ROLE OF MATURATION STAGE ON THE INTERACTION OF OSTEOBLASTS AND MESENCHYMAL STEM CELL DERIVED OSTEOPROGENITORS WITH MICROVASCULAR ENDOTHELIAL CELLS

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INTRODUCTION: Angiogenesis is essential to the development, maintenance, and repair of bone tissue, as well as the survival of engineered tissues. Also, since osteoblasts (OB) are found in close proximity to endothelial cells in areas of new bone formation, it has been postulated that communication exists between these cell populations. Indeed, recent *in vitro* studies have shown that endothelial cells produce growth factors for osteoblasts [1]. In turn, osteoblasts and osteoprogenitors have been found to produce angiogenic factors, while mature osteoblasts also produce anti-angiogenic factors, suggesting that OBs are capable of modulating angiogenesis in bone in a maturation dependent manner [2, 3]. However, the cellular mechanisms behind osteoblast-endothelial cell interactions resulting in angiogenesis and bone formation remain unclear, particularly the changes in osteoblast-endothelial cell interactions with osteoblast maturation. Furthermore, osteoblast maturation stage dependent changes in interactions with endothelial cells may explain the differences in the necessity of direct cellular contact for increased osteoblast anabolic activities reported in the literature [1, 4]. In this study we examined the role of osteoblast maturation stage on the interactions between osteoblast and endothelial cells, both with and without direct cell contact.

METHODS:

Cell Culture: Immature pre-osteoblastic cells (pOB) were obtained by culturing human mesenchymal stem cells (MSC) in 10^{-8} mol/L dexamethasone for 5 d prior to use to initiate osteoblastic differentiation. MSCs were isolated from the bone marrow obtained from patients undergoing total hip replacement surgeries, with NIH Internal Review Board approval. Mature osteoblasts (tOB) were isolated from trabecular bone chips obtained from the same patients, and cultured in 10^{-8} mol/L dexamethasone. Human microvascular endothelial cells (HMVEC) were purchased from Cambrex Bioscience. Cells were then divided into the following groups and cultured in 6-well or 6-well Transwell plates: 1) pOB or tOB alone; 2) pOB or tOB + HMVECs with direct cell contact; and 3) pOB or tOB + HMVECs without cell contact (transwell). Co-cultures were maintained in endothelial cell basal medium supplemented with 10 % fetal bovine serum and 50 µg/mL ascorbate. In order to quantify the fraction of osteoblastic cells in mixed co-cultures, osteoblastic cells were pre-stained with Vybrant CM-DiI membrane dye prior to co-culture, and at each time point, co-cultures were trypsinized and the fraction of dyed cells were counted.

Cell Proliferation and Alkaline Phosphatase (ALP) Assays: Cells were lysed in distilled water using repeated freeze-thaw cycles, and lysates were assayed for DNA content using Picogreen. For mixed co-cultures, the DNA content was corrected for the OB fraction. ALP activity was measured by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol and normalized to the DNA content.

Gene Expression: Real-time PCR analysis using SYBR green was performed for a semi-quantitative assessment of changes in gene expression of type I collagen (COLI), osteocalcin (OC), and bone sialoprotein (BSP), and normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression. GAPDH expression was corrected for the fraction of OBs. There was no expression of COLI, OC, and BSP in HMVECs cultured alone. Changes in gene expression were expressed as fold changes over day 1 OB alone cultures. Standard uncertainties of these analyses are reported in Fig. 1 and 2.

Immunohistochemistry: To visualize HMVECs, immunostaining against platelet endothelial cell adhesion molecule 1 (PECAM/CD31), an endothelial cell specific marker, was performed on co-cultures, and counterstained with 4'-6-Diamidino-2-phenylindole (DAPI).

RESULTS: The presence of HMVECs in co-cultures of immature pOBs significantly increased ALP activity both with and without direct cell contact (Fig. 1) compared to pOBs alone. In contrast, co-cultures of HMVECs and mature tOBs only showed an increase in ALP with direct cell contact. Proliferation of osteoblastic cells was enhanced in co-cultures, both with and without direct cell contact (data not shown). Type I collagen gene expression in osteoblastic cells was significantly upregulated with direct cell contact with HMVECs in both immature pOBs and mature tOBs, but not in co-cultures without direct cell contact (Fig. 2). In contrast, osteocalcin (Fig. 3) and bone sialoprotein (not shown) gene expression was significantly downregulated in co-cultures with direct cell contact, but not in co-cultures without cell contact. Immunostaining for PECAM in mixed co-cultures showed uniform intermixing of HMVECs and osteoblastic cells at day 1, and segregation of HMVECs at day 14 (Fig. 4).

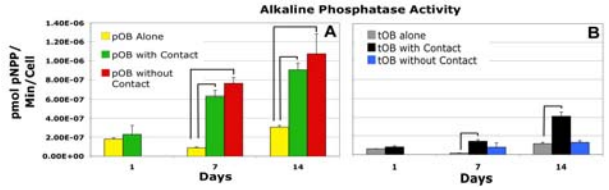

Figure 1. ALP activity of A) immature OBs (pOB) and B) mature OBs (tOB). Mixed co-cultures were normalized to OB fraction. $p<0.05$

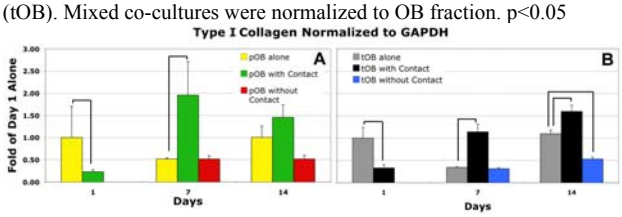

Figure 2. Gene expression of COLI normalized to GAPDH in A) immature osteoblasts (pOB) and B) mature osteoblasts (tOB).

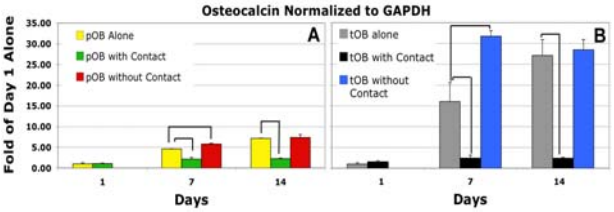

Figure 3. Gene expression of OC normalized to GAPDH in A) immature osteoblasts (pOB) and B) mature osteoblasts (tOB).

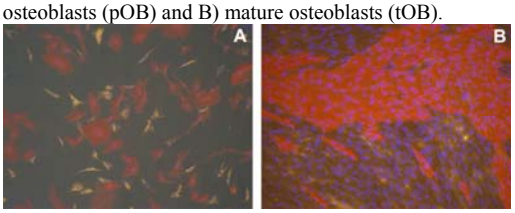

Figure 4. PECAM immunostaining (red), DiI labeled osteoblastic cells (orange), and DAPI nuclear staining (blue) at A) day 1 and B) day 14.

DISCUSSION: In this study we have demonstrated that osteoblast-endothelial cell interactions are altered by the maturation stage of the osteoblasts. Specifically, mature OBs only increased ALP activity with direct contact with HMVECs, while immature OBs showed increased ALP both with and without direct cell contact. Also, ALP and COLI, which are earlier markers of osteoblastic differentiation, were increased with direct cell contact with HMVECs, while mature osteoblastic markers such as OC and BSP were decreased with contact. Contact mediated changes in osteoblastic gene expression may be due to the direct contact itself, such as gap junctional coupling [4], and/or due to changes in factors secreted by the HMVECs. Furthermore, the spontaneous segregation of HMVECs and osteoblastic cells in mixed cultures may further modulate their interactions. Interestingly, such segregation of OBs and endothelial cells has also been observed in 3D co-cultures [5]. A better understanding of the interactions between osteoblasts and endothelial cells may lead to treatments that enhance both bone formation and vascularization, as well as graft and engineered tissue integration.

REFERENCES: 1) Wang, DS, *et al., Endocrinology,* 1997; 2) Goad, DL, *et al., Endocrinology,* 1996; 3) Quan, GM, *et al., Calcif Tissue Int*, 2005; 4) Villars, F, *et al., Am J Physiol*, 2002; 5) Wenger A, *et al., Tissue Eng*, 2004.