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Cell Based Interventions for Therapeutic Angiogenesis: Review of Potential Cell Sources

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Abstract

Alternative therapies are currently being developed to treat patients with chronic limb ischemia who are unable to be revascularized in order to avoid amputation. Cell based therapy using mononuclear cells is gaining attention as many clinical trials are currently underway. We review cell differentiation along with the different potential cell sources for use in therapeutic angiogenesis.

Introduction

Critical limb ischemia (CLI) is defined as chronic ischemic rest pain, or presence of tissue loss such as ulcers or gangrene, as a manifestation of severe peripheral arterial disease (PAD). Chronicity is defined by the presence of symptoms for more than 2 weeks.[1] CLI implies end stage disease and the expectation of limb loss, and therefore revascularization remains the optimal treatment option for CLI patients, with the ultimate goal of limb salvage. However, some patients have no surgical or endovascular options for revascularization and are left to amputation. Others present to vascular surgeons and are poor candidates for surgical repair secondary to multiple medical co-morbidities, increasing the risk of procedures. The concern for this subset of patients has led to exploration for additional therapeutic options to prevent tissue loss.

Gene and cell based therapies have been evaluated both in the laboratory and at the patient's bedside as possible options for patients unable to be revascularized. Initial animal models with gene therapy demonstrated some promising results;[2] however, double blinded, randomized trials, such as RAVEL, failed to duplicate the promising animal and Phase I and II studies, and even showing some negative outcomes.[3]

The Therapeutic Angiogenesis using Cell Transplantation (TACT) trial was the first randomized controlled cell-based study in humans. The authors injected bone marrow-derived mononuclear cells (BM-MNC) into the gastrocnemius muscle of the patient's

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ischemic limb; primary outcomes measured were safety and feasibility of treatment. The ankle-brachial index, transcutaneous oxygen pressure and pain free walking measures were all improved in the treated group and were found to be statistically significant compared to the control group.[4] Since then, several series have been published using BM-MNC in patients with CLI, thromboangiitis obliterans and PAD demonstrating positive results.[5–12]

Review of Cell Differentiation

Traditionally, the components of the hematopoietic system are divided into the myeloid tissues and the lymphoid tissues. The myeloid tissue is comprised of the bone marrow and the cells it produces, whereas the lymphoid tissue consists of the lymph nodes, spleen, and thymus. Despite this distinction, all of the formed elements of the blood – erythrocytes, granulocytes, monocytes, platelets, and lymphocytes – share a common hematopoietic origin (Figure 1). Early transplantation, developmental, and irradiation experiments helped establish the existence of multi-potent hematopoietic progenitor cells.[13–18] Furthermore, various experiments demonstrated the single-cell origin of multi-lineage hematopoiesis with the identification of hematopoietic stem cells (HSC) capable of self-renewal and complete hematopoietic differentiation.[19–22] HSC give rise to all blood cells through a differentiation process where developmental potentials are gradually lost while lineage-specific features are gained.[23]

During fetal development, hematopoietic cellular differentiation begins in the yolk sac and aorta/gonad/mesonephros region then sequentially progresses to the liver, spleen, and bone marrow.[24–27] In adult mammals, hematopoietic cellular differentiation and proliferation occurs in the bone marrow and to a lesser extent in the spleen and thymus.[23, 28] HSC represent up to 0.05% of cells in mouse and human bone marrow and are responsible for the short and long-term multi-lineage reconstitution of blood cells.[28] HSC differentiate into lymphoid and myeloid progenitor cells via several proposed models.[29, 30] Myeloid precursors go on to develop into erythrocytes, megakaryocytes, granulocytes, and monocytes. In contrast, lymphoid precursors develop into three distinct cell populations: T and B lymphocytes and natural killer (NK) cells. The differentiation and lineage commitment of each cell is an intricate process that involves the complex integration of extracellular and internal signals to regulate the cellular composition of blood in homeostasis. Although HSC differentiation and lineage commitment appear to follow a well defined set of steps, significant plasticity exists and there are many ongoing investigations to further clarify this complex process.[31]

Potential Cell Sources

The concept of injecting autologous bone marrow (BM) into ischemic limbs was proposed on the premise that components of the cellular mixture are capable of homing to, and regenerating ischemic tissue.[4, 32, 33] Modern thought regarding the mechanism of tissue regeneration is that some sub-fraction(s) of BM are capable of contributing, perhaps indirectly, to both the cellular components and growth factors required for the expansion of blood supply, including: angiogenesis, arteriogenesis, and perhaps even vasculogenesis, the *de novo* formation of new vessels (Table 1).[34–36] One or more populations of the BM are likely contributing to this circulatory expansion in the ischemic environment. But, there is currently no consensus regarding which population is the “effector population” in therapeutic angiogenesis. A multitude of studies have shown *in vivo* success with the use of several isolated populations including mononuclear cells (MNC), monocytes, endothelial progenitor cells (EPC) and mesenchymal progenitor cell (MPC) fractions of the BM. Questions of which specific cells and what dose remain to be elucidated. In current studies and clinical trials, the composition of the cellular therapy used has great variability. Factors

such as the origin of the cells (bone marrow vs. peripheral blood), the co-morbidities of the donor and the methods used to prepare the cells may lead to major differences in this cellular product. It is important to characterize the precise nature of the "effector population(s)" used for therapeutic angiogenesis before continuing on with large-scale clinical trials. This will allow for optimization and standardization of therapy, and will contribute to the knowledge of involved mechanisms.[37] Overview of potential cell sources for therapeutic angiogenesis can be found in Table 2.

Raw Bone Marrow/Mononuclear Cells

The bone marrow is a heterogeneous mixture of cells. It contains hematopoietic stem cells, including common lymphoid and myeloid precursors and their mature forms (T and B lymphocytes, NK cells, monocytes, EPC, dendritic cells, megakaryocytes, RBC and granulocytes) as well as mesenchymal progenitor cells. Some preparations of cells for use in therapeutic angiogenesis use the MNC population, which are isolated from BM by density centrifugation or using a blood cell separator, which allows for the removal of RBC and granulocytes.[10, 38] After preparation, the mononuclear cell population includes primarily lymphocytes (85%), monocytes (15%) and EPC (0.03%), as well as dendritic cells, NK cells and MPC with even lower levels of hematopoietic stem and progenitor cells.[39] These populations can be identified based on cell surface markers using flow cytometry, including CD45, which identifies lymphocytes, monocytes, NK cells, dendritic cells and a monocyte fraction of EPC that only indirectly promotes endothelial cell growth, but does not directly become endothelial cells. This population can be further divided based on co-expression of CD45 with CD2 (T lymphocytes and NK cells), CD19 (B lymphocytes), and CD14 (monocytes).[37] Additional markers such as CD34 and CD133 are often used to identify EPC, CD 11b and CD115 for monocytes, and CD73, CD90 and CD105 for MPC, as will be discussed below.

Many investigators have successfully demonstrated that after BM-MNC injection into ischemic limbs, there are improved clinical outcomes in patients with PAD and/or CLI.[4, 8, 10, 11, 40, 41] Higashi et al.[8] demonstrated that BM-MNC implantation into ischemic limbs increased the ankle-brachial pressure index, transcutaneous oxygen pressure, basal leg blood flow as well as improved endothelium-dependent vasodilation. Another study investigated the efficacy and safety of autologous BM-MNC implantation in patients with CLI due to thromboangiitis obliterans (Buerger's disease). The patients received multiple injections into the gastrocnemius muscle, the intermetatarsal region, and the dorsum of the foot or forearm. At 6 months, patients demonstrated a statistically significant improvement in rest pain scores, peak walking time, and quality of life. Total healing of the most important lesion was achieved in 83% of patients with ischemic ulcers and angiography studies at 6 months after the implantation showed vascular collateral networks had formed across the affected arteries in 78.5% of the patients.[11] In animal models of ischemia, MNC injections induced collateral sprouting and angiogenesis.[42–44]

Peripheral blood has also been a source of MNC for the therapeutic use in ischemia. Since a large amount of BM is required to obtain an adequate number of MNC, growth factors (granulocyte colony stimulating factor or granulocyte-macrophage colony stimulating factor) have been administered to stimulate the BM to mobilize progenitor and stem cells into circulation.[45, 46] These cells can subsequently be harvested by leukapheresis.

Li et al.[43] studied the effects of the granulocyte colony-stimulating factor (G-CSF) mobilized PB-MNC and CD34-depleted G-CSF-mobilized PB-MNC in an ischemic model in mice. Fluorescence-labeled PB-MNC were intramuscularly injected into the ischemic hind limbs and laser Doppler imaging analysis demonstrated significant increases in blood

perfusion at day 7, 14 and 28 after the operation. Transplanted cells were found to accumulate around arterioles and disperse in capillary networks. Incorporation of transplanted cells into new capillaries was observed in the PB-MNC CD34+ group, but not found in the group deprived of CD34 cells. There was also an elevated expression of vascular endothelial growth factor (VEGF) in ischemic tissue. These findings indicate that G-CSF-mobilized PB-MNC promote vascular growth not only by incorporating into vessel walls but also by supplying angiogenic factors.[43]

Given that similar results have also been shown with peripheral blood PB-MNC,[11, 40–42] these studies suggest that the therapeutic population is not limited to immature precursors in the bone marrow. However, BM-MNC may have a more rich supply of CD34+ cells compared to PB-MNC.[37] It was classically believed that the therapeutic properties of MNC were attributed to the CD34+/EPC component of the BM.[10] In addition to the EPC population, other MNC may be required for the recruitment of EPC,[39] as well as the having additional therapeutic benefits by providing the optimal *milieu* for growth by producing cytokines and growth factors.[47] Though studies involving MNC have produced intriguing results, it remains that MNC contain many cell populations. Further characterization and efficacy testing of each component will provide insight into the mechanisms of their angiogenic properties.

Monocytes

The mononuclear phagocyte system is constituted by three cell types: monocytes, dendritic cells and terminally differentiated macrophages. Monocytes, which are derived from the hematopoietic stem cells in the bone marrow, comprise approximately ten percent of the peripheral leukocytes and have a half-life of three days.[48]

We have previously reported that patients with CLI have elevated preoperative monocyte levels compared to control patients (with PAD but without CLI). This increase in monocytes was independent of an increase in other WBC populations, and was shown to significantly decrease in CLI patients following successful revascularization procedures. These results suggest that monocytes may be a useful perioperative marker in CLI patients undergoing surgery, and that this population of cells plays a critical role in the recovery of ischemic limbs.[39]

The monocyte population is one component of MNC that is likely to contribute to angiogenesis in ischemic tissue. Monocytes are an essential part of the innate immune system capable of phagocytosis and production of inflammatory cytokines. Monocytes are capable of migration between the bone marrow, blood and tissues in response to infection and inflammation, as well as differentiation into dendritic cells and macrophages. The fate of monocytic location and differentiation is thought to depend on the local and systemic microenvironment, including cytokines, growth factors and toxins.[49] Though the complete differentiation pathways of monocytes has yet to be fully elucidated, it is clear that at least two distinct phenotypic classes of monocytes exist that have been characterized by their cell surface markers: $Gr1^{+}/Ly\text{-}6C^{high}$ and $Gr1^{-}/Ly\text{-}6C^{low}$. These two lineages give rise to two distinct macrophage populations, M1 and M2, respectively.[50]

The M1 population, arising from $Gr1^{+}/Ly\text{-}6C^{high}$ monocytes, is part of the “classical” inflammatory cascade stimulated by LPS and INF-γ involved in the innate defense against microbes. This population is not thought to be directly involved in tissue repair and regeneration.[49] M2 macrophages are of particular interest in ischemic injuries as they have been shown to play a role in tissue repair. For instance, Auffray et al. have shown that $Gr1^{-}/Ly\text{-}6C^{low}$ monocytes migrate along the luminal surface of vascular endothelium and may have a role in surveying for local damage and infection to the endothelium.[51] Furthermore,

this population expresses VEGF after being recruited to sites of ischemic myocardium in mice where it promotes angiogenesis and collagen deposition.[52] In a model of skeletal muscle injury, Arnold et al. examined monocyte recruitment. During the early phase of recovery, Ly6C^{hi} monocytes are recruited and an overall inflammatory environment exists with expression of TNF-α and IL-1β. However, these cells evolve into Ly6C^{lo}F4/80^{hi} macrophages. This transition results in an overall anti-inflammatory environment, which allows for membrane repair, myogenesis, and fiber growth.[53]

Endothelial Progenitor Cells

The endothelial progenitor cell phenotype is complex, dynamic, and dependent on several factors including location and length of peripheral circulating time. Classically, bone marrow derived EPC have been identified by the presence of CD34, CD133, and VEGFR-2 markers.[54–56] Once released into the peripheral circulation (Figure 2), differentiation of EPC have been associated with a change in surface marker expression.[57] Peripherally circulating EPC are further classified as either early EPC or late endothelial outgrowth cells (EOC). This maturation process involves the down regulation of BM-derived EPC markers, such as CD133, and a concurrent increase in mature endothelial cell marker expression.[57–59] Early circulating EPC continue to express CD34, CD133, and VEGFR-2 while assuming additional markers such as CD14, CD31, CD45, VE-cadherin, and vWF.[54, 56, 57] In comparison, EOC down regulate CD14, CD45, and CD133 while up-regulating eNOS, a marker of mature endothelial cells.[58, 59] EOC precursors were found to be of the CD34+ CD45− cell fraction, which directly lead to endothelial cells. In contrast, CD34+ CD45+ hematopoietic progenitor cell fraction display characteristics of early EPC and promote angiogenesis via secreted factors.[60]

It should, however, be noted, that despite progress in identifying EPC associated markers, variations in definition persist secondary to lack of a universal specific identifier. In particular, it is misleading to refer to CD45+ pro-angiogenic monocytes as EPC even though they could be considered `early outgrowth' cells; they do not become endothelial cells so the cells that are growing in the early outgrowth are not derived from EPC, but they are monocytes – or perhaps an as yet un-named macrophage subtype. It is similarly misleading to refer to all previous reports as having used EPC even if they claimed to use EPC; rather the differentiation of CD45+ from CD45− cells is now a critical aspect of modern reports and EPC characterization.

Over the last several years, new and emerging roles for EPC have been discovered. Today, it is known that EPC play critical roles in all areas of adult neovascularization including angiogenesis, arteriogenesis, and postnatal vasculogenesis. Angiogenesis, defined as the proliferation of pre-existing capillary networks through the use of native endothelial cells, is augmented by growth factors and cytokines released by EPC.[33, 61–63] In a similar manner, the paracrine-like effects of EPC promote arteriogenesis, the vascular remodeling of resident vessels.[64–66] Until recently, vasculogenesis was believed to be a process restricted to the embryonic period of development. This concept changed with the successful isolation of EPC from adult peripheral blood in 1997 by Asahara et al. which validated the possibility of postnatal vasculogenesis.[32] Vasculogenesis, the de novo formation of blood vessels through recruitment and use of BM-derived EPC, has become an emerging and critical component in therapeutic angiogenesis.[56]

Subsequent to these findings, the use of EPC to increase perfusion and treat ischemia has become a potential alternative for patients who are not candidates for traditional surgical intervention. Growing evidence, both in the form of preclinical and clinical data, has shown

therapeutic angiogenesis to be a safe alternative method for non-surgical enhancement of neovascularization with regard to both limb and myocardial ischemia.[33, 67–70]

Mesenchymal Progenitor Cells

In an analogous manner, similar to that of the EPC, mesenchymal progenitor cells (MPC) are found in adult bone marrow, and have recently been shown to occupy an important augmentory role in therapeutic angiogenesis.[71] MPC likewise lack specific cell surface markers. They are frequently identified by documenting their lack of expression of hematopoietic markers, which include CD14, CD34, and CD45, as well as their positive expression of CD73, CD90, and CD105.[65, 72, 73] MPC are capable of generating most somatic cells, including myoblasts, smooth muscle cells, and hematopoietic stromal support cells, when provided with a suitable environment.[74]

Historically the marrow stromal population was derived by adherence to plastic culture wells; this property actually complicates the matter since it is not clear whether the plastic and/or the in vitro culture induces changes in the cells. Subsequent studies showed that this population consists of many subpopulations including endothelial cells, osteoblasts, monocyte/macrophages, as well as relatively rare stem/progenitor cells that have the ability to self-renew in vitro and to differentiate at the clonal level down the fibroblast, osteoblastic, chondrogenic and adipocyte lineages. The data regarding their ability to become endothelial or smooth muscle cells are not uniformly accepted because antigen profiles of smooth muscle cells and myofibroblasts overlap.

Although original therapeutic angiogenesis studies focused on hematopoietic-derived $CD34^+$ cells, more recent investigations have preliminarily shown that the synchronous injection of both $CD34^+$ EPC and $CD34^-$ MPC leads to heightened neovascularization of ischemic tissues.[71] Questions continue to exist over the exact mechanism through which MPC contribute to neovascularization. Some studies have suggested that the addition of MPC stimulates the recruitment of smooth muscles cells, pericytes, and other stromal support cells which cohesively contribute to neovascularization and the formation of more mature vessels.[32, 75] Other findings suggest that MPC are capable of differentiating into EPC.[76, 77] At present, there are ongoing clinical trials further investigating MPC and their role in therapeutic angiogenesis.

Discussion

Cell based therapy remains a potential therapeutic option for patients with critical limb ischemia to restore blood flow and salvage limbs. Multiple cells in the bone marrow can play a role in angiogenesis, arteriogenesis and vasculogenesis in the postnatal period. Ongoing clinical trials will hopefully demonstrate which cells are optimal, along with the optimal injection site, cell number and whether there is a need for multiple injections. Additional studies are needed to characterize which cell markers are optimal to determine cell phenotypes, and particularly which cells are optimal for therapeutic use. Controlled animal studies may aid in determining these conditions.

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Abbreviations

BM	bone marrow
BM-MNC	bone marrow-derived mononuclear cells
CLI	critical limb ischemia
EOC	endothelial outgrowth cells
EPC	endothelial progenitor cells
HSC	hematopoietic stem cells
MNC	mononuclear cells
MPC	mesenchymal progenitor cells
NK	natural killer cells
PAD	peripheral arterial disease
PB	peripheral blood
PB-MNC	peripheral blood-derived mononuclear cells
VEGF	vascular endothelial growth factor

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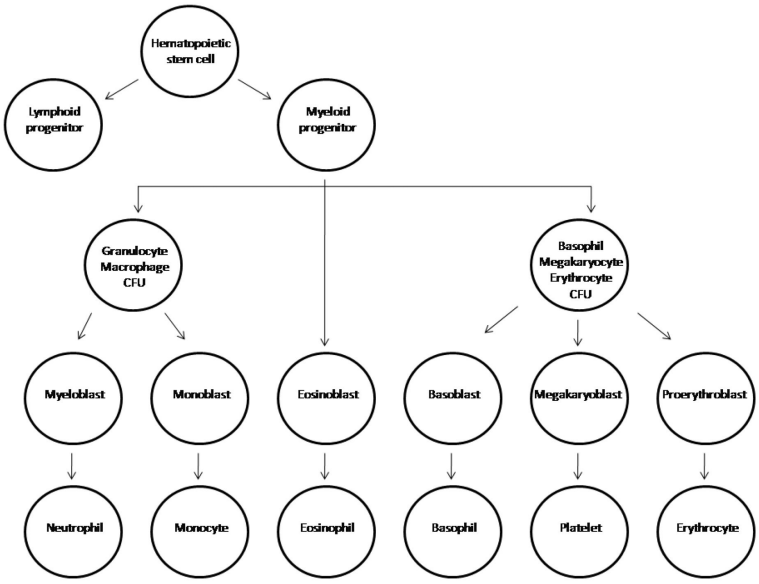

Figure 1.
Hematopoietic stem cell (HSC) differentiation
Legend: CFU=colony forming unit

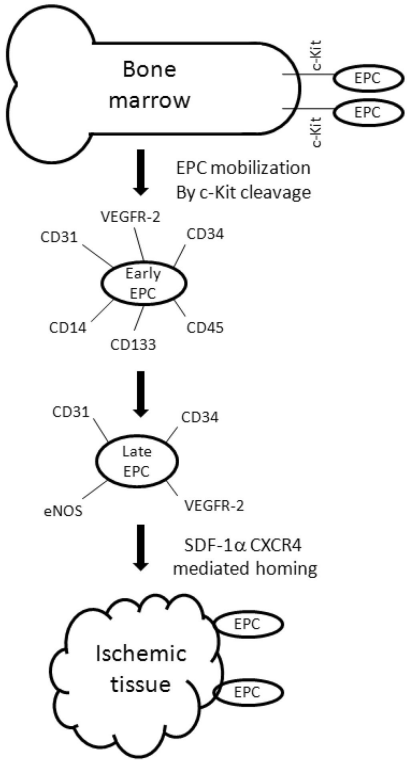

Figure 2.
Endothelial progenitor cell (EPC) transit from the bone marrow to the periphery

Table 1

Angiogenesis, Arteriogenesis, and Vasculogenesis

Process	Vessels	Timing	Cell type involved	Mechanism
Angiogenesis	New capillaries	Embryo + Adult	Endothelial cells	Hypoxia
Arteriogenesis	Enlargement of collaterals	Embryo + Adult	Endothelial cells	Shear stress Forces
Vasculogenesis	Primary development of arteries, veins and capillaries	Embryo	Angioblasts	Embryonic development

Table 2

Overview of potential cell sources for therapeutic angiogenesis

Cell Type	Characteristics	Human Markers	Source
Raw bone marrow	Heterogeneous mixture of cells		BM
Mononuclear cells	Include lymphocytes, monocytes, EPCs, MPCs, dendritic cells & NK cells Promote angiogenesis (CD34+ CD45+)	CD2, CD11b, CD14, CD19, CD34, CD45, CD73, CD90, CD105, CD113, CD115	BM, PB
Monocytes	Give rise to M1 and M2 macrophage population (M2 plays role in ischemia)	CD11b, CD115	BM, PB
Endothelial progenitor cells	Bone marrow derived and peripherally circulating Create endothelial cells (CD34+CD45−)	CD31, CD34	BM, PB
Mesenchymal progenitor cells	Capable of generating most somatic cells (myoblasts, smooth muscle cells & hematopoietic stromal support cells)	CD73, CD90, CD105	BM