Review

Therapeutic neovascularization for peripheral arterial diseases: advances and perspectives

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Summary. Recently, with the better understanding of the mechanisms of neovascularization, many new therapeutic approaches to enhance neovascularization have emerged. Of these diverse emerging methods, use of growth factors and cells are the two major ones. This review will provide an update on the present understanding of the basic mechanisms of angiogenesis, vasculogenesis, and arteriogenesis, as a basis for designing future pro-neovascularization treatments. Several angiogenic factors including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) have been implicated in augmenting the neovascularization process. However, single growth factors are not sufficient to generate functional vessels. In synergistic or complementary manner, these factors may be used in harmony to form long-term functional vessels. Cell therapy has the potential to supply stem/progenitor cells and multiple angiogenic factors to the region of ischemia. However, the efficacy of stem cells transplantation may be impaired by low survival rate, insufficient cell number and impaired function in aging and diseases. Combination of cells or cells primed with growth factor(s) or genetic modification may augment the therapeutic efficacy. This paper reviews critical literature in depth to elucidate the mechanism of therapeutic neovascularization, angiogenic factor therapy and cell transplantation. Based on past experience and actual knowledge, we propose future strategies for clinical application and discuss the problems and controversies that need to be addressed in order to fully exploit the potential of growth factors and/or cell transplantation with clinical relevance.

Key words: Therapeutic neovascularization, Peripheral arterial diseases, Cell transplantation, Growth factor, Stem cells

Introduction

Generally, normal tissue function depends on sufficient supply of oxygen and nutrient delivered via the blood vessels. Inadequate blood perfusion may deprive the tissue of oxygen and nutrient resulting in tissue ischemia. One example is ischemic limb with dysfunction and may progress to gangrene or amputation at late stage. Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year (1991). Psychological testing of such patients has disclosed quality-of-life indices similar to those of patients with terminal cancer (Albers et al., 1992). Despite the use of prosthetics and rehabilitation, reestablishment of full mobility is inconsistently achieved, particularly in the elderly. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling.

Extensive preclinical studies and recent clinical trials have indicated that therapeutic neovascularization is a potential method for cure of ischemic myocardial and limb disease. This review will provide an update on the present understanding of the basic mechanisms of angiogenesis, vasculogenesis, and arteriogenesis, as a basis for designing future pro-neovascularization treatments. We will then focus on the past experience on use of growth factors and cell transplantation in animal models and in human patients as to allow better design of future therapeutic approaches to limb ischemia.

Mechanisms of therapeutic neovascularization

Angiogenesis

The term angiogenesis describes the development of new capillaries from postcapillary venules (Emanueli and Madeddu, 2005). Sprouting of new vessels from pre-existing vessels in the adult depends on proliferation and migration of local vessel wall-associated endothelial cells (ECs) in response to angiogenic stimuli, chief among which is hypoxia (Fig. 1a). The oxygen tension

in tissue is sensed by the proline hydroxylase-hypoxia inducible factor 1 (HIF-1) system. Among many genes induced by HIF-1α, the genes directly involved in angiogenesis include most prominently the VEGF family of genes, angiopoietins, and the inducible form of nitric oxide synthase (iNOS) (Losordo and Dimmeler, 2004). Many excellent reviews have already addressed the topic of angiogenesis (Carmeliet, 2000; Losordo and Dimmeler, 2004; Simons, 2005).

Vasculogenesis

Vasculogenesis refers to the in situ formation of blood vessels from EPCs or angioblasts. Vasculogenesis was previously considered an establishment of the primordial vascular network in the embryo, and ischemia-induced neovascularization postnatally was thought to be solely a result of the angiogenic process (Carmeliet, 2000). However, recent studies have demonstrated that postnatal neovascularization did not rely exclusively on sprouting of pre-existing vessels, as marrow-derived progenitor cells were also involved (Asahara et al., 1997). The demonstration of postnatal circulating bone marrow–derived endothelial progenitor cells (EPCs) that may home to sites of neo-vascularization and differentiate into ECs in situ is consistent with "vasculogenesis". Subsequently, a variety of growth factors including granulocyte macrophage-colony stimulating factor (GM-CSF), and VEGF have been demonstrated to recruit bone-marrow derived angioblasts to sites of neovascularization postnatally (Asahara et al., 1999; Takahashi et al., 1999). Ischemia may increase the number of angioblasts and/or enhances their ability to differentiate (Takahashi et al., 1999). However, endogenous neovascularization is insufficient to overcome the loss of blood flow through occluded arteries in patients with peripheral vascular disease, so that external supply of angioblasts might be used to augment vascularization and restore blood flow to ischemic tissue (Kalka et al., 2000b) (Fig. 1b).

Arteriogenesis

Arteriogenesis, also named collateral vessel formation, denotes the expansive growth of pre-existing vessels forming collateral bridges between arterial networks (Fig. 1c). Unlike angiogenesis, the regulation of arteriogenesis does not depend on local tissue hypoxia. Rather, shear stress and local activation of endothelium seem to play critical roles (Carmeliet, 2000). In this process, vascular smooth muscle cells and pericytes are important, as they serve multiple functions, including modulating blood flow and vascular permeability, regulating growth of blood vessels, and providing signals to endothelium and matrix via secreted and other cellular molecules (Rucker et al., 2000). As a result of the increased collateral flow, ECs express monokines like monocyte chemotactic protein-1 (MCP-1) and monocyte adhesion molecules like intercellular adhesion molecules-1 (ICAM-1). Monocytes/macro-phages, platelets, mast cells and other leukocytes are 'chemoattracted' to sites of inflammation for wound healing. These blood-borne cells produce angiogenic and arteriogenic factors (VEGF, ßFGF, transforming growth factor ß-1/TGFß1, interleukin-8, platelet derive growth factor/PDGF, insulin-like growth factor-1/IGF-1, MCP-1, tumor necrosis factorßα/TNF-α and proteinases) that, in turn, attract endothelial and smooth muscle cells, fibroblasts, leukocytes or platelets. The new milieu leads to endothelial and smooth muscle cell proliferation, migration, vessel enlargement and maturation, as well as synthesis of extracellular matrix (Arras et al., 1998; Heil et al., 2002). Ultimately, the small preexisting arterioles remodel into large functional conducting collaterals, improving the recovery of the ischemic tissue.

Interplay of angiogenesis, vasculogenesis and arteriogenesis

During growth of new vessels, vasculogenesis and angiogenesis occur simultaneously and they probably should not be considered as separate events. Existing
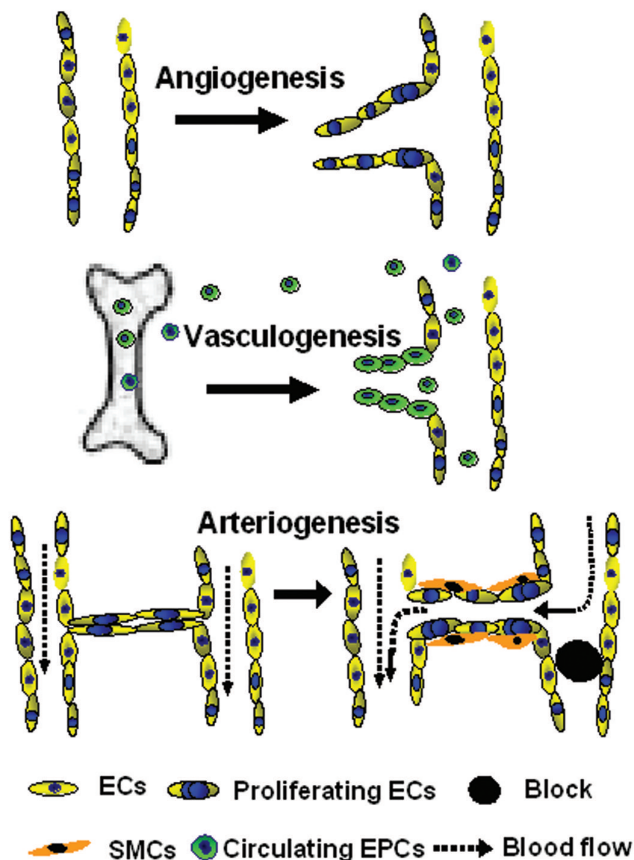

Fig. 1. Neovasculorization consists of angiogenesis, vasculogenesis, and arteriogenesis.

experimental models are used only to test or emphasize one or the other mechanism. In the case of mouse matigel plug and tumor models, angiogenesis is likely to be the predominant process (Iba et al., 2002). Neovascularization in hindlimb ischemia models in animals likely represent arteriogenesis (Cao et al., 2005). In animals systemically receiving a bone marrow transplant after ischemia, vasculogenesis will predominate at the ischemic sites (Kalka et al., 2000b). None of these models, however, are completely selective and in fact these three processes are tightly linked at physiological and biochemical levels in vivo. Recently, we demonstrated in the nude mice with femoral artery ligation that arteriogenesis predominated at the site of ligation (usually adductor muscles), whereas angiogenesis predominated in the ischemic distal bed (usually lower calf muscles) (Li et al., 2006). Nonetheless, we can not rule out the possible contribution of vasculogenesis to reestablishment of distal tissue perfusion, because the up-regulation of VEGF, stromal derived factor-1 (SDF-1α) at ischemia sites will also recruit circulating EPCs to participate in vascular repair.

Vascular progenitors can participate in all three forms of neovascularization. Vascular progenitors can differentiate to ECs in response to VEGF, and into smooth muscle cells in response to PDGF-BB (Yamashita et al., 2000). Therefore, a common vascular progenitor could contribute to the formation of naked endothelial capillaries (angiogenesis) as well as smooth muscle cells-coated vessels (arteriogenesis). Although there may be considerable mechanistic overlap in the two processes, at this time there is insufficient information to definitively distinguish the molecular processes leading to arteriogenesis and those leading to angiogenesis

In therapeutic neovascularization, it is also difficult to distinguish between the therapeutic effects of angiogenesis, arteriogenesis and vasculogenesis. The currently known growth factors are not selective for any of these mechanisms (Cao et al., 2005). In the following sections, we will discuss the most frequently used growth factors purportedly aimed at different mechanisms of neovascularization.

Growth factor administration

In parallel with improvements in the knowledge of the mechanisms of action, the number of agents available to stimulate vascular growth is soaring. Many growth factors are now at our disposal for therapeutic neovascularization. VEGF is the one of the factors that has been used clinical settings. It was originally discovered in the course of investigations designed to identify the tumor-secreted factor responsible for the increased vascular permeability characteristic of nearly all malignant solid tumors (Dvorak et al., 1979), but later was also found to induce proliferation and migration of ECs (Connolly et al., 1989). VEGF increases EC

viability through a combination of its effect on mitogenesis as well as inhibition of EC apoptosis (Senger et al., 1996). Furthermore, VEGF induces the release of GM-CSF by bone marrow ECs and increases the SDF-1 driven transendothelial progenitor cell migration (Bautz et al., 2000), which favors the process of vasculogenesis.

Clinically, intramuscular gene transfer of naked plasmid DNA encoding the 165-amino-acid isoform of human VEGF promotes collateral vessel development in patients with critical limb ischemia (Baumgartner et al., 1998). The recent small, randomized gene therapy study showed that significant and meaningful improvement was found in patients with diabetic limb ischemia treated with a VEGF(165)-containing plasmid (Kusumanto et al., 2006). Additionally, in these patients receiving VEGF gene transfer, augmented circulating EPCs were detected (Kalka et al., 2000a). These findings imply that neovascularization of human ischemic tissues after angiogenic growth factor therapy results in not only angiogenesis but also vasculogenesis. However, the relative contributions of angiogenesis and vasculogenesis to postnatal neovascularization, and the extent to which each is influenced by VEGF, still remain to be clarified.

Combination therapy: synergisms and complement

While treatment with factors such as VEGF and bFGF has produced encouraging results in animal models and in early clinical trials, the results of previous studies must be cautiously interpreted (Henry and Abraham, 2000). It was shown that VEGF induced an angiogenic response characterized by the formation of enlarged, thin-walled vessels that lacked supporting pericytes and were hyperpermeable to plasma proteins (Pettersson et al., 2000). The newly formed vessels were therefore abnormal and provided marginal functional benefit. The establishment of stable and functional blood vessel networks is a complex process that requires a cocktail of angiogenic factors to stimulate vessel sprouting and remodeling of the primitive vascular network (Yancopoulos et al., 2000). Given this complexity, it is questionable whether a single growth factor will be sufficient to initiate the entire cascade of events leading to a mature, functional and stable vascular network in vivo. In addition, questions regarding growth factor dosage and duration of therapy remain. Excessively long period of exposure of ischemic tissue to high doses of exogenously administered angiogenic cytokines (such as VEGF) results in upreguation of its receptors and may lead to aberrant neovascularization (Brown et al., 1997). Normal vessel development requires the proper expression of multiple growth factors rather than over expression of one single factor (Emanueli and Madeddu, 2005). Many other growth factors that are not vascular endothelium-specific are also required for blood vessel formation. These include members of the PDGF or TGF-ß families.

Previous work has suggested that PDGF-BB is centrally involved in vascular network maturation and remodeling by recruiting mural cells (pericytes and smooth muscle cells) onto the nascent endothelium (Lindahl et al., 1997). Indeed, PDGF-BB and also VEGF, FGF-2, are each capable of stimulating angiogenesis in the short term, but none of these factors alone is able to maintain the newly formed vessels. Separate mechanisms may control blood vessel growth, and the decision between maintenance and regression of newly formed vessels is depending upon exposure to a set of angiogenic factors, instead of single factors.

Cao et al. (2003) provided compelling evidence that an early phase of neovascularization by transient exposure to a specific combination of angiogenic factors such as FGF-2 and PDGF-BB could ensure the long-term stability of vascular networks. Their study using various doses and ratios of FGF-2 and PDGF-BB in a rat hind-limb model suggested that the dose of FGF-2 used was critical and the ratio should be optimal between these two agents. While single angiogenic factors, including FGF-2, and PDGF-BB, were unable to establish stable vascular networks, a combination of them could synergistically induce angiogenesis and formation of long-lasting functional vessels.

Many combinations of agents capable of inducing growth and stabilization of functional vessels in adult tissues have been discovered in recent years. A combination of submaximal doses of angiogpoietin 1 (Ang1) and VEGF produces an enhanced growth and functional vascular stabilization effect that is more potent than a maximal dose of VEGF alone (Chae et al., 2000). Moreover, combination therapy permitted administration of less VEGF, circumventing VEGF-induced hyperpermeability. As opposed to VEGF, Ang-1 induces tightening of the vascular endothelial permeability barriers (Thurston et al., 2000), likely by enhancing the interaction between ECs, pericytes, and the surrounding matrix. It could also be advantageous to combine VEGF-A with angiogenesis activators that are VEGF-independent, such as tissue kallikrein (TK) (Emanueli et al., 2004) or proteins of the Wnt family (Dufourcq et al., 2002). Moreover, synergism between VEGF and placental growth factor (PLGF) contributes to angiogenesis (Carmeliet et al., 2001). We may in the future combine three or more like a cocktail of factors to better control the sophisticated process of neovascularization. Thus, a principle that is emerging is that the proper combination of factors can enhance the formation of functional vessels. This suggests that we should consider alternative therapeutic strategies based on a combination of multiple factors, used in a complementary and coordinated manner (Table 1).

Cell-based therapeutic neovascularization

As discussed earlier, there are significant theoretical explanations for the disappointing results of trials using a single angiogenic agent. Cell therapy, mainly by virtue of its potential to supply stem/progenitor cells as well as multiple angiogenic factors to the region of developing capillaries and collaterals, may overcome some of these problems.

Bone marrow cells

Bone marrow cells have been greatly investigated in animal models and in clinical settings. Transplantation of autologous bone marrow mononuclear cells (BMMNCs) augmented neovascularization in response to tissue ischemia in rabbit and rat models of unilateral hindlimb ischemia (Hamano et al., 2001; Shintani et al., 2001). One rationale and proposed advantage of BMMNCs therapy versus single agent therapy is that cell therapy directly increases the number of stem/progenitor cells in ischemic tissue and provide a cocktail of secreted growth factors. The stem/progenitor cells can be delivered systemically or locally. Systemic injection of cells or induction of cells to exit the marrow microenvironment by chemokine administration has been found to substantially increase the incorporation of the targeted cells into the vessel wall (Takahashi et al., 1999). Local implantation of autologous BMMNCs into ischemic limbs has also been shown to be an effective therapeutic strategy in patients (Tateishi-Yuyama et al., 2002). The BMMNC transplant could therefore be a safe and effective form of therapeutic angiogenesis, given the aforementioned natural ability of marrow cells to supply endothelial progenitor cells and to secrete various angiogenic factors or cytokines.

Mobilized peripheral blood mononuclear cells (M-PBMNCs)

Although BM-MNCs are rich in CD34$^+$ cells and have been proven to be effective in animals and patients with ischemic limbs, it requires a general anaesthesia and collection of a large amount of marrow. Non-mobilized PBMNCs have been compared to BMMNCs for the treatment of limb ischemia, with conflicting results. Recently, Osamu et al. (Iba et al., 2002) showed that PBMNCs can augment angiogenesis and promote ischemia recovery. However, compared with BMMNCs, implantation of PBMNCs itself was not as effective as that of BM-MNC owing to its low CD34$^+$ cell concentration (Tateishi-Yuyama et al., 2002). In contrast, Issei Komuro and colleagues who showed that PBMNCs had similar efficacy as BMMNCs (Minamino et al., 2002).

To bolster the potential efficacy of PBMNCs, we used G-CSF stimulation to mobilize mononuclear cells into the peripheral blood (mobilized peripheral blood mononuclear cells, M-PBMNCs). There are several advantages of M-PBMNCs: 1) CD34$^+$ cells are significantly increased after G-CSF mobilization; 2) M-PBMNCs are rich in angiogenic factors and cytokines; 3) M-PBMNCs can be easily sorted and noninvasively obtained from the peripheral blood of patients after G-

CSF administration. Moreover, this therapy does not require anesthesia and need only short hospitalization with low cost. We showed that M-PBMNCs were effective for severe arteriosclerosis obliterans of lower extremities (Huang et al., 2004). More recently, we used this novel therapy for severe diabetic foot ischemia and proved its effectiveness and simplicity in clinical application (Huang et al., 2005).

To investigate the underlying mechanisms of how M-PBMNCs act and what role those stem cells (CD34$^+$) play in vivo, we use a murine hindlimb ischemia model and compared the effectiveness between M-PBMNCs and CD34$^+$ depleted M-PBMNCs (Li et al., 2006). We showed that transplantation of M-PBMNCs without CD34$^+$ cells was still effective in augmenting neovasculogenesis, but its therapeutic efficacy was compromised. In contrast to the observations by others that CD34$^-$ cells could incorporate into the endothelium of blood vessels in mouse ischemic limbs (Harraz et al., 2001), we did not observe incorporation of CD34$^-$ cells into host vasculature. Recently, we demonstrated that M-PBMNCs are as effective as BMMNCs in proneo-vascularization ability both in vitro and in vivo (submitted for publication).

Mechanisms by which locally delivered BMMNCs or M-PBMNCs alleviate limb ischemia are currently being investigated (Han, 2005). Using real time in vivo imaging and immunofluorescence, we could observed the survival, migration, differentiation, fusion, secretion of angiogenic factors of locally delivered mononuclear cells (Li et al., 2006; Zhou et al., 2006a). The pro-neovascularization effect was mediated partly through cell-to-cell contact and cell transdifferentiation. Importantly the effect was also mediated via paracrine mechanisms involving release of cytokines that exert influences on surrounding cells. Taken together, M-PBMNCs might expand the armementarium of therapeutic neovascularization, in particular during senescence or disease when the reparative growth potential of vessel-associated vascular cells becomes limited. Although these approaches, like M-PBMNCs for limb ischemia, are still in their infancy, we believe they are promising and will become a viable therapeutic strategy (Zhou et al., 2006b).

Endothelial progenitor cells from bone marrow (BM), peripheral blood (PB) or cord blood (CB)

In 1997, Asahara et al. (1997) first isolated circulating angioblasts (CD34$^+$) from the human peripheral blood of adult humans. Since these cells had the potential to rapidly proliferate, and to differentiate into ECs in vitro and in vivo, they were named "endothelial progenitor cells" (EPCs). Later, Reyes et al. (Reyes et al., 2002) showed that multipotent adult progenitor cells (MAPCs) could be isolated from BMMNCs, and MAPCs could be differentiated into EPCs, suggesting that EPCs may be derived from MAPCs. Since angioblasts (CD34$^+$) and EPCs express the chemokine receptor CXCR4, they can migrate in response to SDF-1, which is upregulated in ischemic tissues. After they adhere and invade into host vasculature, they begin a processs of vasculogenesis. EPCs can also be isolated from (M-)PBMNCs, and injection of these ex vivo expanded EPCs into mice greatly augmented neovascularization in the ischemic hindlimb (Kalka et al., 2000b). In diabetic mice with limb ischemia, local injection of angioblasts (CD34$^+$) dramatically accelerated the restoration of flow, and ameliorated the hindlimb ischemia (Schatteman et al., 2000). Harvesting circulating EPCs from human subjects therefore appears to be a promising future approach for therapeutic neovascularization.

These EPCs were also shown to express AC133, an orphan receptor which is specifically expressed on EPCs. Recently, we reported that CB AC133$^+$ cell-derived EPCs transplantation could enhance neovascularization in ischemic hindlimbs (Yang et al., 2004). In contrast to adult bone marrow–derived hematopoietic stem cells (HSC), CB progenitors have distinctive proliferative advantages, including the capacity to form a greater number of colonies, a higher cell-cycle rate, and a longer telomere (Vaziri et al., 1994). Recently, we found that CB progentitors were more effective than adult BM or PB cells in therapeutic neovascularization (Zhang et al., 2006). Thus, the use of CB progenitors represents a promising future approach for therapeutic neovascularization.

In spite of the positive findings of the bone marrow derived angioblasts or EPCs in contribution of neovascularization, some lines of evidence questioned the contribution of postnatal vasculogenesis to therapeutic efficacy. Zentilin et al. reported that bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels (Zentilin et al., 2006). Their result was in accordance with the previous results from Ziegelhoeffer group that bone marrow-derived cells do not promote adult vascular growth by incorporating into vessel walls (Ziegelhoeffer et al., 2004), and from Voswinckel group that circulating vascular progenitor cells do not contribute to compensatory lung growth (Voswinckel et al., 2003). The bone marrow-derived cells were proposed to act as supporting cells to promote vascular growth (Ziegelhoeffer et al., 2004). Based on the fact that EPCs or angioblasts can secret angiogenic factors, those circulating cells may not only physically contribute to the repair of injured vessels, but also secret some angiogenic factors that augment local angiogenesis and arteriogenesis in vivo. This was evidenced by O'Neill et al that mobilization of bone marrow-derived cells enhances the angiogenic response to hypoxia without transdifferentiation into endothelial cells (O'Neill et al., 2005). Taking into account the methodological difficulties, the varying or even contradictory results, and the many unanswered questions concerning the possible mechanisms, the role of bone marrow–derived

cells in organ or vascular repair and growth still remains unsolved and awaits further investigation.

Mesenchymal stromal/stem cells (MSC)

Besides mononuclear cells, EPCs and HSC, mesenchymal stem cells (MSC) were recently discovered to be a promising therapeutic alternative for hindlimb ischemia treatment. In vitro studies reveal that after several weeks of culture, MSCs acquire a phenotype that closely resembles smooth muscle cells (Kashiwakura et al., 2003). One study directly addressing MSC therapy for angiogenesis suggested that locally delivered MSCs were able to incorporate into newly formed vessels and displayed endothelial and smooth muscle cell phenotypes (Al-Khaldi et al., 2003). Besides differentiation, MSCs also secrete a wide array of arteriogenic cytokines and local delivery of MSCs can contribute to collateral remodeling through paracrine mechanisms (Kinnaird et al., 2004b). MSC express genes encoding a broad spectrum of arteriogenic cytokines, such as VEGF, bFGF etc. Consequently, MSC conditioned medium (CM), which is enriched for these cytokines, has been shown to promote in vitro and in vivo arteriogenesis (Kinnaird et al., 2004a).

Cell based therapy with growth factor or cytokines: future directions

As mentioned before, MNC transplantation induces therapeutic angiogenesis in ischemic limb; however, some patients fail to respond to this cell therapy. In addition to the host poor response, there are data to suggest that cell therapy itself may also have inherent limitations related to low survival rate, insufficient cell number and impaired function. For example, BMMNCs and M-PBMNCs from diabetic animals or patients are dysfunctional, and diabetes impaires the therapeutic potential of autologous transplantation in limb ischemia (Tamarat et al., 2004; Zhou et al., 2006a). Thus, a novel therapeutic strategy to enhance the angiogenic property of MNCs is desirable (Table 1).

MNC plus adrenomedullin

Adrenomedullin is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (Kitamura et al., 1993). It was recently reported that a combination of adrenomedullin infusion and MNC transplantation resulted in significantly greater improvement in hindlimb ischemia than MNC transplantation alone (Iwase et al., 2005). For bone marrow cell therapy to be more beneficial, the supportive role of adrenomedullin for optimizing the milieu for host vasculature to respond to tissue ischemia appears important. This effect was mediated in part by the angiogenic potency of adrenomedullin itself and the beneficial effects of adrenomedullin on the survival, adhesion, and differentiation of transplanted MNCs. This is a good example of synergistic effect on therapeutic angiogenesis by combination therapy.

EPCs plus SDF-1

At present, enthusiasm for the therapeutic potential of EPC transplantation therapeutic strategy is limited by certain practical considerations. For example, there is an insufficiency of clinically relevant number of EPCs. How to make full potential of these limited EPCs and augment their therapeutic efficacy needs more attention now. It has been well established that SDF-1 is a chemokine considered to play an important role in the trafficking of HSC. Since there is a close relationship between HSC and EPC, the effect of SDF-1 on EPC-mediated vasculogenesis has therefore been investigated. Yamaguchi et al. (2003) showed in animals given a systemic infusion of ex vivo expanded EPCs, that vasculogenesis was greatly augmented by local injection of SDF-1. This cell plus cytokine formula was shown to increase neovascularization in vivo by augmenting EPC recruitment in ischemic tissues. The local delivery of SDF-1 has an additional effect that was later discovered by another group. Local upregulation of SDF-1 enhanced ischemia-induced vasculogenesis and angiogenesis in vivo through a VEGF/eNOS-related pathway (Hiasa et al., 2004). We have also demonstrated that local intramuscular delivery of SDF-1α restored the therapeutic efficacy of OxLDL treated-EPCs by recruiting more cells to ischemic sites (submitted for publication). Thus, cell based therapy with growth

Table 1. Therapeutic allternatives and mechanisms.

Type/Subtype	(Pre-)clinical trial	Mechanisms
Growth Factor		
Single	VEGF, bFGF, et al.	Ang, Vas, Art
Multiple	FGF-2+PDGF-BB	Ang, Vas, Art
	Ang1+VEGF	Ang, Vas, Art
	VEGF+PLGF	Ang, Art
	VEGF/TK or Wnt	Ang, Vas, Art
Cells		
Single	BMMNC	Ang, Vas, Art
	(M-)PBMNCs	Ang, Vas, Art
	EPCs	Vas
	MSCs	Ang, Art
Cells + factor	MNC+adrenomedullin	Ang, Vas, Art
	EPCs+SDF-1	Ang, Vas
	Cells/GM-CSF or VEGF	Vas
	MNC+VitC,E, et al.	Vas, Art
Cells+gene modification	MSC+Akt gene	Ang, Art
	MSC+ HIF-1/VP16	Ang, Art
	EPCs+VEGF gene	Ang, Vas
	EPCs+TRT gene	Vas

Cell or growth factor delivery can be used as therapeutic neovascularization. Ang: Angiogenesis; Vas: Vasculogenesis; Art: Arteriogenesis; TRT: Telomerase reverse transcriptase (TRT).

factors may present another important therapeutic strategy.

Exogenous administration of cytokines for cell mobilization

Another method to strengthen the ischemia-induced vasculogenesis is to increase the circulating cells by exogenous administration of cytokines, such as G-CSF, GM-CSF, VEGF etc. Augmented mobilization of bone marrow-derived EPCs may be achieved by using GM-CSF (Takahashi et al., 1999), and the mobilization improves the recovery of hindlimb ischemia in mice and rabbit. Our group used G-CSF clinically to mobilized PBMNCs for local administration, and this also resulted in more stem cells mobilized and migrated to the ischemic tissue for repair with increased vasculogenesis (Huang et al., 2005). There are many other cytokines that have the ability to mobilize bone marrow cells into circulating EPCs, such as VEGF. Recently another novel cytokine hemagiopoietin (HAPO) we discovered were shown to act on the primitive cells of both hematopoietic and EC lineages (hemagioblast). We showed that HAPO may have a clinical potential in the treatment of various cytopenias and radiation injury and in the expansion of HSC and EPCs (Liu et al., 2004). We also showed that HAPO could mobilize endothelial stem/progenitor cells (Tanii et al., 2006), and this cytokine may be a useful agent for novel chemokine therapy for next generation therapeutic neovascularization.

Cells with genetic modifications

Transplantation of MSC for ischemic cardiovascular diseases has been proposed as an effective strategy. However, poor cell viability associated with transplantation has limited the reparative capacity of these cells in vivo. It is therefore necessary to solve the question of survival and make cells play their roles longer before they died in vivo. Akt is a serine threonine kinase that is a powerful survival signal in many systems (Datta et al., 1999). Mangi et al. (2003) took advantage of this gene for overexpression in MSC, and their result was exciting. Transplantation of Akt-modified MSCs could restore fourfold greater myocardial volume than equal numbers of cells transduced with control genes. This prosurvival method opens a new way for cell therapy in ischemic diseases.

As indicated earlier, transplantation of EPCs could promote the recovery of ischemic limbs through vasculogenesis. The fact that transplanted EPCs migrated and were incorporated into sites of neovascularization in adult tissues suggests a potential use of EPCs as cell vectors for gene delivery to targeted sites in vivo (Murohara et al., 2000). It was recently reported that VEGF gene transfer enhances EPC proliferation, adhesion, and incorporation into EC monolayers (Iwaguro et al., 2002). Interestingly, such phenotypic modulation of EPCs can also facilitate therapeutic neovascularization in ischemic limbs. The dose of EPCs carrying the VEGF gene used was small (30 times less than that required in the control), and this dose would be subtherapeutic if the VEGF gene were not present. Thus, VEGF gene transfer into EPC constitutes one option to address the limited number of EPCs that can be isolated from BM, PB, CB before ex vivo expansion and subsequent administration.

Moreover, the regulatory molecule for cell life span, telomerase, was modified by telomerase reverse transcriptase gene transfer to investigate its effect on EPCs in neovascularization. Strengthened neo-vascularization in ischemic tissues could be achieved by EPCs so transfected (Murasawa et al., 2002). The concept of "rejuvenating" EPCs via delay in senescence and enhanced regenerative properties may therefore have therapeutic implications for vascular disorders, including limb ischemia.

Recently, transcription factors acting as master angiogenesis gene switches has been clarified. Since HIF-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors (Yamakawa et al., 2003), an alternative method can be the use of transcription factors that act upstream of multiple mechanisms of the angiogenic cascade. This area needs more attention and investigation.

Cell plus metabolic intervention

That the beneficial effect of BMMNCs could be amplified by concurrent metabolic intervention as reported by Napoli et al. (2005) suggests that BMMNCs together with metabolic intervention (Vitamin C, E, etc) could be an effective clinical treatment for peripheral arterial disease. Thus, future clinical intervention should combine the advantage of different mechanisms of neovascularization and also other interventions, and customize a protocol that may yield optimal efficacy with lowest side effect.

Concerns and Conclusions

There are still several concerns that must be resolved before we step further. One is undesirable "off-target" angiogenesis, particular in the retina (Zhou et al., 2006b), by VEGF treatment. Another is that systemic administration of some cytokines or cells may promote tumor growth. Given the link between angiogenesis and neurogenesis, and the recent finding that VEGF improves ischemic neuropathy, we should bear in mind that long term angiogenic treatment might cause undesired neuronal effects (Schratzberger et al., 2000). Thus, as we develop new ways of treatment, we should always consider and be cautious of the potential disadvantages and side effect as well as the individual host response to the treatment.

One logic to be emphasized is that prevention is more effective and much easier to accomplish than treatment of ischemia in late stage. The prophylactic

delivery of human tissue kallikrein gene could ameliorate peripheral diabetic ischemia (Emanueli et al., 2004) and could exert long-term protection against the development of diabetic microangiopathy (Emanueli et al., 2002). Therefore, it might be more effective to prevent diabetic microangiopathy than to rescue established ischemia in the late stage.

Recent progress in the understanding of the molecular and cellular mechanisms of angiogenesis, vasculogenesis, arteriogenesis is expected to speed up the clinical development of biological revascularization. Vast and rapidly growing body of data that have been obtained on growth factors and cell based strategies in past years has forced us to gain insight into a more careful translational research program. This program will incorporate the ever-evolving basic understanding of the biology of therapeutic neovasculorization into an effective clinical trials program, which should be customized for individual patients for optimal efficacy with the lowest side effect.

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