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Vascular regeneration in peripheral artery disease

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Abstract

Peripheral artery disease (PAD) is a common disorder and a major cause of morbidity and mortality worldwide. Therapy is directed at reducing the risk of major adverse cardiovascular events, and at ameliorating symptoms. Medical therapy is effective at reducing the incidence of myocardial infarction and stroke to which these patients are prone, but is inadequate in relieving limb-related symptoms, such as intermittent claudication, rest pain and ischemic ulceration. Limb related morbidity is best addressed with surgical and endovascular interventions that restore perfusion. Current medical therapies have only modest effects on limb blood flow. Accordingly, there is an opportunity to develop medical approaches to restore limb perfusion. Vascular regeneration to enhance limb blood flow includes methods to enhance angiogenesis, arteriogenesis and vasculogenesis using angiogenic cytokines and cell therapies. We review the molecular mechanisms of these processes; briefly discuss what we have learned from the clinical trials of angiogenic and cell therapies; and conclude with an overview of a potential new approach based upon transdifferentiation to enhance vascular regeneration in PAD.

Peripheral Arterial Disease and Its Management

Peripheral artery disease (PAD) is the third leading cause of atherosclerotic cardiovascular morbidity, following coronary artery disease and stroke[1]. PAD is a common disease that is underdiagnosed[2], and which has significant adverse effects on over 8 million Americans[3, 4] and more than 200 million individuals worldwide[1, 5]. Age, tobacco use, diabetes, hypertension, hypercholesterolemia and sedentary state are the major risk factors for PAD[1, 6]. PAD may cause leg pain when walking (intermittent claudication, IC) which interferes with the cardiovascular benefit of regular exercise. Indeed, limitation of exercise capacity is a strong predictor of mortality in patients with PAD[7]. Critical limb ischemia (CLI)[8] is the most advanced form of PAD, defined as chronic ischemic rest pain, ulcers, or gangrene of the lower extremity. CLI is a major cause of limb amputation[9], and a harbinger of cardiovascular mortality in PAD[10].

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Stanford University is the assignee, and JPC is one of the inventors, on patents related to therapeutic modulation of innate immunity.

Medical therapy for PAD effectively reduces major adverse cardiovascular events (MACE) in PAD, and includes exercise, smoking cessation, anti-platelet agents, and therapies to restore normal levels of blood lipids, blood sugar and arterial pressure[11]. Medical therapy to relieve IC and CLI is of modest benefit. Cilostazol increases walking distance by 50%, but does not reduce MACE. Prostanoids reduce rest pain and improve ulcer healing[12], and iloprost may reduce the incidence of amputation in CLI. However, vascular surgery and endovascular intervention are more effective than medical therapy at improving limb perfusion, reducing symptoms, and preventing loss of limb[11, 13, 14]. Clearly, there are opportunities for novel medical therapies to improve limb blood flow.

Vascular regenerative strategies include restoration of vascular function (e.g. vasodilation) and structure (e.g. plaque regression) (Figure). Restoration of vascular function, in particular endothelial function, can improve limb blood flow by enhancing vasodilation, reducing vascular inflammation, suppressing platelet aggregation and thrombosis, and promoting endogenous thrombolysis[15, 16]. Lipid-lowering therapy induces plaque regression, and this effect, combined with its benefit on endothelial function, may explain the increase in walking distance in PAD patients treated with statins[17]. In this review however, we will focus on the regenerative processes of angiogenesis, arteriogenesis and vasculogenesis; and describe a new process termed angiogenic transdifferentiation that may participate in vascular regeneration.

Angiogenesis and PAD

Angiogenesis is the formation of new blood vessels from pre-existing vessels[18–20] (Figure). The process begins with endothelial cell (EC) sprouting from existing capillaries, followed by EC migration, proliferation and lumen formation. In addition, intussusception of existing capillaries[18, 21] also contributes to expansion of the microvasculature. In ischemic tissues, hypoxia activates the transcription factor hypoxia-inducible factor 1 (HIF-1), a basic-helix-loop-helix-pas heterodimeric protein that is responsive to oxygen tension[22]. This transcription factor contains two subunits, HIF-1α and HIF-1β. HIF-1α has an oxidation dependent degradation domain. Under normoxic conditions, two prolyl residues in this domain are hydroxylated by prolyl hydroxylase, which leads to the ubiquitination and destruction of HIF-1α. In the setting of hypoxia, this degradation process is inactive, HIF-1α becomes stable, and accumulates in the nucleus. There HIF-1α dimerizes with HIF-1β to activate target genes including angiogenic cytokines such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin, and matrix metalloproteinases MMP2 and MMP9[23].

In PAD animal models, VEGF[24], FGF[25], hepatocyte growth factor (HGF)[26], platelet-derived growth factor (PDGF)[27] and angiopoietins[28], prokineticin 2 (PROK2)[29], and other angiogenic cytokines have been shown to enhance angiogenesis and limb blood flow. VEGF is the most widely studied angiogenic cytokine and is essential for endothelial proliferation, migration and lumen formation mediated by its receptors VEGFR-1 and VEGFR-2[30].

Angiogenic cytokines have also been implicated in the effect of adult stem cell therapy to improve perfusion in preclinical models of PAD. Mesenchymal stem cells derived from bone

marrow or adipose tissue, mononuclear cells isolated from the bone marrow or peripheral blood, and endothelial cells derived from embryonic stem cells or induced pluripotent stem cells, have each been shown to generate angiogenic cytokines and home to sites of ischemia, where they increase microvascular density, and improve perfusion in the ischemic limb in murine models[31–33].

Arteriogenesis

Arteriogenesis is the positive remodeling of preexisting collateral arterioles to generate larger conductance vessels that compensate for occluded arteries[34, 35] (Figure). These preexisting collateral arterioles are narrow and high resistance channels that generally conduct little or no blood flow in healthy tissue[36]. However, severe arterial obstruction or occlusion creates a pressure gradient that favors blood flow through the collateral channels. The increase in blood flow, and shear stress, in the collateral channels induces a positive remodeling. This vascular remodeling process increases both diameter and wall thickness, and is accompanied by alterations in cellular proliferation and extracellular matrix degradation and deposition[37, 38].

Early in the process, monocytes and macrophages are observed adhering to the endothelium and infiltrating into the subintimal space of collateral arterioles[39–41]. These cells play an essential role in the remodeling process by the secretion of growth factors, chemokines and metalloproteinases[42]. This pericollateral macrophage recruitment is mediated in part by an ICAM-1 dependent mechanism[43]. In addition, the CC-chemokine receptor-2 (CCR2) plays a critical role in monocyte/macrophage recruitment to the perivascular space of collateral vessels and is required for the increase in vessel diameter[44]. Monocyte differentiation and maturation into macrophages is required for arteriogenesis, and is controlled by Notch ligand Delta-like 1 (Dll1) expressed on vascular ECs and macrophage Notch effector Rbpj[45]. Granulocyte-colony stimulating factor (G-CSF)[46, 47] and granulocyte macrophage-colony stimulating factor (GM-CSF)[48, 49] also promote arteriogenesis. Other immune cells such as T cells[50] and mast cells[51] have also been reported to play a role in arteriogenesis.

During arteriogenesis, smooth muscle cells in the media of the collateral vessel transform from a contractile to a proliferative phenotype, and form a neo-intima[41]. The P2Y2 nucleotide receptor, which mediates vascular cell proliferation and migration, also participates in this positive remodeling[50]. Importantly, the smooth muscle cells return to a contractile phenotype in the end stage of this remodeling process[37]. It is well known that there are species-specific variation in collateral arterioles[35, 51]. Furthermore, in patients with PAD, there is substantial heterogeneity in the generation of collateral channels in the limb, which may contribute to individual differences in the severity of limb symptoms.

Adult Vasculogenesis

Vasculogenesis refers to the establishment of primary vasculature from mesodermal progenitors during early development. The incorporation of circulating progenitor and stem cells into regenerating blood vessels after development is termed adult vasculogenesis (Figure). Asahara's discovery of "endothelial progenitor cells" (EPCs) in 1997[52] galvanized

interest in adult stem cells for vascular regeneration. These cells originate in the bone marrow and can be isolated from the blood using cell surface markers such as CD34, CD133 and VEGFR2[52]. Ischemia and hypoxia triggers EPC mobilization from the bone marrow, mediated by VEGF[53], stromal-derived factor 1 (SDF-1)[54, 55], FGF and angiopoietin-1. G-CSF and GM-CSF also stimulate mobilization of hematopoietic and progenitor cells from the bone marrow[56]. In the murine hind limb ischemia model, GM-CSF administered by injection or by plasmid transfer augments circulating levels of EPCs and increases capillary density[57].

After mobilization, EPCs home to the ischemic tissue under the influence of VEGF and SDF-1, the latter binding to EPC chemokine receptor CXCR-4[55]. Recently, it has been shown that GDF11 improves angiogenic function of EPCs in diabetic limb ischemia[58]. Inhibition of macrophage inflammatory protein-1β (MIP-1β) improves EPC homing and angiogenesis in diabetic models[59].

In studies of their angiogenic effects, most investigators have used a small set of surface markers to define EPCs[60]. However, the surface markers that are commonly used for identification of human EPCs include markers that are not specific for endothelial lineage, such as CD133 and VEGFR2[61]. Only a small subset of EPCs is of true endothelial lineage in humans[62], most being of hematopoietic lineage. It is unlikely that EPCs differentiate into mature endothelium *in vivo*[60, 63]. Rather, they may promote angiogenesis by secreting angiogenic cytokines and matrix metalloproteinases[64, 65]. Still other bone marrow derived cells can form pericytes, which may associate with and stabilize endothelial networks[66]. Many progenitor cell types such as EPCs[52, 67, 68], endothelial colony forming cells[69, 70], circulating angiogenic cells (CACs)[71, 72] may promote expansion of the microvasculature in preclinical models by generating angiogenic factors. Finally, under the influence of circulating factors generated by ischemia, mature endothelial cells from other sites may be mobilized into the systemic circulation and home to the ischemic tissue[73].

What have we learned from clinical trials?

Angiogenic Cytokines

Administration of angiogenic cytokines (such as VEGF, FGF and HGF) and agents that can mobilize EPCs (such as SDF-1, G-CSF and GM-CSF) have shown benefit in pre-clinical models of PAD. These data precipitated small clinical studies which were encouraging. However, larger randomized clinical trials have been largely negative for their primary endpoints. Intra-arterial or intramuscular administration of adenoviral or plasmid VEGF gene therapy failed to increase walking distance in patients with IC, or reduce amputations in patients with CLI[74–76]. In one phase 2b/3 study in Russia, intramuscular injections of VEGFA-165 increased the primary endpoint of pain-free walking distance at 2 years[77]. However, this study was not blinded, and control subjects did not receive a vehicle injection. By contrast to the Russian study, all other large randomized controlled trials (RCTs) testing angiogenic therapies, whether delivered as gene therapy or recombinant proteins, have not achieved their primary endpoints of increasing walking distance (in IC) or reducing amputations (in CLI). These include trials testing the efficacy of therapies based on FGF,

HGF, HIF1α, SDF-1, G-CSF or GM-CSF[78,79–80]. Accordingly, there is currently no FDA-approved angiogenic therapy for relieving intermittent claudication or ischemic ulcerations.

Cell Therapies: Since the first pilot clinical trial of cell therapy in PAD in 2002[81], numerous cell types have been examined in clinical trials for PAD, including bone marrow derived cells, peripheral blood derived cells, progenitor or stem cells isolated from bone marrow or blood using specific surface markers, adipose vascular stromal cell or mesenchymal stem cells. Whereas early uncontrolled series seemed promising, the initial excitement surrounding cell therapy for PAD has dimmed as larger randomized clinical trials failed to confirm the earlier results, as we and others have previously discussed[63, 82, 83]. However, because CLI is an unmet need, and because there have been some positive data with small trials[84], clinical studies in CLI are ongoing with autologous bone marrow derived mononuclear cells (BM MNC), adipose derived stem cells, and umbilical derived mesenchymal stem cells (MSC)[85,86].

Indeed, there is evidence to support further research into cell therapy for CLI[82]. A meta-analysis of 10 randomized, placebo-controlled trials (499 CLI patients) showed that cell therapy provided significant improvements in ankle-brachial index, resting pain, and pain-free walking time, although there was no improvement in amputation rates or amputation free survival[87]. A more recent meta-analysis of 19 RCT (837 CLI patients) concluded cell therapy modestly reduced the risk of amputation by 37%, improved amputation free survival by 18%, and improved wound healing by 59%[88].

Lessons learned: In brief, angiogenic therapies have failed, whereas cell therapies may yet prove useful. The failure of angiogenic therapies may be related to the fact that we have limited knowledge regarding dosing, delivery and duration of angiogenic cytokines. Angiogenesis is a complex choreographed process that cannot be mimicked by administration of a single angiogenic cytokine. Furthermore, angiogenesis increases microvascular density, which may be insufficient for PAD patients who have long segments of occluded conduit arteries that impair perfusion. Accordingly, master regulators of both angiogenesis and arteriogenesis, such as transcriptional factors that regulate a cascade of genes involved in vascular regeneration, may have greater potential for efficacy.

Furthermore, angiogenic and cell therapies have been based upon flawed animal models. A significant limitation is that much preclinical work is performed in healthy mice subjected to ligation of a femoral or iliac artery. This model induces a very different pathobiology than that in our elderly patients with multiple cardiovascular risk factors, whose vascular disease has progressed over decades. The strengths and limitations of animal models of PAD have been recently reviewed[89], and must be considered in the development of new therapeutics for vascular regeneration. In this regard, autologous cell therapy in a patient with vascular disease may be comprised of fewer and/or dysfunctional stem cells. For example, in 55 patients transplanted with BM MNC, those that had a positive outcome (wound healing and limb salvage, n=33) received a significantly greater number of $CD34^+$ cells with their transplantation compared to those individuals that required limb amputation (n=22)[90].

There is much we don't know about the dosing and delivery of cell therapies. However, there is abundant evidence that cells injected into ischemic zones do not persist. Nevertheless, in the brief time that they survive in the tissue, they may generate angiogenic cytokines that contribute to an increase in microvascular density and improved perfusion. Alternatively or in addition, they may secrete exosomes containing biological activity, e.g. as in the form of angiogenic microRNA. Indeed, therapeutic effects have been observed in preclinical PAD models of exosomes derived from a variety of progenitor cells[91, 92]. In this regard, HIF-1α increases MSC-exosome secretion[93]. Finally, the effect of injected cells may also be due to the local inflammatory signaling that they induce as they undergo cell death in the ischemic zone[94]. In this regard, we have uncovered a novel mechanism by which inflammation may induce adaptive changes in cell identity that could promote angiogenesis.

New Insights into Vascular Regeneration

Inflammation and Transdifferentiation:

Lineage tracing studies indicate that endothelial-to-mesenchyme transition (EndoMT) may contribute to fibrosis[95]. EndoMT may explain the reduced vascular density and increased interstitial fibrosis seen in many fibrotic conditions. The existence of EndoMT begs the question of whether the reverse phenomenon occurs, i.e. the transdifferentiation of mesenchymal cells to endothelial cells (Figure). To be sure, the transdifferentiation of fibroblasts to endothelial cells occurs during development[96]. Whether this phenomenon plays a role in the adult is controversial[97, 98]. We and others have shown that under specific experimental conditions, human fibroblasts can be directly reprogrammed into endothelial cells *in vitro* and *in vivo*[99–102]. Furthermore, our unpublished data suggests that this process may play a role in the recovery from limb ischemia. Specifically, lineage tracing studies combined with single cell RNAseq have provided preliminary support for subsets of fibroblasts which may participate in angiogenic transdifferentiation.

The process of transdifferentiation from one somatic cell to a different lineage requires an increase in DNA accessibility so that new cell identity genes can be activated. We have shown that DNA accessibility is increased by signaling pathways known to be activated during injury and ischemia. In brief, pattern recognition receptors (PRRs; such as Toll-like receptors) can sense damage- or pathogen-associated molecular patterns (DAMPs or PAMPs respectively). Activation of PRRs induces inflammatory signaling, e.g. through NFκB, which subsequently promotes DNA accessibility through global changes in the expression and activity of epigenetic modifiers[99, 100, 103–106]. *It is as though a cell, sensing a challenge, opens up its genetic toolbox so as to adapt and survive.* Such adaptation may include a change in phenotype.

We have shown that this inflammatory signaling increases the expression of histone acetyltransferases and suppresses the expression of histone deacetylases[103, 105] so as to promote epigenetic plasticity and cell fate transitions. Furthermore, inflammatory signaling causes inducible nitric oxide synthase (iNOS) to translocate to the nucleus. There iNOS S-nitrosylates epigenetic modifiers, such as the polycomb[100] and NURD complexes[106], to antagonize their suppressive histone markings. Intriguingly, a glycolytic switch is activated by this inflammatory signaling, and is coupled to epigenetic changes. Specifically,

inflammatory signaling is associated with mitochondrial export of citrate to the nucleus, which increases nuclear acetyl-coA, the substrate for histone acetylation. These processes increase DNA accessibility as shown by micrococcal nuclease assay. In this state, the cell is rendered more tractable to reprogramming. The effect of inflammatory signaling to increase DNA accessibility and thereby facilitate changes in cell identity is called transflammation[107].

Into *what lineage* the cell is reprogrammed is determined by the environmental milieu. For example, we observe that an inflammatory stimulus (the TLR3 agonist polyinosinic cytidilic acid) increases the epigenetic fluidity of fibroblasts so that they can transdifferentiate into endothelial cells under the influence of medium containing high levels of VEGF and other endothelial growth factors[99, 100]. Thus it seems possible that in the setting of ischemic injury, the local injury and inflammatory signaling, together with the release of angiogenic cytokines, might induce transdifferentiation of fibroblasts to endothelial cells, thereby enhancing angiogenesis. There is abundant evidence that an inflammatory response is necessary for tissue regeneration[108]. Future elucidation of this process may provide a novel therapeutic avenue for ischemic syndromes.

Summary

Since Judah Folkman's early work on angiogenesis in the 1970s, much has been learned about angiogenesis and vascular regeneration. Although angiogenic therapy in preclinical models appeared to be very promising, the clinical trials of angiogenic factors have disappointed, failing to show a consistent effect on claudication distance, ischemic pain relief or ulcer healing[78, 109]. Cellular therapies to improve perfusion also showed promise in preclinical studies as well as small trials[110, 111] but larger randomized clinical trials have been in general negative. The lack of benefit may be due to incomplete understanding regarding the appropriate dose, duration, delivery method and/or mechanisms of angiogenic or cell therapies. Angiogenic expansion of the microvasculature is probably insufficient to improve perfusion in human PAD, which is typically characterized by long segments of obstructed vessels. Combined with arteriogenesis, angiogenic therapies might be more effective. Finally, new insights into vascular regeneration, such as transdifferentiation, may provide for effective vascular regenerative strategies in PAD.

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Nonstandard Abbreviations and Acronyms

PAD	peripheral artery disease
IC	intermittent claudication
CLI	critical limb ischemia
MACE	major adverse cardiovascular events

EC	endothelial cell
EPC	endothelial progenitor cell
CAC	circulating angiogenic cell
RCT	randomized controlled trial
BM MNC	bone marrow derived mononuclear cell
MSC	mesenchymal stem cell
EndoMT	endothelial-to-mesenchyme transition
PRR	pattern recognition receptor
DAMP	damage-associated molecular pattern
PAMP	pathogen-associated molecular pattern

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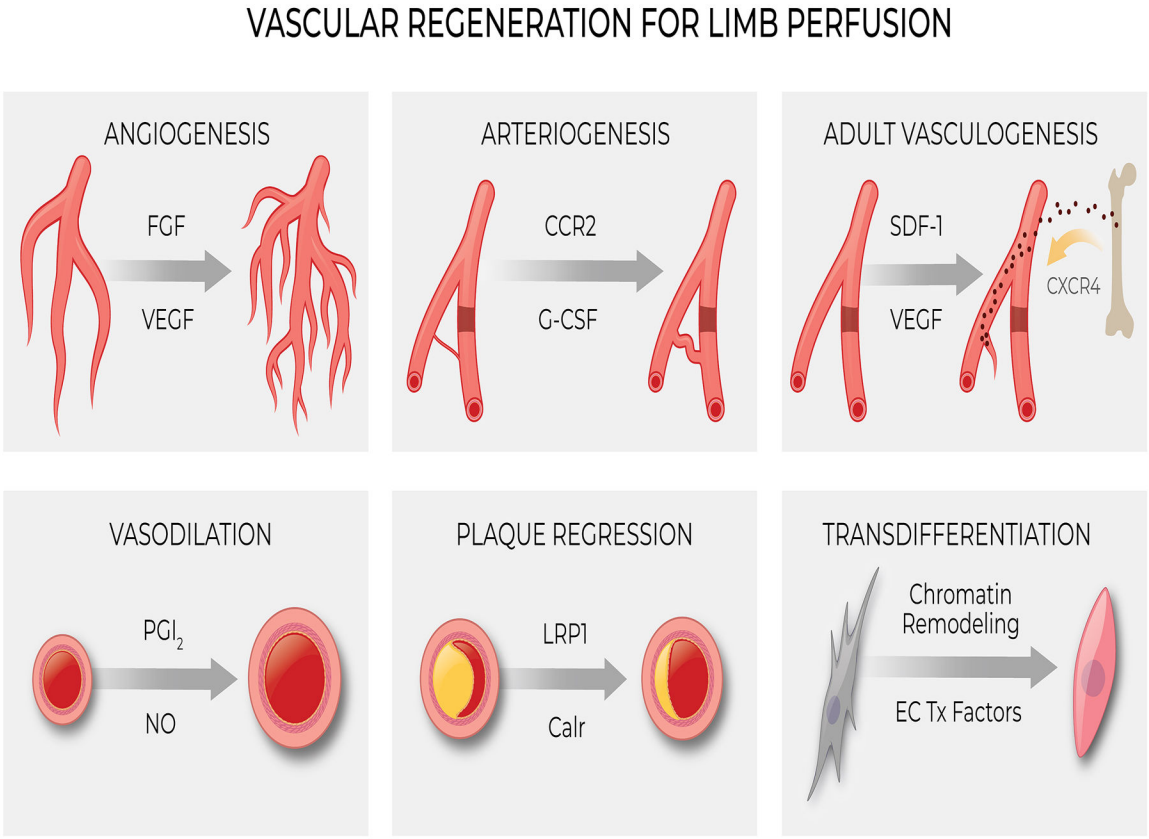
Highlights

- Vascular regeneration comprises angiogenesis, arteriogenesis and vasculogenesis.
- Clinical trials of angiogenic factors for peripheral arterial disease have failed, in part because of imperfect pre-clinical models and incomplete knowledge
- Clinical trials of cell therapies for critical limb ischemia suffer from similar limitations, but some positive results provide encouragement for continued development
- The role of inflammatory signaling and transdifferentiation in vascular regeneration merits further study



Processes involved in Vascular Regeneration. Tx = Transcriptional. See text for additional details.