In Vivo Perfusion of Human Skin Substitutes With Microvessels Formed by Adult Circulating Endothelial Progenitor Cells

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BACKGROUND At present, tissue-engineered human skin substitutes (HSSs) mainly function as temporary bioactive dressings due to inadequate perfusion. Failure to form functional vascular networks within the initial posttransplantation period compromises cell survival of the graft and its long-term viability in the wound bed.

OBJECTIVES Our goal was to demonstrate that adult circulating endothelial progenitor cells (EPCs) seeded onto HSS can form functional microvessels capable of graft neovascularization and perfusion.

MATERIALS AND METHODS Adult peripheral blood mononuclear cells (PBMCs) underwent CD34 selection and endothelial cell (EC) culture conditions. After in vitro expansion, flow cytometry verified EC phenotype before their incorporation into HSS. After 2 weeks in vivo, immunohistochemical analysis, immunofluorescent microscopy, and microfil polymer perfusion were performed.

RESULTS CD34⁺ PBMCs differentiated into EPC demonstrating characteristic EC morphology and expression of CD31, Tie-2, and E-selectin after TNFα induction. Numerous human CD31 and Ulex europaeus agglutinin-1 (UEA-1) microvessels within the engineered grafts (HSS/EPCs) inosculated with recipient murine circulation. Limitation of murine CD31 immunoreactivity to HSS margins showed angiogenesis was attributable to human EPC at 2 weeks posttransplantation. Delivery of intravenous rhodamine-conjugated UEA-1 and microfil polymer to HSS/EPCs demonstrated enhanced perfusion by functional microvessels compared to HSS control without EPCs.

CONCLUSION We successfully engineered functional microvessels in HSS by incorporating adult circulating EPCs. This autologous EC source can form vascular conduits enabling perfusion and survival of human bioengineered tissues.

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stasis ulcers. They act as temporary biologic dressings requiring multiple applications because they lack vascular perfusion necessary for engrafted cell survival.\(^3\),\(^4\) Blood vessel development within avascular HSSs depends on host angiogenesis from vessels in the wound bed and vasculogenesis from circulating endothelial progenitor cells (EPCs).\(^5\)–\(^7\) Under optimal conditions, neovascularization of engrafted tissues require at least 14 days and is likely to be delayed in patients with vascular insufficiency.\(^1\),\(^8\) Therefore, the same conditions that predispose patients to developing nonhealing skin ulcers also reduce their capacity for forming new vessels in engrafted tissues.

Novel strategies for vascularizing HSS by incorporating endothelial cells (ECs) have demonstrated encouraging preclinical results.\(^8\)–\(^10\) Formation of human EC-lined vessels and their inosculation with recipient circulation were demonstrated by seeding human umbilical vein ECs (HUVECs) onto HSS before transplantation.\(^7\),\(^8\),\(^11\) The concentration of mature vessels and the rate of successful engraftment were increased with retroviral transduction of HUVECs to constitutively express Bcl-2, an antiapoptotic protein.\(^12\),\(^13\)

Although the introduction of human ECs improves HSS vascularization, they can induce immunologic rejection by an immunocompetent host. Unlike keratinocytes or fibroblasts, human ECs may initiate an immune response by resting allogeneic T cells.\(^14\) Bcl-2–transduced HUVECs may become more resistant to some but not all forms of immune-mediated injury.\(^15\),\(^16\) Because of their persistence in vivo, however, Bcl-2–transduced HUVECs may theoretically be more potent stimulators of alloimmunity than nontransduced cells. An alternative to allogeneic ECs in constructing HSSs is harvesting autologous ECs from the graft recipient. It is difficult to efficiently generate adequate numbers of ECs from small skin specimens for both experimental as well as therapeutic purposes. To circumvent this problem, autologous ECs from bone marrow–derived circulating EPCs may be propagated in vitro for participation in postnatal vasculogenesis.\(^17\)–\(^20\) Peripheral blood mononuclear cells (PBMCs) coexpress endothelial lineage markers and form cordlike structures in vitro under angiogenic conditions.\(^21\) Hence, hematopoietic and ECs may share a common precursor cell origin referred to as the bone marrow–derived hemangioblast.\(^22\),\(^23\) A critical limitation for the therapeutic application of postnatal EPC is their low number in systemic circulation. Typically, only \(5.0 \times 10^6\) EPCs may be cultured from \(100\) mL of blood from healthy volunteers.\(^24\) To overcome this problem, investigators have explored the use of umbilical cord blood or bone marrow mobilization of EPCs by cytokines (GM-CSF)\(^25\),\(^26\) or growth factors (VEGF).\(^27\),\(^28\) Alternatively, in vitro propagation of cells expressing a common antigen with EPCs such as CD34 may produce an adequate number of cells. This strategy is appealing because protocols for isolating CD34 + PBMCs are well established in the restoration of hematopoiesis after bone marrow ablation.\(^29\) These bone marrow–derived circulating progenitor cells have been shown to actively participate in physiologic and pathologic postnatal vasculogenesis.\(^30\)–\(^35\) They have been used for therapeutic vasculogenesis in vivo by restoring blood flow to ischemic hindlimb\(^21\) and improving ventricular systolic dimension after myocardial infarctions in rats.\(^36\) In this study, we demonstrated the ability of adult circulating EPCs to form functional mature microvessels in HSS that can inosculate with recipient circulation.

Materials and Methods

**Human Keratinocyte Isolation and Culture**

All human cell populations were obtained using protocols approved by the Yale Human Investigation Committee. Keratinocytes were isolated from discarded neonatal human foreskin by dispase 0.025 g/mL in phosphate-bufered saline (PBS) (Roche Diagnostics, Indianapolis, IN) digestion, mechanical separation of the epidermis from the dermis, and further digestion of the isolated epidermis with 0.05% trypsin-EDTA (Life Technologies, Inc., Grand Island, NY) for 3 to 5 minutes at room...
temperature. The keratinocytes were then propagated in culture for one or two passages in KGM-2 medium (Cambrex, Walkersville, MD) before seeding onto engineered grafts.

**Human Endothelial Cell Isolation and Culture**

HUVECs were separated from umbilical veins by collagenase digestion and serially expanded in vitro on 0.1% gelatin-coated flasks in M199 medium containing 20% fetal bovine serum (FBS), 1-glutamine, penicillin/streptomycin (Life Technologies, Inc.), and EC growth supplement (Calbiochem, La Jolla, CA) with heparin. PBMCs were collected from three healthy adults by leukapheresis and enriched by density gradient centrifugation using lymphocyte separation medium (MP Biomedicals, Aurora, OH). CD34+ PBMCs were further enriched by selection with antibody (Ab)-coated magnetic beads (MAC-Separation kit, Miltenyi-Biotec, Auburn, CA). The CD34+ PBMCs were plated onto tissue culture plastic coated with gelatin-human plasma-fibronectin in EGM-2 (Lonza Biologica Inc., Portsmouth, NH) supplemented with additional 10 ng/mL vascular endothelial growth factor (VEGF, National Institutes of Health, Bethesda, MD). Colonies of proliferating spindle-shaped cells were typically observed by 21 to 28 days, at which time the medium was changed to EGM-2 with 15% FBS (Life Technologies, Inc.) but without additional cytokines. Cultures were serially propagated on gelatin-coated flasks for three passages before seeding onto engineered grafts.

**Endothelial Progenitor Cell Morphologic Evaluation and Phenotypic Characterization**

The CD34+ PBMCs isolated and cultured from three separate leukapheresis products were examined with an Olympus inverted phase-contrast microscope after 4 weeks in vitro and analyzed for specific EC markers by flow cytometry. These EPCs and HUVECs, used as positive control, were exposed to TNF-α (10 ng/mL, R&D Systems, Minneapolis, MN) for 3 hours before immunofluorescent labeling. The confluent monolayer was washed in HBSS (Life Technologies, Inc.) and then incubated with trypsin-EDTA for 30 to 60 seconds. Trypsin-EDTA activity was quenched with M199 containing 20% FBS. After centrifugation, the collected cells were incubated with 2 µg/mL primary Ab or isotype control (K16/16), which was conjugated to fluorescein isothiocyanate for 45 minutes. Immunoreactive cells were analyzed with flow cytometry using computer software (CellQuest, BD Biosciences, San Jose, CA). Specific Abs used in these analyses included human CD31, Tie-2, and E-selectin (CD31 and E-selectin, Beckman Coulter Immunotech, Miami, FL; Tie-2, R&D Systems).

**Preparation of Human Skin Equivalent**

Cadaveric donor skin from the Yale Skin Bank was rinsed in PBS (Life Technologies, Inc.) with antibiotics, subjected to three rapid freeze-thaw cycles in liquid nitrogen, and incubated in PBS with antibiotics at 37°C for 2 weeks. After this preparation, the epidermis was gently removed and the dermis was stored at −20°C. At this point, no residual intact cells were identifiable by histologic examination.

The thawed dermis was cut into 1-cm² pieces and placed in 3 mL of KGM-2. The KGM-2 was removed after the dermis was rehydrated for at least 1 hour at 37°C. Keratinocytes (3 × 10⁵), suspended in a 30-µL droplet of KGM-2, were pipetted onto the center of the epidermal side of the devitalized tissue. The keratinocytes were allowed to settle for 3 hours before submerging in KGM-2. Three days after seeding, a differentiation medium was used consisting of KGM-2, Dulbecco’s modified Eagle medium, Ham’s F12 with defined calcium FBS, cholera toxin (1 × 10⁻¹⁰ M; Calbiochem), hydrocortisone (0.4 µg/mL; BD Biosciences), amphotericin B (Life Technologies, Inc.), and gentamycin. The differentiation medium with a final calcium concentration of 1.2 mM was changed every other day for 6 to 10 days until a multilayered epidermis had developed. At this time, the EPCs (8 × 10⁵ per graft) were applied to the reticular side of the dermis analogous to the seeding of the keratinocytes.
The EPCs cultured from each leukapheresis product seeded six 1-cm² HSSs, totaling 18 grafts. They were suspended in a 30-μL droplet of M199 with 20% FBS, EC growth supplement with hydrocortisone (0.4 μg/mL), cholera toxin (1 × 10⁻¹⁰ M), epidermal growth factor (10 ng/mL; BD Biosciences), amphotericin B, and gentamycin. After seeding of EPCs, the grafts were submerged for 3 hours in EGM-2MV before transplantation.

**Transplantation**

All animal procedures were performed using protocols approved by the Yale IACUC (Institutional Annual Care and Use Committee). Graft sites on the backs of 6- to 10-week-old female C.B-17 SCID/beige mice (Taconic, Tarrytown, NY) were prepared by removing all visible hair with a depilatory agent (Nair, Carter Wallace, New York, NY). The mice were anesthetized with chloroform before surgical procedure. A 1-cm² piece of murine skin was excised to the underlying fascia and an engineered HSS was sutured to cover the defect. The grafts were covered with bacitracin and waterproof dressing consisting of two layers of 1.5-cm² nonadherent wound dressing (Telfa, Kendall, Mansfield, MA), transparent dressing (Tegaderm, 3M, St. Paul, MN), foam bandage (Stop and Shop, Boston, MA), and securing tape (Duropore, 3M). During surgery and every other day afterward, 1.0 mL of PBS with L-glutamine, antibiotics, and amphotericin B was injected into the Telfa.

**Immunohistochemical Analysis**

The mice were euthanized by carbon dioxide asphyxiation 2 weeks after grafting. Half of each HSS was formalin fixed while the other portion was snap-frozen in OCT (BD Biosciences). Paraffin-embedded tissue sections (6 μm) or frozen sections (5 μm) were used for immunohistochemical analysis. Primary Ab reactive with mouse CD31, human CD31 (both 1:100, BD PharMingen), human collagen type IV (1:400, Biocompare, San Francisco, CA), smooth muscle cell–specific-actin (SMA, 1:100, Novocastra, Newcastle Upon Tyne, UK) were used. Biotinylated Ulex europaeus agglutinin-1 (UEA-1, 1:4,000, Biocompare) was also used to identify human ECs within the HSS.

**Fluorescence Microscopy**

After 2 and 4 weeks of transplantation, 150 μL of a rhodamine-conjugated UEA-I Ab (Vector Laboratories) was administered by tail vein injection 60 minutes before harvesting HSS/EPC grafts from the recipient mouse. Sections of the grafts, 25-μm thick, were examined on a fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan). The confocal images of the sections were obtained using a confocal microscope (CI, Nikon).

**Microfil Perfusion**

Two weeks after transplantation, recipient mice were anesthetized and perfused with 20 mL of 37°C PBS plus 10 U/mL heparin at a flow rate of 10 to 15 mL/min through the left ventricle followed by 20 mL of 4% paraformaldehyde and 15 mL of microfil (MV-112 [white], Flowtech, Carver, MA). The microfil polymerized overnight at 4°C. The tissue was optically cleared by its immersion in graded concentrations of glycerol solutions (40%–100% in water with 20% increases per 24 hours). The clarified specimens were viewed on a dissecting microscope (SMZ1000, Nikon).

**Results**

**Characterization of EPCs**

CD34 + PBMCs remained static and appeared circular or dendritic (Figure 1A) for 3 to 4 weeks before proliferating, adopting spindled-shape morphology similar to ECs and forming stellate-shaped colonies (Figure 1B). After 6 to 8 weeks in vitro, flow cytometry (Figure 2) demonstrated that, like HU-VECs, these cells expressed CD31 (also known as
platelet EC adhesion molecule-1 [PECAM]), Tie-2 (also known as vascular endothelial receptor 2 [VEGFR-2]), and E-selectin, (EC surface antigen inducible by TNF-α). These cells with EC morphology and characteristic antigen expression (EPCs) were observed in all three CD34 \(^1\) PBMC cultures.

**Immunohistochemical Evaluation of Microvessels in HSS/EPCs**

After 2 weeks in vivo, the HSS/EPCs contained microvessels extending into the mid dermis (Figure 3B) whereas HSS control without EPCs did not (Figure 3A). These microvessels in HSS/EPCs were formed with cells that reacted to human CD31 (Figure 4A), UEA-1 (Figure 5A), and human type IV collagen (Figure 5C) antibodies. HSS control did not show human CD31 Ab reactivity (Figure 4C). Cells that stained positively for murine CD31 were limited to the HSS/EPC edges abutting the wound bed (Figure 4B, inset). The microvessels in HSS/EPCs were composed of smooth muscle cells demonstrated by reactivity to murine SMA Ab (Figure 5B). Ten of 18 (56%) HSS/EPCs had intact stratified epithelium, extension of vessels two-thirds up from the base of the dermis, and more than three EC-lined vessels per high-power field (original magnification, \(\times 400\)).

**Functionality of Microvessels in HSS/EPCs**

The microvessels in HSS/EPCs inosculated with recipient circulation and demonstrated functionality by 2 weeks in vivo. Rhodamine-conjugated UEA-I

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**Figure 1.** Light microscopy: (A) CD34 \(^+\) PBMCs are morphologically round or dendritic in the first 3 to 4 weeks of culture in EC-selective medium (phase contrast micrograph taken at 3 weeks). (B) Afterward, they proliferate rapidly forming stellate-shaped colonies composed of spindle-shaped cells (phase contrast micrograph taken at 6 weeks.)

**Figure 2.** Fluorescent-activated cell sorting (FACS): Immunofluorescent labeling demonstrated that cells cultured from CD34-selected PBMCs (EPCs, blue) expressed EC surface markers such as CD31, Tie-2, and TNF-α—induced E-selectin similar to HUVEC-positive control (blue). K16/16, which is not an EC surface marker, was used as isotype-matched negative control (pink).
perfusion of HSS/EPCs, which highlighted human EC-lined vascular structures, occurred 45 to 60 minutes after intravenous injection (Figure 6A). The engineered microvessels made of UEA-I–immunoreactive cells persisted to at least 4 weeks in vivo (Figure 6B). Furthermore, intraventricular infusion of microfil polymer followed by glycerol clarification casted microvessels in HSS/EPCs (Figure 7B) but not in HSS control (Figure 7A).

**Discussion**

Although the thickness of the HSS may delay effective neovascularization causing nutritional dependence on imbibition for at least 2 weeks, which diminishes the success of engraftment, previous studies demonstrated that acellular dermis seeded with autologous keratinocytes was repopulated by fibroblasts and blood vessels after 2 weeks. Rapid and effective neovascularization in HSS is critical for promoting graft survival for chronic wounds. A bilayered HSS can promote better wound healing than a single-layered one because the epidermis protects the construct from external shearing forces and the dermis prevents significant wound contraction. We hypothesized that adult circulating EPCs, a source of autologous ECs, would form functional microvessel networks in HSS, which could inosculate with the circulation of immunosuppressed (C.B-17 SCID/beige mice) recipients.

This was confirmed by in vivo rhodamine-conjugated UEA-I immunolabeling of human EC-lined vascular structures after intravenous injection as well as microfil polymer casting of these microvessels in HSS/EPCs after intraventricular infusion. Moreover, formation of functional microvessels with circulating adult EPCs persisting to 4 weeks in vivo was not due to recipient (murine) neovascularization except for limited areas abutting the wound bed at 2 weeks.

At present, strategies to enhance vascularization of engineered tissues involve incorporation of various sources of ECs in a dermal scaffold of collagen/fibronectin or a bilayered composite graft. The EC sources include mature human dermal microvascular ECs, allogeneic HUVECs, or autologous progenitors of ECs derived from the bone marrow or peripheral blood. Bcl-2–transfected HUVECs were necessary for organization into functional microvessels that inosculated with murine vasculature in less than 7 days or 14 days. Our data suggest that organized functional microvessels engineered with circulating EPC from healthy human subjects do not require constitutive expression of Bcl-2. Hence, our data are in accord with previously published report of circulating EPCs from umbilical cord and adult peripheral blood developing more human EC-lined blood vessels than non–Bcl-2-transduced HUVECs in skin substitutes. We also demonstrated engineered blood vessels composed
of circulating EPCs recruited recipient smooth muscle cell investiture, a sign of vascular maturity.

Our study was limited by the lack of long term data after transplantation with vascularized HSSs compared to avascular controls. Murine-derived vessels had been shown in HSS by 4 weeks after transplantation as human-derived vessels decrease. Future translational studies should analyze the remodeling of the human EC-lined vessels via in vivo noninvasive confocal imaging technique beyond 2 week transplantation. This timed-course demonstration of human EC-lined vessel formation, inosculation with recipient circulation, and disappearance may help access graft survival advantage of vascularized HSS compared to avascular controls. Another preclinical study should evaluate the utility of vascularized HSS in a diabetic or hyperlipidemic mouse model to simulate common wound healing compromised states. Thus far, it has been reported that rapamycin does not affect the ability of circulating EPCs to vascularize HSS despite drug-induced impairment of angiogenesis and vasculogenesis. In addition, immunogenicity of HSS components (neonatal

**Figure 4.** Immunohistochemical evaluation of HSS’s EC lineage: (A) Two weeks after grafting onto the wound bed of C.B-17 SCID/beige mice, HSS/EPCs contained human EC-lined vessels demonstrated by human CD31 antibody (Ab) reactivity (red). (B) Minimal murine angiogenesis was detectable by murine CD31 Ab reactivity at the lateral and deep margins (inset, red) but not the center of the HSS/EPCs. (C) HSS control did not reveal reactivity to human CD31 Ab (original magnification, ×200).
keratinocytes, devitalized dermis, and autologous ECs) should be studied in an immunocompetent host or an immune reconstituted animal model before clinical trials because our study subjects were immunocompromised mice.

Our study showed that circulating EPCs can be harvested from adult blood, expanded in vitro reliably, and reproducibly formed functional mature human vessels in vivo. Further studies are necessary to demonstrate that inosculation of bioengineered vascular conduits with recipient blood vessels improves survival of tissue-engineered grafts beyond the initial posttransplantation period. Such preclinical studies should utilize CD34+ PBMCs from diabetic and hyperlipidemic patients to access their ability to participate in neovascularization of HSS compared to healthy controls. Besides our HSS, CD34+ PBMCs should be seeded onto commercially available HSSs for transplantation onto donor wound bed to evaluate graft viability. We chose to use acellular cadaveric skin based on an assumption that preservation of vascular channels may facilitate northerly migration of seeded EPCs. Our HSS constructs should be compared to commercially available ones in terms of cost, time needed for graft engineering, and clinical outcomes. The results of these studies may lead to practical applications of transplanting vascularized engineered tissues.

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References


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