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Regenerative Medicine in the Treatment of Peripheral Arterial Disease

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ABSTRACT

The last decade has witnessed a dramatic increase in the mechanistic understanding of angiogenesis and arteriogenesis, the two processes by which the body responds to obstruction of large conduit arteries. This knowledge has been translated into novel therapeutic approaches to the treatment of peripheral arterial disease, a condition characterized by progressive narrowing of lower extremity arteries and heretofore solely amenable to surgical revascularization. Clinical trials of molecular, genetic, and cell-based treatments for peripheral artery obstruction have generally provided encouraging results. J. Cell. Biochem. 108: 753–761, 2009.

KEY WORDS: ANGIOGENESIS; ARTERIOGENESIS; ENDOTHELIAL PROGENITOR CELL

Peripheral arterial disease (PAD) is characterized by progressive narrowing or occlusion of lower extremity arteries, most often secondary to atherosclerosis [Schainfeld and Isner, 1999]. The disease affects approximately 27 million adults throughout the United States and Europe. PAD may be asymptomatic or lead to symptoms such as intermittent claudication, ulceration, rest pain, and critical limb ischemia [Ghosh et al., 2008]. PAD is more common in the elderly, especially those over the age of 70, and in those with risk factors such as hypercholesterolemia, smoking, hypertension, and diabetes. The most common treatments for PAD include surgical bypass procedures or percutaneous angioplasty; however, many patients are ineligible for these procedures upon their first presentation to a physician and 10–40% patients eventually require leg amputation. Thus, PAD currently has a poor prognosis and sharply reduces the quality of life.

These circumstances have driven the pursuit of novel therapeutic alternatives for PAD. The recent discovery of gene products able to stimulate endothelial cells, and hence blood vessel growth, as well as the identification of stem and progenitor cells that participate in post-ischemic vascular repair has generated an interest in using molecular, genetic, and/or cellular-based regenerative therapies for the treatment of PAD. This Prospects will first review physiological mechanisms underlying angiogenesis and arteriogenesis, the two processes that comprise the post-ischemic vascular response, and then review some of the outcomes of preclinical and clinical trials that have evaluated these novel approaches in the treatment of PAD.

ANGIOGENESIS

Angiogenesis is the formation of new capillaries within tissue downstream from the site of conduit artery obstruction; for example, angiogenesis occurs in the calf muscles following femoral artery occlusion [Heil et al., 2006]. The net consequence of angiogenesis is an expansion of capillary density, that is, the number of capillaries per unit volume of tissue parenchyma, such as skeletal muscle. This process increases the capillary surface area available for oxygen diffusion, increases the net diffusive flux of oxygen from capillary-to-cell, and thus attenuates the cellular hypoxia generated by loss of tissue perfusion.

The driving stimulus for angiogenesis is the cellular hypoxia generated by the interruption of tissue oxygen delivery secondary to perfusion deficit. Cellular hypoxia interrupts the oxygen-dependent proteolysis of the α subunit of hypoxia inducible factor-1, a critical transcription factor capable of transactivation of >60 genes relevant to the process of angiogenesis; as well, cellular hypoxia increases expression of RNA binding proteins that stabilize specific mRNA transcripts [Pugh and Ratcliffe, 2003]. An early consequence of these actions is a dramatic increase in the expression vascular endothelial growth factor (VEGF) and its receptors.

VEGF is a family of growth factors comprised of five members (designated A–E), as well as a homologue called placental growth factor (PIGF). The most relevant VEGF isoform in initiating and sustaining angiogenesis is VEGF-A which exerts its actions by

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binding to VEGFR2, a tyrosine kinase receptor with multiple downstream targets; other isoforms and receptors play supportive roles [Bouloumié et al., 1999]. VEGF-A is an endothelial cell mitogen, induces migration of endothelial cells in vitro, and generates the formation of tubular structures by endothelial cells embedded within a Matrigel matrix. A critical downstream target of VEGFR2 is endothelial nitric oxide synthase (eNOS), whose activation yields a sharp rise in the signaling molecule nitric oxide (NO) [Murohara et al., 1998]. VEGFR2-induced activation of eNOS is mediated via the phosphoinosotide-3 kinase/Akt pathway. eNOS-derived NO mediates many of the established effects of VEGF-A, including EC proliferation and migration, and post-ischemic angiogenesis.

Expression of other growth factors also increase during angiogenesis and these agents carry out well defined roles in this process [Jain, 2003]. The creation of durable and mature vessels during angiogenesis requires the enhancement of cell–cell contact between ECs, as well as the generation of new ECM and the recruitment of pericytes that surround and support the new capillary endothelium. Several growth factors participate in these processes, including angiopoietin-1 (Ang1), platelet derived growth factor (PDGF), and transforming growth factor-β1 (TGF-β1). Ang1, largely produced by ECs, facilitates cell–cell linkage between nascent ECs by upregulating expression of the adhesion molecules PECAM, occludin, and VE-cadherin. Ang1 also acts in concert with VEGF in the recruitment of pericytes. PDGF is secreted by ECs in response to VEGF and facilitates pericyte proliferation and migration to emerging capillary. TGF-β1 is expressed by several vascular cell types and mediates remodeling of the provisional ECM surrounding nascent capillaries. As well, several members of the fibroblast growth factor (FGF) are upregulated in the post-ischemic hindlimb; chief among these is basic FGF (bFGF or FGF-2), which serves as a mitogen for endothelial, vascular smooth muscle, and stromal cells.

Generation of new capillaries requires modification of the subendothelial basement membrane and surrounding extracellular matrix (ECM) to permit migration of endothelial cells, a task accomplished by members of the matrix metalloproteinase family and urokinase plasminogen activator [Huang et al., 2009]. The ECM is not simply dissolved, as excessive disruption would destroy guidance cues for EC migration; instead, the composition of the ECM is modified to become permissive to vessel sprouting. The critical role of proteases such as MMP-9 is underscored by the aberration of angiogenesis that occurs in mice deficient in the genes coding for these agents. Not surprisingly, these proteolytic systems are upregulated by HIF-1α in a manner coordinate with the VEGF system [Pugh and Ratcliffe, 2003].

For many years it was believed that proliferation of existent, differentiated endothelial cells provided the necessary reservoir for regeneration of blood vessels and for post-natal neovascularization, for example, angiogenesis. A paradigm shift occurred following the report of Asahara which demonstrated that $CD34^+$ or $Flk\text{-}1^+$ subsets of peripheral blood mononuclear cells could differentiate toward an endothelial cell phenotype in vitro, and that these cells engrafted into sites of hindlimb angiogenesis when transfused into mice that had undergone expiration of the femoral artery [Asahara et al., 1997]. This seminal finding indicated that circulating cells could participate in post-natal, post-ischemic blood vessel regeneration and growth.

These cells were designated endothelial progenitor cells (EPC). More than a decade has passed since their introduction, yet the origin and full characterization of EPC remains somewhat unsettled. At least two EPC subsets have been recognized [Asahara and Kawamoto, 2003]. The first group appears to be derived from the mesodermal hemangioblast believed to be the common precursor for hematopoietic stem cells (HSC) and vascular stem cells within the bone marrow. These EPCs express the surface phenotype $CD34^+$, $AC133^+$, and $Flk\text{-}1^+$ (Flk-1 is also called VEGFR2 or KDR), exhibit clonal expansion in vitro, and can be made to differentiate to a mature EC phenotype by VEGF (e.g., development of the expression of eNOS, Tie-2, CD31, E-selectin, VE-cadherins). The second group appears to be derived from more differentiated myeloid progenitor cells. These EPC generally express the surface phenotype $CD34^{low}$, $AC133^-$, $Flk\text{-}1^-$, and $CD14^+$. The relative roles of these EPC subsets in angiogenesis remains ill-defined, although it is clear that both $CD34^+$ and $CD14^+$ EPC participate and that these cells are primarily derived from bone marrow.

Another mononuclear cell involved in angiogenesis merits description, the mesenchymal stem cells (MSC) [Kinnaird et al., 2004]. MSC are derived from the bone marrow stromal elements, but have also been identified within the stromal elements in a variety of tissues. MSC defy clear phenotypic distinction as they lack consistent expression of specific surface antigens. They are primarily identified by their capacity to adhere to bare plastic in vitro and by the absence of surface markers characteristic of EPC; moreover, these cells exhibit clonal expansion in vitro and clearly participate in post-ischemic angiogenesis.

Participation of bone marrow-derived progenitor and stems cells in angiogenesis requires their mobilization from the marrow niche, and trafficking and adhesion at the sites of angiogenesis [Aicher et al., 2005]. Circulating growth factors (VEGF, Ang-1) and cytokines (granulocyte/monocyte colony stimulating factor, erythropoietin) participate in post-ischemic EPC mobilization, as do the chemokines SDF-1 and IL-8; indeed, SDF-1 expression within ischemic tissue is enhanced by HIF-1α, providing further evidence of the critical role of HIF-1α in orchestrating angiogenesis [Pugh and Ratcliffe, 2003]. Another essential party to EPC mobilization is eNOS-derived NO, which mediates proteinase-induced disengagement of EPC from bone marrow stromal cells. Trafficking of EPC to the sites of angiogenesis also involves SDF-1 and IL-8 via the chemokine receptors CXCR4 and CXCR2, respectively. The adhesion molecules which mediate EPC rolling, firm adhesion, and transendothelial migration are similar to those used by leukocytes: P- and E-selectin, and β1 and β2 integrins and their counter ligands, VCAM-1, and ICAM-1.

The fate of vascular stem and progenitor cells at the site of angiogenesis remains controversial. Initial reports indicated engraftment and differentiation of EPC to mature endothelial cells within the growing capillary network [Asahara et al., 1997]. Later studies, however, failed to confirm this fate. Instead, it has been proposed that the principal role of vascular stem and progenitor cells is paracrine, that is, that these cells promote proliferation and migration of existing endothelial cells and pericytes, as well as

produce additional cytokines and chemokines to continue stem and progenitor mobilization, trafficking, and adhesion [Gnecchi et al., 2008]. Production of several growth factors and chemokines by MSC is significantly increased when these cells exist within a relatively hypoxic environment, as would be anticipated to occur within the ischemic hindlimb. Moreover, transfection of MSC with constitutively active Akt results in their sustained production of SDF-1, VEGF, and FGF, a circumstance that might prove useful in stem cell transplantation.

ARTERIOGENESIS

Obstruction of a large conduit artery, for example, the femoral artery, results in enlargement of preexisting collateral arteries that run parallel to the much larger conduit artery. Under normal conditions collateral arteries have a small diameter that generates a high vascular resistance, a circumstance which precludes a significant flow rate. However, obstruction of the conduit artery substantially increases the pressure gradient across the collaterals, sharply raising their flow rate. Flow rate is a crucial determinant of wall shear stress, a mechanostimulus generated by the movement of blood across the static endothelial surface of the vessel. It is this stimulus of shear stress that induces arteriogenesis. In this context, arteriogenesis differs from angiogenesis: arteriogenesis occurs within preexisting collaterals that bypass the site of arterial obstruction and is stimulated by shear stress, whereas angiogenesis occurs well downstream and is stimulated by tissue hypoxia [Heil et al., 2006].

An increase in shear stress alters the state of the endothelium [Heil et al., 2006]. An immediate effect is activation of eNOS, resulting in a substantial, albeit transient NO-based collateral artery vasodilation. Mechanical deformation of the endothelial cell surface enhances transcription of chemokines and adhesion molecules via mechanoreceptors; a critical consequence of this process is release of monocyte chemo attractant protein-1 (MCP-1), GM-CSF, and SDF-1, which recruit $CD14^{+}$ monocytes the activated endothelial cell surface. Monocytes adhere and undergo transendothelial migration to the sub-endothelial space, where they become activated and, in some instances, assume a macrophage phenotype. These cells produce chemokines, cytokines, and growth factors favorable to vascular remodeling, including VEGF, NO, basic fibroblast growth factor (bFGF), and hepatic growth factor (HGF). Proliferation of existing endothelial cells during arteriogenesis occurs rapidly and, by most accounts, is sufficient to generate the mass of cells requisite for collateral artery enlargement. Most existing vascular smooth muscle cells undergo apopotsis, to be replaced with new cells that express a proliferative phenotype, assuming a contractile phenotype after enlargement of arterial diameter.

Platelets may also participate in arteriogenesis (as well as angiogenesis) via their capacity to produce SDF-1 [Stellos et al., 2008]. Accordingly, platelets adhere to the exposed subendothelial space or to activated endothelial cells, whereupon they release the potent chemokine SDF-1 and also express P-selectin. The latter serves to initiate rolling of monocytes and vascular progenitor cells (EPC, MSC), the first phase of the adherence and transmigration process.

The final consequence of arteriogenesis is the permanent enlargement of collateral artery diameter, an effect designed to reduce vascular resistance through these vessels to restore downstream perfusion [Heil et al., 2006]. Interestingly, while the net diameter of these enlarged collaterals generally exceeds that of the obstructed conduit artery that they bypass, the summated flow through these vessels falls short of baseline. This deficiency reflects two physical properties of collaterals: first, they are longer than the conduit they have replaced, a factor that increases vascular resistance; second, collaterals are tortuous, with repeated sharp curves that generate turbulent flow. It remains, however, that arteriogenesis is a critical component in the natural vascular adaptation to the type of conduit artery obstruction characteristic of PAD.

PRECLINICAL STUDIES

The identification of pro-angiogenic growth factors in the 1980s (e.g., VEGF, bFGF) led to their application in preclinical models of PAD in attempts to enhance angiogenesis and arteriogenesis. Most commonly, these agents were injected into the muscle (thigh, calf) following induction of acute hindlimb ischemia by extirpation of the femoral artery. It was generally concluded that while exogenous VEGF or bFGF improved hindlimb perfusion, the effects of a single agent were often transient, dissipating once the level of the exogenous protein within the target tissue decreased. This outcome led to the simultaneous application of multiple growth factors, based on the established capacity of agents such as Ang-1 and PDGF to enhance maturation of nascent, VEGF-induced capillary outgrowths [Jain, 2003]. Clear evidence of synergism was noted when multiple growth factors were applied after induction of hindlimb ischemia [Carmeliet et al., 2001].

Significant problems were also observed [Epstein et al., 2001]. Hindlimb muscle capillaries generated in response to exogenous VEGF or bFGF delivered by intramuscular injection were frequently disconnected from existing capillaries and hence not effectively perfused; moreover, these vessels were dysmorphic and leaky. Some of the administered growth hormones gained systemic access. This action caused systemic hypotension, but more importantly initiated neovascularization at sites other that the intended target, the hindlimb. VEGF also destabilized atheromatous plaques in hypercholesterolemic mice.

These concerns, coupled with advances in genomic technology, led to the use of gene transfer as a means to introduce pro-angiogenic factors to enhance the post-ischemic vascular response [Ylä-Herttuala and Alitalo, 2003]. Advantages of gene transfer include more selective local treatment of affected tissues, for example, ischemic muscle or collateral arteries, and the possibility of more sustained release of growth factors that could be achieved by the direct injection of the proteins themselves. Two chief categories of vector have used to maximize gene transfer: plasmids and viruses. Plasmids do not generate a host immune response, although they generally produce low gene transfer efficiency and

short-lived gene expression. Viral vectors, for example, adenovirus, lentivirus, Sendai virus have more effective gene transfer, but risk generation of local or systemic inflammation. An alternative approach has been to transfect a cell line (e.g., NIH-3T3, myoblasts) in vitro using retrovirus and then injecting the transfected cells to thigh or calf muscle as a means to introduce exogenous genetic material.

Gene transfer of VEGF was used with variable success in rodent and rabbit models of hindlimb ischemia. Surprisingly, Masaki et al. [2002] observed acceleration, not amelioration of hindlimb tissue loss in response to VEGF gene transfer into the thigh and calf muscles following femoral artery excision. Interestingly, improvement was noted by the same group following gene transfer of bFGF (FGF-2) using a similar viral vector (Sendai virus). Chang et al. [2003] reported effective VEGF gene transfer using an adenovirus vector delivered via an intra-arterial route during transient occlusion of the hindlimb vasculature. This approach improved hindlimb perfusion and generated normalization of tissue oxygenation for 1 month after treatment. Repeated intramuscular injections of a plasmid encoding VEGF also produced sustained vascular recovery in the ischemic hindlimb [Olea et al., 2009]. Simultaneous administration of plasmids encoding FGF-2 and PDGF-BB generated more sustained restoration of hindlimb perfusion that achieved with VEGF, as well as greater enhancement of capillary density [De Paula et al., 2009].

Other attempts at gene transfer to treat experimental hindlimb ischemia have capitalized on the established interactions among transcription factors, growth factors, and their downstream targets. Patel et al. [2005] administered an adenovirus-linked, constitutively active HIF-1α into the adductor muscle (thigh) of rabbits following femoral artery occlusion. This approach generated a sustained increase in the expression of MCP-1, placental growth factor, PDGF, SDF-1α, and VEGF mRNA; as well, evidence of arteriogenesis (improved hindlimb perfusion, collateral artery diameter) and angiogenesis (increased capillary density) was noted that was sustained for at least one month following treatment. Alternatively, eNOS cDNA has been administered, insofar as eNOS-derived NO has been demonstrated to mediate the mitogenic effects of VEGF. Smith et al. [2002] administered adenovirus-linked human eNOS cDNA directly into the adductor muscle of rats following femoral artery extirpation and noted expression of human eNOS, increased muscle cGMP, improved hindlimb perfusion, and increased capillary density in the gastrocnemius muscle for 1 month thereafter. Brevetti et al. [2003] administered adenovirus-linked eNOS cDNA via an intra-arterial route and noted that eNOS overexpression increased muscle oxygen tension in a titer-dependent fashion. Moreover, this group reported a fourfold increase in hindlimb perfusion that persisted even after eNOS expression had returned to pretreatment levels. Qian et al. [2006] used an alternate approach: eNOS null C57Bl/6 and Balb/c mice, both refractory to VEGF-induced post-ischemic repair, were administered plasmid-based, constitutively active eNOS cDNA that was introduced into the adductor and gastrocnemius muscles by electroporation. These mice demonstrated marked improvement in post-ischemic hindlimb perfusion.

Gene transfer has also been used with the specific intent of increasing recruitment of vascular progenitor cells to the ischemic hindlimb. The chemokine SDF-1α is a critical participant in EPC mobilization, homing, and attachment. Hiasa et al. [2004] administered pcDNA3-SDF-1α into the thigh muscle of mice immediately after femoral extirpation; these mice had undergone lethal irradiation followed by complete bone marrow transplant with LacZ-expressing cells to permit tracking of EPC from marrow to peripheral blood, to the ischemic hindlimb. Treated mice demonstrated an increased mobilization of marrow-derived EPC, improved hindlimb perfusion and capillary density, as well as increased expression of activated eNOS and Akt in the ischemic hindlimb than mice receiving empty vector. Moreover, administration of the NOS inhibitor L-NAME to SDF-1α treated mice significantly attenuated the beneficial effects of SDF-1α, suggesting that eNOS-derived NO was an important downstream mediator. Another interesting observation that has great clinical potential is the synergism between SDF-1α gene transfer and pharmacological treatment with 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (HMG-CoA reductase inhibitors, or statins) in the treatment of experimental hindlimb ischemia [Kureishi et al., 2000]. Statins upregulate eNOS and enhance mobilization of EPC, likely via eNOS-derived NO; as well, these drugs enhance EC migration and tube formation in vitro and, given as a sole agent, improve post-ischemic angiogenesis. Shao et al. [2008] combined SDF-1α gene transfer, achieved by intramuscular injection of transfected NIH3T3 cells, with intraperitoneal fluvastatin following femoral artery extirpation in mice. Treated mice demonstrated increased EPC incorporation, increased EC proliferation and less EPC and EC apoptosis, and improved perfusion and capillary density than control mice. Treated mice also manifest greater expression of activated Akt and eNOS, better NO production, and increased expression of MMP within ischemic muscle than controls.

Vascular progenitor and stem cells (EPC, MSC) have been used to enhance post-ischemic angiogenesis and arteriogenesis with great success. Kalka et al. [2000] first demonstrated that human EPC, expanded in vitro, improved hindlimb perfusion and capillary density when transplanted into immunodeficient mice after induction of hindlimb ischemia. Similar reports followed, including those which demonstrated that coupling of EPC transplantation with gene transfer for bFGF [Jeon et al., 2005] or Ang1 [Kobayashi et al., 2006] into the ischemic muscle generated a degree of hemodynamic and tissue injury improvement that exceeded that seen with either therapy alone. An alternative approach was pre-transplant modification of EPC. Jiang et al. [2008] transfected EPC with HIF-1α and then cultivated the cells under hypoxic or normoxic conditions. Hypoxia-cultivated EPC expressed more VEGF and demonstrated greater differentiation toward an EC phenotype than normoxia-cultivated EPC; moreover, hypoxia-cultivated cells were more effective in restoring hindlimb perfusion after transplantation. Shiba et al. [2009] enhanced expression CXCR4 on EPC by sustained cultivation. CXCR4 is the receptor for the angiogenesis chemokine SDF-1α and it is thus not surprising that these modified cells demonstrated increased in vitro migratory capacity to SDF-1α. More importantly, however, the recruitment of these modified EPC to the post-ischemic hindlimb significantly exceeded that noted using freshly harvested EPC, leading to a marked improvement in recovery of hindlimb hemodynamics. Transplantation of bone

marrow-derived MSC also significantly improved post-ischemic hindlimb hemodynamics. Interestingly, these cells appeared to primarily function in a paracrine manner, enhancing the endogenous production of growth factors and chemokines requisite for angiogenesis and arteriogenesis [Gnecchi et al., 2008]. MSC harvested from non-bone marrow sites have also been successfully used to augment angiogenesis [Kondo et al., 2009]. An important reservoir for these cells is adipose tissue, a fact that makes them particularly attractive as a possible candidate in the therapy for human PAD.

HUMAN TRIALS OF GENE THERAPY FOR PAD

At this writing, the results from 23 Phase I and/or Phase II trials of gene therapy for PAD have been published. Some of these reports document safety of gene therapy in the setting of PAD, while others describe proof-of-concept studies wherein intramuscular (IM) or intra-arterial (IA) gene transfer was undertaken in limbs just prior to their planned amputation to document the efficacy of gene into vascular or muscular tissue within the ischemic limb. Still other studies were uncontrolled or included too few patients (generally <30) to satisfactorily power effective analysis of the stated primary endpoint. Space constraint precludes a detailed description of these studies; the reader is referred to Ghosh et al. [2008] for literature citations of these studies.

Seven studies of gene transfer of established angiogenic growth factors have been carried out using a randomized, placebo-controlled, double-blind experimental format (Table I). Only one of these studies, Mäkinen et al. [2002] demonstrated improvement in the primary study endpoint of improvement in the angiographic indices 3 months after VEGF was given via intra-arterial infusion in conjunction with endovascular repair. Interestingly, these patients failed to demonstrate significant improvement in the secondary study endpoints: the ankle brachial index (a ratio of blood pressures in the foot) and peak walking distance were not significantly improved by VEGF gene transfer. Similar negative findings were reported in two other studies of VEGF gene transfer. Rajagopalan et al. [2003] failed to detect an improvement in peak walking time or ABI in patients receiving low- or high-dose adenovirus-linked VEGF in patients with symptoms of claudication (calf pain noted when walking, 2° to calf muscle tissue hypoxia). These patients also reported no differences in the quality of life. Kusumanto et al. [2006] reported that patients receiving VEGF gene transfer healed chronic ischemia-induced foot ulcers at a rate similar to control patients and showed no improvement in ABI over a 25 week follow-up, although the VEGF-treated group experienced fewer amputations than the control group during this period. Gene therapy of PAD with HGF [Powell et al., 2008], FGF-1 [Nikol et al., 2008], and FGF-2 [Lederman et al., 2002] failed to significantly improve the primary study endpoints, which included improvement in lower extremity oxygenation, peak walking time, and healing of chronic, ischemia-induced foot ulcers. Gene transfer with Del-1, an extracellular matrix protein that contains epidermal growth factor-like repeats, also proved unsuccessful [Grossman et al., 2007]. Indeed, a meta-analysis that included five of these studies concluded that gene therapy did not confer improvement in PAD [Ghosh et al., 2008].

There is an obvious discrepancy between the outcomes of experimental trials using gene transfer of angiogenic growth factors in animal models, which have consistently demonstrated significant benefit, and human trials, which have been disappointing. Several explanations for this disparity are tenable. First, experimental hindlimb ischemia is most commonly induced by femoral artery extirpation, an acute procedure that generates an immediate, robust intrinsic stimulus for post-ischemic vascular repair; in contrast, PAD develops very slowly, over decades. This difference may be particularly relevant in the study of human arteriogenesis because the dramatic, instantaneous rise in collateral artery shear stress (the principal stimulus for arteriogenesis) does not occur in PAD. One means to circumvent this problem is by gradual occlusion of the femoral artery. Tang et al. [2005] compared gradual versus acute arterial occlusion in the hindlimb perfusion in rats and noted that chronic femoral artery occlusion resulted in less complete recovery of perfusion, as well as a smaller increase in the normal post-ischemic rise in collateral artery diameter (both observations indicative of reduced arteriogenesis following chronic occlusion). This observation is clearly consistent with the clinical observation that arteriogenesis is relatively ineffective in PAD, even in the presence of regenerative therapies. Second, experimental hindlimb ischemia is studied in young, otherwise healthy animals that are highly inbred and kept in a tightly controlled environment. This circumstance sharply contrasts with the human experience. PAD patients are older and most commonly have at least one of several co-morbidities, including hypertension, smoking, diabetes, and hypercholesterolemia; as well, genetic susceptibility has been identified as an important factor in the development and

TABLE I. Selected Clinical Trials of Gene Therapy for Peripheral Arterial Disease

Agent	Route	Doses	# Pt	Entry criteria	Duration	1° endpoint	2° endpoint	Outcome	Refs.
$hVEGF_{165}$	IA	1	54 (30♀)	PAD; angiographic stenosis	1, 3 m	Angiography	Restenosis rate; ΔABI	+/−	Mäkinen et al. [2002]
hFGF-2	IA	1–2	201 (47♀)	PWT ≤12 min; ABI < 0.8	3 m	ΔPWT	ΔABI	+	Lederman et al. [2002]
$VEGF_{121}$	IM	1	105 (22♀)	PAD; angiographic stenosis	3 m	ΔPWT	ΔABI	−	Rajagopalan et al. [2003]
$VEGF_{165}$	IM	2	54 (23♀)	CLI; rest pain; non-healing ulcer	100 d	Amputation rate	ΔABI	−	Kusumanto et al. [2006]
Del-1	IM	1	105 (16♀)	PAD; PWT ≤10 min; ABI ≤ 0.8	1–6 m	ΔPWT	ΔABI; ΔCOT	−	Grossman et al. [2007]
HGF	IM	2–3	104 (39♀)	CLI; rest pain; T_cpO_2 <40 mm Hg; ulcer; ABI ≤ 0.8	6 m	ΔT_cpO_2	Ulcer healing; ΔABI; pain relief	−	Powell et al. [2008]
FGF-1	IM	4	107 (32♀)	CLI with non-healing ulcer	1 m	Ulcer healing	Amputation, death	−	Nikol et al. [2008]

d, day; m, month; IA, intra-arterial; IM, intramuscular; PWT, peak walking time; ABI, ankle brachial index; COT, time to onset of claudication (time of pain free walking); PAD, peripheral arterial disease; CLI, critical limb ischemia; T_cpO_2, transcutaneous pO_2 level. Note that the +/− designation for study outcome refers to the 1° outcome variable for the study. (+) Indicates statistically significant improvement, (−) indicates that the change in the 1° outcome variable as not statistically relevant.

progression of PAD [Knowles et al., 2007]. Control over environmental factors potentially relevant to PAD progression, for example, diet (including vitamin intake) and exercise can be difficult to attain in a human study population. Another point merits discussion. Many human trials of gene transfer for PAD have been carried out in patients deemed unsuitable for conventional therapeutic alternatives, that is, surgical reconstruction or endovascular treatment with stents. Stated otherwise, gene transfer therapy has not undergone a vigorous comparison with currently accepted treatments for PAD, but has been relegated to patients with very advanced disease, a circumstance that might bias gene transfer to failure. Use of gene transfer as an first line treatment for PAD, or used in conjunction with endovascular reconstruction might significantly improve outcome. One of the unfortunate realities of PAD, however, is that the correlation between the degree of vascular occlusion and the onset of symptoms (e.g., claudication) is limited; thus, many patients demonstrate very advanced atherosclerotic obstruction of the leg arteries at the time of their first presentation to a physician, a circumstance that might limit the efficacy of therapy.

HUMAN TRIALS OF STEM AND PROGENITOR CELL THERAPY FOR PAD

The putative utility of stem or progenitor cell therapy for vascular occlusive disease, including PAD, is underscored by the relationship between the number of circulating $CD34^+$ EPC and the risk for adverse cardiovascular events [Vasa et al., 2001]. This observation, coupled with the marked improvement in experimental post-ischemic angiogenesis noted following EPC or MSC transplant, has led to several human studies. Most of these trials, have included small sample sizes and have not been effectively controlled and patients offered inclusion into these studies often have very advanced disease. Unlike the outcome of gene transfer studies, however, the results of cell-based therapy for PAD have been consistently encouraging.

Tateishi-Yuyama et al. [2002] was the first to report the efficacy of bone marrow mononuclear cells (BM-MNC) in PAD. Twenty patients were randomized to receive autologous BM-MNC or peripheral blood MNC (PB-MNC), injected into the gastrocnemius muscle. These cells were not purified with regards to surface phenotype, although it was determined that ~2.5% of the BM-MNC were $CD34^+$ (a similar phenotypic description was not offered for the PB-MNC population). Statistically relevant improvements in ABI, pain-free walking time, rest pain, and TcO_2 were noted, although the actual magnitude of these differences were relatively small. A major problem with this study is that it utilized the PB-MNC transplant group as the control group and while it is now clear that CD34+ EPC are present in peripheral blood, so that the PB-MNC-treated patients did, in fact, receive EPC.

Three additional uncontrolled studies have been published. Lenk et al. [2005] harvested PB-MNC following stimulation G-CSF to mobilize marrow-derived progenitor cells. Fibronectin-adherent cells were expanded ex vivo for 4 days prior to autologous re-infusion; these cells were $CD34^+$ (31%), $AC133^+$ (22%), $VEGFR2^+$ (59%), and VE-$cadherin^+$ (49%), identifying them as EPC. Three months after intra-arterial infusion of these cells, seven patients demonstrated improvement in pain-free walking distance, ABI and calf TcO_2, as well as increased flow reserve in response to adenosine. Gu et al. [2008] compared intramuscular injection of freshly harvested PB-MNC at multiple sites along the length of leg conduit arteries to intra-arterial infusion of these cells. Improvement in rest pain, ABI, and TcO_2 were noted regardless of the route of BM-MNC administration.

Two controlled trials of cell-based therapy for PAD have been reported. Huang et al. [2005] stimulated bone marrow EPC mobilization with G-CSF for 5 days prior to peripheral blood harvest in PAD patients. These cells were subsequently injected into 40 sites in the thigh and calf, while control subjects underwent saline injections. After 3 months, cell-treated patients demonstrated significantly greater healing of pre-existing foot ulcers than control subjects (78% vs. 39%), as well as greater improvement in limb perfusion and ABI. Importantly, these patients were diabetic, a point of potentially great relevance insofar as it demonstrates that progenitor cells harvested from diabetic patients can be effectively used for autologous transplant. Bartsch et al. [2007] administered freshly harvested PB-MNC by the intra-arterial and intramuscular routes in the same patient group, while control patients received saline. In intra-arterial infusion was given into the femoral artery following exercised-induced dilation of the leg to maximize capillary perfusion, so as to increase the potential of exposure of PB-MNC to existing lower extremity capillaries. One year later, cell-treated patients demonstrated better pain free walking distance, ABI, and reactive hyperemia than control patients.

FUTURE DIRECTIONS

Existing studies substantiate the need for a large scale trial of cell-based therapy for the treatment of PAD and, while human trials of gene transfer of growth factors for angiogenesis have met with limited success, it is possible that the combination of gene-based and cell-based therapeutic interventions may prove useful. Several issues require resolution:

(i) *Source of the cells*: Both peripheral blood and bone marrow have been used as sources of $CD34^+$ and $CD14^+$ EPC. While mobilization of $CD34^+$ progenitor cells from the bone marrow can be safely achieved using G-CSF, it is unclear if these mobilized cells include a substantial percentage of EPC (CD34 is a common stem cell antigen expressed by HSC-derived progenitor cells and by itself does not itself delineate an EPC). One means to specifically increase the number of EPC is by ex vivo cultivation. EPC adhere to fibronectin and proliferate in response to VEGF. It is thus feasible that PB-MNC, isolated by gradient centrifugation, could be cultured in the presence of VEGF and the adherent cells harvested and used as a source of cells for autologous transplant. Another source of vascular stem cells, specifically MSC, is adipose tissue [Kondo et al., 2009]. MSC are present in great abundance within the adipose stroma and can be readily isolated; moreover, these

cells can be differentiated to EC and vascular smooth muscle phenotypes in vitro and significantly improve post-ischemic angiogenesis when transplanted in vivo [Cao et al., 2005].

(ii) *Effect on the co-morbidities on vascular stem and progenitor cells*: PAD most commonly occurs in the presence of diabetes and hypercholesterolemia [Schainfeld and Isner, 1999] conditions that exert an adverse effect on the number and efficacy of vascular stem and progenitor cells [Couffinhal et al., 1999; Tepper et al., 2002]. This circumstance may limit the number of cells harvested from the patient, compromise the capacity of these cells to expand ex vivo, and reduce their efficacy following transplantation. Hypercholesterolemia and diabetes generate systemic oxidative stress, and the deleterious effects of oxidants on regulation of stem and progenitor cells, including EPC and MSC, has been established [Hosokawa et al., 2007]. It remains to be determined if these effects generate permanent impairment of the cells, or if post-harvest treatment of vascular stem or progenitor cells will be requisite to restore their angiogenic efficacy. In a similar context, is likely that cardiovascular risk factors such as hypertension, diabetes, hypercholesterolemia impair the endogenous capacity for post-ischemic arteriogenesis and angiogenesis, even in the presence of therapeutic endeavors (gene transfer, progenitor cell infusion) designed to maximize these processes [Kinnaird et al., 2008]. Aging also plays an important role in the efficacy of vascular stem and progenitor cells [Dimmeler and Leri, 2008]; thus, the number of circulating EPC, the in vitro migratory and tube forming capabilities of these cells, and their ability to participate in angiogenesis in vivo is reduced in older animals, suggesting that an elderly population may prove less amenable to vascular regenerative therapy.

(iii) *Genetic modification of vascular stem and progenitor cells*: A strategy currently evolving in the field of cardiac regeneration could be applied to cell-based treatment of PAD, specifically genetic modification of stem/progenitor cells prior to transplant. At least two advantages have been identified, enhancement of stem/progenitor cell survival and improvement of homing and engraftment [Penn and Nangi, 2008]. For example, enhancement of CXCR4 expression by ex vivo transfection of $CD34^+$ progenitor cells significantly enhances their proliferation and homing in vivo, as well as their beneficial effect on the post-ischemic myocardium [Kahn et al., 2004]. It is likely that this paradigm can be applied to the use of $CD34^+$ progenitor cells for the treatment of PAD, particularly if it were coupled with gene transfer of SDF-1α to the ischemic limb.

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