NECROSIS FOLLOWED BY APOPTOSIS IN ARTICULAR CARTILAGE AFTER REPETITIVE IMPACTS

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INTRODUCTION

Reductions in cartilage collagen/GAG deposition and in chondrocyte numbers (Burton-Wurster et al., 1982) are hallmarks of osteoarthritis (OA). A recent study (Blanco et al., 1998) suggests these changes in OA lesions are closely related to in situ apoptosis. Apoptosis (cell suicide) is characterized by chromatin condensation, DNA fragmentation, and budded cell membranes. This contrasts with necrosis, another type of cell death (cell murder), characterized by ruptured cell membranes and swollen nuclei, (Majno and Joris, 1995; DeFrancesco, 1999).

Excessive loading occurs in unstable joints. In vitro cartilage studies show that excessive load/impact can produce structural damage and OA-like changes including tissue swelling (Borelli, et al., 1997; Farquhar et al., 1996), increased collagen denaturation (Chen et al., 1999) and reduced GAG synthesis (Farquhar et al., 1996). It is important to know 1) if cell death occurs in impact-damaged cartilage; 2) the relationship between cell death and the severity of impact damage; and 3) the mode of cell death. Recently, apoptosis (Leoning et al. 1999) was shown in excessive sinusoidal loaded cartilage. We hypothesized that impact damage also would induce apoptosis in cartilage explants and that the number of dead cells would increase with the severity of injury.

MATERIALS AND METHODS

Cartilage: Normal cartilage was excised from the canine humeral joint with a 4 mm biopsy punch. Damage to normal cartilage was induced by 5MPa cyclic impacts at 0.3 Hz with a 30% loading cycle for 2, 20, or 120min (described in Chen et al., 1999), and incubated for 4, 48 or 144hr. Necrosis was induced in cartilage by four freeze (liquid N2) / thaw cycles. Apoptosis was induced by treatment with 50µg/ml mitomycin C (MMC) for 48hrs. Cartilage with OA lesions was obtained from 2-year-old canine dysplastic hips.

Cell Viability: Necrosis in normal, loaded, and freeze/thawed cartilage was evaluated less than 2hr post treatment by uptake of the cell membrane impermeable dye, propidium iodide (PI). The cellular hydrolysis activity dye, fluorescein diacetate (FDA), was used to assess the cell viability. The (live) explants were immersed in 40µg/ml PI and 1µM FDA for 5min before being sliced into ~200µm and viewed with a fluorescent microscope.

TUNEL and Nucleus Morphology Assay: To determine DNA fragmentation, explants were cryo-embedded in OCT and then cut into 6mm sections. The sections were then immobilized onto glass slides, air-dried, fixed, and stained with TUNEL assay (Boehringer-Mannheim, Indianapolis, IN) for DNA nicking. Since the cell membrane was compromised after TUNEL assay, the total numbers of nuclei could be determined by counter-staining with 50µg/ml PI. Fluorescence of TUNEL and PI was captured using a CCD camera. The percentage of TUNEL+ (v.s. PI+) cells was analyzed using NIH image 1.62. The adjacent slide was stained with 0.2mM Hoechst dye (#33342) for 5 min, and viewed with a fluorescent microscope at 1000x to determine the morphology of the nucleus.

Biosynthesis and Statistical Analysis: GAG synthesis in explants was measured via a 4 hr radiolabel with 40µC/ml S35-sulfate. Sample was then digested with papain to determine the total DNA (flurometric assay) and GAG content (DMMB assay). The GAG synthesis in live cells was determined by normalizing to the percentage of live (non-TUNEL+) cells. Non-paired Student's t-test was performed to determine the statistical significance (P<0.05).

RESULTS

When incubated for just 4 hours post loading, only a few cells in the superficial zone and fracture site of cartilage loaded for 120min were TUNEL+. But, after 48hr of incubation, TUNEL+ cells were seen in all regions (Fig. 1). TUNEL+ cells were also found in the superficial and middle zones of 2 and 20min-loaded cartilage after 48hr of incubation (Fig. 1). The percentage of TUNEL+ cells increased from 7.8% to 73.4% when the duration of loading increased from 2 to 120min. No further increase in TUNEL+ cells in loaded explants occurred between 48hr and 144hr of incubation post loading. Eighty-eight percent of cells in a freeze/thawed cartilage after 48hrs of incubation and 28% of cells in an OA lesion (Fig. 2a) were also TUNEL+. The GAG synthesis (normalized
to live cells) of loaded explants was consistently increased (Fig. 2b).

Vital stains showed that 32.4±8.3% cells in 120min-loaded cartilage and all cells in freeze/thawed cartilage were (PI+/FDA-) necrotic (Fig. 3a). Hoechst dye showed swollen nuclei in all regions of freeze/thawed explants (Fig. 3b) and many condensed and fragmented nuclei in the middle and deep zones of MMC–treated and 120min-loaded cartilage (Fig. 3c, d).

**DISCUSSIONS**

Although the TUNEL assay has been reported to be specific to apoptotic cells (Gold *et al.*, 1995), at least in the early stage, in this study we found that the TUNEL assay also stained necrotic cells in freeze/thawed cartilage as evidenced by cell vital stains and swollen nuclei. To avoid false-positives in the TUNEL assay, a second independent method (nuclear morphology, cell vital stain, and DNA ladder) should be used (DeFrancesco, 1999). Since by vital staining and nuclear morphology, 40% of dead cells in the loaded tissue were necrotic, we reject our hypothesis. But, some cells in the deep zone of the loaded explant after 48hrs of incubation were TUNEL+ and showed clear features of apoptosis (nuclear fragmentation). This result indicates repetitive impacts induce necrosis (PI+/FDA-) followed by apoptosis in damaged cartilage. The percentage of dead cells increases with the severity of impact-injury. Work is in progress to determine the relative contribution of necrosis and/or apoptosis to characteristics of OA lesions.

**REFERENCES**


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