Comparative Evaluation of Apoptotic and Necrotic Cell Death After Injurious Compression of Bovine Articular Cartilage Using Electron Microscopy and TUNEL Staining

Introduction
Chondrocyte death by apoptosis may play a role in the development of osteoarthritis (OA). Several recent studies have identified an increased number of apoptotic chondrocytes in osteoarthritic cartilage [1-4]. In addition to a reduced ability to repair the extracellular matrix, it has been proposed that chondrocyte apoptosis may contribute to cartilage calcification or matrix degradation [1,3], although conflicting results have been reported [4].

Our previous studies have used an in vitro cartilage explant model to study aspects of traumatic joint injury, an event which leads to an increased risk for the development of OA. In particular, Loening et al. [5] demonstrated a dose-dependent increase in apoptosis with increasing injurious compression as detected by TUNEL staining. D’Lima et al. also reported an increase in apoptosis after an injurious loading protocol by TUNEL staining [6]. However, questions remain as to the specificity of TUNEL and how it relates to the total number of dead cells assessed by in situ viability dye methods [7].

Methods
Cartilage harvest: Cartilage disks (3 mm diameter x 1 mm thick) were obtained from the femoropatellar groove of 1-2 week old calves and cultured in low-glucose DMEM with 10% FBS, 20 µg/ml ascorbic acid, proline, nonessential amino acids, HEPES buffer, and antibiotics at 37°C and 5% CO2.

Injurious compression: Three days after harvest, cartilage explants were compressed three at a time in a custom-designed chamber placed in an incubator-housed compression apparatus [5]. A single compression was applied under displacement control at a strain rate of 1 s-1 (corresponding to a velocity of 1 mm/s) to a strain of 50%. Compression was held for 5 minutes and released. Cartilage was then either returned to culture in fresh medium (1x-load protocol), or the compression was repeated 6 times with 25 minutes rest between each compression (6x-load protocol). TUNEL staining and nuclear morphology: As reported previously [5], cartilage explants were flash-frozen in liquid nitrogen 4 days after injury and sectioned. At least 100 cells were assessed on each section for apoptotic cell death by TUNEL staining and, on the same sections, by morphology on light microscopy (by the presence of nuclear blebbing). Electron microscopy: A separate group of explants from nearby locations on the same joint were subjected to injurious compression and fixed 4 days later in 0.1 M sodium cacodylate, 2% glutaraldehyde, and 0.7% ruthenium hexamine trichloride [8]. The disks were prepared for EM analysis by post-fixation in a solution of osmium tetroxide followed by dehydration in ethanol and embedding in Epon 812. Thin sections (65 nm) were then cut and stained with uranyl acetate and lead citrate. Two sections were made from each explant disk and in each section approximately 250 cells from the central and edge portions of the section were classified by morphology as either normal, necrotic, or apoptotic.

Results
The peak stress produced during injurious compression was 11 ± 4 MPa (mean ± st. dev.). As previously reported for this experiment [5], in cartilage explants subjected to the 6x-load compression protocol, 70 ± 4% (mean ± sem, N = 4) of chondrocyte nuclei were stained positive for TUNEL and were positive for the presence of nuclear blebbing, compared to 14 ± 4% of chondrocyte nuclei in unloaded control explants (p < 0.001), (Fig. 1). In the cartilage explants assessed by EM, 28 ± 4% (mean ± sem, N = 4) of chondrocytes were found to be apoptotic after 6x-load injurious compression, compared to 8 ± 2% of the chondrocytes in control explants (p < 0.005), (Fig. 2). Results for apoptosis after the 1x-load protocol were very similar (31 ± 4% after injury vs. 5 ± 2% in controls). Necrotic cells were found to comprise approximately 1% of the total dead cell populations. Examples of some of the criteria used for cell classification by EM are shown in Fig. 3. In contrast to the intact membranes and normal organelle structure of a viable cell (Fig. 3C, 2500x), an apoptotic cell (Fig. 3A, 3300x) has a nucleus with condensed chromatin along the nuclear membrane and prominent nuclear blebbing. The cytoplasm is condensed and clear and the cell membrane appears intact though with blebbing present. In contrast, a necrotic chondrocyte (Fig. 3B, 3300x) has an amorphous, osmophilic, and vacuolated cytoplasm with no clear remaining structural organization.

Discussion
Our previous studies demonstrated an increase in apoptosis after injurious mechanical compression by TUNEL staining and by nuclear morphology on light microscopy [5]. The present study extends these data by, first, confirming the increase in apoptotic cells by examination of cell morphology on electron microscopy. In this experiment, a significant increase in apoptosis was seen by all three methods used. Secondly, and most interestingly, the cell death seen on EM was identified as predominantly apoptotic for these loading conditions. In this study, the percentage of apoptotic cells was higher as assessed by TUNEL compared to those assessed by EM. It seems unlikely that this is due to false-positive TUNEL staining because of the high concordance with nuclear morphology on light microscopy in this experiment [5]. As different cartilage explants were used for the two analyses, this is most likely due to disk-to-disk variability of the effects of the loading protocol (e.g., due to variable compressive moduli giving rise to different peak stresses when subjected to the same final strain). It is also interesting that the relative increase in apoptotic cells appeared similar in the two groups. Electron microscopy images verified that increased apoptotic cell death was seen after either the 1x or the 6x loading protocols. This suggests that cell death was caused by the initial compressive injury and was not substantially affected by subsequent loading to the same level of displacement. Taken together, our data confirm that injurious compression of bovine calf articular cartilage produces an increase in apoptotic cell death. Whether traumatic joint injury or osteoarthritic chondrocytes also respond to mechanical insults via an apoptotic pathway requires further study as the mechanical and biological environment of the cell will differ from the model used here. However, this study demonstrates that chondrocytes have the ability to sense mechanical injury, either through direct effects of compression, or through altered extracellular matrix interactions, and to respond with programmed cell death.

References

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