Bone-Vascular Microdevice Highlights Importance of Fluid Flow in Investigating Inflammatory Environments

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Disclosures: The authors have nothing to disclose.

INTRODUCTION: Bone degrades and becomes increasingly susceptible to fracture as we age and with diseases like cancer, inflammatory arthritis, diabetes, and cardiovascular disease.[1,2] Bone fragility is also common with ischemic stroke[3,4] and disuse with prolonged bedrest is commonly blamed, as bone is highly mechanosensitive.[5] However, the amount of bone mineral density loss with prolonged bedrest (1-2% in 3 months)[6,7] cannot fully explain the density loss in stroke patients (13% in 1 year).[3] Inflammatory cytokines are elevated in serum of stroke patients compared to healthy controls for months after the infarct,[8,9] including interleukin (IL)-1β and IL-6, which could contribute to stroke-related bone loss. Microfluidic devices recreate tissue microenvironments (e.g., multiple cell lines, shear stresses, 3D biomimetic cell scaffolds), providing more physiologically relevant *in vitro* systems.[10,11] Previous microfluidic platforms with mineralized bone either do not include vasculature[11] or use bulk media perfusion and interstitial flow[10] instead of perfusion through an endothelium with diffusive nutrient delivery, which is more similar to native nutrient exchange. The goal of our study was to use our new bone-vascular microdevice to determine cell responses to inflammatory factors IL-1β and IL-6, which are present in the acute phase of stroke recovery, using secreted factor assays and gene expression.

METHODS: Cancellous bone scaffolds (8:2 wt% type I collagen:chitosan) were made by freeze drying, carbodiimide crosslinking, and passive mineralization. Microdevices (Fig. 1A) were fabricated using molded polydimethylsiloxane (PDMS) in a 10:1 elastomer:hardener ratio, with bone scaffolds placed in the top chamber during assembly, and plasma-treated to bond chambers when assembled. A 10-µm thick polycarbonate membrane with 5-µm pore size separated top and bottom chambers. Devices were sterilized by rinsing channels with isopropyl alcohol, and the bottom chamber was coated with collagen and fibronectin in PBS (50 µg/mL each) for 1 hour prior to cell seeding. Human umbilical vein endothelial cells (HUVEC, Lonza, 300,000 cells/device) and human mesenchymal stem cells (hMSC, Rooster Bio, 100,000 cells/device) were seeded in 2:1 (v/v) endothelial:osteogenic media (EM:OM) in devices, inverted 1 hr to promote HUVEC attachment to the membrane, and flow-conditioned 3 days to mature the endothelial barrier, with a final flow rate of 1000 µL/min to achieve 8 dynes/cm^2 shear stress required for endothelial polarization. To simulate inflammatory conditions post-stroke, cytokines were perfused through devices in fresh 2:1 EM:OM for 4 days using 4 groups (n=3): no cytokines (control), interleukin (IL)-6 only (15 pg/mL), IL-1β only (185 pg/mL), or both IL-6 and IL-1β. Also, a 24-well Transwell® plate control was prepared with HUVECs seeded (25,000 cells/insert) in the insert and hMSCs seeded (50,000 cells/well) in the well on top of a 5 x 5 mm section of fabricated cancellous bone scaffold. The control plate was set up to match the groups used in devices. Conditioned media was collected at 1, 6, 24, and 96 hours after cytokine introduction and analyzed via alkaline phosphatase (ALP) activity and ELISAs for secreted factors RANKL, OCN, TNF-α, VEGF-A, SPARC, and MCSF. Pooled RNA from the two chambers or Transwell® condition was collected and purified for gene expression using NanoString (PanCancer IO 360™ panel and custom code sets of *BGLAP, CSF1, RUNX2, SP7, SPARC, TNFSF11*). Metrics were compared between groups and over time with two-way ANOVAs and Tukey post hoc tests (α=0.05), using repeated measures for ALP and ELISA data.

RESULTS: No significant differences were found between groups for ALP activity, macrophage colony stimulating factor (MCSF) secretion, or vascular endothelial growth factor A (VEGF-A) secretion. For main effects, osteonectin (SPARC) secretion differed in the Transwell® plate group over time (p=0.0001) and with cytokine treatment (p=0.0018). At 1 hour perfusion, SPARC secretion was higher with dual infusion (IL-1β + IL-6) compared to IL-1β only (p=0.017) and IL-6 only (p=0.0075). Gene expression did not differ across groups for osteocalcin (BGLAP), type 1 collagen (COL1A1), osterix (SP7), and Dickkopf-related protein 1 (DKK1). Broadly, expression for many genes changed with the addition of inflammatory cytokines, with most differences occurring between Transwell® plate and devices rather than between cytokine treatment groups. Compared to devices, expression was significantly higher in Transwell® plates for runt-related transcription factor 2 (RUNX2, p=0.0002), VEGFA (p=0.0037), fibronectin (FN1, p<0.001), and IL-8 (p<0.0001), and lower in Transwell® plates for transforming growth factor beta 1 (TGFB1, p=0.0002), β-catenin (CTNNB1, p<0.0001), and SPARC (p<0.0001). IL-8 (Fig. 1B) expression showed main effect differences between Transwell® plates vs. devices and between cytokine treatments (p=0.0005). IL-8 expression was highest in the Il-1β perfusion group and was higher with dual infusion compared to control and IL-6 infusion only.

DISCUSSION: We designed and fabricated a 3D bone-vascular microdevice that incorporated bone cells grown on a 3D biomimetic cancellous bone scaffold and endothelial cells to determine the effects of inflammation on cell responses. Inflammatory cytokines impacted expression of several genes, with most differential expressions seen between the Transwell® plates and devices, not cytokine treatment groups. Interestingly, genes most associated with pro-osteoblastic phenotypes (BGLAP, COL1A1, SP7) were not different between cytokine treatment groups or in plates vs. devices. Despite a lack of differences between groups, expression of these genes confirms the presence of osteoblasts in the devices. Gene expression elevated in Transwell® plates (RUNX2, VEGFA, FN1, IL-8) suggests a strong drive toward bone remodeling via proangiogenic, osteogenic, and osteoclastogenic expression, whereas gene expression elevated in devices (CTNNB1, THBS1, TGFB1, SPARC) suggests a pro-osteogenic environment in bone with a quiescent endothelium. Taken together, these data indicate a difference in cell response to inflammation that is related to flow, which is present only in devices.

SIGNIFICANCE: The findings of this study demonstrate the feasibility of this bone-vascular microdevice to sustain HUVEC and hMSC co-cultures during inflammatory stimulation. The development of this platform enables further study of bone-vascular interactions in a highly controlled manner to further investigate inflammatory bone loss with stroke and other applications such as cancer metastasis and drug delivery.

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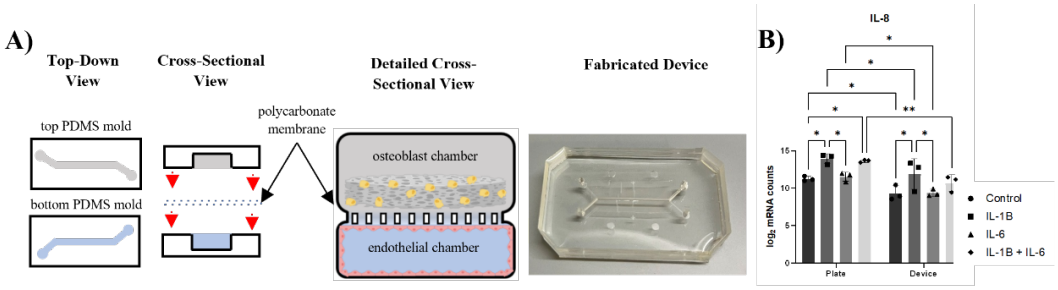

Figure 1. A) Schematic overview of device assembly illustrating the individual cell chambers in the cross-sectional views, and the fabricated device. B) Gene expression of IL-8 Transwell plates and devices after cytokine infusion. * p<0.05, ** p< 0.01