ENGINEERING ROBUST AND FUNCTIONAL VASCULAR NETWORKS IN VIVO WITH HUMAN ADULT AND CORD BLOOD-DERIVED PROGENITOR CELLS

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ABSTRACT

The success of therapeutic vascularization and tissue engineering (TE) will rely on our ability to create vascular networks using human cells that can be obtained readily, expanded safely *ex vivo* and produce robust vasculogenic activity *in vivo*. We hypothesized that blood-derived endothelial progenitor cells (EPCs) have the required proliferative and vasculogenic activity to create vascular networks *in vivo*. To test this, EPCs were isolated and expanded from human umbilical cord blood or from adult peripheral blood. EPCs were then combined with human saphenous vein smooth muscle cells (HSVSMCs) as a source of perivascular cells, suspended in Matrigel and implanted subcutaneously into immunodeficient mice. Evaluation of implants at one week revealed an extensive network of human-specific lumenal structures containing erythrocytes, indicating formation of functional anastomoses with the host vasculature. This study demonstrated that human blood-derived EPCs were able to form the endothelial lining of vascular networks in vivo (Melero-Martin et al. 2007). To obtain a blood-derived progenitor cell that could perform the role of the mature smooth muscle cells, we isolated, expanded and characterized human mesenchymal progenitor cells (MPCs) from umbilical cord blood and from adult human bone marrow. We showed that MPCs differentiated towards a smooth muscle cell phenotype in vitro when co-cultured with EPCs, indicating the MPCs had the appropriate differentiation potential to substitute for mature smooth muscle cells. Indeed, we found that EPCs combined with MPCs formed robust, extensive vascular networks in vivo in the Matrigel model described above(Melero-Martin et al. 2008). The rapid formation of long-lasting microvascular networks by human post-natal progenitor cells, the EPCs and MPCs, constitutes an important step in the development of clinical strategies for tissue vascularization.

1. INTRODUCTION

Our goal is to build vascular networks from human endothelial progenitor cells (EPCs) and mesenchymal progenitor cells (MPCs) to re-build damaged tissues and organs. A vascular network is required within most tissues and organs to ensure adequate nutrients, gas exchange and elimination of waste products. Hence, it is widely accepted in the field of tissue-engineering (TE) that a vascular network must be incorporated into a TE construct, or its formation rapidly induced after in vivo implantation, in order for the nascent organ or tissue to survive (Nomi et al. 2002). For tissue regeneration, it is well-established that formation of new blood vessels is required. For example, liver regeneration that occurs after hepatectomy requires angiogenesis (Drixler et al. 2002) and the extent of angiogenesis in the regenerating liver may even dictate the eventual organ mass (Folkman 2007). Bone formation requires a vascular network, with recent studies showing hypoxia-inducible factor- α (HIF-α) as a critical link between osteogenesis and angiogenesis (Wang et al. 2007a). In TE experiments, co-implantation of endothelial cells with bone marrow stromal cells (MSCs) has been reported to stimulate bone formation by the MSCs (Kaigler et al. 2005), suggesting that the endothelium provides signals for tissue regeneration that go beyond the delivery of oxygen and nutrients. Further evidence that the vasculature provides inductive cues comes from studies on organ development during embryogenesis: two landmark papers showed

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that signals derived from endothelial cells are required for proper development of the pancreas and liver (Lammert et al. 2001; Matsumoto et al. 2001). Taken together, these studies provide specific examples of the endothelium driving tissue development. The fundamental role of the vasculature in TE and tissue regeneration forms the rationale for the studies we embarked upon.

We envision use of a soldier's own EPCs and MPCs for in situ regeneration of vascular networks in ischemic tissue and possibly for a variety of tissue-engineering (TE) applications. For tissue regeneration in situ, a combination of EPC and MPCs would be delivered to the site in vivo where the cells would undergo vasculogenesis, as we have demonstrated occurs in an in vivo Matrigel model(Melero-Martin et al. 2008). For TE, vascular networks created from EPCs and MPCs would be incorporated into TE constructs in vitro such that upon implantation in vivo, anastomoses with the host circulation would occur rapidly to establish blood flow. In summary, our overall hypothesis is that EPCs and MPCs applied either in situ to ischemic tissue or to a TE organ will establish an adequate blood supply and thereby promote resident cells to undergo appropriate tissue development and regeneration. Soldiers are well-suited for this proposed strategy because it would be feasible to isolate EPCs and MPCs from their peripheral blood or bone marrow because of overall good health and relatively young age. Below, we provide a brief introduction to use of patient-derived cells for TE, to EPCs and MPCs, and to previously published approaches for creating vascular networks for TE.

1.1 Successes in TE.

To date, cell-based TE therapies for humans have been limited to two types of tissue – cartilage and skin. Carticel, approved in 1997, has been used in 14,000 patients in the US with long-term success. Carticel represents an important example of the feasibility and success of human autologous cell-based therapy. In this procedure, a sample of the patient's articular cartilage is removed for isolation and expansion of chondrocytes, which are then placed back into damaged cartilage by a surgical procedure (De Bie 2007). The ex vivo culture time is approximately 5 weeks. Epicel, a TE skin construct, produced as a humanitarian product by Genzyme, has been used since 1987 to treat severe burns in approximately 1300 patients. Epicel involves expansion of autologous keratinocytes, taken from a patch of the patient's healthy skin. A number of similar constructs are used to treat non-healing ulcers and superficial burns (MacNeil 2007). Further improvement of these TE skin products will require improvements in angiogenesis (MacNeil 2007). This brief description of FDA-approved TE applications highlights the feasibility of using autologous cells and in vitro cell expansion to obtain sufficient cell numbers for therapy.

1.2 Endothelial Progenitor Cells (EPCs)

EPCs can be isolated from human peripheral blood by us (Melero-Martin et al. 2007; Wu et al. 2004) and by others (Ingram et al. 2004; Lin et al. 2000; Peichev et al. 2000; Yoder et al. 2006), and from the wall of mature human vessels(Ingram et al. 2005). Importantly, these human EPCs are defined by their endothelial functions; they have the ability to proliferate robustly in culture and form the lining of functional blood vessels in vivo. EPCs (also called ECFCs for endothelial colony forming cells) are distinct from monocytes and other cells of the hematopoietic lineage(Yoder et al. 2006). There has been considerable controversy and debate about the identity, differentiation, and functional roles of EPCs for nearly a decade. To briefly summarize the current view, three types of endothelial cells from blood have been described. The first is the circulating endothelial cell (CEC); this is a damaged endothelial cell released from the vessel wall due to injury or disease. CECs have low proliferative capacity in vitro (Lin et al. 2000) and are thought to have little therapeutic potential. The second is the "early EPC" or "angiogenic EPC". This is a hematopoietic cell of the myeloid lineage that can increase angiogenesis in vivo by secreting angiogenic factors. It does not form the endothelial lining of vessels (Yoder et al. 2006) and therefore does not fit the nomenclature "EPC". Third is the EPC or ECFC, which is present at 2-3 cells/ml in adult blood and ~15 fold higher in cord blood. It has enormous expansion potential in vitro and the ability to form the endothelial lining of functional blood vessels in vivo (Au et al. 2008b; Melero-Martin et al. 2007; Melero-Martin et al. 2008; Yoder et al. 2006).

1.3 Smooth muscle progenitors/bone marrow mesenchymal progenitor cells (MPCs)

Human bone marrow stromal cells were shown to share phenotypic properties with smooth muscle cells in studies dating back to 1993 (Galmiche et al. 1993). Subsequently, smooth muscle progenitor cells from adult peripheral blood were cultured and shown to express α-smooth muscle actin (α-SMA), calponin, and smooth muscle myosin heavy chain in a pattern consistent with human vascular smooth muscle cells(Simper et al. 2002). Cells with a similar phenotype were isolated from murine bone marrow stromal cells (Kashiwakura et al. 2003), indicating that bone marrow and blood are sources of smooth muscle progenitor cells. In support of this, many studies have shown bone marrow cells to be the source of intimal smooth muscle cells in a variety of vascular diseases (Metharom; Caplice 2007; Shimizu et al. 2001). The cellular origin of bone marrow and blood-derived smooth muscle progenitors has not been precisely determined but studies have shown both phenotypic and functional differentiation of smooth muscle cells from bone marrow-derived multipotent adult progenitor cells in response to TGF-β1 and PDGF-BB(Ross et al. 2006). We isolated, expanded and characterized MPCs from umbilical cord blood and from adult human bone marrow, and showed ability of both types of MPCs to differentiate towards a smooth muscle phenotype in vitro and to function as smooth muscle/perivascular cells in vivo(Melero-Martin et al. 2008). The MPCs we describe are similar in growth and differentiation properties to bone marrow-derived mesenchymal stem cells (MSCs); however, we refer to these cells as mesenchymal progenitor cells (MPCs) because we feel this is most consistent with their limited multi-lineage differentiation potential and rate of growth in vitro.

1.4 Vascular networks for TE constructs

A variety of approaches have been proposed to vascularize TE constructs. These strategies include embedding an angiogenic factor, e.g. basic FGF(Lee et al. 2002), or receptors needed for vascular development, e.g., ephrin-A1 (Moon et al. 2007) into the scaffold to promote in-growth and assembly of host microvessels. Another approach is self-assembling nanostructures coated with heparin which in turn can bind bFGF or VEGF, which were shown to stimulate new blood vessel formation in vivo, indicating these nanostructures could be used to promote neovascularization for TE (Rajangam et al. 2006). Technologies to create microfluidic networks seeded with endothelial cells suggest the possibility of creating pre-fabricated vascular channels (Chrobak et al. 2006; Golden; Tien 2007). Recently, endothelial cells cultured on patterned substrates generated by optical lithography were shown to form tubular networks when transferred to tissue and implanted into mice (Kobayashi et al. 2007) Several microfabrication technologies are being used to create endothelial-lined channels directly within a biocompatible scaffold, as reviewed by Langer, Vacanti and colleagues (Khademhosseini et al. 2006).

The feasibility of using human endothelial cells to pre-construct a microvascular network in vitro has been shown by embedding endothelial cells in collagen/fibronectin gels. Tubular structures formed in vitro, and then upon implantation into immunodeficient mice, a microvascular network formed after 31 days in vivo (Schechner et al. 2000; Shepherd et al. 2006). Similar results have been reported with human ECs seeded on biopolymer matrices, where functional microvessels were evident 7-10 days after implantation (Nor et al. 2001). These studies established proof-of-principle: preconstructed networks can integrate with the host vasculature. However, the clinical use of mature ECs derived from healthy autologous vascular tissue presents some important limitations: the isolation relies on an invasive procedure and sacrifice of healthy tissue, mature ECs show relatively low proliferative potential and it is difficult to obtain a sufficient number of cells from a small biopsy of autologous tissue. These limitations have prompted the search for other sources of ECs such as those derived from embryonic and adult progenitor cells (Rafii; Lyden 2003). For example, human ES-derived endothelial cells combined with a murine mesenchymal cell line were shown to result in durable vascular beds in vivo (Wang et al. 2007b). However, ethical considerations along with a nascent understanding of the mechanisms controlling the differentiation of embryonic stem cells are hurdles that need to be overcome before these cells can be used in a clinical setting. We suggest that the highly proliferative EPCs and MPCs that can be obtained from peripheral blood or bone-marrow are well-suited for creating vascular networks because the cells can

be obtained safely, repeatedly, and without sacrificing healthy tissue

2. RESULTS

2.1 In vivo vasculogenesis using EPCs and mature SMCs as a perivascular support cell

We hypothesized that blood-derived endothelial progenitor cells (EPCs) have the required proliferative and vasculogenic activity to create vascular networks *in vivo*. To test this, EPCs isolated from human umbilical cord blood or from adult peripheral blood as described(Melero-Martin et al. 2007). The EPCs were combined with human saphenous vein smooth muscle cells (HSVSMCs) as a source of perivascular cells, suspended in Matrigel and implanted subcutaneously into immunodeficient mice. The details of the in vivo assay are as follows: Adult peripheral blood or cord blood EPCs were combined with HSVSMCs at a ratio 4:1 (EPCs:HSVSMCs) and a total of 1.9×10^6 cells in 200 ul of ice-cold Matrigel. The cell/Matrigel suspension was injected subcutaneously into immunodeficient male mice 6 weeks of age. Matrigel forms a gel at 37°C, effectively localizing the cells in vivo. At specified time points, the Matrigel implants were harvested from the mice, processed for paraffin-sectioning, and stained with hematoxilin and eosin (H&E). Functional blood vessels were counted as lumenal structures containing red blood cells (RBCs). Matrigel containing either cbEPCs or HSVSMCs alone failed to form any detectable microvessels after one week. Importantly, this two-cell system contained no added growth factors or supplements. Prior to injection, the cell monolayers were washed, harvested by trypsinization, resuspended in Matrigel and kept ice-cold until injection. Matrigel injected alone was inert. A detailed description of the methods used to isolate the EPCs and the in vivo model will be published as a chapter in Methods in Enzymology (Melero-Martin; Bischoff 2009).

To verify that vessel lumens were composed of human endothelial cells, sections were stained with a human-specific CD31 antibody(Melero-Martin et al. 2007). The location of the smooth muscle cells was detected using anti-α-SMA. Evaluation of implants at one week revealed an extensive network of human-specific lumenal structures containing erythrocytes, indicating formation of functional anastomoses with the host vasculature. Quantitative analyses showed the microvessel density was significantly superior to that generated by human dermal microvascular endothelial cells (HDMECs). The average vessel density achieved with EPCs:HSVSMCs after 7 days in vivo was 48 ± 8 microvessels/mm^2. We found that as EPCs were expanded in culture, their morphology, growth kinetics and proliferative responses toward angiogenic factors progressively resembled those of HDMECs, indicating a process of *in vitro* maturation. This maturation correlated with a decrease in the degree of vascularization *in vivo*, which could be compensated by increasing the number of EPCs seeded into the implants.

2.2 In vivo vasculogenesis using EPCs and MPCs from blood or bone marrow.

The experiments in our 2007 *Blood* paper (Melero-Martin et al. 2007) used mature SMCs for the in vivo experiments. However, our goal is to build vascular networks from cells that can be obtained without sacrifice of healthy tissue. Therefore, we set out to isolate a cell that could differentiate into smooth muscle phenotype from adult human bone marrow (purchased from Cambrex, Inc.) and cord blood. Cord blood was obtained as non-identified, normally discarded tissue from the Brigham and Women's Hospital, Boston under an IRB-approved protocol. In vitro expansion of such cells was achieved using the same methods we use to isolate EPCs but the growth media was modified by removing VEGF, bFGF and heparin to reduce EPC growth. Colonies with mesenchymal morphology were isolated from the primary cultures and expanded. Following this procedure, both cord blood and bone marrow sources yielded cells that resemble mesenchymal stromal cells, which we refer to as cbMPCs and bmMPCs, respectively. The phenotypic characterization of both sources of MPCs, in comparison to cbEPCs, and the robust cell expansion potential of the MPCs is reported in our 2008 Circulation Research paper(Melero-Martin et al. 2008).

The ability of MPCs to fully differentiate towards a smooth muscle phenotype was investigated by 1) treating cells with TGF-β1 and 2) by co-culture with EPCs. TGF-β1 and contact with endothelial cells has been shown to promote smooth muscle differentiation (Hirschi et al. 1998; Sinha et al. 2004). TGFβ-treatment increased expression of PDGF-Rβ in MPCs, as

shown by Western blot (data not shown). Importantly, co-culture of bmMPCs or cbMPCs with EPCs was very effective in inducing expression of smooth muscle myosin heavy chain (smMHC), a definitive marker of the smooth muscle phenotype(Melero-Martin et al. 2008). MPCs cultured in the absence of EPCs do not express smMHC.

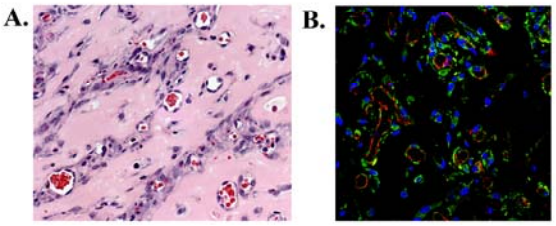
2.3 EPCs combined with MPCs are vasculogenic in vivo.

When either bmMPCs or cbMPCs were combined with cbEPCs in Matrigel and injected into immunodeficient mice.Matrigel, extensive and uniform vascularization was seen at 7 days (Figure 1). Erythrocyte (e.g. red blood cell)-filled lumens seen in H&E sections indicating that the nascent vessels had formed connections with the host vasculature. The lumens of the vessels stained positive for human CD31 and the perivascular cells stained positive for the smooth muscle marker α-smooth muscle actin (α-SMA) indicating the proper assembly of the two cell types. Additional studies using green fluorescent protein (GFP)-labeling of the EPCs and MPCs allowed us to track the cellular fate of each population and verify the cellular assembly and orientation: EPCs lining the vessel lumen and MPCs surrounding the lumen in a perivascular location. These results, fully reported in our 2008 publication (Melero-Martin et al. 2008), demonstrate the feasibility, from a cellular standpoint, of creating vascular networks from *clinically relevant* blood or bone marrow-derived progenitor cells.

2.4 Adult EPCs combined with adult MPCs are also vasculogenic in vivo.

We previously showed that adult blood EPCs (abEPCs) were vasculogenic in vivo when combined with mature human SMCs, but required higher seeding densities (Melero-Martin et al. 2007). An apparently lower vasculogenic capacity of abEPCs has been reported recently by others(Au et al. 2008b). We hypothesized that abEPCs combined with bmMPCs at an optimized ratio would yield high density vascular networks. Therefore, we tested abEPCs + bmMPCs in parallel with cbEPCs + bmMPCs in our in vivo model. The results show that extensive vascularization occurred when abEPCs and bmMPCs (40:60 ratio) were implanted. Quantification of vessels/mm^2 showed the vessel density achieved with abEPCs and cbEPCs was

Figure 1. Human EPCs and MPCs in vivo.



Legend: Cord blood EPCs and bone marrow-derived MPCs (ratio 40:60) were suspended in 200 µl of Matrigel and implanted on the back of six-week-old nu/nu mice. Implants were harvested after 7 days and stained with H&E (panel A). Numerous blood vessels containing erythrocytes were seen. The vessels stained positive for human CD31 (red, panel B) and α-SMA (green, panel B). Nuclei were stained blue with DAPI.

similar (P=0.158) indicating that adult blood EPCs represent a valuable source of EPCs for tissue vascularization(Melero-Martin et al. 2008). In summary, we demonstrated the feasibility of engineering vascular networks in vivo with clinically relevant human post-natal progenitor cells that can be obtained by non-invasive procedures. We propose that our in vivo model of human vasculogenesis, and variations thereof, are well-suited for the development of strategies for neovascularization of engineered tissues and regenerative therapies.

CONCLUSIONS

We show that human postnatal EPCs and MPCs isolated from either blood or bone marrow have an inherent vasculogenic ability that can be exploited to create functional microvascular networks *in vivo*. Using Matrigel as a supporting 3-dimensional extracellular matrix, we show that co-implantation of EPCs with either bmMPCs or cbMPCs into immunodeficient mice resulted in formation of extensive vascular networks after one week. The presence of human EPC-lined lumens containing erythrocytes (>100 vessels/mm^2) throughout the implants indicated not only a process of vasculogenesis from the two cell types, but also the formation of functional anastomoses with the host circulatory system. The extent of the engineered vascular networks was highly influenced by the ratio of EPCs to MPCs, with a progressive increase in vessel density and consistency of vascularized implants achieved when the ratio of EPC:MPCs was 40:60. Adult blood EPCs showed a similar degree of vessel development when combined with human MPCs in the same ratio. The use of

blood-derived endothelial progenitor cells and mesenchymal progenitor cells to create arteries and blood vessel networks to repair injured tissues and organs could be used to treat patients of all ages and all walks of life – whether their injury is due to trauma or to a chronic condition. Hence, this research project has the potential to benefit not only soldiers but their families and society as a whole.

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