**INTRODUCTION:**

Fatigue damage in bone results in localized osteocyte apoptosis, which plays a direct role in the recruitment/activation of osteoclasts that remodel the damaged tissue. Although osteocyte apoptosis is limited in scope, the region of cell death extends well beyond the area directly contacting microcracks, suggesting that indirect cellular insults may produce the apoptotic events that ultimately trigger bone resorption. However, little is known about the nature of those acute signals. Previously, we demonstrated that microcracks in bone impair local fluid transport in the osteocyte lacunar-canalicular system. Such a disruption in transport could produce hypoxic stress causing osteocytes either to undergo apoptosis or to produce pro-resorptive cytokines. Here we test whether osteocytes in areas of microdamage acutely express i) HIF-1α, indicating a cellular response to hypoxia, and ii) VEGF-A, a cytokine inducible by HIF-1α and known to stimulate expression of RANKL, a factor necessary for differentiation of osteoclasts.

**METHODS:**

Right ulnae of adult Sprague-Dawley rats (4-5 months old, n=12) were fatigue loaded in vivo to activate intracortical remodeling. Ulnae from non-loaded animals were used as naive controls. Ulnae were harvested 24 hours following fatigue to assess acute responses to microdamage.

**Gene expression:** 6 mm segments of ulnar mid-diaphysis were removed, stripped of periosteum, flash-frozen and pulverized prior to RNA extraction. These segments, which sustain the majority of fatigue microdamage in this model, contain virtually no marrow cavity; hence, after removal of periosteum, osteocytes comprise the principal cell population in these tissue samples. Total RNA was assayed for expression of hypoxia-inducible factor (HIF)-1α and VEGF-A (144kDa and 164kDa splice variants, Accession # AY702972) using RT-PCR. Amplifiers were separated on 2% agarose gels; ethidium bromide-stained amplifier bands were quantified by NIH Image J. Differences were evaluated by Kruskal-Wallis analysis of variance with post-hoc testing using the Dunn procedure.

**Immunohistochemistry (IHC):** was performed on 5 µm thick longitudinal sections of decalcified ulnar diaphyses. VEGF-A protein was localized in osteocytes using a mouse monoclonal antibody (Santa Cruz, SC-7269) against VEGF-A, visualized with an HRP-linked secondary antibody and DAB enhancement. Point count stereological methods were used to count VEGF-A positive-staining osteocytes near microcracks and at comparable locations in control tissues. Differences between experimental groups were evaluated using the Friedman Test with distance from microcrack and treatment (fatigue vs. control) as variables; post-hoc testing was performed using Dunnett's procedure. Significance is reported at p<0.05. All procedures were approved by the Mount Sinai School of Medicine IACUC.

**RESULTS:**

**Gene expression:** There was a marked increase in HIF-1α and VEGF-A expression in bone after fatigue loading (Fig. 1). Both splice variants were upregulated after fatigue with a significantly greater increase in VEGF-A144 expression than VEGF-A164.

**Immunohistochemistry:** Strong VEGF-A staining was present in osteocytes surrounding microdamage (Fig. 2). Within 125 µm of the microcrack almost 50% of the osteocytes stain for VEGF-A, which is greater than a 4-fold increase over controls (Fig. 2). VEGF-A staining decreased exponentially with distance from microcracks (Fig. 2B).

**DISCUSSION:**

HIF-1α is characteristically expressed by cells experiencing hypoxic stress. Its greatly elevated expression by osteocytes 24 hours after fatigue supports the contention that fatigue microdamage results in impaired oxygen delivery to bone by disruptions in fluid flow. In addition, co-expression of VEGF-A with its upstream regulator HIF-1α by osteocytes in conjunction with fatigue-induced apoptosis resembles the situation in epiphyseal growth plates, where HIF-1α and VEGF-A expression is linked to apoptosis of hypertrophic chondrocytes and the subsequent resorption and remodeling of calcified cartilage.

The exponential decline of osteocytes expressing VEGF-A with distance from microcracks parallels the previously observed pattern of apoptotic osteocytes. However, the VEGF-A-expressing cells at 24 hours post-fatigue appeared to be non-apoptotic; furthermore the number of VEGF-A-positive cells (nearly 50%) approximates the fraction of non-apoptotic cells seen by Verborgt et al at 24 hours (approximately 60%) after fatigue. This pattern suggests that while osteocyte apoptosis may be necessary for recruitment of osteoclasts to microdamage induced remodeling sites, transmission of critical pro-resorptive cytokine signals like VEGF-A may be carried out by the cells that survive.

**CONCLUSIONS:** Fatigue induces an acute hypoxic response in osteocytes near microdamage sites, in conjunction with a spatially-restricted upregulation of VEGF-A, a known upstream regulator of pro-resorptive factors like RANKL.

**ACKNOWLEDGEMENTS:** NIH AR41210 & AR47628

**REFERENCES:**