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THERAPEUTIC ANGIOGENESIS

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1. ABSTRACT

Therapeutic angiogenesis constitutes a fundamental survival mechanism that acts to preserve the integrity of tissues subjected to ischemia. Supplemental administration of angiogenic cytokines - as recombinant protein or plasmid DNA - have been shown to augment collateral development when endogenous angiogenesis is suboptimal for organ function, and thus constitute a novel therapeutic option for the treatment of cardiovascular disease. These angiogenic cytokines, all of which share in common the ability to act as mitogens for endothelial cells, do not promote angiogenesis in an indiscriminate fashion; thus angiogenic cytokines selectively produce neovascularization in the ischemic tissues. The purpose of this review is to consider the mechanisms responsible for therapeutic angiogenesis which develops endogenously as well as strategies which have been devised to augment this response. The development of blood vessels is considered from the context of the embryonic paradigm; certain principles which have emerged from studies of pathologic neovascularization; and, principally, the development of collateral blood vessels supplying ischemic tissues, either endogenously or in response to administered growth factors.

2. INTRODUCTION

The development of blood vessels may be considered in several contexts. Vasculogenesis and angiogenesis are the processes responsible for the development of the circulatory system, the first functional unit in the developing embryo (1). Pathologic angiogenesis includes the role of post-natal neovascularization in the pathogenesis of arthritis, diabetic retinopathy, and, most notably, tumor growth and metastasis (2). Therapeutic angiogenesis involves the development of collateral blood vessels supplying ischemic tissues, either endogenously or in response to administered growth factors. The purpose of this review is to consider the mechanisms responsible for therapeutic angiogenesis which develops endogenously as well as novel strategies which have been devised to augment this response. Because recapitulation of the embryonic paradigm forms the conceptual basis for therapeutic, as well as pathologic angiogenesis, selected aspects of embryonic blood vessel development are included. While pathologic angiogenesis is beyond the scope of the current paper, certain principles which have emerged from studies of pathologic neovascularization are considered for the implications they may have for cardiovascular disease.

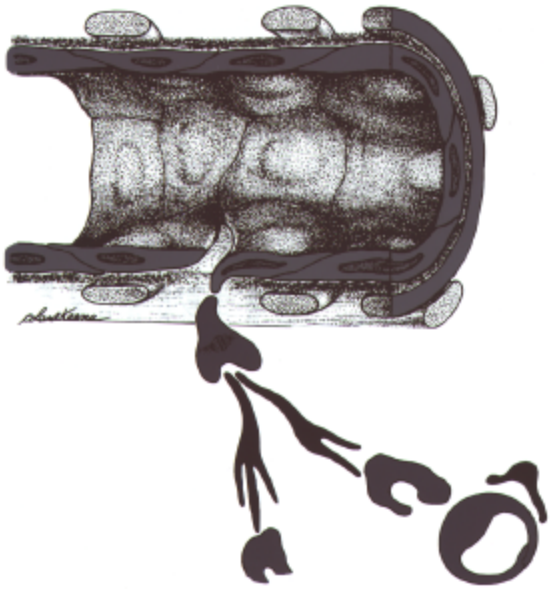

Figure 1. Classic paradigm for angiogenesis. ECs break free from their basement membrane and surrounding extracellular matrix, migrate, proliferate, and remodel (i.e. form a lumen), thus generating new blood vessels or "sprouts" from the parent vessel. (After D'amore and Thompson[10]).

3. DISCUSSION

3.1. Vasculogenesis and Angiogenesis

Vasculogenesis refers to the in situ formation of blood vessels from progenitor endothelial cells (ECs), or angioblasts (3). It is necessary to distinguish between vascular development which takes place in the yolk sac of the embryo from that which occurs in the embryo proper. Extraembryonic vasculogenesis begins as a cluster formation, or blood island. Growth and fusion of multiple blood islands in the yolk sac of the embryo ultimately give rise to the yolk sac capillary network (4); after the onset of blood circulation, this network differentiates into an arteriovenous vascular system (5). The integral relationship between the elements which circulate in the vascular system - the blood cells - and the cells which are principally responsible for the vessels themselves - ECs - is implied by the composition of the embryonic blood islands. The cells destined to generate hematopoietic cells are situated in the center of the blood island and are termed hematopoietic stem cells (HSCs). Endothelial progenitor cells, or angioblasts, are located at the periphery of the blood islands. In addition to this spatial association, HSCs and angioblasts share certain antigenic determinants, including Flk-1, Tie-2, and CD-34. These progenitor cells have consequently been considered to derive from a common precursor, putatively termed a hemangioblast (6-8).

Vasculogenesis which occurs within the embryo proper is currently considered to involve differentiation of so-called "solitary" angioblasts, i.e. angioblasts which are not intimately associated with concomitantly differentiating HSCs (3). These angioblasts may migrate and fuse with other angioblasts and capillaries or form vessels *in situ*. In contrast to *in situ* differentiation of progenitor cells required to establish the primordial vacular network, extension of the primitive vasculature involves angiogenesis, i.e. sprouting of new capillaries from the pre-existing network; by definition, this implicates differentiated ECs as the responsible cellular element. Full development of the circulatory system involves recurrent remodeling as some vessels regress, presumably by apoptosis, and others branch and/or are invested with a multilayer architecture characteristic of medium to large arteries and veins. The extensive EC proliferative activity, which constitutes the basis for angiogenesis in the embryo, contrasts with extraordinary EC quiescence in the adult, where the interval for EC turnover is estimated to be >1000 days (9).

3.1.1. Classic paradigm for angiogenesis

Until recently (see below), vasculogenesis was considered retricted to the embryo, while new blood vessel formation in adult species was inferred to be the exclusive consequence of angiogenesis. The full paradigm for angiogenesis has been suggested to begin with "activation" of ECs within a parent vessel, followed by disruption of the basement membrane, and subsequent migration of ECs into the interstitial space, possibly in the direction of an ischemic stimulus (10) (figure 1). Concomitant and/or subsequent EC proliferation, intracellular-vacuolar lumen formation, pericyte "capping", and production of a basement membrane complete the developmental sequence.

During angiogenesis, migration always precedes proliferation by approximately 24 hrs (11). Sholley *et al* (12) documented the critical if not exclusive roles of migration and redistribution of pre-existing ECs in the commencement of neovascularization. Subsequent studies have established the critical role played by plasmin and other proteases in promoting migration through pre-existing matrix (13,14).

In contrast to these *in vivo* inflammatory and *in vitro* organ culture models, angiogenesis which develops in response to experimental vascular obstruction, i.e. collateral vessel development, has been shown by several previous investigators to involve proliferation of not only ECs, but SMCs as well. Peak EC proliferation which contributes to naturally occurring collateral development in the setting of vascular occlusion varies from 2.6 % to 3.5 % in the canine coronary circulation (15,16); from 5 to 6 % in the rodent renal vasculature (17); and is < 1 % in swine coronaries (18). Proliferation of SMCs, the additional requisite cell type for the formation of larger blood vessels, is an implicit component of angiogenesis, regardless of animal species or circulatory site. Schaper *et al* in fact speculated nearly 25 years ago that "... it is tempting to assume that EC proliferation not only serves the purpose of forming the endothelium of a finally larger artery but rather actively participates in the development of the tunica media" (19). Proliferative activity - for SMCs as well as ECs - is highest at the level of the smallest-diameter collateral vessels, the so-called midzone collateral segments (15,16,20,21). Fifth, while evidence of EC and SMC proliferation alone does not

necessarily distinguish new vessel development from an increase in the size of pre-existing vessels, adjunctive data regarding increased capillary density (22,23) support the notion that proliferative activity does in fact reflect true angiogenesis.

3.1.2. Ligand-receptor systems modulate vasculogenesis and angiogenesis

A series of gene targeting studies have elucidated the role of certain ligands and/or their receptors in vasculogenesis and angiogenesis. The phenotypic characteristics of these "knockout" mice are relevant to adult cardiovascular disease because of the implications they may have for the role played by these same ligand-receptor systems in promoting post-natal angiogenesis.

As indicated above, KDR (the murine equivalent is known as flk-1 or VEGFR-2), the principal receptor for vascular endothelial growth factor (VEGF), is expressed by both angioblasts and HSCs. It is perhaps not surprising, therefore, that mice deficient in this gene die in utero between 8.5 and 9.5 days post-coitum due to an early defect in the development of hematopoietic and ECs. Yolk-sac blood islands were absent at 7.5 days, organized blood vessels could not be observed in the embryo and yolk sac at any stage, and hematopoietic progenitors were severely reduced (24). Markers of early endothelial precursors such as flt-1, flt-4, and tie-2 were expressed, but a marker of later endothelial development, tie-2, could not be detected, indicating a deficiency of mature ECs (24). Expression of CD34, a marker of HSCs, was greatly reduced as well. The absence of blood islands and blood vessels in these mice established that the flk-1 signalling pathway is required very early in the development of endothelial lineage, and may be important for blood cell development as well.

Findings in the flk-1 knockout mouse predicted what was to be found when the ligand, VEGF, was deficient. Mice deficient in even one of two VEGF alleles die in utero between days 10.5 and 12. Both blood island formation (vasculogenesis) and vascular sprouting from pre-existing vessels (angiogenesis) were again impaired (25,26). The failure of blood-vessel ingrowth was accompanied by apoptosis and disorganization of neuroepithelial cells. The heterozygous lethal phenotype was interpreted as evidence for tight dose-dependent regulation of embryonic vessel development by VEGF. Parenthetically, the aortas of VEGF-deficient mice have been noted to be hypoplastic, similar to that observed in mice deficient in endothelial nitric oxide (NO) synthase (P. Huang, personal communication). It is interesting to speculate that this may reflect the role of NO in VEGF-modulation of EC function (see below).

The tyrosine kinase flt-1 receptor (VEGF-R1) constitutes a second high-affinity binding receptor for VEGF. Mouse embryos homozygous for a targeted mutation in the flt-1 locus formed fully differentiated ECs in both embryonic and extra-embryonic regions, but assembled these cells into abnormal vascular channels and died in utero (27). Blood islands, for example, were disorganized, consisting of intermixed angioblasts and HSCs. In the head mesenchyme, instead of progressive development of individual small vessels, large fused vessels were seen which contained internally localized groups of ECs. These findings were interpreted as evidence that the flt-1 signalling pathway may regulate normal EC cell-cell or cell-matrix interactions during vascular development.

Tie-1 and tie-2 (28) comprise a second family of receptor tyrosine kinases, other than the VEGF family, in which expression is nearly specific for ECs. Mice embryos deficient in tie-1 fail to establish structural integrity of otherwise differentiated ECs (29); consequently, erythrocytes extravasate through the blood-vessel EC (but not between ECs) leading to death immediately after birth with widespread hemorrhage. Embryos homozygous mutant for tie-2 die earlier (day 10.5 in utero) with dilated vessels lacking distinction between small and large vessels; absence of ordered branching has been inferred as evidence of disordered angiogenesis. Vasculogenesis per se was not disrupted.

Successful disruption of the ligand for tie-2, angiopoietin-1 (30,31), resulted in embryonic lethality by day 12.5 with defects similar to those seen in the tie-2 receptor knockout. These included defects in organized branching, so that vessels remained dilated and almost syncitial. There was no change, however, in the total number of ECs.

The critical roles played by VEGF and its receptors in governing vasculogenesis and angiogenesis, and the tie receptor/ligand family in maturation of the vascular network, have implications for the roles of these EC mitogens in promoting angiogenesis under circumstances of tissue ischemia in adults. In contrast to the lethal consequences of VEGF and tie deficiencies, it is interesting to note that mice in which gene targeting has been used to disrupt the gene for basic fibroblast growth factor survive to maturity with no apparent phenotypic abnormalities of either the vascular or hematopoietic systems (Gerald Dorn and Tom Deutschman, personal communication).

3.1.3. Remodeling

Remodeling in angiogenesis refers to the formation of a vascular lumen. Whereas multiple cell types, including ECs, grown *in vitro* on a collagen-matrix gel will form cords, the presence of a lumen distinguishes vessels, or tubes, from solid cords. The presence of a lumen is clearly fundamental to the function of the circulatory system; yet the mechanisms responsible for tube formation are perhaps the least well understood aspect of angiogenesis (32). As Risau has pointed out, since isolated ECs may

combine to form a lumen *in vitro* (33), lumen formation must represent an intrinsic feature or differentiation program of these cells (3). Mechanisms which have been discussed include the joining of polarized ends of capillary ECs in a ring-like fashion, or alternatively, simple deletion of a portion of the cell (vacuole formation) (34). Adding further to the complexity is the requirement to form qualitatively differing luminal and abluminal surfaces. Finally, it is inferred that remodeling comprises a co-ordinated process of lumen formation and vessel extension with fusion of individual cells and their lumina via cell-cell adhesion molecules. The specific molecules responsible for vessel extension remain ambiguous. It is presumed that this aspect of vessel formation is subject to the regulatory factors responsible for vascular development in the embryo, as discussed above. In this respect, it is interesting to note that VEGF, for example, upregulates gap junction expression (specifically connexin 43) in ECs (35).

3.1.4. Nascent vessels

Capillary growth rates (i.e. the velocity of neovascularization) range from 0.23 to 0.8 mm/day, depending on the experimental system used and/or the type of tumor (36). The light microscopic features of newly formed vessels have been distinguished from those of native vessels (37). Whereas a histologic section of a capillary blood vessel in the normal brain reveals one or two ECs per lumen, in a brain tumor such as a glioblastoma, 5-10 ECs may occupy one lumen. Tumor-induced vessels often appear dilated and saccular. Moroever, tumors may contain giant capillaries and arteriovenous shunts without intervening capillaries, so that blood may even flow from one venule to another.

Ultrastructural analysis of newly formed vessels has focussed on features potentially responsible for augmented permeability. Dvorak *et al* (38,39), for example, found that vascular leakage could not be attributed to passage of molecules through inter-endothelial cell junctions or injured tumor endothelium, but instead involved transendothelial transport via a novel cytoplasmic organelle which they termed the vesicular-vacuolar organelle (VVO). Others (40) have reported VEGF-induced ultrastructural features consistent with endothelial fenestration.

3.1.5. Matrix-integrin interactions

Activated or proliferative ECs have been shown to express high levels of $\alpha v\beta 3$ (41,42). In non-human primates subjected to focal cerebral ischemia, for example, microvascular expression of $\alpha v\beta 3$ was noted in ischemic, but not non-ischemic, tissues (43). Ligation of $\alpha v\beta 3$ on proliferating ECs promotes a critical adhesion-dependent cell survival signal leading to inhibition of p53 activity, decreased expression of $p21^{WAF1/CIP1}$ and suppression of the bax cell death pathway (44). The intra-cellular molecular conflict which results from blocking $\alpha v\beta 3$ thus leads to unscheduled apoptosis and the abrogation of angiogenesis (42,44,45). Failure to ligate $\alpha v\beta 3$ may therefore inhibit the ability of actively cycling cells to ligate extracellular matrix proteins including fibronectin, vitronectin, fibrinogen, and osteopontin, thereby influencing adhesion, migration, and ultimately survival of these cells (46).

Consistent with this notion, the integrin $_{v}$ $_{3}$ has been shown to be required for angiogenesis *in vivo* (41) and antagonists of this integrin have been shown to inhibit angiogenesis by inducing apoptosis.(42) Basic FGF, which has been shown to protect ECs from apoptosis,(47) is known to modulate integrin expression by ECs.(48-50) VEGF, by up-regulation of both the $\beta 3$ integrin and fibronectin, may similarly inhibit apoptosis by enhancing EC adhesion to matrix proteins (51). This notion is thus consistent with the concept that VEGF may exert a survival effect on ECs (41,42,52-54). These findings suggest that the net increase in EC viability following VEGF administration is not limited to the mitogenic effects of VEGF on ECs, but is supplemented by the potential for VEGF to inhibit apoptosis.

3.1.6. Vasculogenesis in the adult

Postnatal neovascularization has been previously considered to result exclusively from the proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels, i.e. angiogenesis (3,9,15). The formation of blood vessels from EC progenitors, or angioblasts - i.e. vasculogenesis - has been considered restricted to embryogenesis (5,55).

We reasoned, however, that the use of HSCs derived from peripheral blood in lieu of bone marrow to provide sustained hematopoietic recovery constituted inferential evidence for circulating stem cells (56). Given the common ancestry of HSCs and angioblasts, we investigated the hypothesis that stem cells circulating in peripheral blood might under selected circumstances differentiate into ECs (57,58). Flk-1 and a second antigen, CD-34, shared by angioblasts and HSCs (24,59-69) were used to isolate putative angioblasts from the leukocyte fraction of peripheral blood. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EC progenitors incorporated into sites of active angiogenesis (figure 2). These findings thus suggest that circulating EC progenitors may contribute to neoangiogenesis in adult individuals, consistent with vasculogenesis. Parenthetically, these findings may have implications for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis, see below) and for delivery of anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis. A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (70) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (71). This issue may be successfully addressed

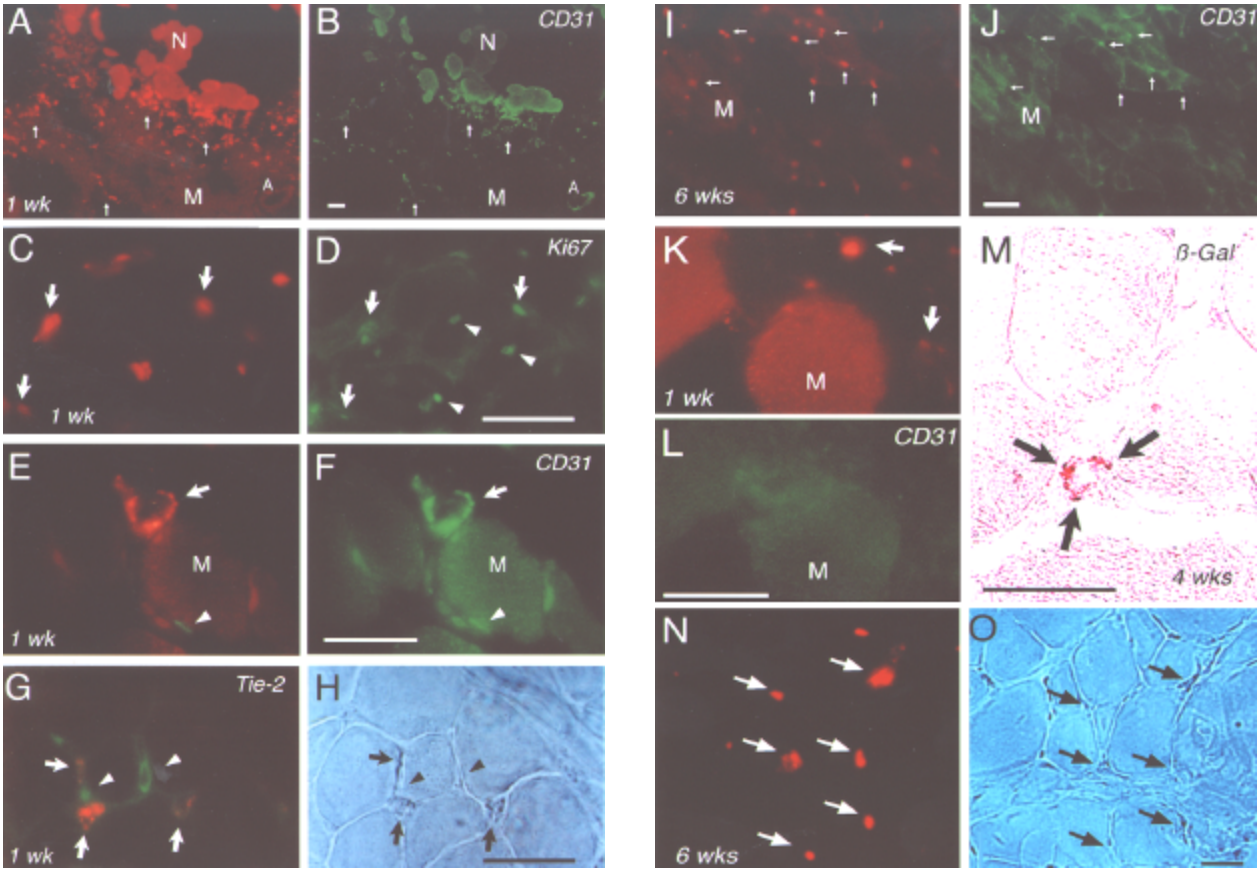

Figure 2. Heterologous (A to L), homologous (M), or autologous (N and O) EC progenitors incorporate into sites of angiogenesis *in vivo*. (A and B) CD34+ mononuclear peripheral blood cells (MB^{CD34+})(red, arrows), labeled with the floursecent dye DiI, between skeletal myocytes (M), including necrotic (N) myocytes 1 week after injection; most are co-labeled with CD31 (green, arrows). Note pre-existing artery (small A), identified as CD31 positive, but DiI-negative. (C and D) Evidence of proliferative activity among several DiI-labeled MB^{CD34+} derived cells (red, arrows), indicated by coimmunostaining for Ki67 antibody (green). Proliferative activity is also seen among DiI negative, Ki67 positive capillary ECs (arrowhead); both cell types comprise neovasculature. (E) DiI (red) and CD31 (green) in capillary ECs (arrows) between skeletal myocytes, photographed through double filter 1 week after DiI-labeled MB^{CD34+} injection. (F) Single green filter shows CD31 (green) expression in DiI-labeled capillary ECs, integrated into capillary with native (DiI negative, CD31 positive) ECs (arrowheads). (G) Immunostaining 1 week after MB^{CD34+} injection showing capillaries comprised of DiI-labeled MB^{CD34+} derived cells expressing Tie-2 receptor (green). Several MB^{CD34+}-derived cells (arrow) are Tie-2 positive and are integrated with some Tie-2 positive host capillary cells (arrowhead) identified by the absence of red fluorescence**.** (H) Phase contrast photomicrograph of same tissue section shown in G indicates corresponding DiI-labeled (arrows) and -unlabeled (arrowheads) capillary ECs**.** (I and J) Six weeks after administration, MB^{CD34+} derived cells (red) colabel for CD31 in capillaries between preserved skeletal myocytes. (K and L) One week after injection of MB^{CD34-}, isolated MB^{CD34-} derived cells (red, arrows) are observed between myocytes, but do not express CD31. (M) Immunostaining of β-galactosidase in tissue section harvested from ischemic muscle of B6,129 mice 4 weeks after administration of $MB^{Flk\text{-}1+}$ isolated from transgenic mice constitutively expressing -gal. (Flk-1 cell isolation was used for selection of EC progenitors due to lack of a suitable anti-mouse CD34 antibody.) Cells overexpressing -gal (arrows) have been incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 and anti-BS-1 lectin. (N and O) Sections of muscles harvested from rabbit ischemic hindlimb 4 weeks after administration of autologous MB^{CD34+}. Red fluorescence indicates localization of MB^{CD34+}-derived cells in capillaries, seen (arrows) in phase-contrast photomicrograph (O). Each scale bar indicates 50 μm. (From Asahara *et al*[58]).

Table 1. Angiogenic cytokines

ANGIOGENIC CYTOKINE	ABBREVIATION	REFERENCES
Acidic fibroblast growth factor	aFGF	124,159,164,167,168
*Angiopoietin		30,31
Basic fibroblast growth factor	bFGF	97,125,126,135,156,160,162,169-172
Heparin-binding epidermal growth factor	HB-EGF	101
Insulin-like growth factor	IGF	173
*Placental growth factor	PlGF	88
Platelet derived growth factor	PDGF	173
Scatter factor hepatocyte growth factor	HGF	117
Transforming growth factor-beta	TGF-beta	101
*Vascular endothelial growth factor	VEGF	23,25,26,70,127,128,157,158,163,169,172-175

*Specific for endothelial cells

with autologous EC transplants**.** The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For anti-neoplastic therapies, CD34-positive cells could be isolated and transfected with or coupled to anti-tumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} to achieve constitutive expression of angiogenic cytokines and/or provisional matrix proteins (72).

3.2. Angiogenic Cytokines

Beginning a little over a decade ago (73), a series of polypeptide growth factors (table 1) were purified, sequenced, and demonstrated to be responsible for natural as well as pathologic angiogenesis. These angiogenic cytokines all share in common the ability to act as mitogens for ECs.

Among the various growth factors which have been shown to promote angiogenesis, VEGF (74), also known as vascular permeability factor (VPF) (75) and vasculotropin (VAS) (76), is an EC-specific mitogen. Moreover, a plethora of studies have documented upregulation of VEGF in various cell types following exposure to other angiogenic cytokines. VEGF may thus be considered a prototypical angiogenic cytokine and for this reason will be discussed here in further detail. Specific aspects of the remaining angiogenic cytokines in Table 1 may be found in the accompanying lists of citations.

Four homodimeric species of VEGF have been identified, each monomer having 121, 165, 189, or 206 amino acids respectively (77). The secretion pattern of the four isoforms differs markedly. $VEGF_{121}$ is a weakly acidic polypeptide that does not bind to heparin, and is freely soluble in the conditioned medium of transfected cells. The heparin-binding capabilities of the remaining three isoforms are progressively augmented as the result of a step-wise enrichment in basic residues. Thus $VEGF_{165}$, the predominant form secreted by a variety of normal and transformed cells (38), is a basic heparin-binding glycoprotein with an isoelectric point of 8.5; while secreted, a significant portion remains bound to the cell surface or extracellular matrix. The $VEGF_{189}$ isoform includes 24 additional amino acids and has been shown not to be freely secreted, but instead remains nearly completely bound to the cell surface and/or extracellular matrix (78). $VEGF_{206}$ is a rare isoform so far identified only in a human fetal liver cDNA library.

The possibility of hierarchial efficacy among these three isoforms by performing arterial gene transfer of $phVEGF_{121}$, $phVEGF_{165}$, and $phVEGF_{189}$ was investigated in the rabbit ischemic hindlimb model (79). Remarkably, no differences with regard to anatomic or physiologic evidence of angiogenesis could be demonstrated - although all three isoforms yielded statistically significant improvement in every parameter measured compared to the LacZ controls. Moreover, separate experiments performed using 100, 200, and 400 µg of each plasmid failed to disclose a differential dose-response curve among the three isoforms with respect to angiographic score, calf blood pressure ratio, resting/maximum flow or capillary/myocyte ratio (Y. Tsurumi, unpublished data).

These findings may be interpreted to support the observation made previously by Houck *et al* (78) regarding the proteolytically clipped VEGF species which result from the action of plasmin on the 165 and 189 isoforms. The size of the resulting monomers, which in each case are mitogenic for ECs and enhance vascular permeability in a Miles assay (80), is similar to the size of the intact 121 isoform. It is therefore possible that the proteolytic cascade of plasminogen activation, a key step during angiogenesis (13), cleaves the longer forms of VEGF, releasing a soluble $VEGF_{121}$-like species that is the final common mediator of angiogenesis *in vivo*.

Besides VEGF, also referred to as VEGF-1 or VEGF-A, two additional proteins with particular abundancy in heart and skeletal muscle, VEGF-2 or VEGF-C (81) and VEGF-3 or VEGF-B (82) have been isolated.

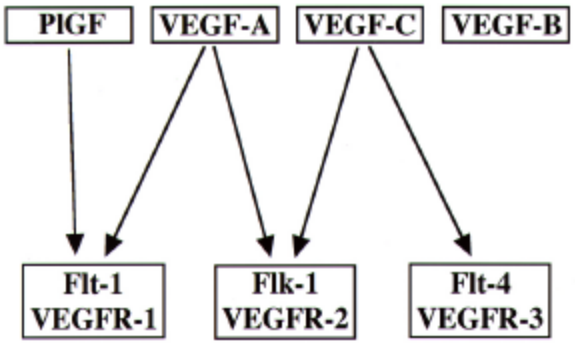

Figure 3. Ligand-receptor relationships for placental growth factor (PlGF) and proteins encoded for by the three vascular endothelial growth factor (VEGF) genes. (VEGFR= vascular endothelial growth factor receptor; alternative receptor nomenclature is shown directly above). (After Carmeliet and Collen[123])

In contrast to the widespread distribution of these VEGFs, the fourth member of the VEGF-family, placenta growth factor (PlGF) (83) appears to be restricted *in vivo* to placenta and certain tumors .(84,85) All four proteins share structural homology among themselves as well as with the platelet-derived growth factor A and B polypeptides (PDGF-A and -B), in particular a conserved motif, including eight cysteine residues in the putative receptor-binding domain (86). Similar to the a nd b chains of PDGF, VEGF-1/VEGF-A and PlGF can form heterodimers with biological activity (87-89). VEGF-3/VEGF-B is also able to heterodimerize with VEGF-1/VEGF-A in cells expressing both factors (82). Although not shown thus far, it seems reasonable to assume that VEGF-2/VEGF-C, by virtue of its shared homology to the other members of the VEGF family, can form heterodimers in a similar manner, therefore adding another level of complexity to processes related to angiogenesis.

Whereas VEGF-1/VEGF-A is a high-affinity ligand for the receptors flt-1 and KDR/flk-1, VEGF-2/VEGF-C was shown to bind to another recently identified endothelial specific receptor tyrosine kinase, flt-4, as well as to flk-1/KDR, but not to flt-1 (81,90) (figure 3). PlGF was shown to bind with high affinity to flt-1, but not to flk-1/KDR (88) or flt-4 (91). The receptor(s) for VEGF-3/VEGF-B (82) have not yet been characterized.

The constitutive coexpression of all three VEGF types in many adult tissues(81,82,92) suggests an interactive or at least redundant capacity of the VEGF members to regulate angiogenesis and modulate EC function. Data regarding the bioactivity of VEGF-2/VEGF-C and VEGF-3/VEGF-B, however, are currently limited. *In vitro*, both factors exhibit mitogenic activity (81,82,92), and VEGF-2/VEGF-C stimulated EC outgrowth in a collagen gel assay (81). In contrast, the fact that expression of PlGF is restricted to placental tissue and certain tumors (84,85) suggests that this protein plays a minor role in the maintanance of vascular integrity.

3.2.1 Autocrine loop as a feature of angiogenic cytokines

Certain angiogenic cytokines, notably aFGF and bFGF, lack a secretory signal sequence (93,94). This prompted previous investigators to study the possibility that these cytokines act via an alternative pathway. Indeed, several groups established evidence to support the notion that aFGF and bFGF can indeed modulate EC behavior via an autocrine pathway (95-98).

Evidence for a similar autocrine pathway in the case of VEGF was established by Namiki *et al* (99). Under quiescent conditions, VEGF mRNA was not detected in either HUVECs or human microvascular ECs (HMECs). Because ECs also express the high-affinity VEGF receptors, flt-1 and KDR, these cells in humans appear to include the requisite elements for an autocrine pathway. The finding that hypoxia induces activation of KDR in HUVECs is consistent with an external autocrine pathway (100) given that all VEGF isoforms include a secretory signal sequence.

The extent to which such an autocrine pathway may complement endogenous production of VEGF from SMCs, macrophages and tumor cells, or facilitate the response to exogenous administration of VEGF remains to be determined. With regard to the latter, it has been recognized that single bolus administration of VEGF may stimulate development of new collateral vessels over a period of several days, despite the fact that the circulating half-life of VEGF is <3 min. One explanation for this observation is that as VEGF is rapidly cleared from the circulation, it binds avidly to heparan sulfate proteoglycans present on the luminal surface of the vascular endothelium. Experimental evidence to support this concept in the case of bFGF has been previously reported by Fuks *et al* (47). Alternatively, an autocrine loop, activated under hypoxic conditions known to stimulate angiogenesis, might also serve to amplify and thereby protract the response in ECs stimulated by exogenously administered VEGF (figure 4). Such autocrine amplification may in fact represent a common motif shared by angiogenic cytokines in general.

3.2.2. Direct versus indirect cytokines

Certain stimuli capable of inducing the development of neovessels *in vivo*, specifically certain cytokines (101-104) and hypoxia, fail to stimulate EC proliferation *in vitro*, suggesting a role for additional mediators and/or cell types. We considered whether these so-called indirect angiogenic growth factors might stimulate vascular SMCs to express genes encoding direct EC mitogens. Such a sequential cascade would provide a mechanism by which growth factors which are otherwise non-mitogenic or frankly inhibitory for EC proliferation *in vitro* could instead stimulate angiogenesis *in vivo*.

Although PDGF BB, for example, can directly stimulate proliferation of selected populations of

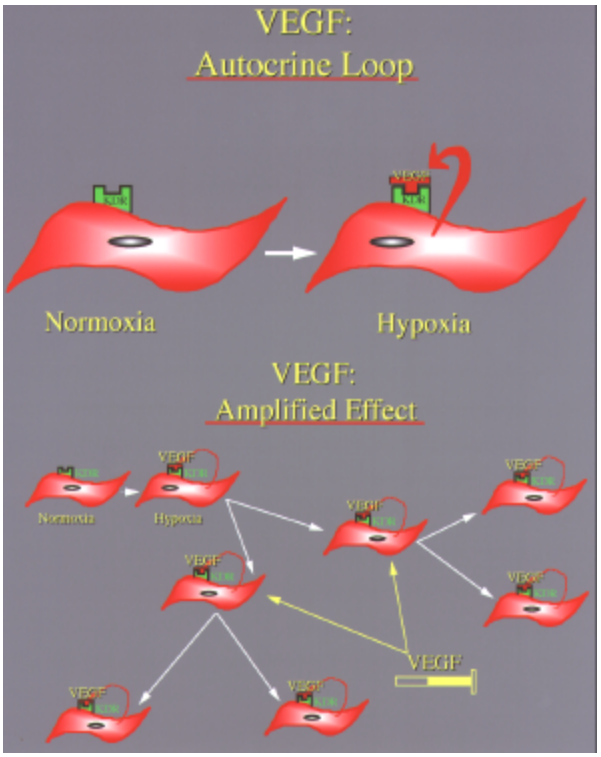

Figure 4. Under conditions of hypoxia, ECs upreglate VEGF, which, once secreted, may then interact with its receptor. Such an autocrine loop provides the basis for amplification of any given VEGF secreted or administered into an ischemic territory. ECs simtulated to proliferate in response to VEGF may then serve as additional sources of VEGF synthesis, thus amplifying the effect of the initial dose of VEGF.

microvascular ECs *in vitro* (105-107), ECs from most vascular districts do not respond to treatment with PDGF BB (108). Nonetheless, *in vivo* PDGF BB has been clearly demonstrated to induce supportive angiogenesis in both a wound healing model (103) and chorioallantoic membrane assay (104). Furthermore, microvascular ECs isolated from rat epididymal fat pads and not directly responsive to PDGF BB stimulation, when co-cultured with myofibroblasts from the same tissue source form capillary chords in the presence of PDGF BB (102). The angiogenic action of PDGF BB documented in this way was attributed to production of soluble EC mitogen(s) by myofibroblasts in response to stimulation with PDGF BB.

We demonstrated that PDGF BB treatment of human vascular SMCs concurrently induces VEGF and bFGF mRNA species (109). This finding indicates that PDGF BB, a known mitogen for SMCs, could also stimulate SMCs to produce direct angiogenic factors and thereby promote angiogenesis.

Likewise, the plurality of mechanisms that contribute to the angiogenic activity of transforming growth factor-ß1 (TGF-ß1) (110) has been the subject of controversy. Although clearly inhibiting growth and migration of subconfluent ECs *in vitro* (111), TGF-ß1 facilitates capillary formation *in vivo* (110). Furthermore, TGF-ß1 modulates the composition of the extracellular matrix (112) and may therefore act in part by locally creating a pro-angiogenic environment. While TGF-ß1 directly inhibits EC proliferation (111), it may nevertheless direct organization of ECs into tube-like structures, depending on conditions of culture tested (113). TGF-ß1 in high concentrations was found to inhibit the formation of capillary-like structures in an *in vitro* model of angiogenesis, whereas low doses were observed to potentiate VEGF- or bFGF-induced neovessel formation in the same assay system (111). This phenomenon was interpreted by Pepper *et al*. as an example of "contextual" angiogenesis to indicate that the angiogenic response to a given cytokine is dependent upon the presence and concentration of other mediators in the peri-cellular environment of the target ECs. Winkles *et al*. have also observed that TGF-ß1 stimulates bFGF expression by SMCs, thus supporting the notion that direct angiogenic mediators (114) might contribute in part to TGF-ß1-induced angiogenesis. Our finding (109) that TGF-ß1 stimulation induces VEGF as well as bFGF gene expression, in concert with the above-described mechanisms, suggests that both EC mitogens are likely instrumental for mediating the indirect angiogenic effects of TGF-ß1 *in vivo*.

Subsequently this same motif has been demonstrated for insulin-like growth factor (IGF (115)), bFGF (116), and scatter factor (hepatocyte growth factor (117)). Basic FGF and scatter factor, however, are further distinguished by the fact that they exert a direct effect on EC proliferation and migration, in addition to upregulating VEGF synthesis in vascular SMCs.

In the context of factors which upregulate VEGF expression, it is important to emphasize the critical role played by hypoxia, since this explains at least in part the clinical observation that tissue ischemia is a fundamental stimulus for angiogenesis. Increased expression of VEGF has been documented at the periphery of necrotic foci of certain neoplasms, as well as hypoxia-stimulated induction of VEGF in cultured cells from glial tumor and rat skeletal muscle (118). We have shown that low O2 tension selectively modulates VEGF but not bFGF in vascular SMCs (109). This observation is intriguing in light of homology between a sequence in the VEGF promoter (77) and a nucleotide sequence in the erythropoietin promoter identified as a binding site for an hypoxia-specific transcription factor (HIF-1) (119). It is now clear that while transcriptional upregulation of VEGF does occur in response to hypoxia, post-translational mechanisms constitute the dominant basis for hypoxia-induced synthesis of VEGF protein (120-123).

3.2.3. Site-specific effects of angiogenic cytokines

From a teleologic perspective, it would appear critical that EC mitogens not promote angiogenesis in an

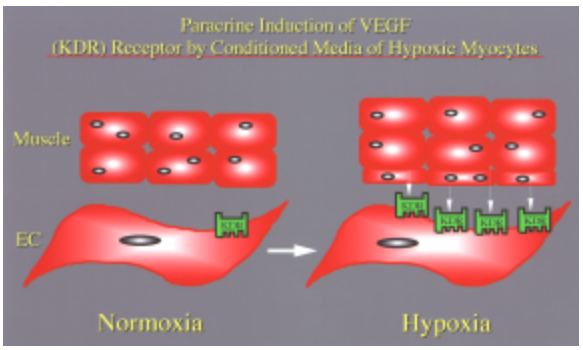

Figure 5. Factors secreted by hypoxic myocytes upregulate VEGF receptor expression on ECs within the hypoxic milieu. Such localized receptor expression may explain the finding that angiogenesis does not occur indiscriminately, but rather at sites of tissue ischemia. (From Horowitz *et al* (176))

indiscriminate fashion; it is clearly preferable for the survival of the organism that angiogenesis be limited to sites of wound healing and tissue ischemia where it may have facilitatory effects. There is in fact evidence to indicate that this is the case. Studies in a variety of animal models using aFGF (124), bFGF (125,126), VEGF (23,127,128), and scatter factor (117) have shown that systemic administration of angiogenic cytokines selectively produces neovascularization in the ischemic limb (129); neither in the contralateral normal limb nor in any other organs were foci of neovascularization observed. In fact, when rhVEGF was injected into the normal or ischemic limb of rabbits with unilateral hindlimb ischemia, angiogenesis was observed only in the ischemic limb.

Furthermore, patients with peripheral vascular disease have been shown to have detectable levels of circulating bFGF (130), yet evidence of angiogenesis appeared limited in these patients to collateral vessel development in the ischemic lower extremities. More recently, following intramuscular injection of the gene encoding VEGF, we have documented circulating levels of VEGF (I. Baumgartner and J. Isner, unpublished data, see below), yet, again, evidence of angiogenesis was limited to the ischemic limb.

There is evidence to suggest that regional ischemia is the principal factor responsible for localization of angiogenesis. In rabbits, for example, treated with rhVEGF, the magnitude of augmented collateral development is inversely related to the extent of hindlimb collateral arteries seen prior to rhVEGF therapy (23). The same is true for blood flow in the ischemic limb (131). Thus, if a paucity of angiograpically visible collaterals and reduced blood flow are indicative of limb ischemia, then it would appear that the extent to which angiogenesis is enhanced is related to the severity of ischemia.

Experiments performed on ECs *in vitro*, suggest that the basis for localized bioactivity of angiogenic cytokines such as VEGF is due to upregulation of the VEGF receptor in response to hypoxia. In certain types of ECs, notably bovine retinal ECs, this may occur as a direct response to hypoxia (132); in our experience, direct upregulation of receptor expression does not occur in human umbilical vein ECs (HUVECs) or microvascular ECs. The difference in these observations may be due to the fact that bovine retinal ECs possess three-fold more VEGF receptors (132).

In the case of HUVECs and microvascular ECs, receptor upregulation appears to occur indirectly in response to hypoxia (figure 5). Specifically, we observed an increase in KDR mRNA levels in HUVECs treated for 3 hours with conditioned medium from hypoxic myoblasts, as compared to KDR mRNA levels of HUVECs treated for the same time with conditioned medium from normoxic myoblasts (133). Such increase suggests new receptor synthesis, in conjunction with mobilization of preformed receptors from the cellular pool. Scatchard analysis of 125I-VEGF binding indicated no substantial change in affinity of the KDR receptor in HUVECs treated with myoblast medium conditioned either in normoxia or hypoxia. Thus, increased binding observed using hypoxia-conditioned medium was due to a 13-fold increase in the number of KDR receptors. These data suggest that a "factor" secreted from hypoxic myocytes in ischemic tissues upregulates VEGF receptor expression on adjacent ECs; consequently the ECs in these ischemic tissues act as a magnet for circulating VEGF.

3.3. Therapeutic Angiogenesis

The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Folkman and colleagues over two decades ago (134). Their work documented the extent to which tumor development was dependent upon neovascularization and suggested that this relationship might involve angiogenic growth factors which were specific for neoplasms.

More recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. This novel strategy for the treatment of vascular insufficiency has been termed "therapeutic angiogenesis" (23). The angiogenic growth factors first employed for this purpose comprised members of the FGF family. Baffour *et al* administered bFGF in daily intramuscular (IM) doses of 1 or 3 µg to rabbits with acute hindlimb ischemia; at the completion of 14 days of treatment, angiography and necropsy measurement of capillary density showed evidence of augmented collateral vessels in the lower limb, compared to controls (125). Pu *et al* used aFGF to treat rabbits in which the acute effects of surgically-induced hindlimb ischemia were allowed to subside for 10 days before beginning a 10-day course of daily 4-mg IM injections; at the completion of 30 days follow-up, both angiographic and hemodynamic evidence of collateral development was superior to ischemic controls

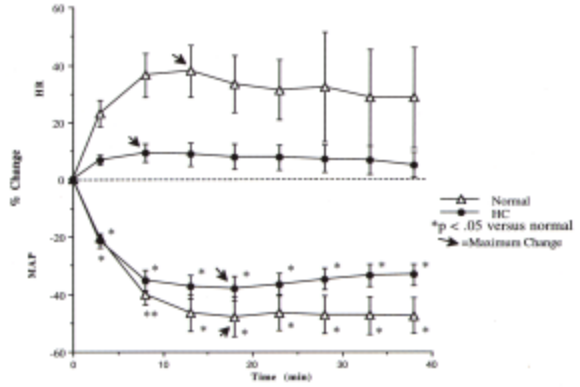

Figure 6. Angiogenesis at level of medium-sized arteries.Selective internal iliac angiography of control rabbit witrh unilateral hindlimb ischemia performed at (a) day 10 (baseline), (b) day 20, and (c) day 40, and of VEGF-treated rabbit at (d) day 10 (baseline), (e) day 20, and (f) day 40. The angiograms shown here yielded angiographic scores of (a) 0.10, (b) 0.11, (c) 0.17, (d) 0.12, (e) 0.36, and (f) 0.41. Distal reconstitution, barely apparent in control group (b, c, arrows), was evident in the VEGF-treated group (e, f, arrows). Direct and linear extension of internal iliac artery to popliteal and/or saphenous arteries was also more evident in VEGF -treated group (b, c, e, f, open arrows). (From Takeshita *et al* [25]).

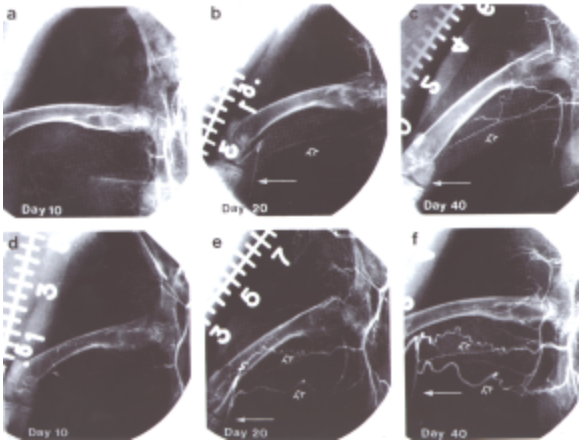

Figure 7. Angiognesis at the capillary level. Alkaline phosphatase staining of rabbit ischemic hindlimb muscle harvested at day 40. (a) Control group and (b) VEGF-treated group. Dark blue dots indicate capillaries (arrows). (Counterstained with eosin) (From Takeshita *et al* [25]).

treated with IM saline (124). Yanagisawa-Miwa *et al* likewise demonstrated the feasibility of bFGF for salvage of infarcted myocardium, but in this case growth factor was administered intra-arterially at the time of coronary occlusion, followed 6 hrs later by a second intra-arterial bolus (135).

Evidence that VEGF stimulates angiogenesis *in vivo* had been developed in experiments performed on rat and rabbit cornea (136,137), the chorioallantoic membrane (74), and the rabbit bone graft model (137). The finding that VEGF could be employed to achieve angiogenesis that was therapeutic was first demonstrated by Takeshita *et al* (23). The 165-amino acid isoform of VEGF (VEGF165) was administered as a single intra-arterial bolus to the internal iliac artery of rabbits in which the ipsilateral femoral artery was excised to induce unilateral hindlimb ischemia. The severity of hindlimb ischemia in this animal model has been shown in previous studies to include reduced tissue oxygen saturation, (125), increased femoral venous lactate (138), and skeletal muscle necrosis (125). Doses of 500-1,000 µg of VEGF produced statistically significant augmentation of angiographically visible collateral vessels (figure 6), and histologically identifiable capillaries (figure 7); consequent amelioration of the hemodynamic deficit in the ischemic limb was significantly greater in animals receiving VEGF than in non-treated controls (calf blood pressure ratio=0.75 ± 0.14 vs 0.48 ± 0.19, $p<0.05$). Serial (baseline, as well as 10 and 30 days post-VEGF) angiograms disclosed progressive linear extension of the collateral artery of origin (stem artery) to the distal point of parent-vessel (reentry artery) reconstitution in 7 of 9 VEGF-treated animals. Similar results were achieved in a separate series of experiments in which VEGF was administered by an IM route daily for 10 days (127). By 30 days post-VEGF165, flow at rest, as well as maximum flow velocity and maximum blood flow provoked by 2 mg papaverine were all significantly higher in the VEGF-treated group (131). These findings thus established proof of principle for the concept that the angiogenic activity of VEGF is sufficiently potent to achieve therapeutic benefit.

3.3.1. Therapeutic angiogenesis preserves vasomotor reactivity of collateral vessels

Abnormal vascular reactivity may limit the facilitatory effects of collateral vessels on tissue perfusion. (139-143) Previous studies have established that chronic perfusion through native coronary collateral vessels produces endothelial dysfunction in the recipient, downstream reconstituted vasculature (139,142). Therapeutic angiogenesis may promote recovery of endothelium-dependent flow. In the rabbit model of hindlimb ischemia, endothelium-independent and endothelium dependent hindlimb blood flow were essentially restored 30 days following administration of a single intra-arterial bolus of VEGF (144).

At least three mechanisms could explain an improvement in endothelium-dependent flow responses of the collateral-dependent limb after VEGF therapy. The first possibility relates to the characteristics of flow and perfusion pressure in arterioles distal to collaterals. We have previously demonstrated that VEGF therapy produces a significant increase in the calf blood pressure of the ischemic limb (23); it is entirely possible that such improved perfusion pressure may lead to repair of dysfunctional endothelium in the collateral-perfused distal vasculature. A second and intriguing possibility relates to a direct improvement of endothelial function by VEGF. In the case of bFGF, for example, *in vitro* studies have

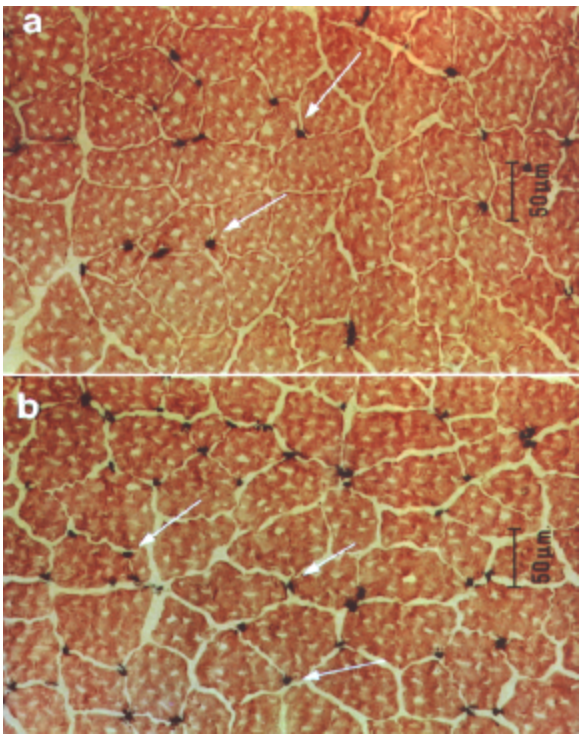

Figure 8. Selective digital subtraction angiograms performed in patient with critical limb ishemia due to occlusion of all three infrapopliteal vessels at mid-calf level. (A) immediately prior to, and (B) 1 month post-gene therapy with 2000 µg of naked DNA encoding VEGF. The latter angiogram disclosed plethora of new collateral vessels in ischemic limb. (Isner *et al* (177))

recently demonstrated that endothelial function in the coronary microcirculation perfused via collateral vessels is preserved by chronic administration of this EC mitogen (145). The fact that VEGF may also modulate qualitative aspects of EC function (146) suggests that it too may directly repair ECs presumed damaged by protracted ischemia in the collateral-dependent limb, and thereby restore normal endothelium-dependent flow. Third, the possibility that the documented improvement in endothelium-dependent flow is the result of the newly formed, VEGF-induced collateral vessels, cannot be discounted.

The finding that angiogenic growth factors restore the responses of the ischemic hindlimb to endothelium-dependent vasodilators may have important clinical implications. The hypersensitivity to serotonin of the collateral circulation is not limited to animal models. Platelet activation releases vasoactive quantities of serotonin *in vitro* (147), and the S2-receptor antagonist ketanserin dilates limb collaterals in over 50% of human patients with advanced atherosclerosis (148). Ketanserin has also been reported to improve limb perfusion in selected patients with peripheral artery disease (149), suggesting that abnormal reactivity may largely limit the beneficial consequences of collaterals in humans.

3.3.2. Therapeutic angiogenesis achieved by arterial gene transfer

No recombinant protein formulation of any of the three principal VEGF isoforms or any other angiogenic cytokine is currently approved or available for human clinical application. Arterial gene transfer constitutes an alternative strategy for accomplishing therapeutic angiogenesis in patients with limb ischemia. In the case of VEGF this is a particularly appealing strategy because, as indicated previously, the VEGF gene encodes a signal sequence which permits the protein to be naturally secreted from intact cells (77). Previous studies from our laboratory (150,151) indicated that arterial gene transfer of cDNA encoding for a secreted protein could potentially yield meaningful biological outcomes in spite of a low transfection efficiency. We therefore performed pre-clinical animal studies to establish the feasibility of site-specific gene transfer of $phVEGF_{121}$, $phVEGF_{165}$, and $phVEGF_{189}$ applied to the hydrogel polymer coating of an angioplasty balloon (152), and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischemia (138).

Site-specific transfection of phVEGF165 was confirmed by analysis of the transfected internal iliac arteries using reverse transcriptase-polymerase chain reaction (RT-PCR) (153) and then sequencing the RT-PCR product. Augmented development of collateral vessels was documented by serial angiograms *in vivo*, and increased capillary density at necropsy. Consequent amelioration of the hemodynamic deficit in the ischemic limb was documented by improvement in the calf blood pressure ratio (ischemic/normal limb) to 0.70 ± 0.08 in the VEGF-transfected group vs 0.50 ± 0.18 in controls ($p<0.05$). Similar findings were acheived with the 121 and 189 VEGF isoforms (79). These findings thus established that site-specific arterial gene transfer can be used to achieve physiologically meaningful therapeutic modulation of vascular disorders, including therapeutic angiogenesis.

The relevance of these findings in the rabbit ischemic hindlimb model is supported by recent clinical application of arterial gene therapy (154). Using a dose-escalating design, treatment was initiated with 100 µg of phVEGF. Three patients presenting with rest pain (but no gangrene) and treated with 1000 µg were subsequently shown at 1-year follow-up to have improved blood flow to the ischemic limb and remain free of rest pain. With the increase in dose of $phVEGF_{165}$ to 2000µg, angiographic (figure 8) and histologic evidence of new blood vessel formation became apparent (70) (figure 9). Subsequently, the use of intramuscular gene transfer, employed initially as a means of treating patients in whom vascular disease in the ischemic limb was too extensive to permit an intra-arterial approach, resulted in circulating levels of VEGF

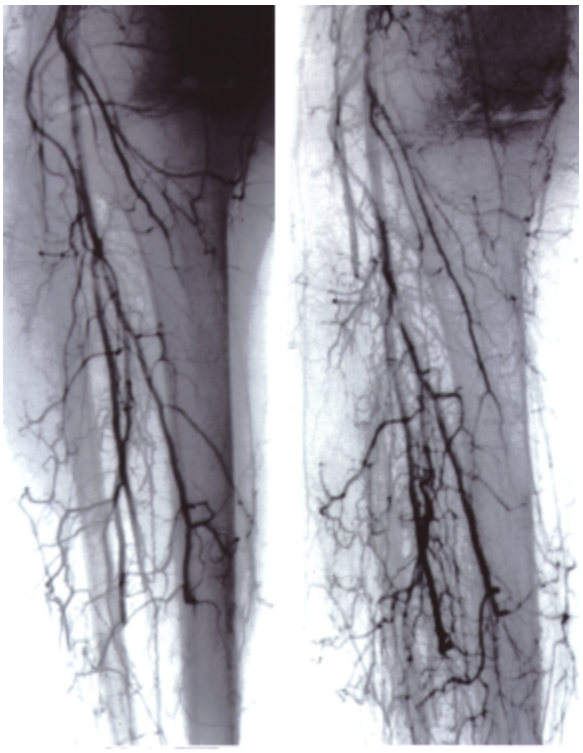

Figure 9. One of 3 spider angiomata which developed approximately 1 week post-gene therapy in distal portion of ischemic limb of patient whose angiograms are shown in Figure 8. Photomicrographs of tissue sections immunostained with antibody to endothelial antigen CD31 indicate vascularity of lesion, while immunostain of adjacent section for proliferating cell nuclear antigen (PCNA) indicates extent of proliferative activity among ECs in lesion. (From Isner *et al* (177))

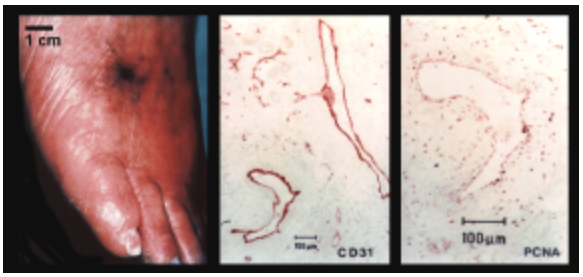

Figure 10. Successful placement of split-thickness skin graft in first patient treated with intramuscular (IM) administration of naked DNA encoding VEGF ($phVEGF_{165}$). Following two IM injections of 2000 µg of $phVEGF_{165}$, patient's ankle-brachial index increased from 0.28 to 0.55, angiography disclosed new collateral vessels in ischemic limb, and 9X3cm wound at site of vein harvest in medial calf healed sufficiently to permit successful grafting.

detectable by ELISA assay, as well as reproduciblehemodynamic and angiographic improvement (I. Baumgartner and J. Isner, unpublished observations). In at least one case, therapeutic angiogenesis administered in this fashion accomplished genuine limb salvage (figure 10). The clinical reproducibility of these findings may ultimately be influenced by certain features of host diversity, including the extent of native VEGF and VEGF receptor expression among patients with peripheral and/or myocardial ischemia.

3.3.3. Recombinant protein versus gene therapy

There is now abundant data in animal models to support the potential utility of both recombinant protein therapy and arterial gene transfer for myocardial angiogenesis (135,145,155-161). The same is true for lower extremity vascular insufficiency (23,117,124-126,126-128,162-164). It is therefore reasonable to ask why, if the recombinant protein has or can be manufactured, consider transferring the gene encoding that protein (i.e. gene therapy)? There are at least four issues to consider in this regard. First, and perhaps most critical in the case of arterial gene therapy, is the potential requirement to maintain an optimally high and local concentration over time. In the case of therapeutic angiogenesis, for example, it may be preferable to deliver a lower dose over a period of several days or more from an actively expressing transgene in the ischemic limb, rather than a single or multiple bolus doses of recombinant protein. Second, there is the matter of economics; namely, which therapy would ultimately cost more to develop, implement, and reimburse, particularly for those indications requiring multiple or even protracted treatment? Third, in certain species - namely rats, rabbits, and swine - both rhbFGF and rhVEGF have been shown to produce varying degrees of systemic hypotension, even in the setting of hypercholesterolemia (20,157,165,166) (figure 11). And fourth, the route of administration may be a factor depending upon the target site for neovascularization. For example, the ready access to ischemic skeletal muscle together with the promising results of IM gene therapy make this approach eminently suitable for the treatment of lower extremity vascular insufficiency. In contrast, IM administration for myocardial angiogenesis cannot be accomplished as readily, and may therefore be more amenable to recombinant protein therapy.

Resolution of these issues will almost certainly require the empirical experience of human clinical trials. Recombinant bFGF and VEGF are now being investigated in human clinical trials for patients with lower extremity vascular disease and myocardial ischemia respectively, so that the magnitude of bioactivity that can be achieved with either of these should be ultimately clarified.

4. PERSPECTIVES

The identification of angiogenic growth factors has generated the opportunity for novel therapies in the treatment of a variety of diseases. This includes pathologic angiogenesis, such as diabetic retinopathy, rheumatoid arthritis, and cancer. In these cases, antibodies and/or

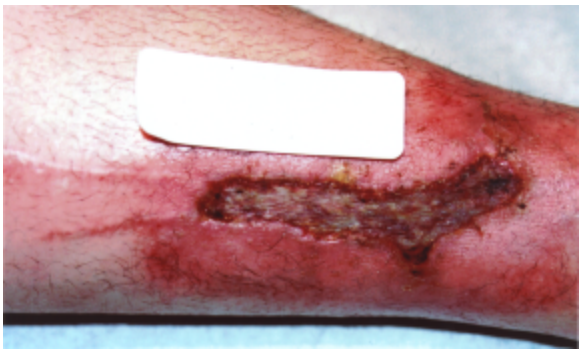

Figure 11. A) Administration of rhVEGF to normal and hypercholesterolemic (HC) rabbits leads to prompt reduction in mean arterial pressure (MAP), with compensatory increase in heart rate (HR). The magnitude and duration (≥ 40 minutes) of hypotension are not significantly different in HC versus normal rabbits. B) MAP does not drop below baseline when administration of VEGF is preceded by administration of an inhibitor of nitric oxide synthase (L-NNA).

naturally occuring angiogenesis inhibitors are being investigated clinically to antagonize key angiogenic factors. The strategy is designed to eliminate the vascular infrastructure and thereby minimize the extent of pathological consequences to the patient.

A complementary strategy is likely to emerge for the treatment of cardiovascular diseases. Clinical trials of therapeutic angiogenesis have already been initiated in patients with myocardial ischemia and peripheral vascular disease. These include trials of recombinant protein therapy as well as gene transfer. While preliminary applications of gene therapy have established proof of concept that angiogenic growth factors can augment collateral artery development in human subjects, many questions remain to be answered. What are the relative advantages of protein versus gene therapy, both in terms of bioactivity as well as cost and safety? What is the maximum extent of clinical improvement that can be expected? How is the therapeutic outcome affected by certain features of the host?

It is also likely that future investigations may clarify the impact on established risk factors for vascular disease, such as lipid dyscrasias and diabetes, on native angiogenesis; thus risk factor modifications may be useful for indirectly aiding natural angiogenesis in ischemic territories. It is intriguing to consider the possibility that one may be able to identify certain genotypic characteristics that may indicate why some patients from robust collateral networks, while others fail to do so.

The role of endothelial cell (EC)progenitors in both natural as well as therapeutic angiognesis will be clarified by future studies. Recent studies have forced us to modify the classic concept that collateral development occurs solely by migration of fully differentiated ECs. It is now clear that circulating stem cells contribute to such neovascularization. What remains to be determined is the proportion of new vessel growth that results from circulating stem cells versus parent vessel ECs. Finally, it is possible that EC progenitors may be employed in strategies of "supply side" therapuetic angiogenesis; specifically can administration of EC progenitors, including cells engineered to secrete pro-angiogenic agents, complement the impact of angiogenic cytokines on collateral vessel growth?

5. ACKNOWLEDGEMENTS

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