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REVIEW

Angiogenesis gene therapy to rescue ischaemic tissues: achievements and future directions

[1]Costanza Emanueli & *,[1,2]Paolo Madeddu

[1]Cardiovascular Medicine and Gene Therapy Section, National Laboratory of the National Institute of Biostructures and Biosystems, Osilo, Italy and [2]Department of Internal Medicine, Medical University of Sassari, Sassari, Italy

Ischaemic diseases are characterized by an impaired supply of blood resulting from narrowed or blocked arteries that starve tissues of needed nutrients and oxygen. Coronary-atherosclerosis induced myocardial infarction is one of the leading causes of mortality in developed countries. Ischaemic disease also affects the lower extremities. Considerable advances in both surgical bypassing and percutaneous revascularization techniques have been reached. However, many patients cannot benefit from these therapies because of the extension of arterial occlusion and/or microcirculation impairment. Consequently, the need for alternative therapeutic strategies is compelling. An innovative approach consists of stimulating collateral vessel growth, a natural host defence response that intervenes upon occurrence of critical reduction in tissue perfusion (Isner & Asahara, 1999). This review will debate the relevance of therapeutic angiogenesis for promotion of tissue repair. The following issues will receive attention: (a) vascular growth patterns, (b) delivery systems for angiogenesis gene transfer, (c) achievements of therapeutic angiogenesis in myocardial and peripheral ischaemia, and (d) future directions to improve effectiveness and safety of vascular gene therapy.
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Abbreviations: AV, adenovirus; AAV, adeno-associated virus; Ang-1 and Ang-2, angiopoietin-1 and -2; Tie-1 and Tie-2, angiopoietin receptors; ACE, angiotensin converting enzyme; CMV, cytomegalovirus; EC, endothelial cells; ECP, endothelial cell precursors; FGF-1 and FGF-2, fibroblast growth factors-1 and -2; GF, growth factors; HK, human tissue kallikrein; LV, lentivirus; NO, nitric oxide; RV, retrovirus; RSV, rous sarcoma virus; VEGF, vascular endothelial growth factor; VEGFR-1 (flt-1), VEGFR-2 (flk-1/KDR), VEGFR-3 (flt-4), VEGF receptors

Different models of vascular growth

Vasculogenesis and angiogenesis are two distinct biological processes that mediate the formation of the vascular system through partially overlapping molecular pathways (Carmeliet, 2000):

(1) Vasculogenesis, the *de novo* formation of blood vessels from immature EC precursor (ECP), has been considered restricted to embryogenesis. Essential steps of vasculogenesis are the establishment of the endothelial cell (EC) lineage (angioblasts) from mesodermal precursors in extra-embryonic areas, the assembly of angioblasts into cord-like vascular structures, the formation of vascular lumens and the organization of vascular networks which centripetally invade embryonic endodermic structures. It is now clear that vasculogenesis plays an important role also in neovascularization induced by ischaemia. In adults, ECP have been identified not only in bone marrow but also in peripheral blood. ECP seem to be rapidly mobilized to colonize ischaemic areas, where they differentiate into mature ECs (Asahara *et al*., 1997). The molecular events regulating vasculogenesis are largely unknown, but it has been well established that fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) participate in the differentiation of mesodermal precursors in angioblasts and in the formation of a primitive vascular network (Carmeliet *et al*., 1999). Differentiation and mobilization of such elements by VEGF, FGF-2 and insulin-like growth factor-1 (IGF-1) is followed by colonization of sites of vascular growth (Asahara *et al*., 1997; 1999).

(2) Angiogenic sprouting from pre-existing vessels is essential for vascularization of ectodermal and mesenchymal organs and, after birth, for the development of several physiological and pathological settings, such as endometrial proliferation, wound healing, and cancer growth and dissemination (Risau & Flamme, 1995). Angiogenesis occurs in stages, which orchestrate a network of cooperative interactions. The following steps have been determined: (i) vasodilatation and increase in vascular permeability allow extravasation of plasma proteins that lay down a provisional scaffold for migrating ECs; (ii) in order to migrate, vascular ECs need to loose their mutual contact and interaction with basement membrane. Various proteinases influence angiogenesis by destabilizing the vascular structure and degrading the extra-cellular matrix, thus favouring EC sprout from pre-existing capillaries. (iii) Once activated, ECs migrate, assemble in primitive vascular

*Author for correspondence at: Gene Therapy Section, National Laboratory of the National Institute of Biostructures and Biosystems, Viale S. Antonio, 07033 Osilo, Italy;
E-mail: madeddu@ssmain.uniss.it

structures, and subsequently acquire a lumen. (iv) The final step consists of EC differentiation (according to the functional needs of host tissue), stabilization, and remodelling of newly formed vessels into correct three-dimensional networks. In general, growth factors (GF) that guide EC proliferation and migration are named direct angiogenic factors. Indirect angiogenic GF modulate the release of direct factors from cells. Mediator molecules such as FGF released from ECs promote the recruitment of mesenchymal cells to vessel walls (Bussolino *et al*., 1997; Hanahan, 1997; Risau, 1997).

Angiogenesis is strongly regulated by hypoxia (Semenza, 1999). Hypoxia-inducible transcription factors trigger a coordinated response by inducing expression of endothelial GF. Angiogenesis is also induced by metabolic stimuli, including hypoglycaemia and acidosis. Modulators of vascular growth often intervene synergically and at more than one step of the angiogenic process: for instance, the initial vasodilation and increase in vascular permeability involve VEGF and nitric oxide (NO). By interacting with endothelial tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (flk-1/KDR), VEGF-A regulates the progression phase of angiogenesis and induces ECs to proliferate, migrate and survive (Ferrara & Devis-Smyth, 1997). VEGF-C, with related receptors VEGFR-2 and VEGFR-3 (flt-4), represents an apparently redundant pathway for post-natal angiogenesis. VEGF-C was shown to stimulate NO release from ECs and to induce neovascularization in a rabbit model of hindlimb ischaemia (Witzenbichler *et al*., 1998). Recent evidence indicates a role for VEGF-C in pathologic angiogenesis (Ferrara & Alitalo, 1999). Angiopoietins constitute another family of GF relatively specific for the endothelial lineage. Angiopoietin-1 (Ang-1) was the first ligand to be cloned for the Tie family of receptors. Acting through the Tie-2 receptor, Ang-1 is essential for normal embryonic development, while in the adult it decreases vascular permeability and stabilizes networks initiated by VEGF, presumably by stimulating an interaction between ECs and pericytes (Suri *et al*., 1998; Chae *et al*., 2000). Ang-2 is highly expressed at sites of normal and pathological vascular remodelling in the adult. It contributes to stabilizing the shape of native vessels, rendering a quiescent capillary responsive to VEGF-A; however, in the absence of the appropriate stimulus for vessel growth, expression of Ang-2 in endothelium is associated with vessel regression (Maisonpierre *et al*., 1997). Recently, a role for the kallikrein-kinin system (KKS) in angiogenesis has been proposed. Kinin B_1 receptor and tissue kallikrein gene expression is tightly regulated by myocardial or limb ischaemia (Tschope *et al*., 2000; Emanueli *et al*., 2001a). The functional importance of these expression changes is documented by the fact that native angiogenic response to ischaemia is blunted by chronic B_1 receptor antagonism or knockout, but not by blockade of constitutive kinin B_2 receptor (Emanueli *et al*., 2001a). The number of newly discovered angiogenic substances is rapidly increasing. Table 1 shows a list of the most representative members of separate families of GF with angiogenic activity.

(3) Arteriogenesis consists of the formation and 'muscolarization' of collaterals alongside pre-existing vessels, using these as guiding cues. Members of the FGF family seem to be involved in *in situ* differentiation of mesenchymal elements into vascular smooth muscle cells, thus leading to branching and elongation of the arterial tree (Carmeliet, 2000). Mechanical forces are involved in arteriogenesis. For instance, remodelling of collaterals after arterial occlusion depends on blood flow and shear stress changes (Schaper & Ito, 1996).

Delivery systems for angiogenesis gene transfer

Therapeutic angiogenesis represents a novel strategy for the treatment of vascular insufficiency (Isner & Asahara, 1999). It is based on supplementation with angiogenic GF for enhancing native angiogenesis in critical myocardial or peripheral ischaemia, not susceptible to conventional revascularization. This approach seems to be particularly suitable under conditions of advanced age, diabetes, atherosclerosis or hypertension, in which impairment of ischaemia-induced activation of endogenous GF is responsible for delayed haemodynamic recovery and deficient tissue healing (Rivard *et al*., 1999a, b; Couffinhal *et al*., 1999; Emanueli *et al*., 2001b).

Angiogenic GF can be administered as recombinant proteins or by way of gene transfer. With the latter strategy, the relevant protein is delivered to targeted tissue in form of encoding gene and under conditions whereby the gene is expressed, thereby allowing cells to produce and possibly release the protein for a certain period, generally a few weeks (Leiden, 2000). The gene can be delivered as naked DNA or in a vector *via* a variety of approaches, including direct injection, electroporation, by way of transfected cells, or commercially available liposome preparations. The expression vector, usually consisting of a replication-deficient adenovirus (AV), retrovirus (RV), lentivirus (LV), and/or an adeno-associated virus (AAV), is taken up by the host cells *via* receptor-mediated mechanisms or endocytosis.

AV have been widely used for gene therapy, due to their ability to transduce non-dividing cells very efficiently (Gilgenkrantz *et al*., 1995; Muhlhauser *et al*., 1996; Crystal, 1997). They are made replication deficient by producing point mutations, deletions, insertions and combinations directed toward a specific AV gene or genes, such as the El gene. AV vectors can be amplified and produced in high titres ($10^{11}-10^{13}$ virus particle ml^{-1}), they infect post-mitotic cells, and accommodate transgene cassette up to 10 kb. Usually, the relevant gene is positioned such that an AV promoter is

Table 1 Angiogenic molecules potentially applicable for therapeutic angiogenesis

Angiogenic molecule	*Abbreviation*	*EC mitogenicity*
Angiopoietin-1	Ang1	permissive
Acidic fibroblast growth factor	aFGF, FGF-1	yes
Basic fibroblast growth factor	bFGF, FGF-2	yes
Epidermal growth factor	EGF	yes
Hepatocyte growth factor	HGF	yes
Human tissue kallikrein	HK	n.d.
Insulin-like growth factor	IGF	yes
Kinins		yes
Platelet derived growth factor	PDGF	yes
Transforming growth factor-beta	TGF-beta	yes
Vascular endothelial growth factor	VEGF	yes

operatively linked to the insert for direct transcription of the transgene (Chao *et al*., 1996). Similarly, the transgene insert can be positioned such as to use other AV regulatory sites, including splice junctions and polyadenylation signals. Alternatively, the transgene may contain a different enhancer/promoter (e.g., CMV or RSV-LTR enhancer/promoter sequences) that confers a wide expression pattern, or derive sequences necessary for expression from the host, with the purpose of targeting specific tissues or organs.

Due to the lack of integration in host genome, AV-mediated transgene expression is not sustained. Furthermore, repeated administration is precluded by the imunogenicity of the AV proteins. Adverse reactions may derive from incomplete inactivation of replication and include life-threatening systemic inflammatory-like reactions more likely occurring with high titres of AV (Leiden, 2000). Relevant to the issue of this review, AV vectors *per se* can stimulate the formation of new capillaries at the site of injection (Emanueli *et al*., 2000). This may be explained by the fact that AV transfer is able to recruit monocytes and T lymphocytes, which are important sources of GF (Newman *et al*., 1999). From the above, it seems extremely unlikely that AV vectors will have a significant future in the clinical field.

AAV is a small single-strand DNA parvovirus that is defective, non-pathogenic, and largely diffused in the general population. More than 90% of the parental viral genome is deleted in AAV-based vectors. Consequently, no viral proteins are expressed from AAV vectors in transduced cells. AAV are much less inflammatory than their AV counterparts and indeed appear to be capable of programming long-term gene expression in immunocompetent animals *in vivo*. As for AV, any desired promoter can be included in the construct for constitutive, regulated, or tissue specific transgene statement. Recent advances enhanced the production capability of high-titre stocks of AAV (Grimm and Kleinschimidt, 1999). Accordingly, vectors that express different genes can be mixed before transduction in order to obtain the simultaneous statement of two or more different proteins in the same tissue. Finally, AAV has a broad host range, but displays an exquisite tropism for nervous and muscular tissue (Pruchnic *et al*., 2000). The efficiency of AAV transduction in skeletal and cardiac muscle cells as well as in smooth muscle cells is very high and transgene statement persists for long periods of time (Donahue *et al*., 1999). All these properties make AAV vectors valuable tools for delivery of single factors or of their combinations in therapeutic angiogenesis.

Vectors derived from LV offer major advantages over other viral vectors as they do not depend on the proliferation of target cells for 'stable' transduction and induce very little immune response. These two features, together with the relative ease of production, make LV vectors an ideal tool for the design of experimental models involving gene transfer into progenitor populations. Recently, LV vectors have been used for stable expression of marker genes in haematopoietic progenitors, thus opening new perspectives to stem cell gene transfer studies (Kay *et al*., 2001). This is an extremely attractive opportunity for angiogenesis gene therapy. Indeed, bone marrow-derived ECP directly harvested from peripheral blood could be engineered to secrete pro-angiogenic substances so as to be employed for *ex-vivo* angiogenesis gene therapy (Isner & Asahara, 1999). However, concerns have been raised that LV-mediated transgene integration into the host genoma may induce gene mutations or unregulated, continuous transgene expression leading to generation of vascular tumours in the implantation site (Lee *et al*., 2000).

At variance with traditional drugs, the effective dosage of gene therapy is hard to predict as it depends on infectivity of the viral vector, the half-life of the angiogenic molecule and its ability to diffuse to adjacent tissue, a peculiarity of those transgenes carrying a secretory signal sequence. In relation to the last assumption, it should be noted however that even angiogenic GF lacking a signal sequence might be released as a result of lysis of infected cells or by a non-classic pathway (Mignatti *et al*., 1991; Fujita *et al*., 1996).

Current achievements of angiogenesis gene therapy

Pre-clinical studies have documented that angiogenic GF can promote collateral growth in animal models of peripheral and myocardial ischaemia (Ware & Simson, 1997; Isner & Ashara, 1999). Histological analysis indicates that such neovascularization encompasses a wide range of vessel calibre, the median range, however, being skewed toward vessels less than 180 μm in diameter (Isner & Asahara, 1999). It is presently unknown if remodelling of larger arteries, defined by Arras *et al*. (1998) as arteriogenesis occurs as a direct result of GF modulation or as flow-mediated response to augmented down-stream capillary capacitance.

Among angiogenic GF used in pre-clinical studies, VEGF (Takeshita *et al*., 1996; Banai *et al*., 1994; Tsurumi *et al*., 1997), FGF1 (Banai *et al*., 1991; Pu *et al*., 1993), FGF2 (Baffour *et al*., 1992; Lazarous *et al*., 1996) and hepatocyte growth factor (HGF) (Van Belle *et al*., 1998) have all shown significant improvement of native angiogenic response to ischaemia, resulting in accelerated rate of perfusion recovery. In general, these results have been documented under conditions in which experimental ischaemia was applied to otherwise normal animals. However, recent evidence indicates that the endothelial dysfunction which is typical of senescence, diabetes, hypercholesterolemia and hypertension does not preclude a favourable response to cytokine replacement therapy. Indeed, the absolute magnitude by which haemodynamic recovery and capillary density were increased in the above conditions was equivalent to that of normal animals (Rivard *et al*., 1999a, b; Couffinhal *et al*., 1999; Emanueli *et al*., 2001a).

Besides GF, a number of old molecules have been revisited for their putative angiogenic potential. Because angiotensin converting enzyme (ACE) inhibitors may favourably affect endothelial function, the hypothesis has been advanced that their administration could enhance spontaneous angiogenic response to ischaemia. With this in mind, Fabre *et al*. (1999) employed two ACE inhibitors, quinaprilat and captopril, both of which may achieve equivalent ACE inhibition in plasma but only one of which, quinaprilat, inhibits ACE activity at the tissue level. Both haemodynamic and morphological outcomes indicated augmented angiogenesis in the ischaemic limb of quinaprilat-treated rabbits, while captopril failed to exert any protective effect. The response to quinaprilat was similar to that observed with VEGF supplementation. Angiotensin II reportedly stimulates vascular NADH oxidase, thus increasing the generation of superoxide anions capable of degrading NO (Rajagopalan *et*

al., 1996) and consequently the bio-availability of this important modulator of angiogenesis. Furthermore ACE inhibitors may stimulate vascular growth by enhancing the expression of VEGF and/or by protecting kinin from degradation. In support of this theory, the authors referred to previous reports showing that ACE-inhibitor-induced augmentation in intracellular cyclic GMP levels and myocardial capillarity is prevented by the kinin B_2 receptor antagonist, icatibant (Gohlke *et al*., 1997).

We directly addressed the angiogenic potential of kinins by the use of a gene transfer approach. A replication-defective AV vector containing the HK gene was injected into the adductor skeletal muscle of mice which had been previously submitted to unilateral limb ischaemia (Emanueli *et al*., 2001a). Transgene expression in injected muscle and plasma peaked between 3 and 7 days and vanished within 28 days. By increasing muscular kinin levels, HK significantly augmented the native angiogenic response to ischaemia, accelerated the rate of haemodynamic recovery, and preserved muscular energetic charge (Figure 1). Putative mechanisms of HK-induced angiogenesis are shown in Figure 2. Angiogenesis gene therapy with HK proved to be useful also in pathological conditions characterized by endothelial dysfunction and progressive vascular rarefaction. Expression of endogenous tissue kallikrein is reportedly defective in the ischaemic hindlimb muscle of SHR (Emanueli *et al*., 2001b). Local supplementation with HK proved to be able to correct impaired native angiogenic response to ischaemia and allowed for complete haemodynamic recovery, without any change in systemic blood pressure (Emanueli *et al*., 2001b).

Diabetic microangiopathy represents another potential target for modulators of vascular growth. This condition is characterized by enhanced vascular EC turnover and death leading to microvascular loss and ischaemia. We found that a single intra-muscular injection of HK gene is effective in preventing microvascular rarefaction occurring in the limb skeletal muscle of streptozotocin-induced diabetic mice, independently from superimposed insulin treatment. Potentiation of limb microvasculature by HK might prove to be useful for prevention of peripheral vascular complications, which represent a major clinical and therapeutic problem in diabetes.

Therapeutic angiogenesis has been successfully extended from the bench to the bedside. Isner *et al*. (1996) was the first to demonstrate that arterial gene transfer of naked DNA encoding for VEGF165 (phVEGF165) at dosages from 100 to 2000 μg improves limb perfusion and relieves rest pain in patients with critical peripheral ischaemia. At the highest dose, angiographic and histological evidence of new blood vessel formation became apparent. Marked improvement in collateral vessel development was also achieved in critical peripheral ischaemia by the use of intra-muscular VEGF gene transfer (Baumgartner *et al*., 1998). VEGF reportedly increases circulating ECP in human subjects, thus suggesting that neovascularization promoted by this approach is not limited to angiogenesis but involves bone marrow derived cell-induced vasculogenesis (Asahara *et al*., 1997).

The successful application of recombinant protein and gene transfer for the treatment of myocardial ischaemia has been reported by Losordo *et al*. (1999) with improvement in treadmill exercise time and resting nuclear perfusion scans. These results were confirmed in phase 1 assessment of direct intra-myocardial administration of AV expressing VEGF121

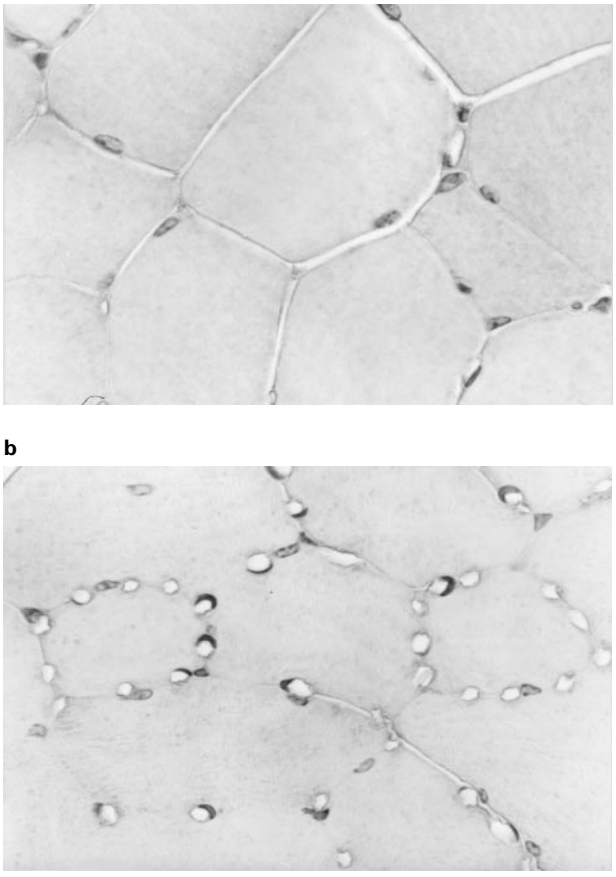

Figure 1 Immunohistochemical identification of vascular endothelial cells using antibodies against von Willebrand factor. Skeletal muscle sections were harvested from ischaemic hindlimbs of mice, 21 days after surgical removal of the femoral artery. Representative pictures show higher capillary density in adductor muscle injected with an adenoviral vector carrying the human tissue kallikrein gene (B) or reporter gene encoding for beta-galactosidase (A). (Emanueli *et al*., 2001a; reproduced with permission of publishers).

cDNA in patients with severe coronary artery disease (Rosengart *et al*., 1999). The effect appears to be dose-related, at least on the basis of results obtained with examination of myocardial perfusion by positron emission tomography (Hendel *et al*., 2000). Administration of FGF-1 has also been reported to improve myocardial perfusion in patients undergoing coronary bypass surgery (Schumacher *et al*., 1998). Similarly, administration of recombinant FGF-2 to patients with advanced coronary disease resulted in an attenuation of stress-induced ischaemia and improvement in resting myocardial blood flow (Udelson *et al*., 2000). In one of the few randomized, double-blind, placebo controlled studies completed so far, local peri-vascular delivery of FGF-2 (at 10 or 100 μg versus placebo) to patients undergoing coronary bypass surgery demonstrated the safety and feasibility of angiogenesis gene therapy (Laham *et al*., 2000). This was documented by an improvement of symptoms and at stress nuclear perfusion imaging. Magnetic resonance of the target zone showed a trend toward reduction of ischaemic area in the high dosage sub-group. The progress of therapeutic angiogenesis was recently summarized by the Angiogenesis Foundation and Angiogen-

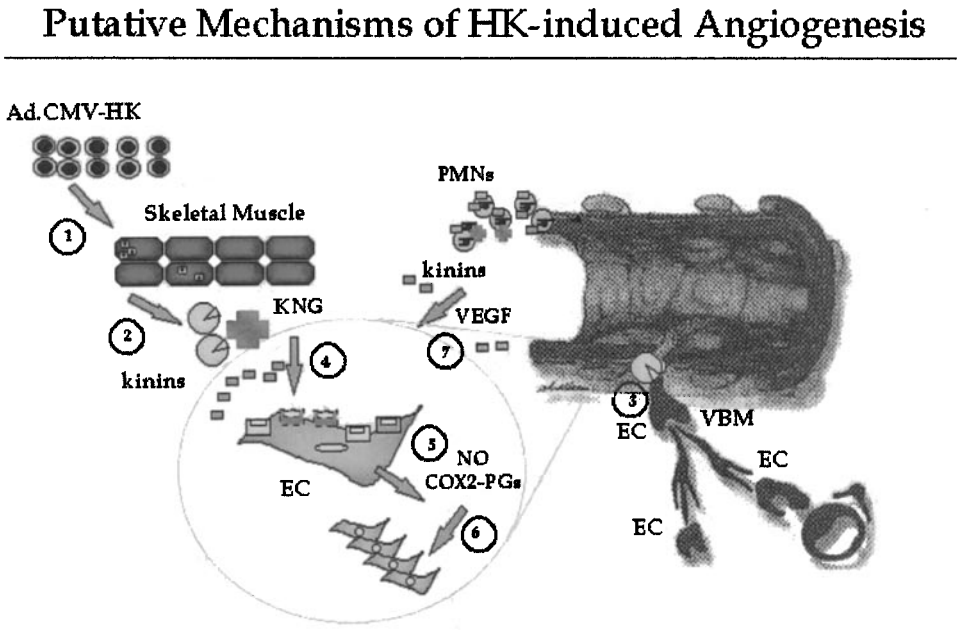

Figure 2 Representative sketch showing putative mechanisms (numbered in circles) of the angiogenic action exerted by human tissue kallikrein (HK). After delivery to targeted tissue by an adenoviral vector (Ad.CMV-HK, step 1), HK is released from infected skeletal muscle into the interstitial space and the blood stream (step 2). HK may contribute to digest vascular basal membrane (VBM), thus favouring vascular endothelial cell (EC) detachment and migration (step 3). Furthermore, as indicated in magnification of an EC, TK cleaves kininogen (KNG) to generate kinins which, *via* activation of inducible B_1 (B_1R, dashed squares on EC surface) and constitutive B_2 (B_2R, full squares on EC surface) receptors (step 4) and subsequent release of nitric oxide and cyclooxygenase 2 (COX2)-formed prostaglandins (step 5), stimulate vascular EC proliferation (step 6). Binding of kinin receptors may shift the receptor repertoire in favour of the inducible B_1R (inducibility is indicated by dashed line-squares reproducing the B_1R). Thus, the B_1R can act as magnets for TK-generated kinins. Other growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), could be up-regulated by kinins. Furthermore, kinin-attracted leukocytes (PMNs) may contribute to stimulation of angiogenesis by providing an additional source of growth factors, kininogen, and kinin generating enzyme (step 7). VEGF released by leukocytes stimulates endothelial proliferation by interacting with its own receptors on EC.

esis Research Center at the occasion of the 72nd Meeting of the American Heart Association. The statement also attempted to define a consensus on the challenges facing development of therapeutic angiogenesis for coronary disease (Simons *et al*., 2000).

Future directions to improve effectiveness and safety of vascular gene therapy

There are few other fields that are attracting the expectancy of public opinion so much as vascular gene therapy. To translate scientific promises into clinical results a series of problems have to be rigorously assessed.

Structural problems of neo-vascularization induced by gene therapy

The mechanisms and the molecular cues, which dictate the spatial tridimensional distribution of nascent vessels during neo-angiogenesis in the adult organism, constitute a deep hole in our knowledge. The processes involved in the regulation of the shape and dimension of vessels also remain largely unexplored. Delivery of angiogenic inducers (e.g. VEGF-A or FGF) in ischaemic tissues allows rescue of blood perfusion. However, angiographic studies clearly show that the newly formed vasculature is abnormal and not well organized as in normal tissues (Isner *et al*., 1996; Murohara *et al*., 1998; Shyu *et al*., 1998), resembling the characteristics of leaky haemangiomas (Detmar *et al*., 1998; Lakatos, 1998; Suri *et al*., 1998). Furthermore, unregulated expression of VEGF or aFGF might promote pathological angiogenesis in distant tissues and stimulate the formation of vascular tumours in skeletal muscle and myocardium (Banai *et al*., 1991, Lee *et al*., 2000). From all of the above, it appears wise to deliver GF in minimal angiogenic quantities. This requires assessment of dose-response titration curves and evaluation of toxicity, possibly in large animals so as to allow extrapolation of results to the clinical field. Combination strategies are envisaged to be better than a single angiogenic molecule, yet only few studies have been conducted with this in mind. For instance, Ang-1 and Ang-2 appear uniquely suitable for stabilization of VEFG induced angiogenesis (Chae *et al*., 2000). Particularly attractive seems the association of endothelial GF with agents such as FGF capable of providing the nascent microcirculation with a muscular layer.

The ideal device for gene transfer

There is a substantial lag between the possibilities offered by the increasing number of angiogenic molecules and the slow progress in the area of vector and device development. Indeed, at the present time, transfer methodology represents

a major barrier to the successful application of therapeutic angiogenesis. At variance with hindlimb skeletal muscle, that is easily accessible for direct injection, other organs including the heart require the use of physical devices for gene delivery. Unfortunately, local vascular catheters proposed to achieve this aim tend to produce an uneven pattern of vector delivery, to leak vector into coronary side branches, or even to rapidly inactivate AV vectors (Leiden, 2000). Advances in nano-technologies would hopefully help to overcome these limitations. The ideal device for treatment of ischaemia would consist of a vascular stent engineered so as to release nanomolar quantities of active agiogenic principle only upon occurrence of conditions of tissue acidosis or hypoxia.

Prevention of vascular insufficiency by potentiation of collateral vasculature

As indicated above, most pre-clinical and clinical studies consisted in rescuing ischaemic tissue by enhancing the native host defence response to interruption in blood flow. This approach is based on the paradigm that 'regional tissue ischaemia is still the only situation which leads to collateral vessel formation' (Schaper *et al*., 1993). No drug or GF was believed to accomplish this result in the absence of ischaemia. In fact, chronic infusion of VEGF into the canine coronary arteries does not lead to endothelial proliferation. Moreover, endothelial growth factor, regarded as an effective angiogenic agent in ischaemic tissue, is instead ineffective in the normoperfused rabbit hindlimb (Pu *et al*., 1993). In contrast, in the ischaemic setting, even endothelial GF devoid of mitogenic effect on vascular smooth muscle cells can indirectly improve collateral circulation as the result of an increased microvascular capacitance. Furthermore, under conditions of hypoxia, an autocrine loop provides the basis for amplification of the angiogenic effect of any given VEGF secreted or administered into an ischaemic territory (Isner & Asahara, 1999). Recent evidence however supports the view that at least VEGF and HK can promote *de novo* angiogenesis in normoperfused skeletal muscle (Gowdak *et al*., 2000; Emanueli *et al*., 2000). A similar effect was observed by Safi *et al*. (1999) following FGF-1 gene transfer into non-ischaemic rabbit heart. These results suggest that patients with intermittent ischaemia might take advantage of preventive angiogenesis. Indeed, potentiation of collateral circulation may provide an anatomical basis for reduction in the risk region upon occurrence of vascular occlusion and also relieve exercise-induced ischaemic symptoms. To achieve this goal, it is mandatory that the newly developed vasculature persists and is haemodynamically efficient. We know from previous experiments that the neoangiogenesis response to HK is still at plateau 28 days after gene transfer in the mouse normoperfused hindlimb muscle (Emanueli *et al*., 2000). These discoveries open new avenues for prevention of ischaemic vascular disease.

Therapeutic angiogenesis with autologous transplantation of ECP

The resident population of EC that is competent to respond to angiogenic GF may potentially limit the response to therapeutic angiogenesis. This limitation has been circumvented by transplanting human EPC (Kalka *et al*., 2000; Shintani *et al*., 2001). EPC can be isolated from peripheral blood in quantities sufficient to permit their harvest, and, after *ex vivo* expansion, be re-administered for the purpose of enhancing neovascularization. Consistently, intra-muscular injection of blood-derived angioblasts reportedly accelerates post-ischaemic blood-flow restoration in healing-impaired diabetic mice (Schatteman *et al*., 2000).

Summary and conclusions

Angiogenesis gene therapy is emerging as a new therapeutic strategy for prevention and treatment of ischaemic vascular disease. Both pre-clinical and clinical results speak in favour of its possible use for the treatment of patients in which traditional revascularization procedures are either precluded or ineffective. However, due to the rapid evolution in the field there is some concern that clinical applications become too obvious and will be undertaken without the most critical pharmacological scrutiny. The death of Jessie Gelsinger, a patient treated with systemic AV gene therapy at very high doses for a genetic enzymatic defect, has seriously compromized public confidence and scientific credibility (Leiden, 2000). Yet, the most desperately ill patients are considered to display the best risk/benefit ratio for evaluating gene therapy effectiveness. We argue that by sticking to this strategy one might underestimate the real advantages of angiogenesis gene therapy. On the other side, it should be admitted that a more extensive application is premature. Therefore, the current status of the field effectively mandates the most rigorous and punctilious analyses in closely defined experimental systems so as to accurately assess (and optimize) such a ratio before any clinical application.

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References

ARRAS, M., ITO, W.D., SCHOLZ, D., WINKLER, B., SCHAPER, J. & SCHAPER, W. (1998). Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J. Clin. Invest.,* **101,** 40–50.

ASAHARA, T., MASUDA, H., TAKAHASHI, T., KALKA, C., PASTORE, C., SILVER, M., KEARNE, M., MAGNER, M. & ISNER, J.M. (1999). Bone marrow origin of endothelial progenitor cells responsible for post-natal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.,* **85,** 221–228.

ASAHARA, T., MUROHARA, T., SULLIVAN, A., SILVER, M., VANDERZEE, R., LI, T., WITZENBICHLER, B., SCATTEMAN, G. & ISNER, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science,* **275,** 965–967.

BAFFOUR, R., BERMAN, J., GARB, J.L., RHEE, S.W., KAUFMAN, J. & FRIEDMAN, P. (1992). Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. *J. Vasc. Surg.,* **16,** 181–191.

BANAI, S., JAKLITSCH, M., CASSCELLS, W., SHRIVASTAV, S., CORREA, R., EPSTEIN, S.E. & UNGER, E.F. (1991). Effects of aFGF on normal and ischemic myocardium. *Circ. Res.,* **69,** 76–85.

BANAI, S., JAKLITSCH, M.T., SHOU, M., LAZAROUS, D.F., SCHEINOWITZ, M., BIRO, S., EPSTEIN, S.E. & UNGER, E.F. (1994). Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation,* **89,** 2183–2189.

BAUMGARTNER, I., PIECZEK, A., MANOR, O., BLAIR, R., KEARNEY, M., WALSH, K. & ISNER, J.M. (1998). Constitutive expression of phVEGF$_{165}$ after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation,* **97,** 1114–1123.

BUSSOLINO, F., MANTOVANI, A. & PERSICO, G. (1997). Molecular mechanisms of blood vessel formation. *Trends Biochem. Sci.,* **22,** 251–256.

CARMELIET, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.,* **6,** 389–395.

CARMELIET, P., NG, Y.S., NUYENS, D., THEILMEIER, G., BRUSSELMANS, K., CORNELISSEN, I., EHLER, E., KAKKAR, V.V., STALMANS, I., MATTOT, V., PERRIARD, J.C., DEWERCHIN, M., FLAMENG, W., NAGY, A., LUPU, F., MOONS, L., COLLEN, D., D'AMORE, P.A. & SHIMA, D.T. (1999). Imparied myocardial angiogenesis and ischemic myocardiopathy in mice lacking the vascular growth factor isoforms VEGF164 and VEGF188. *Nat. Med.,* **5,** 495–502.

CHAE, J.K., KIM, I., LIM, S.T., CHUNG, M.J., KIM, W.H., KIM, H.G., KO, J.K. & KOH, G.Y. (2000). Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. *Arterioscler. Thromb. Vasc. Biol.,* **20,** 2573–2578.

CHAO, J., JIN, L., CHEN, L.M., CHEN, V.C. & CHAO, L. (1996). Systemic and portal vein delivery of human kallikrein gene reduces blood pressure in hypertensive rats. *Hum. Gene Ther.,* **7,** 901–911.

COUFFINHAL, T., SILVER, M., KEARNEY, M., SULIVAN, A., WITZENBICHLER, B., MAGNER, M., ANNEX, B., PETERS, K. & ISNER, J.M. (1999). Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE−/−mice. *Circulation,* **99,** 3188–3198.

CRYSTAL, R.G. (1997). Phase I study of direct administration of a replication deficient adenovirus vector containing E. choli cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the pro-drug 5-fluorocytosine. *Hum. Gene Ther.,* **8,** 985–1001.

DETMAR, M., BROWN, L.F., SCHON, M.P., ELICKER, B.M., VELASCO, P., RICHARD, L., FUKUMURA, D., MONSKY, W., CLAFFEY, K.P. & JAIN, R.K. (1998). Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J. Invest. Dermatol.,* **111,** 1–6.

DONAHUE, B.A., MCARTHUR, J.G., SPRATT, S.K., BOHL, D., LAGARDE, C., SANCHEZ, L., KASPAR, B.A., SLOAN, B.A., LEE, Y.L., DANOS, O. & SNYDER, R.O. (1999). Selective uptake and sustained expression of AAV vectors following subcutaneous delivery. *J. Gene Med.,* **1,** 31–42.

EMANUELI, C., MINASI, A., ZACHEO, A., CHAO, J., CHAO, L., SALIS, M.B., STRAINO, S., TOZZI, M.G., SMITH, R., GASPA, L., BIANCHINI, G., STILLO, F., CAPOGROSSI, M.C. & MADEDDU, P. (2001a). Local delivery of human tissue kallikrein gene accelerates spontaneous angiogenesis in mouse model of hindlimb ischemia. *Circulation,* **103,** 125–132.

EMANUELI, C., SALIS, M.B., STACCA, T., GASPA, L., CHAO, J., CHAO, L. & MADEDDU, P. (2001b). Rescue of impaired angiogenesis in spontaneously hypertensive rats by intramuscular human tissue kallikrein gene transfer. *Hypertension,* (in press).

EMANUELI, C., ZACHEO, A., MINASI, A., CHAO, J., CHAO, L., SALIS, M.B., STACCA, T., STRAINO, S., CAPOGROSSI, M.C. & MADEDDU, P. (2000). Adenovirus-mediated human tissue kallikrein gene delivery induces angiogenesis in normoperfused skeletal muscle. *Arterioscler. Thromb. Vasc. Biol.,* **20,** 2379–2385.

FABRE, J.E., RIVARD, A., MAGNER, M., SILVER, M. & ISNER, J.M. (1999). Tissue inhibition of angiotensin-converting enzyme activity stimulates angiogenesis in vivo. *Circulation,* **99,** 3043–3049.

FERRARA, N. & ALITALO, K. (1999). Clinical applications of angiogenic growth factors and their inhibitors. *Nat. Med.,* **5,** 1359–1364.

FERRARA, N. & DEVIS-SMYTH, T. (1997). The biology of vascular endothelial growth factor. *Endocr. Rev.,* **18,** 4–25.

FUJITA, M., IKEMOTO, M., KISHISHITA, M., OTANI, H., NOHARA, R., TANAKA, T., TAMAKI, A.-I., YAMAZATO, A. & SASAYAMA, S. (1996). Elevated basic fibroblast growth factor in pericardial fluid of patients with unstable angina. *Circulation,* **94,** 610–613.

GILGENKRANTZ, H., DUBOC, D., JUILLARD, V., COUTON, D., PAVIRANI, A., GUILLET, J.G., BRIAND, P. & KAHN, A. (1995). Transient expression of genes transferred in vivo into heart using first-generation adenoviral vectors: role of the immune response. *Hum. Gene Ther.,* **6,** 1265–1274.

GOHLKE, P., KWER, J., SCHNELL, A., AMANN, K., MAE, G. & UNGER, T. (1997). Blockade of bradykinin B_2 receptors prevents the increase in capillary density induced by chronic angiotensin-converting enzyme inhibitor treatment in stroke-prone spontaneously hypertensive rats. *Hypertension,* **29,** 478–482.

GOWDAK, L.H., POLIAKOVA, L., WANG, X., KOVESDI, I., FISHBEIN, K.W., ZACHEO, A., PALUMBO, R., STRAINO, S., EMANUELI, C., MARROCCO-TRISCHITTA, M., LAKATTA, E.G., ANVERSA, P., SPENCER, R.G., TALAN, M. & CAPOGROSSI, M.C. (2000). Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. *Circulation,* **102,** 565–571.

GRIMM, D. & KLEINSCHMIDT, J.A. (1999). Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. *Hum. Gene Ther.,* **10,** 2445–2450.

HANAHAN, D. (1997). Signaling vascular morphogenesis and maintenance. *Science,* **277,** 48–50.

HENDEL, R.C., HENRY, T.D., ROCHA-SINGH, K., ISNER, J.M., KEREIAKES, D.J., GIORDANO, F.J., SIMONS, M. & BONOW, R.O. (2000). Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect. *Circulation,* **101,** 118–121.

ISNER, J. & ASAHARA, T. (1999). Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J. Clin. Invest.,* **103,** 1231–1236.

ISNER, J.M., PIECZEK, A., SCHAINFELD, R., BLAIR, R., HALEY, L., ASAHARA, T., ROSENFIELD, K., RAZVI, S., WALSH, K. & SYMES, J.F. (1996). Clinical evidence of angiogenesis after arterial gene transfer of phVEGF 165 in patient with ischemic limb. *Lancet.,* **348,** 370–374.

KALKA, C., MASUDA, H., TAKAHASHI, T., KALKA-MOLL, W.M., SILVER, M., KEARNEY, M., LI, T., ISNER, J.M. & ASAHARA, T. (2000). Trasplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 3422–3427.

KAY, M.A., GLORIOSO, J.C. & NALDINI, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat. Med.,* **7,** 33–40.

LAHAM, R.J., CHRONOS, N.A., PIKE, M., LEIMBACH, M.E., UDELSON, J.E., PEARLMAN, J.D., PETTIGREW, R.I., WHITEHOUSE, M.J., YOSHIZAWA, C. & SIMONS, M. (2000). Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study. *J. Am. Coll. Cardiol.,* **36,** 2132–2139.

LAKATOS, L. (1998). Transgenic mice model of ocular neovascularization driven by vascular endothelial growth factor (VEGF) overexpression. *Am. J. Pathol.,* **152,** 1397–1398.

LAZAROUS, D., SHOU, M., SCHEINOWIZ, M., HODGE, E., THIRUMURTI, V., KITSIOU, A.N., STIBER, J.A., LOBO, A.D., HUNSBERGER, S., GUETTA, E., EPSTEIN, S.E. & UNGER, E.F. (1996). Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. *Circulation,* **94,** 1074–1082.

LEE, R.J., SPRINGER, M.L., BLANCO-BOSE, W.E., SHAW, R., URSELL, P.C. & BLAU, H.M. (2000). VEGF gene delivery to myocardium. Deleterious effects of unregulated expression. *Circulation,* **102,** 898 – 901.

LEIDEN, J.M. (2000). Human gene therapy. The good, the bad, and the ugly. *Circ. Res.,* **86,** 923 – 925.

LOSORDO, D.W., VALE, P.R. & ISNER, J.M. (1999). Gene therapy for myocardial angiogenesis. *Am. Heart J.,* **138,** (2 Pt 2), S132 – S141.

MAISONPIERRE, P.C., SURI, C., JONES, P.F., BARTUNKOVA, S., WIEGAND, S.J., RADZIEJEWSKI, C., COMPTON, D., MCCLAIN, J., ALDRICH, T.H., PAPADOPOULOS, N., DALY, T.J., DAVIS, S., SATO, T.N. & YANCOPOULOS, G.D. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science,* **277,** 55 – 60.

MIGNATTI, P., MORIMOTO, T. & RAFKIN, D.B. (1991). Basic fibroblast growth factor released by single, isolated cells stimulates their migration in an autocrine manner. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 11007 – 11011.

MUHLHAUSER, J., JONES, M., YAMADA, I., CIRIELLI, C., LEMARCHAND, P., GLOE, T.R., BEWIG, B., SIGNORETTI, S., CRYSTAL, R.G. & CAPOGROSSI, M.C. (1996). Safety and efficacy of in vivo gene transfer into the porcine heart with replication-deficient, recombinant adenovirus vectors. *Gene Ther.,* **3,** 145 – 153.

MUROHARA, T., HOROWIZ, J.R., SILVER, M., TSURUMI, Y., CHEN, D., SULLIVAN, A. & ISNER, J.M. (1998). Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation,* **97,** 99 – 107.

NEWMAN, K.D., DUNN, P.F., OWENS, J.W., SCHULICK, A.H., VIRMANI, R., SUKHOVA, G., LIBBY, P. & DICHEK, D.A. (1999). Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointima hyperplasia. *J. Clin. Invest.,* **96,** 2955 – 2965.

PRUCHNIC, R., CAO, B., PETERSON, Z.Q., XIAO, X., LI, J., SAMULSKI, R.J., EPPERLY, M. & HUARD, J. (2000). The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. *Hum. Gene. Ther.,* **11,** 521 – 536.

PU, L.Q., SNIDERMANN, A.D., BRASSARD, R., LACHAPELLE, K.J., GRAHAM, A.M., LISBONA, R. & SYMES, J.F. (1993). Enhanced revascularization of the ischemic limb by angiogenic therapy. *Circulation,* **88,** 208 – 215.

RAJAGOPALAN, S., KURZ, S., MUNZEL, T., TARPEY, M., FREEMAN, B.A., GRIENDLING, K.K. & HARRISON, D.G. (1996). Ang II mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone. *J. Clin. Invest.,* **97,** 1916 – 1923.

RISAU, W. (1997). Mechanism of angiogenesis. *Nature,* **386,** 671 – 674.

RISAU, W. & FLAMME, I. (1995). Vasculogenesis. *Annu. Rev. Cell. Develop. Biol.,* **11,** 73 – 91.

RIVARD, A., FABRE, J.E., SILVER, M., CHEN, D., MUROHARA, T., KEARNEY, M., MAGNER, M., ASHARA, T. & ISNER, J.M. (1999a). Age-dependent impairment of angiogenesis. *Circulation,* **99,** 111 – 120.

RIVARD, A., SILVER, M., CHEN, D., KEARNEY, M., MAGNER, M., ANNEX, B., PETERS, K. & ISNER, J.M. (1999b). Rescue of diabetes related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. *Am. J. Pathol.,* **154,** 355 – 364.

ROSENGART, T.K., LEE, L.Y., PATEL, S.R., KLIGFIELD, P.D., OKIN, P.M., HACKETT, N.R., ISOM, O.W. & CRYSTAL, R.G. (1999). Six-month assessment of a phase I trial of angiogenic gene therapy for the treatment of coronary artery disease using direct intramyocardial administration of an adenovirus vector expressing the VEGF121 cDNA. *Ann. Surg.,* **230,** 466 – 470.

SAFI, J., DIPAULA, A.F., RICCIONI, T., KAJSTURA, J., AMBROSIO, G., BECKER, L.C., ANVERSA, P. & CAPOGROSSI, M.C. (1999). Adenovirus-mediated acidic fibroblast growth factor gene transfer induces angiogenesis in the nonischemic rabbit heart. *Microvasc Res.,* **58,** 238 – 249.

SCHAPER, W. (1993). Coronary collateral development: concepts and hypotheses. In: Schaper, W., Shaper, J. eds. *Collateral Circulation: Heart, Brain, Kidney*. Boston, Mass: Kluewer Academic Publishers; pp 46 – 58.

SCHAPER, W. & ITO, W.D. (1996). Molecular mechanisms of coronary vessel growth. *Circ. Res.,* **79,** 911 – 919.

SCHATTEMAN, G.C., HANLON, H.D., JIAO, C., DODDS, S.G. & CHRISTY, B.A. (2000). Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J. Clin. Invest.,* **106,** 571 – 578.

SCHUMACHER, B., STEGMANN, T. & PECHER, P. (1998). The stimulation of neoangiogenesis in the ischemic human heart by the growth factor FGF: first clinical results. *J. Cardiovasc. Surg.,* **39,** 783 – 789.

SEMENZA, G.L. (1999). Hypoxia inducible factor 1: master regulator of O_2 homeostasis. *Curr. Opin. Genet. Dev.,* **8,** 588 – 594.

SHINTANI, S., MUROHARA, T., IKEDA, H., UENO, T., SASAKI, K., DUAN, J. & IMAIZUMI, T. (2001). Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation,* **103,** 897 – 903.

SHYU, K.G., MANOR, O., MAGNER, M., YANCOPOULOS, G.D. & ISNER, J.M. (1998). Direct intramuscular injection of plasmid DNA encoding angiopoietin-1 but not angiopoietin-2 augments revascularization in the rabbit ischemic hindlimb. *Circulation,* **98,** 2081 – 2087.

SIMONS, M., BONOW, R.O., CHRONOS, N.A., COHEN, D.J., GIORDANO, F.J., HAMMOND, H.K., LAHAM, R.J., LI, W., PIKE, M., SELLKE, F.W., STEGMANN, T.J., UDELSON, J.E. & ROSENGART, T.K. (2000). Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary. *Circulation,* **102,** E73 – E86.

SURI, C., MCCLAIN, J., THURSTON, G., MCDONALD, D.M., ZHOU, H., OLDMIXON, E.H., SATO, T.N. & YANCOPOULOS, G.D. (1998). Increased vascularization in mice overexpressing angiopoietin-1. *Science,* **282,** 468 – 471.

TAKESHITA, S., TSURUMI, Y., COUFFINAHAL, T., ASAHARA, T., BAUTERS, C., SYMES, J., FERRARA, N. & ISNER, J.M. (1996). Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo. *Lab. Invest.,* **75,** 487 – 501.

TSCHOPE, C., HERINGER-WALTHER, S., KOCH, M., SPILLMANN, F., WENDORF, M., LEITNER, E., SCHULTHEISS, H.P. & WALTHE, T. (2000). Upregulation of bradykinin B1-receptor expression after myocardial infarction. *Br. J. Pharmacol.,* **129,** 1537 – 1538.

TSURUMI, Y., KEARNEY, M., CHEN, D., SILVER, M., TAKESHITA, S., YANG, J., SYMES, J.F. & ISNER, J.M. (1997). Treatment of acute limb ischemia by intramuscular injection of vascular endothelial growth factor gene. *Circulation,* **96** (Suppl I), II-382 – 388.

UDELSON, J.E., DILSIZIAN, V., LAHAM, R.J., CHRONOS, N., VANSANT, J., BLAIS, M., GALT, J.R., PIKE, M., YOSHIZAWA, C. & SIMONS, M. (2000). Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease. *Circulation,* **102,** 1605 – 1610.

VAN BELLE, E., WITZENBICHLER, B., CHEN, D., SILVER, M., CHANG, L., SCHWALL, R. & ISNER, J.M. (1998). Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor. The case for paracrine amplification of angiogenesis. *Circulation,* **97,** 381 – 390.

WARE, J.A. & SIMSON, M. (1997). Angiogenesis in ischemic heart disease. *Nat. Med.,* **3,** 158 – 164.

WITZENBICHLER, B., ASAHARA, T., MUROHARA, T., SILVER, M., SPYRIDOPOULOS, I., MAGNER, M., PRINCIPE, N., KEARNEY, M., HU, J.-S. & ISNER, J.M. (1998). Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am. J. Pathol.,* **153,** 381 – 394.

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