Figure 3C shows that VEGF-B was detected in dorsolaterally located cells of the diaphragm at E 12.5, and the positive area expanded until by E15.5 the entire diaphragm was strongly stained.

Expression in the developing bones and cartilage

Several types of developing bones were also positive for VEGF-B, i.e. the ribs, vertebra and the long bones of the extremities. The staining was confined to the dorsal aspects of the developing ribs and the perichondrium surrounding the hypertrophic chondrocytes. In the ribs, the staining was transiently detected starting at E14.5, with a peak at E15.5 (Figure 3E) and weaker expression again at E17.5. The same pattern of expression was observed in the vertebra where strong expression was detected at E15.5 and weaker at E17.5. As seen in Figure 3D, the long bones on the other hand showed a strong expression of VEGF-B at E14.5. A weaker expression was seen in E15.5 and 17.5. The stained area was cap-like and covered the epiphysis of the growing bone. The chondrocytes of the cartilage and the ossifying bone appeared negative. No

staining for VEGF-B was obtained in association with the developing skull bones.

During tooth development, staining for VEGF-B was seen in the vascular plexus surrounding the enamel differentiation zone of the incisor primordia during E14.5 to E17.5.

Staining of VEGF-B was obtained in the cytoplasm of chondrocytes within Meckel's cartilage and the hyoid cartilage, starting at E12.5 and ending between E15.5 and E17.5 when the cartilage became ossified (Figure 3F).

Most bones of the developing embryo are formed by endochondral ossification, i.e. the cartilage is replaced by bone in ossification centers, starting in several locations in the embryo from E14.5 onwards. During this process, the avascular cartilage is replaced by the highly vascularized bone tissue in a process requiring angiogenesis and capillary ingrowth. With few exceptions (Meckel's and the hyoid cartilages), VEGF-B is not expressed by the cartilage but instead in the surrounding perichondrium. The perichondrium provides a matrix for capillaries invading the ossifying bone and thereby has an essential role during bone formation. The expression of VEGF-B in the perichondrium of developing bones of the vertebral column and the extremities as well as the epiphysis of the growing long bone supports an important role played by VEGF-B in the angiogenic processes which underlie capillary ingrowth into the developing bone tissue.

Expression in the central nervous system, brown fat, pancreas and adrenal gland

Specific staining of VEGF-B was found in the choroid plexus beginning at E12.5, when this structure develops, and continuing throughout further development. VEGF-B expression in other sites of the brain was confined to the smooth muscle cells of the arteries, such as the basilar artery. This

expression started at E12.5, corresponding with the appearance of the smooth muscle cells of the carotid and subcalvian arteries. No expression was detected in the pericytes surrounding the capillary network of the brain.

At E17.5, the brown fat expressed VEGF-B, most intensely in the vascular smooth muscle cells surrounding the larger vessels. In Figure 3G, a strong immunoreactivity was seen at this time in the cortex of the fetal adrenal gland, identified by the arrow. Expression of VEGF-B in the pancreatic primordium started around E12.5. Figure 3H shows that at E17.5, the staining was confined to clusters of glandular cells which constitute the future exocrine pancreas.

Abundant VEGF-B mRNA and protein was consistently observed in the developing heart, various other striated muscles including many skeletal muscles and smooth muscle cells of the larger vessels. It is notable that high levels of VEGF-B mRNA have been observed in the corresponding tissues also in the adult animal (Olofsson *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996 **93**: 2576-2581; Olofsson *et al.*, *J. Biol. Chem.*, 1996 **271**: 19310-19317). In addition, VEGF-B was expressed in the developing bone and cartilage, brown fat and in several organs including some exocrine and endocrine glands such as the pancreas and adrenal gland. No expression of VEGF-B was seen in the endothelial cells of the larger vessels or in capillaries of the different tissues.

A common feature of several of the tissues which abundantly express VEGF-B, e.g. heart, muscle and brown fat, is a high metabolic turnover rate and these tissues also display a high capillary density. This raises the possibility that VEGF-B might have a role in the angiogenic processes which govern capillary ingrowth into these tissues. VEGF-B might also regulate some other functions of the endothelium in these tissues in addition to serving as a potential growth

regulatory molecule involved in the angiogenic processes during embryogenesis. Support for such a role comes from the observation that targeted inactivation of its receptor VEGFR-1, which resulted in failure to assemble endothelial lumens in the otherwise well developed vascular system.

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.

WHAT IS CLAIMED IS:

1. A method of stimulating vascularization of a bone by angiogenesis, comprising the step of administering an effective angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or fragment thereof having the biological activity of VEGF-B.

2. A method according to claim 1, wherein the bone is a long bone.

3. A method according to claim 1, wherein the bone is a vertebra.

4. A method of stimulating the healing of bone fractures or breaks, comprising the step of administering an effective angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

5. A method of enhancing the acceptance and/or securing of an implant to a bone, comprising the steps of:

applying an implant to a bone; and

administering an effective angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

6. A method of treatment or alleviation of the inflammation of the epiphysis of a long bone, comprising the step of administering an effective inflammation inhibiting

amount of an antagonist to a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4.

7. Use of a pharmaceutical composition comprising an effective angiogenesis stimulating amount of a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B, and at least one pharmaceutical carrier or diluent to stimulate vascularization of a bone by angiogenesis.

8. A method of stimulating vascularization of a bone by angiogenesis, comprising the step of administering an effective angiogenesis stimulating amount of a polynucleotide coding for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

9. A method according to claim 8, wherein the bone is a long bone.

10. A method according to claim 8, wherein the bone is a vertebra.

11. A method of stimulating the healing of bone fractures or breaks, comprising the step of administering an effective angiogenesis stimulating amount of a polynucleotide coding for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

12. A method of enhancing the acceptance and/or securing of an implant to a bone, comprising the steps of:

applying an implant to a bone; and

administering an effective angiogenesis stimulating amount of a polynucleotide coding for a polypeptide having an amino acid sequence of at least 85% sequence identity with the sequence of SEQ ID NO:2 or 4, or a fragment thereof having the biological activity of VEGF-B.

FIG. 1

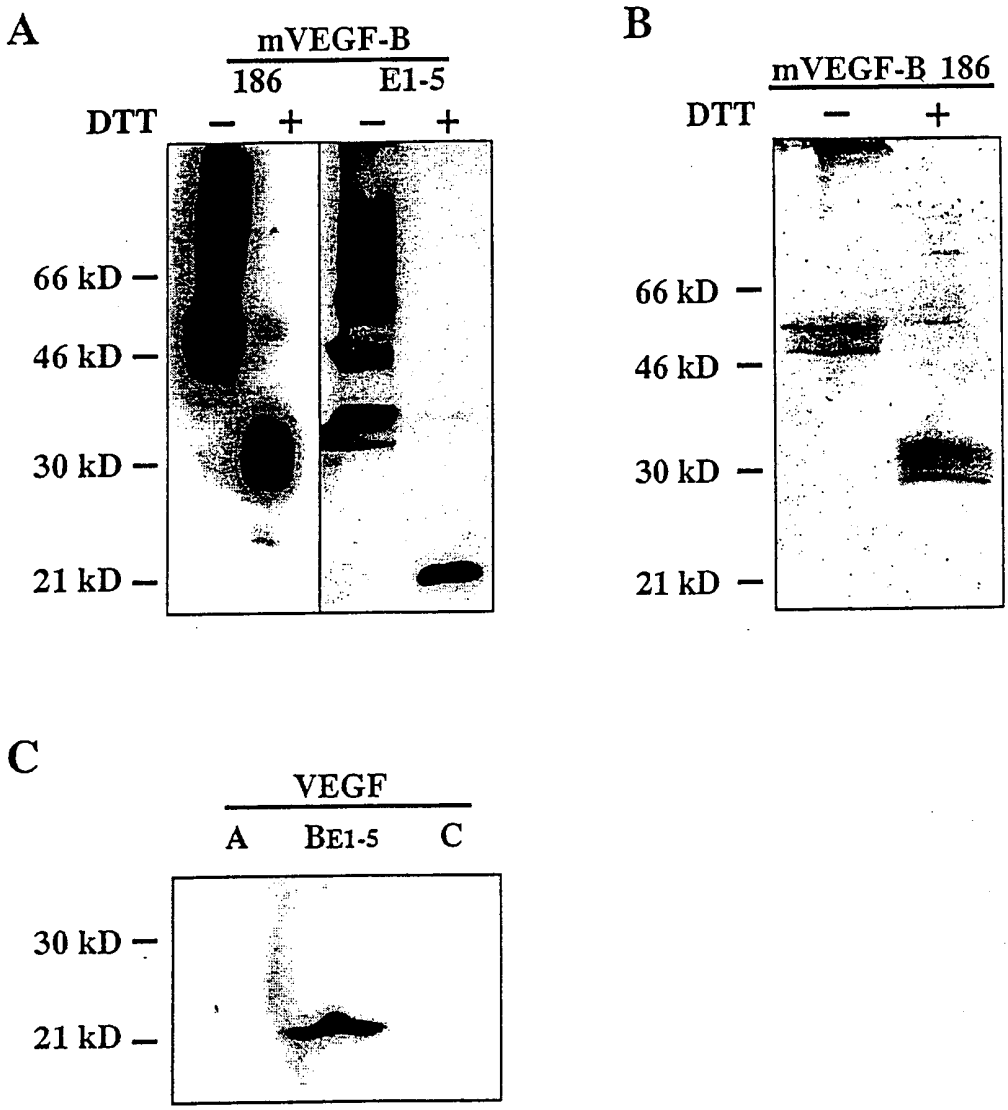
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FIG. 2

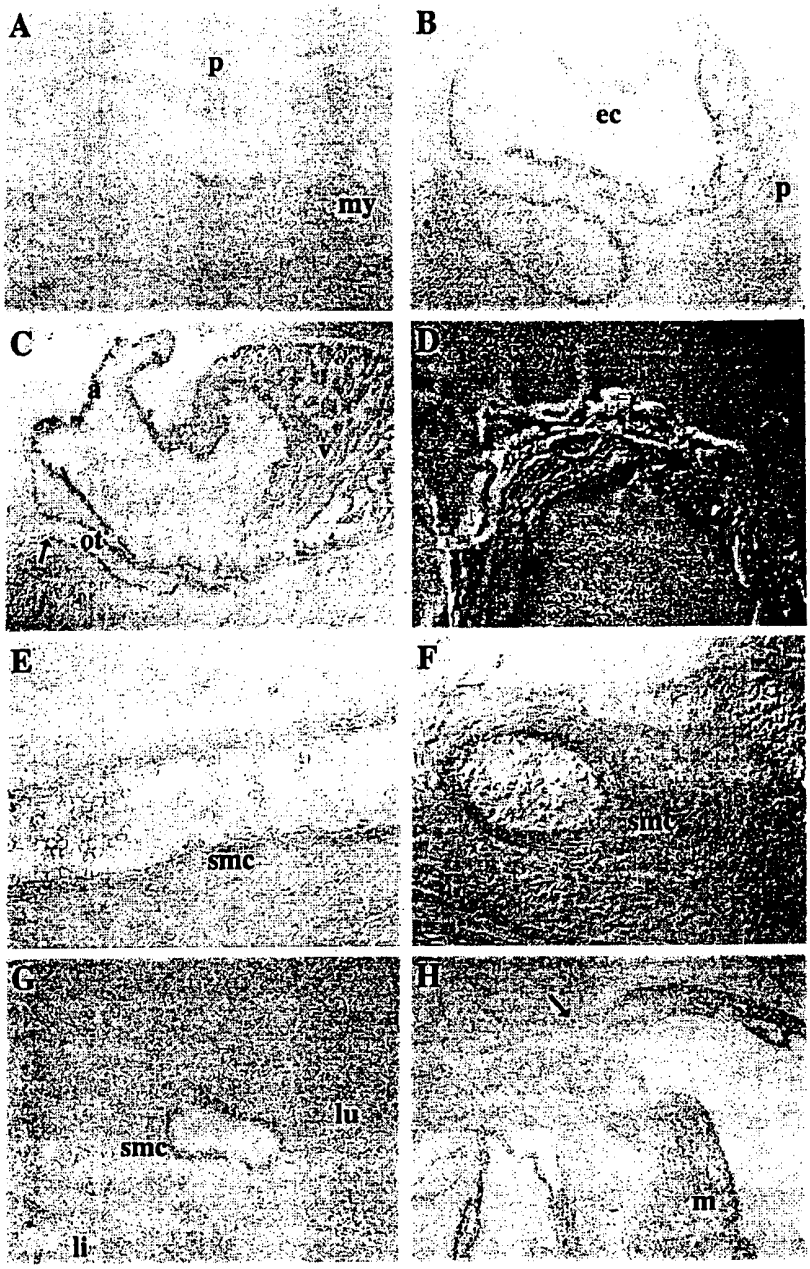
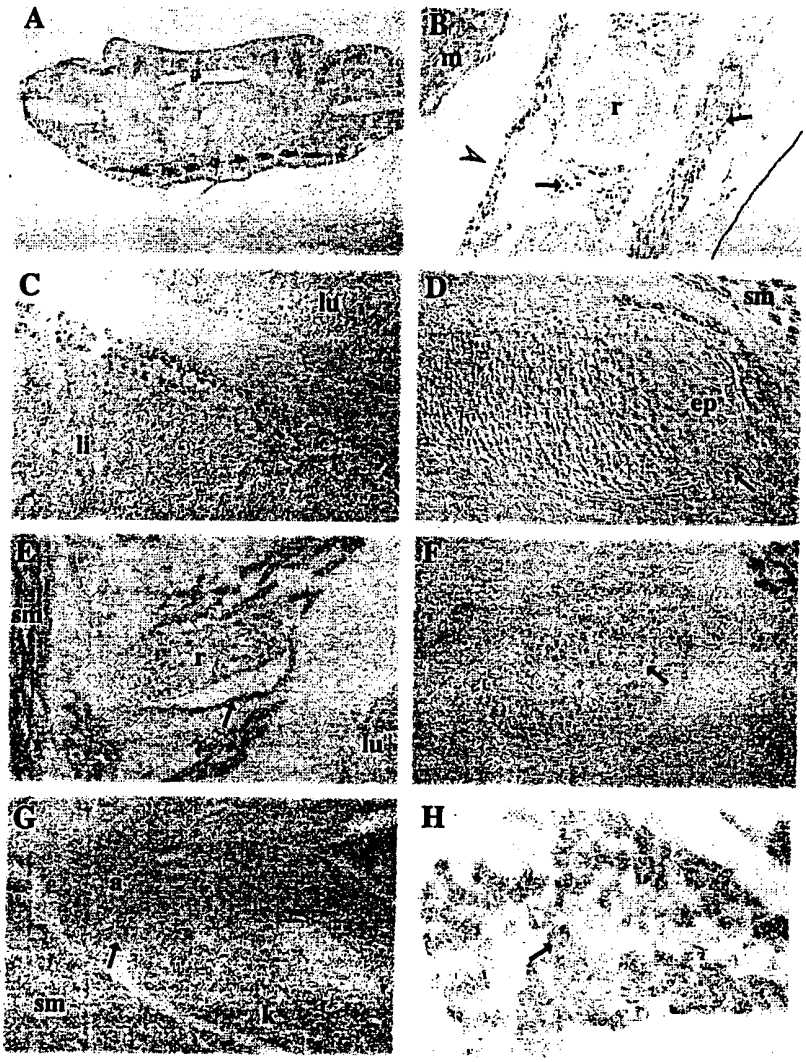


FIG. 3



SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11096

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 44; 435/6, 69.1, 91.1, 455, 366, 375; 530/300, 350, 388.24; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequence search, Dialog, West

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RISTIMAKI et al. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endotheilial growth factor-C. Biol. Chem. 03 April 1998, Vol. 273, No. 14, pages 8413-8418, entire text.	1-12
A	TOWNSON et al. Characterization of the murine VEGF-related factor gene. Biochem. Biophys. Res. Comm. 1996, Vol. 220, pages 922-928, (see especially: Abstract and Introduction, page 922-top of 923)	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 JUNE 2000	21 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JANE ZARA
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Form PCT/ISA/210 (second sheet) (July 1998)★

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PCT/US00/11096

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OLOFSSON et al. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc. Natl. Acad. Sci. USA. 1996, Vol. 93, pages 2576-2581. (Abstract and Introduction, page 2576; figures 1 and 2, pages 2577 and 2588; last paragraph of Results, page 2578; Discussion, pages 2578-2581).	1-5, 7-12
Y	US 5,194,596 A (TISCHER et al.) 16 March 1993, entire text, especially col. 10, line 63-col. 1,2 line 55.	1-5, 7-12
Y	US 5,607,918 A (ERIKSSON et al.) 04 March 1997, entire text, col. 11, line 6-col. 12, line 10	1-12
Y	US 5,840,693 A (ERIKSSON et al.) 24 November 1998, entire text, especially col. 26, line 30-col. 27, line 18, col. 19, lines 5-41 and col. 30, line 26-col.31, line 4.	1-12
Y,P	US 5,928,939 A (ERIKSSON et al.) 27 July 1999, entire text, especially col. 3, line 14-col. 4, line 12.	1-5, 7-12

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IPC (7):

A01N 37/18, 43/04; A61K 38/00, 31/70; C12Q 1/68; C12P 21/06, 19/34, 21/08; C07H 21/02, 21/04; C07K 1/00, 2/00, 4/00, 7/00, 14/00, 16/00, 17/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/2, 44; 435/6, 69.1, 91.1, 455, 366, 375; 530/300, 350, 388.24; 536/23.1, 24.5