OSTEOCYTE ENHANCEMENT OF ANGIOGENESIS IN VITRO

*,**Takai, E; *Taboas, J; **Hudson, S; +*Tuan, R S
+*National Institutes of Health, Bethesda, MD; **National Institute of Standards and Technology, Gaithersburg, MD
tuanr@mail.nih.gov

INTRODUCTION: It is well known that an intimate relationship exists between vascularization and bone formation during development and fracture healing, where new bone formation is preceded by local vascularization. A recent rat exercise model has demonstrated that mechanical loading induced increases in bone formation are also preceded by increased bone vascularization, and correlated to blood vessel number [1]. This exercise induced increase in bone formation and vascularization was abrogated by the addition of antibodies against vascular endothelial growth factor (VEGF), a potent angiogenic agent. Despite the important relationship between vascularization and bone formation, the cellular cues that lead to increased or maintained vascularization of bone remain unclear. Specifically, it is not clear whether osteoblasts, osteocytes (OCY), and/or osteoclasts are involved in the recruitment of new vasculature. Since OCYs form extensive interconnected cellular networks throughout the bone tissue and respond to a variety of mechanical and chemical stimuli, they have been proposed to be the mechano/chemostat cells of bone tissue [2]. Furthermore, osteoporotic bone, which has decreased OCY density and a disorganized osteocyte network [3], has reduced number of capillaries and fewer vessels closely apposed to the bone surface [4]. Thus OCYs may play an important role in vascular recruitment both with and without mechanical loading. In this study we have examined the ability of OCYs subjected to various physiologic stimuli to enhance angiogenesis *in vitro*.

METHODS:

Cell Culture:

Osteocytic MLO-Y4 cells were cultured on collagen type I coated tissue culture plastic in alpha minimum essential medium supplemented with calf serum, fetal bovine serum (FBS), and penicillin/streptomycin (5 %, 5 % and 1 % mass fraction, respectively) prior to experimentation. Human microvascular endothelial cells (HMVEC) were purchased from Clonetics and cultured in endothelial cell basal medium (EBM) with endothelial cell supplements.

Conditioned Medium:

OCYs grown to 90 % confluence were subjected to the following treatments and cultured under static conditions in EBM supplemented with 10 % FBS and 1 % penicillin/streptomycin for 24 h to generate conditioned medium (CM): 1) static culture; 2) hypoxia (5 % mole fraction oxygen, 24 h); 3) dynamic hydrostatic pressure loading (DHP); or steady fluid shear. DHP loading was applied to OCYs using a custom-made feedback controlled pressure chamber at 1 Hz for 1 h with a physiologic peak magnitude of 40 kPa. Steady laminar flow resulting in a physiologic fluid shear of 8 dynes/cm^2 was applied to OCYs for 15 min using a parallel plate flow chamber. CM was centrifuged at 5000 g for 15 min to remove cell debris and stored at -80 °C until use.

HMVEC Proliferation Assay:

HMVECs were seeded into 96-well plates at a density of 5,000 cells/cm^2, and treated with EBM supplemented with 10 % FBS and 1 % penicillin/streptomycin (control medium) or one of the CM described above. Cells were fixed with 10 % buffered formalin on days 0, 3 and 6. HMVECs were then stained with 0.2 % crystal violet, rinsed in distilled water, and air-dried. To quantify cell number, the dye was eluted using a 10 % acetic acid solution, and the absorbance at 590 nm was measured. Standard uncertainties are reported.

Matrigel Assay:

A layer of growth factor reduced Matrigel was formed in 24-well plates and rinsed with control medium for 24 h to remove unbound growth factors. HMVECs were then seeded on top of the Matrigel in control medium or CM, and observed after 24 h to assess the formation of capillary-like structures.

Quantification of VEGF:

The amount of VEGF secreted by OCYs under the various conditions and the amount of VEGF in the control medium was quantified using a commercial VEGF enzyme-linked immunosorbent assay kit. Standard uncertainties are reported.

RESULTS: At day 3, HMVECS exposed to DHP or flow CM showed a trend of increased proliferation compared to control medium, static CM, and hypoxia CM (Fig. 1A). At day 6, all CM treated groups except hypoxia CM showed significantly increased cell proliferation compared to the control group. There was a trend of increased proliferation in the mechanically loaded CM groups compared to the static CM group. Capillary-like network formation was similar in HMVECs cultured in control medium, static CM, and hypoxia CM (Fig. 1). However, HMVECs cultured in DHP or flow CM showed more extensive capillary-like network formation compared to control media. All CM had higher VEGF concentrations than the control medium, with the highest concentrations in mechanically loaded groups (Table 1).

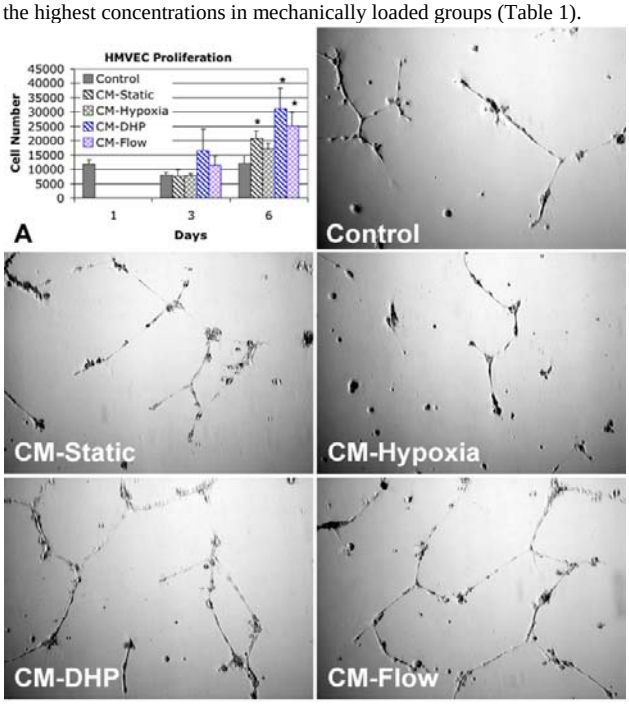

Figure 1. (A) HMVEC proliferation assessed by crystal violet staining. *p<0.05. Light micrographs of HMVECs cultured on Matrigel with control or conditioned medium.

Control	CM-Static	CM-Hypoxia	CM-DHP	CM-Flow
25.4 ± 0.4	47.2 ± 4.6	42.9 ± 0.3	74.6 ± 12.3	97.1 ± 11.0

Table 1. VEGF concentration in control and OCY conditioned medium

DISCUSSION: In this study we have demonstrated the ability of OCYs to enhance proliferation and capillary-like network formation of HMVECs *in vitro*. The mechanically loaded CM groups showed trends of enhanced HMVEC proliferation compared to static conditions, which were paralleled by increased VEGF secretion. Although VEGF has been shown to increase both proliferation and tube formation in endothelial cells, since the concentration of VEGF in the various CM were lower than the concentrations previously found to be mitogenic [5], the observed increases in HMVEC proliferation and tube formation when cultured in CM may be due to other secreted factors. OCY recruitment of vasculature may lead to increased bone formation by increasing endothelial cell-osteoprogenitor cell interactions, which have been shown to enhance osteoblastic differentiation *in vitro* [6]. Also, endothelial cells have been shown to produce CD40 ligand, which is known to protect OCYs from apoptosis [7]. Thus, a better understanding of the interactions between OCYs and endothelial cells may lead to treatments that can mitigate the effects of osteoporosis and/or enhance bone formation.

REFERENCES: 1) Yao, Z, *et al, J Bone Miner Res*, 2004; 2) Burger, EH *et al, FASEB J*, 1999; 3) Knothe-Tate, ML, *J Biomech*, 2003; 4) Burkhardt, R *et al, Bone*, 1987; 5) Wang, DS, *et al, Endochrinology*, 1997; 6) Kaigler D, *et al, FASEB J*, 2005; 7) Bonewald LF, *et al, J Musculoskel Neuron Interact*, 2004.

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