Pre-Seeding Bone Marrow Cells-Derived Endothelial Cells Promotes *in vivo* Osteogenesis of Bone Substitutes

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Statement of Purpose: Besides a cell population capable of creating new bone and a biocompatible scaffold, tissue-engineered bone regeneration requires an appropriate vascular bed to provide nutrient and oxygen transport to 3-dimentional bone matrix substitutes. Bone marrow-derived endothelial progenitor cells (EPCs) can differentiate into endothelial cells *in vitro* and promote neovascularization in animal models of ischemia. To test the hypothesis that addition of EPCs-derived endothelial cells (ECs) may improve osteogenesis and prevent necrosis of tissue engineered bone through efficient neovascularization, we pre-seeded mouse EPC-derived ECs on polycaprolactone-hydroxyapatite (PCL-HA) scaffold before seeding marrow stromal cells (MSCs)-derived osteoblasts, and implanted this cell-scaffold complex to repair mice with a femoral defect.

Methods: Bone marrow from male BALB/c mice (6-8 weeks old) was used as the source of both MSCs and EPCs. The phenotypes of both induced osteoblasts and endothelial cells were confirmed by the expression of osteocalcin and alkaline phosphatase or VEGFR-2 and vWF respectively. Cylindrical PCL-HA scaffolds (4 mm long and 5mm in diameter) were prepared using a particulate leaching technique. Three groups were divided according to different cell types used for seeding on the scaffolds. The EC-OB group consisted of pre-seeding 5×10^3 cells/mm^2 of EPC-derived ECs on scaffolds for 3 days before applying 10^4 cells/mm^2 of MSC-derived osteoblasts. In the OB group, non-ECs-loaded scaffolds were pre-wetted in medium for 3 days, and sequentially seeded with osteoblasts. The scaffold control group consisted of PCL-HA scaffolds immersed in osteogenic medium without cell seeding. All groups were cultured at 37˚C, 5%CO_2 for 6 days before implantation. A 0.4cm-long segmental defect of femur diaphysis of BALB/c mouse was created, and grafts were fixed at the site of the bone defect. The animals were observed for 6 weeks post-implantation and then sacrificed. The grafts were retrieved for histological process, and stained with H&E and Masson's Trichrome. Immunohistochemistry using anti-osteocalcin was performed to determine the bone formation. The capillary density, osteogenesis and necrosis were quantified using a computerized image analysis system. Data were expressed as means ± standard deviations and analyzed by ANOVA, and statistical significance defined as $p<0.05$.

Results/Discussion: Histological examination of the EC-OB group at 6 weeks post-op revealed a widely distributed network of capillaries (Figure 1A, 400×), and ubiquitous extracellular matrix, with osteoid laid down by osteoblasts (identified by the blue regions of Masson's Trichrome staining) coupled with the positive staining for osteocalcin in series sections (Figure 1C, 200×). This finding indicated vascularization, graft survival and bone formation. In contrast, the OB group (Figure 1B, 400×) exhibited few capillaries and apparent ischemic necroses, especially at the central region of grafts, with concomitant impairment of new bone formation. In sections from the scaffold control group there was no inflammation but biomaterial debris (Figure 1D, 200×).

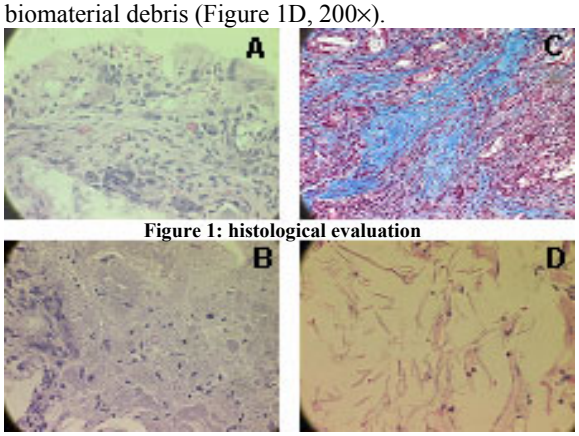

Figure 1: histological evaluation

A strong negative correlation between the capillary density and the amount of necrosis, and a positive correlation between capillary density and osteogenesis in the bone graft were detected between experimental groups. Vascularization in EC-OB group was increased 8 times over that in OB group (Figure 2A, $p<0.007$), with increased osteogenesis (Figure 2B, $p<0.001$) and no detectable ischemic necrosis (Figure 2C). On the other hand, insufficient capillary development in the OB group resulted in significant necrosis accompanied by a paucity of osteoid, although some osteogenesis were observed at regions out of the center of grafts (Figure 2B). This suggested impaired new bone formation and deterioration of the graft survival. The seeded EPCs-derived ECs increased neovascularization through vasculogenesis. Once this functional vascular network was in place, it appears that oxygen, nutrients, growth factors, calcium, and recruited progenitor cells were available at the site of osteogenesis.

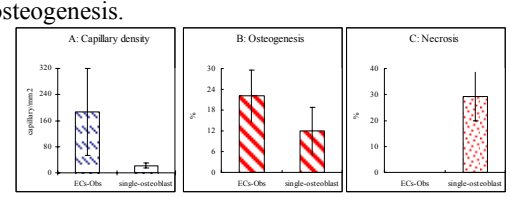

Figure 2: quantify vascularization, osteogenesis and necrosis

Conclusions: Our data demonstrated that pre-seeding a scaffold with EPCs-derived ECs effectively promoted neovascularization in tissue-engineered bone, obviated the ischemic necrosis, and improved osteogenesis. EPCs were also demonstrated to be an innovative source of ECs to promote tissue-engineered vascularization. The integration of bone marrow derived ECs and osteoblasts into a PCL-HA scaffold is a useful strategy to promote the survival of engineered bone and achieve successful bone grafting.