OSTEOCYTE APOPTOSIS PRECEDES BONE RESORPTION AFTER FATIGUE

INTRODUCTION:
Recent studies have shown osteocyte apoptosis in regions of bone where osteoclastic resorption follows the induction of bone fatigue (1,2), leading to the hypothesis that osteocyte apoptosis may identify bone for removal by osteoclasts after fatigue-induced matrix injury. If osteocyte apoptosis does provide a mechanism for "targeting" osteoclastic activity, then cell death must precede the onset of bone resorption; however, the timing of osteocyte apoptosis in relation to the occurrence of fatigue microdamage in bone is unknown. In the current studies, we used the rat in vivo fatigue model to examine the time course of changes in osteocyte integrity after bone fatigue and to determine whether programmed cell death is initiated prior to the onset of bone remodeling.

METHODS:
Right ulna of adult female Sprague-Dawley rats (5-6 months old) were subjected to fatigue loading in vivo using a modification of end-load bending developed by Bentolila et al (2). This model activates intracortical remodeling in fatigued ulnar cortices. Under inhalation anesthesia, fatigue loading of ulnae was performed under load control, as in our previous studies (1,2). Ulnae were fatigued to a single stopping point based on loss of bone stiffness, which reflects microdamage formation in bone. Left ulnae were not loaded and served as paired internal controls. Changes in osteocyte integrity over time after fatigue loading were studied at baseline (0 days) and 1, 3, 7 and 10 days after loading (8-10 animals/time). Procedures were conducted with approval from the IACUC of the Henry Ford Health Sciences Centers and Mount Sinai School of Medicine.

Ulnar diaphysis were sectioned longitudinally at 5 µm thickness and stained using the end-labeling approach to distinguish osteocytes undergoing DNA fragmentation characteristically associated with apoptosis. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-digoxigenin end-labeling (TUNEL) was used to identify the DNA fragments generated by endonuclease activity characteristic of cells undergoing apoptosis. Peroxidase-labeled antidigoxigenin with DAB-cobalt-nickel staining was used to localize TUNEL stained cells. To assess time dependent changes in osteocyte integrity, cell densities for TUNEL negative staining and TUNEL positive staining osteocytes and empty lacunae were determined in bone immediately around microcracks (=100µm). Measurements were performed using point count stereological methods.

ANOVA was used assess TUNEL negative and TUNEL positive staining osteocytes and empty lacunae among groups over time, with post-hoc comparison performed against baseline (0 day) values using the Mann-Whitney U test.

RESULTS:
At baseline, immediately after fatigue loading, the numbers of TUNEL positive stained (apoptotic) osteocytes and empty lacunae immediately were unchanged from control levels. At 1 day after fatigue loading, the number of TUNEL positive osteocytes was increased nearly four-fold over baseline (p<.01). TUNEL positive osteocyte number remained constant at this elevated level for all subsequent time periods examined. In contrast, the number of normal (TUNEL negative) osteocytes decreased continually at 1, 3 and 7 days after loading (p<.01, relative to baseline at each time period), with no subsequent change observed at day 10. The number of empty lacunae showed an inverse pattern to that observed for TUNEL negative osteocytes, with significant increases from baseline at 3, 7 and 10 days after loading. Data for changes in osteocyte TUNEL staining and empty lacunae over time are summarized in Figure 1.

DISCUSSION:
The current experiments indicate that osteocyte apoptosis around bone microdamage was initiated by 1 day after fatigue loading and remained elevated throughout the study period. Maintenance of high levels of apoptosis in the tissue over relatively long time periods after fatigue (10 days after loading) seems somewhat unexpected, since apoptosis at a unit cell level in vitro is a very rapid process, progressing over time periods of several hours (4). However, similar long-term maintenance of high apoptosis levels in tissues in vivo have been reported after focal injury in both brain and cardiac infarct models (5,6). While it possible that apoptosis in vivo is much more protracted process than in vitro, the time course data in these experiments suggests another possibility. In the current experiments, the number of empty lacunae increased throughout the study period, while the number of normal osteocytes around microcracks decreased commensurately. Thus, over time, early apoptotic cells "die" giving rise to additional empty lacunae. High levels of osteocyte apoptosis appear to be maintained as additional cells from the normal cell compartment undergo apoptosis.

Previous electron microscopy studies report that osteocytes juxtaposed to resorbing osteoclasts undergo degeneration (3), with subsequent phagocytosis by osteoclasts. It has been widely interpreted from these studies that osteoclasts causes the involutional changes in osteocytes. However, the results of the current studies support the opposite sequence of events -- that osteocyte degeneration precedes bone resorption and may provide an important local signal to remodel an area of bone.

**References:**
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