Promotion of Osteogenesis in Tissue-Engineered Bone by Pre-Seeding Endothelial Progenitor Cells-Derived Endothelial Cells

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ABSTRACT: In addition to a biocompatible scaffold and an osteogenic cell population, tissue-engineered bone requires an appropriate vascular bed to overcome the obstacle of nutrient and oxygen transport in the 3D structure. We hypothesized that the addition of endothelial cells (ECs) may improve osteogenesis and prevent necrosis of engineered bone via effective neovascularization. Endothelial progenitor cells (EPCs) can improve osteogenesis and prevent necrosis of engineered bone via effective neovascularization. We hypothesized that the addition of endothelial progenitor cells (EPCs) may improve osteogenesis and prevent necrosis of engineered bone via effective neovascularization. We hypothesized that the addition of endothelial progenitor cells (EPCs) may improve osteogenesis and prevent necrosis of engineered bone via effective neovascularization. We hypothesized that the addition of endothelial progenitor cells (EPCs) may improve osteogenesis and prevent necrosis of engineered bone via effective neovascularization.

Bone development is a highly complex process, involving interplay among various tissues, cells, proteins, and minerals. Among these determinants, vascularization plays a critical role in promoting endochondral and intramembranous ossification during bone growth and fracture healing.1,2 Similarly, tissue-engineered bone requires not only cell populations capable of creating new bone and a biocompatible scaffold, but also formation of an appropriate vascular bed to support the metabolic needs of bone. In practice, slow ingrowth of blood vessels leads to cell loss due to hypoxia in the early postimplantation stage.3 Thus, mass transport currently limits the size of engineered tissues to a millimeter scale that is clinically insufficient to repair large tissue defects. To overcome the obstacle of transporting nutrients and oxygen into a 3D engineered bone, a vascular network must be established throughout the newly formed construct. Incorporation of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to enhance vascularization appears the most common approach.4–7 Due to the short half-life of these proteins and their rapid diffusion out of target tissues, frequent and high dose administrations are required to achieve a significant response. However, high levels of growth factors can lead to uncontrolled side effects locally and at distant sites, including hyperpermeable vessels, stimulation of tumor growth, abnormal vascular function, and hypervascularity.8 Another promising approach to enhance neovascularization is to incorporate endothelial cells (ECs) into the engineered tissue.9,10 Physiologically, a variety of cell populations with a series of growth factors and mediators are involved in bone repair, including local vascularization and bone formation, thus the introduction of vasculogenic cells besides osteogenic cells may help the survival and function of engineered bones.

Bone marrow-derived endothelial progenitor cells (EPCs) from both children and adults can differentiate into endothelial lineage cells in vitro. Cell therapies with culture-expanded EPCs can promote neovascularization of ischemic tissues, even absent angiogenic growth factors.11,12 The use of EPCs removes the need to sacrifice a blood vessel or normal tissue to obtain ECs, because EPCs originating from marrow have a doubling ability much greater than mature ECs.13 So EPCs were suggested as an innovative source of ECs for tissue-engineered vascularization. Bone marrow stromal cells (MSCs), multipotent stem cells originating from the marrow stroma, are a particularly promising cell source in bone tissue engineering. MSCs were used as sources of osteogenic precursors in this project. Using a polymer-ceramic composite scaffold made of polycaprolactone (PCL), a degradable polyester and hydroxyapatite (HA), the inorganic part of naturally occurring bone, we explored whether addition of EPCs-derived ECs could improve osteogenesis and prevent necrosis of tissue-engineered bone in vivo through efficient neovascularization.

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bovine serum (FBS) (Invitrogen, Grand Island, NY), 10 mM β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO), 10 mM L-ascorbic acid (Sigma-Aldrich, St. Louis, MO), and 2 mM glutamine (Invitrogen, Grand Island, NY). To promote the endothelial phenotype of ECs in them, MNCs were plated onto flasks coated with fibronectin (2 μg/cm²) (Sigma-Aldrich, St. Louis, MO) and cultured in endothelial cell basal medium [6,2] (Canorex, Walkerville, MD) supplemented with EGM™-2 SingleQuotes. After 4 days of culture, nonadherent cells were removed by washing with PBS. The cells at 2nd passage were used for cell characterization and construct engineered bone.

Immunocytofluorescence studies were performed to detect the induced endothelial phenotypes. Monoclonal antibodies against mouse vascular endothelial growth factor receptor-2 (VEGFR-2) and von Willebrand factor (vWF) (Santa Cruz, Santa Cruz, CA) were used as primary antibodies. The induced ECs were fixed in 4% paraformaldehyde, permeated with 0.01% Triton X-100 in PBS, and incubated in 1% block serum for 1 h at 37°C. The cells were then incubated with either primary antibody (2 μg/ml) for 1 h. Bound antibodies were detected by incubation with FITC-conjugated ABC kit (Santa Cruz Immuno Research, West Grove, PA) (for VEGFR-2) or Alexa Fluor 488-conjugated (Molecular Probes, Eugene, OR) (for vWF) secondary antibody. The cells were examined in fluorescence microscope. For analysis of capillary formation in vitro, 300 μl of Matrigel™ (BD Biosciences, Bedford, MA) mixed with 4 × 10⁴ EPCs-derived ECs at 4°C was dispensed into a 24-well plate and incubated at 37°C until solid. Photographs of capillary-like formation were taken at 7 days of culture in normal condition.

Osteoblast phenotypes were detected by means of immunocytochemistry. After fixation, permeation, and blocking, bone marrow-derived osteoblasts were incubated with anti-osteocalcin antibody (Santa Cruz, Santa Cruz, CA), and visualized using avidin-peroxidase complex (ABC kit, Santa Cruz, CA). The presence of calcium deposits was demonstrated by von Kossa staining. Induced osteoblasts were fixed in citrate-acetone-formaldehyde solution, followed by incubation in alkaline-dye solution. The induced osteoblasts were incubated with anti-osteocalcin antibody (Santa Cruz, Santa Cruz, CA) and 50-μm-thick sections were cut, stained with silver nitrate solution (Sigma-Aldrich, St. Louis, MO) under ultraviolet light for 20 min. Un-reacted silver was removed by washing with 5% sodium thiosulfate (Sigma-Aldrich, St. Louis, MO). The induced osteoblasts were fixed in citrate-acetone-formaldehyde solution, followed by incubation in alkaline-dye mixture for 15 min. The slides were counterstained with hematoxylin solution.

Cell morphology on the biomaterials was evaluated by scanning electron microscopy (SEM). Fifty-microliter aliquots of induced osteoblasts or endothelial cell suspension (3.5 × 10⁶ cells/ml) were respectively added to PCL-HA scaffold (prepared as below). The culture of cell-scaffolds complexes were continued for 7 days. Then complexes were sequentially fixed with 1.5% glutaraldehyde (Fisher Scientific, Pittsburgh, PA), exposed to 2% osmium tetroxide (Sigma-Aldrich, St. Louis, MO) for 30 min, and dehydrated through a graded series of ethanol (50%, 70%, 90%, and 100%). After air-drying, the samples were mounted onto SEM stubs and sputter coated with gold. The images were obtained using SEM (Hitachi S-2400, Japan) at 10 kV.

Preparation of HA-PCL Scaffolds

PCL-HA scaffolds were prepared using a particulate leaching technique. HA (Sigma-Aldrich, St. Louis, MO) and PCL (Aldrich, St. Louis, MO) were mixed at a 1:1 wt/wt ratio and dissolved in tetrahydrofuran (Sigma-Aldrich, St. Louis, MO). NaCl particles (particle size 355-600 μm) nine times as much as the total weight of HA and PCL were used to generate a porosity of 80% ± 3.4% in the scaffold. The mixture was infused into glass tubes with a 5-mm diameter. After evaporation of the solvent, the cylinders were segmented into parts as long as 4 mm, and NaCl was leached out in distilled water. The materials were then sterilized in 70% ethanol before cell seeding. The ultrastructures of PCL-HA scaffolds were observed using a SEM at 15 kV.

Construct Engineered Bone and Animal Model

After trypsinization and resuspension, 10⁵ endothelial cells in 50-μl medium were pinpointed on six spots evenly into one end of the cylindrical scaffolds (at approximately 5 × 10³ cells/mm²). After cell suspension was soaking in, the process was repeated on the other end of the scaffold. The ECs-seeded scaffolds were kept in culture for 3 days. Before seeding MSCs-derived osteoblasts, all scaffold surfaces were gently dried on sterile delicate tissues (Kimberly-Clark, Roswell, GA) to remove the portion of media out of the pores. The 2 × 10⁵ osteoblasts in 100 μl suspension were evenly extended on the end (at approximately 10⁴ cells/mm²), and siphon and gravity favored the cells’ suspension moving into pores as stationed in an incubator. The loading was repeated for the other end. The EC-OB group was generated by seeding osteoblasts ECs-loaded scaffold. The OB group was prepared by dispensing osteoblasts alone at 10⁴ cells/mm² on both ends of non-ECs-loaded scaffolds that were pre-wetted in media. Cell-free scaffolds were generated as a control group by immersing the scaffolds in osteogenic medium. For all three groups, the cultures were continued for another 6 days in osteogenic media prior to surgical implantation.

All animal experiments were approved by the Animal Investigation Committee of Wayne State University. Male BALB/c mice aged 10–5 weeks were randomly divided into three groups (eight mice per group) and anesthetized by i.p. injection of a mixture of Xylazine (8 mg/kg) and Ketamine (100 mg/kg). A 0.4-cm-long bone segment was removed from the femoral shaft using a wheel saw driven by a Lynx™ electric motor (MTI, Greenville, IN). A 26-gauge needle was employed as a fixation pin. Seated in the opposing metaphysis, this support integrated the proximal femur, the graft, and the distal femur (Fig. 1A). X-rays scanning confirmed successful graft implantation (Fig. 1B). At 6 weeks postoperation, the grafts were retrieved for histological evaluation.
Histological Evaluation of Neovascularization, Osteogenesis, and Necrosis
Specimens were decalcified in formic acid (25%, w/v) and sodium citrate solution (100 mg/ml) and embedded in paraffin. Longitudinal 5 µm-thick sections were cut in the center of the grafts and stained with H&E for visualization of cells and tissue structure. Five random fields of each section were captured at 400× magnification, in which typical necrotic areas were identified and calculated as the ratio of necrotic area over total tissue area of the section. Immunohistochemistry was used to identify CD31 positive endothelium, using avidin-biotin-peroxidase complex. The microvessels that were lined by a dark-staining endothelial monolayer were counted manually in 400× fields, and divided by the field area using Image Pro Plus Software (Media Cybernetics, Bethesda, MD), which was denoted by the capillary density. The grafts sections were stained with Masson’s Trichrome to visualize osteoid. Five random fields per section were captured at 400× magnification. Osteogenesis percentage was defined as a ratio of the osteoid areas against the total field area. At least 10 sections for eight samples per group were, respectively, stained for CD31, Masson's Trichrome, and H&E, and quantitatively evaluated for vascularization, osteogenesis, and necrosis. Image collection and quantification were performed by one author blinded to the surgery and group.

Statistical Analysis
All measurements were calculated as mean ± standard deviation. Across groups of EC-OB and OB, independent-sample t-test from SPSS™ (student version 10.0.5) was used to compare results with p < 0.05 considered significant.

RESULTS
Cell Culture and Characterization
Cell colonies were observed in the primary passage of cultures using either osteogenic or endothelial medium. As differentiate, cells established specific morphologies. The differentiated cells in osteogenic medium displayed osteoblast-like spindle morphology (Fig. 2A); ECs presented the characteristic cobblestone morphology (Fig. 2F). Cells retained a stable morphology for more than five passages.

The expression of osteocalcin was prominent in induced osteoblasts (Fig. 2B) by immunocytochemistry. These cells were also positive for ALP activity (Fig. 2C).

Figure 2. Morphology and characterization of differentiated osteoblasts and ECs. The osteoblasts exhibited spindle morphology (A, ×200). Over 95% of induced osteoblasts expressed osteocalcin (B, ×200); and the ALP activity of osteoblasts was revealed as pink color (C, ×200). Von Kossa staining visualized ossification nodules in the culture dishes of induced cells, as an indication of normal osteoblast function (D, ×100). SEM images showed the induced osteoblasts attached, spread, and proliferated on the scaffolds with details of meshwork of ECM, pseudopodia, and cellular projections (E, ×1,500). Panels (F–J) characterize the EPC-derived ECs: typical cobblestone morphology (F, ×200), lightening up for VEGFR-2 (G, ×200) and vWF (H, ×200) with fluorescent secondary antibodies, and possessing ability to form capillary-like 3D structures (I, ×200). SEM exhibits the ECs proliferating to a cell sheet on the scaffolds and retaining their cobblestone shape and pseudopodia extensions (J, ×1,500).

An important phenotype of osteoblasts, ability to mineralize matrix, was demonstrated by von Kossa stain, which revealed darkly stained calcium deposition (Fig. 2D). The differentiation status of EPCs-derived ECs were confirmed by endothelial markers VEGFR-2 and vWF (Fig. 2G,H). Further, in Matrigel™, 3D capillary-like networks were established by the differentiated ECs in 1-week culture (Fig. 2I), indicating normal endothelial phenotype.

The PCL-HA scaffolds exhibited a porous structure and high level of interconnections (Fig. 1A, inset). The continuous culture of MSCs-derived osteoblasts on PCL-HA scaffolds for 7 days resulted in cells retaining spindle morphology (Fig. 2E). Fibrous extracellular matrix (ECM) was present between cells where osteoblast projections and pseudopodia were evident. SEM examination revealed that the scaffolds were entirely covered with a layer of EPCs-derived ECs following a week of culture (Fig. 2J). The ECs retained a typical cobblestone-like appearance with high cell-to-cell contact. Pseudopodia extensions on the cells surface were observed. The cell behavior including adhesion, spread, secretion, and proliferation on the scaffolds indicated normal cell metabolism and satisfactory biocompatibility of PCL-HA scaffold.

Histological Evaluation
Histological examination of the implanted grafts from the EC-OB group (Fig. 3A) at 6-weeks postoperation showed widely distributed capillaries and persistent ECM embedding the osteoblasts, suggesting viable cells, vascularization, and graft survival. CD31 positive cells developed an irregular round or tubular morphology, indicating endothelial monolayers lining the walls of newly formed blood vessels (Fig. 3B). These microvessels were observed throughout the graft on all sections. Loading scaffold with osteoblasts and ECs in the EC-OB group dramatically contributed to new bone formation during the healing of the defect. Associated with cell proliferation, osteoblasts laid down osteoid identified by the blue regions of Masson's Trichrome-stained sections (Fig. 3C). The osteogenesis distributed throughout the grafts in the EC-OB group. In contrast,
poorer neovascularization was observed in the OB group evidenced by the scant CD31 positive microvessels (Fig. 3E). In addition, patches of ischemic necrosis were observed in the OB group, as characterized by the cell nucleus atrophy and cell membrane rupture, with the infiltration of inflammatory cells into the graft (Fig. 3D). Sparse osteogenesis occurred at the graft of the OB group, whereas necrosis and osteogenesis were interlaced within the central region (Fig. 3F). Quantifying capillary density showed that the extent of the vascularization in the EC-OB group (203 ± 97/mm²) was five times higher than that in the OB group (40 ± 7/mm²) (Fig. 4A) (p = 0.007). Capillary density was highly negatively correlated with necrosis and positively correlated with osteogenesis percentage. In the EC-OB group, the higher capillary density favored osteogenesis (22% ± 8%), and eliminated the appearance of ischemic necrosis (0%) (Fig. 4B,C). In contrast, dramatically fewer capillaries were observed in the OB group, with a significantly higher level of necrosis (29 ± 9) (p < 0.0001) and
lower expression of osteoid (12% ± 7%) \( p < 0.001 \), which could be interpreted as impaired bone formation and failure of healing the bone defect (Fig. 4B,C).

Due to the decalcification and clearance during histological preparation, the only remnants detected in control groups were biomaterials debris and a few nucleated cells (Fig. 3G). The biocompatibility of PCL-HA composite was supported by the lack of inflammatory cells except for a paucity of lymphocytes and the lack of fibrous capsules formed around the scaffolds. Most importantly, the host bone marrow cells and stromal cells did not migrate into the scaffolds, and no ECM was produced, obviously distinguished from the cell-loaded scaffolds in which a number of nucleated cell and ECM were observed.

**DISCUSSION**

Currently, most engineered tissues lack the essential microvascular network required for survival and the maintenance of in vivo function. Ultimately due to lack of oxygen and nutrients (especially in the central regions of grafts), the tissue-engineered constructs are limited to an extremely small 3D size that reduces their clinic application. One promising approach for the creation of a microvascular network is the promotion of neovascularization in situ by seeding ECs within the scaffold construct.9,10,16 Mature ECs originating from most adult tissues are not easy to expand due to cell cycle senescence, and only limited numbers of ECs can be attained. In contrast, EPCs isolated from bone marrow are capable of more than 1,000 divisions,13 which means that comparatively low numbers of EPCs may provide the sufficient number of seeding-cells. Furthermore, in comparison with adult ECs, the EPCs displayed higher potency to sprout out of co-culture spheroids in response to the inhibition of osteoblasts.17 Therefore, EPCs may represent a useful source of ECs.

The stem/progenitor cells in vitro possess the capacity of self-renewal and differentiation into organ-specific cell types. Our results indicate that MSCs and EPCs can be harvested from mouse marrow and induced to differentiate into osteoblasts and ECs. Besides the expression of specific molecular markers such as osteocalcin, VEGFR-2, and vWF, these induced MSCs and EPCs functioned normally, as indicated by the fine crystals produced during ossification, the ALP activity of osteoblasts, and the 3D capillary formation of ECs in Matrigel™.

Our data demonstrate that pre-seeding EPCs-derived ECs effectively promoted neovascularization in tissue-engineered bone, preventing ischemic necrosis, and improving osteogenesis. The cells probably increased neovascularization as mimicking vasculogenesis, the mechanism whereby de novo capillary assembly occurs in situ by migration and proliferation of EPCs-derived ECs. Pre-seeding EPCs-derived ECs in scaffolds formed plentiful capillary-like structures throughout the scaffold, at far higher capillary densities than observed in the osteoblast alone group. Theoretically, host vessels could also grow into scaffolds. However, the distinct distribution and quantity of the capillaries in the OB-EC and OB groups suggested that invasion of host vessels probably did not sufficiently contribute to neovascularization. The OB group, which depends only on the host blood vessel ingrowth, did not obtain sufficient blood supply to support cell viability and bone formation, which resulted in impaired osteogenesis and increased necrosis among groups. Additionally, in the control group, the absence of nucleated cells and ECM formation indicated that cell migration from recipient tissue was minimal.

Inadequate vascularization results in hypoxia and contributes to tissue necrosis. Even autogenic trabecular bone grafts, the clinical “gold standard,” cannot obviate transplantation failure from ischemic necrosis.18 The same principle is true for engineered bone, the insufficient vascularity results in hypoxic cell death. Thus, enhancing vascularization has positive effects to prevent necrosis. Our results clearly show that advanced vascularization due to ECs seeding significantly protected against ischemic necrosis, especially at the center of the grafts. We postulate that tissue necrosis may be secondary to the proliferation of seeded osteoblasts and deposition of ECM. After “overlay-seeding” osteoblasts, the cell proliferation and matrix deposition and mineralization in porous scaffolds conferred lower porosity for media diffusion and vascular growth. In the circumstance of extreme low oxygen pressure and accumulation of metabolite, ingrowing cells will undergo degeneration, and progressive necrosis of the graft would ensue, which was shown in the OB group. On the other hand, pre-seeded ECs established their residence in scaffolds and initiated the process of vasculogenesis prior to the ingrowth of osteoblasts. The lower cell amount and “spot-seeding” permitted lots of void space for endothelial cell proliferation. Via these pores and interconnections, media could diffuse inside the scaffold to support endothelial cell survival. During osteoblast proliferation and ECM deposition, the vascular network developed to a sufficient level to anastomose with the host circulatory system. Despite abundant cell proliferation, the hypoxic stimuli might serve as a catalyst for endothelial cells participating in neovascularization to some extent.

Besides accelerated vascularization and obviated ischemic necrosis, the mechanisms by which seeded ECs contribute to osteogenesis are complex. Stahl et al. found that co-culture of ECs and osteoblasts in vitro could enhance VEGFR-2 expression in ECs and ALP activity in osteoblasts,16 with possible communication between the two by soluble factors and intercellular gap junctions.19–21

In conclusion, the addition of EPCs-derived ECs to PCL-HA scaffold prior to the seeding of MSCs-derived osteoblasts promoted the formation of blood vessels. The shortened time course of neovascularization conserved the vitality of osteoblast and increased osteogenesis. The study suggested that the multi-cell seeding technique improved the fate of engineered bone and facilitated bone repair.
REFERENCES