Promoting the formation of new collateral vessels in ischemic tissues using angiogenic growth factors (therapeutic angiogenesis) is a 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 ones[1]. 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 healing[2]. Neovascularization is also important in pathological processes such as tumor growth and metastasis[2].

The known endothelial cell specific growth factors and their receptors can be classified into vascular endothelial growth factor (VEGF) and angiopoietin (Ang) families[3] (Fig. 1).Among the various angiogenic factors, VEGF is probably the most essential for the development and differentiation of the vascular system[4]. 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 ($VEGF_{120}$), is insufficient for the proper development of the cardiovascular system and results in myocardial ischemia and perinatal or early postnatal lethality[7]. Also, other angiogenic factors, such as FGFs may work more indirectly, some of them through the VEGFs and their receptors[8], 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 therapy[9] .

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 $rhVEGF_{165}$ augmented perfusion and development of collateral vessels in a rabbit model of hindlimb ischemia in which the femoral artery was surgically removed[10]. 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 mediators[15]. Arterial gene transfer with cDNA encoding VEGF isoforms also led to revascularization to an extent comparable to that achieved with the recombinant protein[16]. Moreover, administration of a $VEGF_{165}$ 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 disease[17]. As little as 2 μg rhVEGF delivered over 4 weeks periadventitially, distal to the occlusion, resulted in a significant increase in coronary blood flow and functional improvement in a pig model of chronic myocardial ischemia[18]. 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 area[20]. 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 tissues[21]. Adenovirus-mediated gene transfer of $VEGF_{121}$ (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 $VEGF_{165}$, 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 $VEGF_{165}$ 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 transfer[24]. In a subsequent study, the $VEGF_{165}$ plasmid cDNA was injected intramuscularly[25]. 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 reported[25]. Additional small trials by the same group have also shown that local injection of the $VEGF_{165}$ plasmid DNA resulted in clinical improvement in patients affected by myocardial ischemia[26] 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 $VEGF_{121}$ 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 $VEGF_{165}$ 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 $VEGF_{165}$ and bFGF are also ongoing. In a phase I study in patients with coronary ischemia in which $rhVEGF_{165}$ was administered by intracoronary infusion, the molecule was safely tolerated at all doses tested[28]. 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 benefit[29]. The treatment was not better than placebo in treadmill time and pain relief, at least at 60 days[29]. Brief exposures to $rhVEGF_{165}$, 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 $rhVEGF_{165}$ 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 circumstances[1]. 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 patients[30]. 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 this[31]. 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 arteries[34]. Intravascular gene transfer in the arterial wall was not very efficient[35], but secreted proteins such as VEGF could be used for therapeutic gene transfer trials using infusion-perfusion catheters[36] or histamine-induced increase of endothelial permeability[37]. Because VEGF and VEGF-C share one receptor (VEGFR-2) but differ in the other receptor, VEGF-C and $VEGF_{165}$ 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 $VEGF_{165}$ gene transfer[38].

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 supply[39]. 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 begun[40].

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 tumors[4]. Although tumor cells represent the main source of VEGF, tumor-associated stroma is also an important site of VEGF production[44]. There is a correlation between VEGF expression and microvessel density in primary breast cancer sections[45]. A similar correlation has been described in several other malignancies, including gastric carcinoma[46]. 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 outcome[47].

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

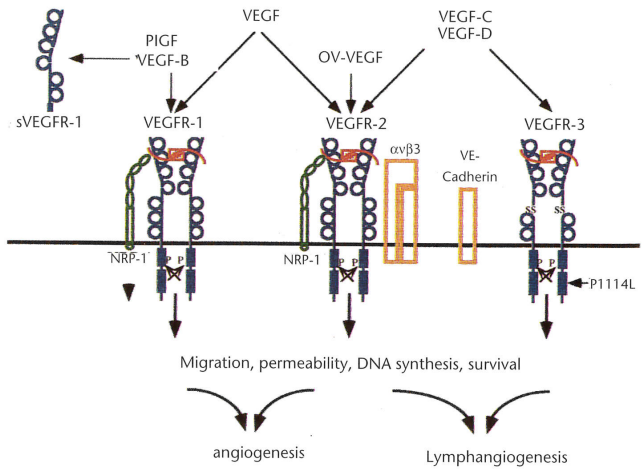

Fig. 1 VEGFs, their receptors and some of their endothelial effects in cells and tissues. Ligand binding induces receptor dimerization and subsequent auto/transphosphorylation, activates various signal transduction pathways and leads to differential cellular responses. sVEGFR-1, soluble VEGFR-1; HSPG, heparan sulphate proteoglycan; NP-1, neuropilin-1; a_vb_3, integrin a_vb_3 (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 pathway[96]. P1114L, point mutation of VEGFR-3 affecting patients in a family with lymphoedema[97].

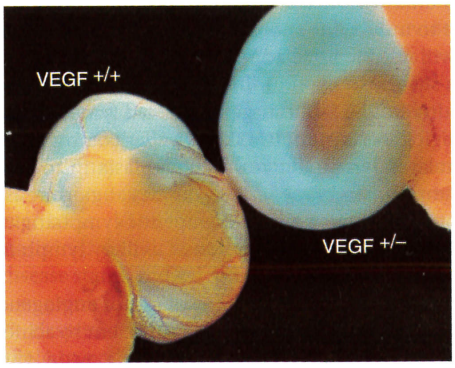

Fig. 2 Yolk sac of E10.5 VEGF$^{+/+}$ and VEGF $^{+/-}$ mouse embryos[5]. There is an apparent absence of vasculature in the yolk sac of the heterozygous, which die around E11. This is probably the only example among vertebrates of lethality after inactivation of a single allele of a gene that is not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

VEGF in human xenografts in nude mice[48]. These initial studies showed that several tumor cell lines can be substantially growth-inhibited by this treatment[48]. These findings were extended to a broad variety of tumor cell lines, including carcinomas, sarcomas and gliomas[4]. 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 treatment[49]. Intravital microscopy techniques have also been used to investigate the effects of VEGF on the permeability and other properties of tumor vessels[50]. 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 cells[50]. 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 described[54]. 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 growth[55]. 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 patients[56]. 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 neovascularization[57]. A common denominator among these conditions is retinal ischemia[57]. 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 prematurity[58]. 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 veins[59]. Likewise, soluble VEGFR-1 or VEGFR-2 extracellular domains fused to the immunoglobulin γ Fc domain suppress retinal angiogenesis in the mouse model[60]. There is also evidence that growth hormone/insulin-like growth factor-1 is involved in ischemia-induced retinal neovascularization[61].

Neovascularization is a principal cause of visual loss also in the wet form of age-related macular degeneration (AMD), the overall leading cause of blindness[62]. Several studies have documented the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients[63]. 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 ischemia[65]; 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 rodents[66] and primates[55]. VEGF inhibitors might be used to treat conditions characterized by ovarian hyperplasia and hypervascularity, such as the polycystic ovary syndrome[66]. 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 gonadotropins[67].

Perspectives

$VEGF_{165}$ 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 cones[68]. Neuropilin is expressed in endothelial cells and enhances the mitogenic effects of VEGFR-2 upon $VEGF_{165}$ 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 system[69-71]. 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 tumors[74]. 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 heterogenous set of genes coordinating angiogenic functions may be provided by active ongoing research of hypoxia-regulated gene expression in mammalian cells[75]. Also, some virus-encoded proteins, such as the VEGFR-2 activating HIV Tat protein[76], Kaposi sarcoma herpesvirus-associated G-protein-coupled receptor[77] or Orf virus encoded VEGF-E[78-80] 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 neovascularization[81], 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 deleterious[83]. 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 skin[84], and recombinant VEGF-C also stimulated lymphatic vessel hyperplasia in mature chick chorioallantoic membrane[85]. 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 imaging[86].

VEGF is not only a mitogen but also a potential survival factor for endothelial cells[4]. Such a 'maintenance' function seems to be developmentally regulated, as it is very dependent on the age of the animal[87]. 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 lethality[87]. 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

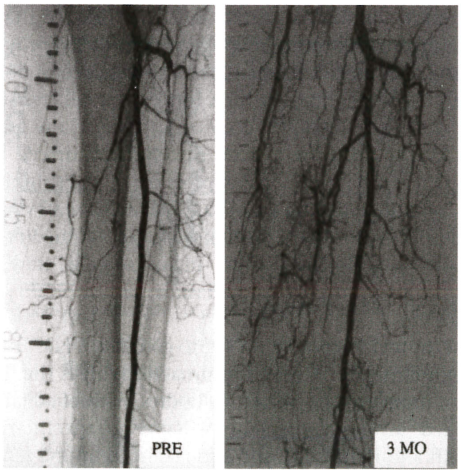

Fig. 3 Angiography of the lower extremity of a patient with limb ischemia before (PRE) and 3 months after (3 MO) the transfection of a VEGF165 plasmid/liposome expression vector, showing strongly increased vascular density after the treatment. Courtesy H. Manninen, P. Matsi, K. Mäkinen, M. Hilpeläinen, 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/intracelullar nature of this switch may be more complex and mostly still to be determined[87]. 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. Neverthless, 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 primates[55] or rodents[87,90], despite the strong constitutive expression of the VEGF mRNA in podocytes and other cell types in the adult kidney[4]. However, administration of VEGF inhibitors to rats with mesangioproliferative nephritis results in impaired glomerular endothelial regeneration and increased endothelial cell death[90].

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 surface[91], and VEGFR-2 is expressed on human hematopoietic stem cells[92]. 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 cells[91–93]. The endothelial progenitor cells from bone marrow may be mobilized using the stromal-derived factor 1 chemokine, the GM-CSF cytokine or tissue hypoxia[94]. As these cells may be capable of participating in active angiogenesis after entry into the circulatory system[94], 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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