

Promoting the formation of new collateral vessels in ischemic tissues using angiogenic growth factors (therapeutic angiogenesis) is an exciting frontier of cardiovascular medicine. Conversely, inhibition of the action of key regulators of angiogenesis, such as VEGF, constitutes a promising approach for the treatment of solid tumors and intraocular neovascular syndromes. These concepts are being tested now in clinical trials.

Clinical applications of angiogenic growth factors and their inhibitors

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In embryos, blood vessels form through two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis involves the de novo differentiation of endothelial cells from mesodermal precursors, whereas in angiogenesis new vessels are generated from pre-existing ones¹. Vasculogenesis takes place only during embryonic development and leads to the formation of a primary vascular plexus. Later these rather uniformly sized endothelial channels are remodeled into a mature system consisting of a tree-like hierarchy of large and small vessels. New capillaries then form through angiogenesis, either by sprouting or by splitting (intussusception) from their vessels of origin. In adults, angiogenesis is essential for the female reproductive cycle, and for repair, remodeling and regeneration of tissues, for example during wound healing². Neovascularization is also important in pathological processes such as tumor growth and metastasis².

The known endothelial cell specific growth factors and their receptors can be classified into vascular endothelial growth factor (VEGF) and angiopoietin (Ang) families³ (Fig. 1). Among the various angiogenic factors, VEGF is probably the most essential for the development and differentiation of the vascular system⁴. Loss of a single VEGF allele results in embryonic lethality^{5,6} (Fig. 2). Even selective inactivation of the heparin-binding isoforms of VEGF, leaving one functional isoform (VEGF₁₂₀), is insufficient for the proper development of the cardiovascular system and results in myocardial ischemia and perinatal or early postnatal lethality⁷. Also, other angiogenic factors, such as FGFs may work more indirectly, some of them through the VEGFs and their receptors⁸, so that a thorough knowledge of the signal transduction pathways of VEGFs and angiopoietins is essential for their use in therapeutic settings.

Therapeutic angiogenesis and inhibition of arterial restenosis

An exciting frontier of cardiovascular medicine is therapeutic angiogenesis. Promoting the formation of new collateral vessels on the ischemic myocardium, leg muscles and other tissues would have an important effect on the treatment of disorders for which pharmacological intervention has been ineffective in controlled trials and for which therapy is now limited to surgical revascularization or endovascular interventional therapy⁹.

Several angiogenic molecules have been tested in animal models, including bFGF, aFGF, FGF-5, VEGF isoforms, VEGF-C, HGF/SF and Ang-1/Ang-2. The factors tested most extensively are VEGF and bFGF. In some cases, the recombinant protein was tested. In others, gene transfer using naked DNA or adenoviral vectors was used. A single intra-arterial administration of 500–1000 µg of rhVEGF₁₆₅ augmented perfusion and development of collateral vessels in a rabbit model of hindlimb ischemia in which the femoral artery was surgically removed¹⁰. Similar results were obtained in the same model

with intramuscular or intra-arterial administration of aFGF, bFGF, HGF/SF and VEGF-C (refs. 11–14). VEGF administration after removal of the femoral artery

not only resulted in increased vascularization but also led to recovery of the normal endothelial reactivity to various mediators¹⁵. Arterial gene transfer with cDNA encoding VEGF isoforms also led to revascularization to an extent comparable to that achieved with the recombinant protein¹⁶. Moreover, administration of a VEGF₁₆₅ adenovirus vector shortly after common iliac artery ligation in the rat was capable of stimulating an angiogenic response that protects against subsequent occlusion of the femoral artery, indicating that gene transfer of VEGF might be useful in the prophylaxis of advancing arterial occlusive disease¹⁷. As little as 2 µg rhVEGF delivered over 4 weeks periaortally, distal to the occlusion, resulted in a significant increase in coronary blood flow and functional improvement in a pig model of chronic myocardial ischemia¹⁸. Very similar results were obtained using bFGF (ref. 19). Unexpectedly, even a single intracoronary administration of VEGF (or bFGF) was efficacious in this model to an extent comparable to that of 4-week infusion, despite the fact that only a small fraction of protein localizes to the ischemic area²⁰. Given such results, it is conceivable that young and otherwise healthy animals are very responsive to exogenous growth factors in the context of ischemia. At least some of this responsiveness may be due to the upregulation of VEGF receptors in the endothelia of ischemic tissues²¹. Adenovirus-mediated gene transfer of VEGF₁₂₁ (ref. 22) or FGF-5 (ref. 23) also resulted in collateral vessel growth and functional improvement in porcine models of myocardial ischemia.

These encouraging animal studies led to clinical trials using recombinant VEGF₁₆₅, aFGF, bFGF or gene therapy with plasmid or with adenoviral vectors. There is considerable debate whether gene therapy or administration of recombinant protein would be preferable. Delivery of angiogenic proteins by gene therapy might not only minimize their systemic side effects, such as hypotension (VEGF) or nephrotoxicity (bFGF), but also provide a slow release of the encoded factor for 1–2 weeks, leading to a more lasting angiogenic response. However, slow release of the recombinant protein, using microspheres or heparin-alginate formulations, might achieve the same results, without the potential risks associated with the use of viral vectors.

Arterial gene transfer of naked plasmid DNA encoding VEGF₁₆₅ in a patient with severe limb ischemia produced angiographic and histologic evidence of angiogenesis in the knee, mid-tibial and ankle levels 4 weeks after the transfer²⁴. In a subsequent study, the VEGF₁₆₅ plasmid cDNA was injected intramuscularly²⁵. Gene transfer was done in ten limbs of nine patients with nonhealing ischemic ulcers and/or rest pain due

to peripheral arterial disease. Improvement in the ankle-brachial index and distal flow in eight limbs were reported²⁵. Additional small trials by the same group have also shown that local injection of the VEGF₁₆₅ plasmid DNA resulted in clinical improvement in patients affected by myocardial ischemia²⁶ or Burger's disease (thromboangiitis obliterans)²⁷. However, none of these studies were placebo-controlled. Clinical trials using VEGF-C naked DNA or adenovirus mediated gene transfer of VEGF₂₁ in myocardial ischemia patients are now in phase I. Femoral angiograms from a patient with limb ischemia, before and 3 months after transfection of a VEGF₁₆₅ plasmid/liposome expression vector, show increased vascular density after the treatment (Fig. 3). However, the trial is ongoing and some caution should be used in interpreting such data, until more patients and the effect of placebo are more extensively evaluated.

Clinical trials using recombinant VEGF₁₆₅ and bFGF are also ongoing. In a phase I study in patients with coronary ischemia in which rhVEGF₁₆₅ was administered by intracoronary infusion, the molecule was safely tolerated at all doses tested²⁸. There was evidence of improvement in perfusion in seven of fifteen subjects and improved collateralization in five of seven who underwent follow-up coronary angiography. However, a subsequent placebo-controlled phase II study, in which rhVEGF was delivered as a single intracoronary infusion, followed by three intravenous infusions, has not demonstrated clinical benefit²⁹. The treatment was not better than placebo in treadmill time and pain relief, at least at 60 days²⁹. Brief exposures to rhVEGF₁₆₅, such as those achieved in this trial, may be insufficient to trigger and maintain a therapeutically meaningful angiogenic response, especially in the context of extensive atherosclerotic disease. Also, systemic administration of rhVEGF₁₆₅ or other factor may fail to generate an appropriate angiogenic concentration gradient from ischemic to non-ischemic areas, a requisite aspect of angiogenesis in a variety of physiological and pathological circumstances¹. Moreover, the placebo effect is probably greater than initially suspected, and even patients with very compromised myocardial function may show a substantial improvement with placebo. A phase II study with bFGF for coronary ischemia is now ongoing.

Local gene transfer into the vascular wall offers a promising alternative for the treatment of the complication of restenosis after percutaneous transcoronary angioplasty (PTCA) and coronary stenting. Restenosis occurs in many treated patients in 6 months, leading to obstruction in 20–35% of the patients³⁰. The pathogenesis of restenosis depends on endothelial damage, which also predisposes arteries to other pathological conditions, such as spasms or thrombosis. Prophylaxis of restenosis could therefore be based on strategies for endothelial protection or enhancement of endothelial repair and endothelial growth factors or vascular gene transfer could be used for this³¹. Re-endothelization in balloon-injured rat carotid artery was accelerated by a single dose of recombinant VEGF injected into the bloodstream or locally^{32,33}. Vessel status was also improved by injection of VEGF plasmid into adventitial surface of rabbit carotid arteries³⁴. Intravascular gene transfer in the arterial

wall was not very efficient³⁵, but secreted proteins such as VEGF could be used for therapeutic gene transfer trials using infusion-perfusion catheters³⁶ or histamine-induced increase of endothelial permeability³⁷. Because VEGF and VEGF-C share one receptor (VEGFR-2) but differ in the other receptor, VEGF-C and VEGF₁₆₅ might have overlapping but distinct effects in the vessel wall. However, VEGF-C gene transfer inhibits intimal thickening early, and the protective effect is at least equal to that seen with VEGF₁₆₅ gene transfer³⁸.

Therapeutic inhibition of vascular endothelial growth factor Tumors

The growth of tumor xenografts in transparent chambers in mice is preceded by an increase in vascular density, indicating that the rapid growth of tumors depends on the development of a neovascular supply³⁹. In 1971, inhibition of angiogenesis was proposed as a valid strategy for the treatment of solid tumors and the search for the mediator(s) of tumor angiogenesis was begun⁴⁰.

Although inhibition of bFGF (ref. 41) or angiopoietin/Tie2 (refs. 42,43) may inhibit tumor growth, so far VEGF and its receptors constitute the most extensively investigated system in tumor angiogenesis and are now a main target of anti-cancer strategies. VEGF mRNA is substantially upregulated in most human tumors⁴. Although tumor cells represent the main source of VEGF, tumor-associated stroma is also an important site of VEGF production⁴⁴. There is a correlation between VEGF expression and microvessel density in primary breast cancer sections⁴⁵. A similar correlation has been described in several other malignancies, including gastric carcinoma⁴⁶. Furthermore, there are increases in plasma levels of VEGF in tumor patients compared with tumor-free individuals, and high VEGF levels before chemotherapy are associated with a poor outcome⁴⁷.

Direct evidence for involvement of VEGF in tumorigenesis was first demonstrated using monoclonal antibodies against

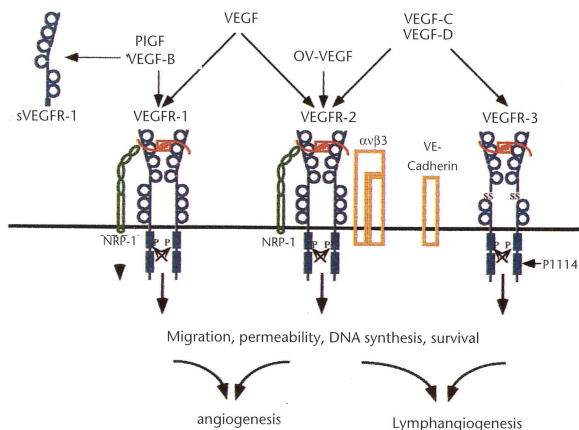


Fig. 1 VEGFs, their receptors and some of their endothelial effects in cells and tissues. Ligand binding induces receptor dimerization and subsequent auto/transphosphorylation, activates various signal transduction pathways and leads to differential cellular responses. sVEGFR-1, soluble VEGFR-1; HSPG, heparan sulphate proteoglycan; NP-1, neuropilin-1; α₅β₃ integrin α₅β₃ (reported to make a molecular complex with activated VEGFR-2; ref. 95). VE-cadherin is also able to form a complex with VEGFR-2, a requirement for VEGF-dependent anti-apoptotic signals involving the PI3-kinase/Akt pathway⁹⁶. P1114L, point mutation of VEGFR-3 affecting patients in a family with lymphoedema⁹⁷.

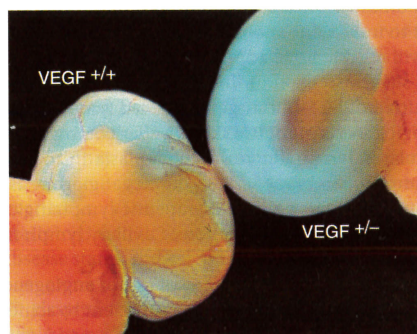


Fig. 2 Yolk sac of E10.5 VEGF^{+/+} and VEGF^{+/-} mouse embryos⁵. There is an apparent absence of vasculature in the yolk sac of the heterozygous, which die around E11. This is probably the only example among vertebrates of lethality after inactivation of a single allele of a gene that is not maternally imprinted.

VEGF in human xenografts in nude mice⁴⁸. These initial studies showed that several tumor cell lines can be substantially growth-inhibited by this treatment⁴⁸. These findings were extended to a broad variety of tumor cell lines, including carcinomas, sarcomas and gliomas⁴. Intravital videomicroscopy techniques have augmented our understanding of VEGF in tumorigenesis^{49,50}. Non-invasive imaging of the vasculature demonstrated a nearly complete suppression of tumor-associated angiogenesis in animals treated with monoclonal antibodies against VEGF compared with controls, providing a direct verification that inhibition of angiogenesis is the mechanism of tumor suppression after anti-VEGF treatment⁴⁹. Intravital microscopy techniques have also been used to investigate the effects of VEGF on the permeability and other properties of tumor vessels⁵⁰. Treatment with antibodies against VEGF resulted in time-dependent reductions in vascular permeability, in the diameter and tortuosity and eventually to a regression of tumor blood vessels; thus, VEGF is also an essential survival factor for tumor endothelial cells⁵⁰. Further evidence that VEGF action is required for tumor angiogenesis has been provided by the finding that retrovirus-mediated expression of a dominant negative VEGFR-2 mutant, which inhibits signal transduction through wild-type VEGFR-2 receptor, suppresses the growth of glioblastoma multiforme as well as other tumor cell lines *in vivo*⁵¹. Furthermore, high local expression of the soluble extracellular domain of VEGFR-1 or VEGFR-2, achieved by administration of the recombinant proteins, adenoviral-mediated gene transfer or by stable transfection of tumor cells, may significantly inhibit tumor growth, metastasis and mortality rate in nude mice^{52,53}.

Several strategies have been used to generate VEGF inhibitors suitable for clinical trials. One approach involves the 'humanization' of mouse monoclonal antibodies. A chief advantage of 'humanized' antibodies is a high degree of specificity, combined with a long half-life and little or no immunogenicity. A 'humanized' high-affinity monoclonal antibody against VEGF (rhuMab VEGF) with the same affinity and biological properties as the original murine antibody has been described⁵⁴. Toxicological studies in primates have shown that the effects of rhuMab VEGF are limited to inhibition of angiogenesis in the female reproductive tract and in the epiphyseal growth plate in

sexually immature animals that have not completed statural growth⁵⁵. rhuMab VEGF is now in phase II clinical trials for the treatment of non-small cell lung carcinoma and colorectal carcinoma in conjunction with standard chemotherapy and for breast and renal cell carcinoma as a single agent. In addition, small molecules that inhibit VEGFR-2 signal transduction are undergoing phase II clinical trials in cancer patients⁵⁶. Furthermore, monoclonal antibodies against VEGFR-2 are entering clinical trials.

Retinal ischemia and other conditions

Diabetes mellitus, occlusion of the central retinal vein or prematurity with subsequent exposure to oxygen can all be associated with intraocular neovascularization⁵⁷. A common denominator among these conditions is retinal ischemia⁵⁷. The new blood vessels may lead to vitreous hemorrhage, retinal detachment, neovascular glaucoma, and eventual blindness. Diabetic retinopathy is the leading cause of blindness in the working population. The hypothesis that ischemia-induced VEGF may be pathogenic in these conditions was initially tested by measuring VEGF levels in the eye fluids of patients. In a large series with 165 patients, a strong correlation was found between concentrations of VEGF in both aqueous and vitreous and active proliferative retinopathy associated with diabetes, occlusion of central retinal vein or prematurity⁵⁸. Direct evidence for the role of VEGF as a mediator of intraocular neovascularization has been generated in several animal models, including a primate model of iris neovascularization and a mouse model of retinopathy of prematurity. In the former, intraocular administration of monoclonal antibodies against VEGF substantially inhibits the neovascularization that follows the occlusion of central retinal veins⁵⁹. Likewise, soluble VEGFR-1 or VEGFR-2 extracellular domains fused to the immunoglobulin γ Fc domain suppress retinal angiogenesis in the mouse model⁶⁰. There is also evidence that growth hormone/insulin-like growth factor-1 is involved in ischemia-induced retinal neovascularization⁶¹.

Neovascularization is a principal cause of visual loss also in the wet form of age-related macular degeneration (AMD), the overall leading cause of blindness⁶². Several studies have documented the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients⁶³. These findings suggest involvement of VEGF in the progression of AMD-related choroidal neovascularization. Anti-VEGF strategies for AMD are now being explored in clinical trials. One approach consists in the intravitreal administration of a recombinant humanized anti-VEGF Fab antibody fragment. Another strategy involves the injection of 2'-fluoropyrimidine RNA oligonucleotide ligands (aptamers)⁶⁴.

VEGF inhibition may also have therapeutic value for the treatment of ischemic-reperfusion related brain edema and injury. VEGF antagonism has shown beneficial effects in a mouse model of cortical ischemia⁶⁵; reducing acutely the volume of edematous tissue and resulting in a significant sparing of cortical tissue.

VEGF is important in angiogenesis in the female reproductive tract. VEGF inhibition results in suppression of corpus luteum angiogenesis in rodents⁶⁶ and primates⁵⁵. VEGF inhibitors might be used to treat conditions characterized by ovarian hyperplasia and hypervascularity, such as the polycystic ovary syndrome⁶⁶. VEGF-dependent angiogenesis may also be important pathogenically in endometriosis. Furthermore, VEGF is a

mediator of the ovarian growth and increased vascular permeability of ovarian hyperstimulation syndrome, a potentially fatal condition characterized by massive ovarian enlargement that may follow medical induction of ovulation with gonadotropins⁶⁷.

Perspectives

VEGF₁₆₅ binds to neuropilin-1, which functions as a ligand binding subunit of putative transmembrane receptors mediating specific signals for different semaphorins, the molecules mediating the collapse of axonal growth cones⁶⁸. Neuropilin is expressed in endothelial cells and enhances the mitogenic effects of VEGFR-2 upon VEGF₁₆₅ stimulation. Thus, there may be an as-yet ill-defined cross-regulation of cellular signals between these two families of factors. These findings lead to the intriguing conclusion that the processes of axon guidance and development of a network of capillary tubes share at least some common molecular mechanisms. In addition, the angiopoietin receptor/Tie and ephrin families of endothelial tyrosine kinases have important functions in the formation and maintenance of the vascular system⁶⁹⁻⁷¹. Endothelial cell-specific members of the TGF- β receptor and Notch families have also been described^{72,73}. Given this complexity of vascular endothelial signaling, therapies using VEGF alone or any other single angiogenic factor may produce incompletely functioning or unstable endothelial channels with defective arteriovenous and pericellular differentiation, characteristic of many tumors⁷⁴. Combinations of growth factors may be preferable in future therapies directed to neovascularization of tissues, with an adequate investment of the formed vessels with periendothelial matrix and pericyte/smooth muscle cells. In fact, a more heterogeneous set of genes coordinating angiogenic functions may be provided by active ongoing research of hypoxia-regulated gene expression in mammalian cells⁷⁵. Also, some virus-encoded proteins, such as the VEGFR-2 activating HIV Tat protein⁷⁶, Kaposi sarcoma herpesvirus-associated G-protein-coupled receptor⁷⁷ or Orf virus encoded VEGF-E⁷⁸⁻⁸⁰ may offer new insights into the mechanism of regulation of angiogenesis.

Although recent research has focused on the combination of VEGF and Ang-1 as being especially promising, it is not known now which growth factor combinations will prove to be the most effective therapeutically. VEGF and bFGF have a very synergistic effect in the induction of angiogenesis, both *in vitro* and *in vivo*⁴. The interaction between VEGF and HGF/SF is also being actively investigated. Although transgenic expression of Ang-1 in the skin epidermis under the keratin (K)14 promoter has been associated with neovascularization⁸¹, other studies, using defined amounts of the recombinant protein in a model of adult neovascularization, have failed to demonstrate strong angiogenic responses to Ang-1, unless it is used in combination with VEGF (refs. 71,82). This discrepancy may be explained by the fact that the expression of the K14 promoter is initiated already at midgestation, and thus the results may reflect persistence of the fetal neovascularization. It is possible, however, that Ang-1 may provide a co-factor for combination therapies. A further unresolved issue is the correct dosage of growth factor(s). This seems particularly important for a molecule like VEGF, which has several isoforms and such a tight dose-response effect that a 50% reduction in expression results in lethality during embryonic life^{5,6}. Conversely, continuous local overexpression of VEGF may result in a hemangioma-like vasculature and thus can be deleterious⁸³.

Also, it is unknown whether an angiogenic treatment may be sufficient to induce functional blood vessels for prolonged periods or will need to be re-administered periodically in order to maintain such vessels.

A K14-driven VEGF-C transgene induced lymphangiogenesis but no angiogenesis in mouse skin⁸⁴, and recombinant VEGF-C also stimulated lymphatic vessel hyperplasia in mature chick chorioallantoic membrane⁸⁵. Thus, besides angiogenesis, it may also become possible to direct therapeutic lymphangiogenesis in patients, such as after evacuation of axillary lymph nodes in breast carcinoma surgery.

Despite the potential redundancy of tumor angiogenesis factors, inhibition of VEGF alone seems sufficient to achieve considerable tumor growth suppression in a wide variety of models. However, it remains to be established whether tumors are able to activate, after prolonged therapy, alternative angiogenic pathways that might confer resistance to the treatment. These issues should be addressed in the current clinical trials with various VEGF inhibitors. A challenge now in anti-VEGF (and anti-angiogenic) therapy is devising appropriate and reliable markers to monitor tumor progression. There is considerable debate whether blood vessel count in biopsy specimens^{45,46} may provide a reliable indicator of response to the treatment. There are also efforts to identify surrogate endpoints, applying non-invasive approaches, such as magnetic resonance imaging⁸⁶.

VEGF is not only a mitogen but also a potential survival factor for endothelial cells⁴. Such a 'maintenance' function seems to be developmentally regulated, as it is very dependent on the age of the animal⁸⁷. VEGF inactivation during early postnatal life, achieved by *Cre-loxP*-mediated inducible gene targeting of by administration of a soluble VEGFR-1 chimeric protein, results in regression of the vasculature, kidney failure and lethality⁸⁷. However, in adult animals a similar treatment has no effects on the existing vasculature. Therefore, a process of maturation occurs in endothelial cells such that VEGF eventually is not essential for survival. This switch seems to take place in the mouse around the fourth postnatal week. Absence of pericyte

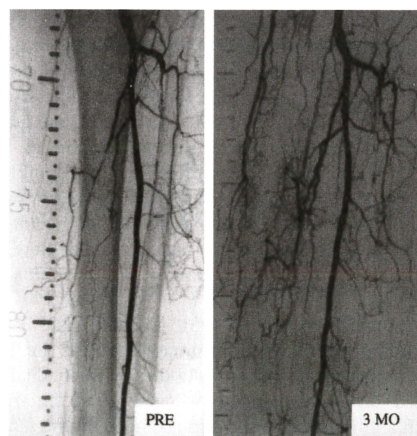


Fig. 3 Angiography of the lower extremity of a patient with limb ischemia before (PRE) and 3 months after (3 MO) the transection of a VEGF165 plasmid/liposome expression vector, showing strongly increased vascular density after the treatment. Courtesy H. Manninen, P. Mätsi, K. Mäkinen, M. Hilpeläinen, M. Laitinen, E. Alhava and S. Ylä-Herttuala, A. I. Virtanen Institute and Kuopio University Hospital (Kuopio, Finland).

coverage in immature vessels may be a factor determining their dependence on VEGF (ref. 88). However, other evidence suggests that the molecular/intracellular nature of this switch may be more complex and mostly still to be determined⁸⁷. In juvenile animals, VEGF is essential for endochondral bone formation and longitudinal growth^{89,55}. In the fully developed animal, VEGF may be required mainly for active angiogenic processes such as corpus luteum development or wound healing. Nevertheless, VEGF may be important for endothelial homeostasis in the adult in certain circumstances; for example, during disease states. Indeed, prolonged VEGF inhibition failed to induce glomerular damage in normal primates⁵⁵ or rodents^{87,90}, despite the strong constitutive expression of the VEGF mRNA in podocytes and other cell types in the adult kidney⁴. However, administration of VEGF inhibitors to rats with mesangioproliferative nephritis results in impaired glomerular endothelial regeneration and increased endothelial cell death⁹⁰.

Some CD34⁺ hematopoietic progenitor cells mobilized by GM-CSF from human peripheral blood, bone marrow, fetal liver or umbilical cord blood were shown to express VEGFR-2 on their surface⁹¹, and VEGFR-2 is expressed on human hematopoietic stem cells⁹². Endothelial progenitor cells expand and differentiate into endothelial cells after addition of bFGF and VEGF to the cultures, and they can thus be considered to provide endothelial progenitor cells⁹¹⁻⁹³. The endothelial progenitor cells from bone marrow may be mobilized using the stromal-derived factor 1 chemokine, the GM-CSF cytokine or tissue hypoxia⁹⁴. As these cells may be capable of participating in active angiogenesis after entry into the circulatory system⁹⁴, they provide an interesting possibility for the delivery of cellular or gene therapy to sites of neovascularization.

Finally, the first placebo-controlled clinical study with rhVEGF may have brought a more realistic assessment of the potential of therapeutic angiogenesis and raised a number of questions. For example, how can one explain the discrepancy between the considerable efficacy observed even with very small amounts of growth factors in animal models of coronary or limb ischemia and the rather disappointing clinical results? An essential difference may lie in the fact that young and otherwise healthy animals are able to mount an effective endogenous angiogenic response that can be maximized by an additional stimulus provided by a recombinant protein or gene therapy. In contrast, patients with extensive atherosclerotic disease may have poor responses. It is possible, however, that a more persistent exposure to an individual growth factor or to a combination of growth factors may be effective. Clinical trials now ongoing should answer at least some of these questions over the next 2-3 years.

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