INJURIOUS COMPRESSION OF BOVINE ARTICULAR CARTILAGE INDUCES CHONDROCYTE APOPTOSIS BEFORE DETECTABLE MECHANICAL DAMAGE

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Introduction: Traumatic joint injury is a known risk factor for development of secondary osteoarthritis (OA). *In vitro* articular cartilage (AC) injury models have produced OA-like degenerative changes, including tissue swelling [2,3,5], elevated sulfated glycosaminoglycan (sGAG) release [5], reduced cell viability [4,6] and reduced biosynthetic activity [2,4]. These models emulate high-energy injuries, with loading sufficiently severe to induce macroscopic tissue damage. Relatively little is known about the effects of less severe loading, which may induce more subtle mechanical and biological changes. Quinn *et al.* [5] observed condensed nuclei in severely injured AC explants, and increased numbers of apoptotic chondrocytes have been found in human OA tissue compared to normal cartilage[1], suggesting that apoptotic cell death may be an important event in OA pathogenesis. To investigate whether compressive injury could induce apoptosis in healthy tissue, we assayed for apoptosis and other biological and mechanical measures of tissue damage for graded levels of injurious compression.

Methods: Cartilage disks (3x1mm) were obtained from the femoro-patellar groove of 1-2 week old calves and maintained in DMEM with 10% FBS, 25 μg/ml gentamicin, and 0.1 mM ascorbic acid at 37°C, 5% CO_2. Anatomically matched disks were assigned to free-swelling control or experimental groups. ***Compression protocol:*** Graded levels of injury were applied to experimental disks in an unconfined compression mode by ramping at 1 mm/s (100%/s) to a final strain level of 30-50% (peak stresses from 2-30 MPa) using an incubator housed compression apparatus. Six compression-release cycles (5 min. on, 25 min. off) were applied, except for the most severely loaded group for mechanical testing (1 cycle). Disks were then cultured free-swelling for an additional 0-6 days. ***Apoptosis:*** Disks from a relatively mild loading condition (30% strain; 4 MPa; n=4) were flash-frozen in liquid N_2 four days after compression and serially sectioned into 8 μm sections (~125 sections/disk). The sections were then immobilized onto glass slides, air-dried, fixed, and stained via TUNEL for the presence of apoptotic nuclei (ApopTag peroxidase *in situ* apoptosis detection kit, Oncor, Gaithersburg, MD). All sections were scored blind (0-3) for stained nuclei, with staining only at the periphery considered negative (cutting artifact, 0 or 1), and staining in the bulk of the tissue considered positive (2 or 3). The number of positive sections (a score of 2-3) was expressed as a percentage of the total number of sections from each cartilage plug. ***Vital dye staining:*** Multiple ~200 μm slices from experimental and control disks were immersed 2-6 days after compression in an 18 μM ethidium bromide, 250 μM fluorescein diacetate PBS solution and viewed with a fluorescent microscope.

Biochemistry/Metabolism: Changes in wet weight and release of sGAG and nitric oxide (NO) into the media were measured for relatively mild (35% strain; 7-8 MPa; n=12) and severe (50% strain; 16 MPa; n=6) compressions over 6 days after loading. Wet weights were measured daily by patting the disks with sterile gauze and weighing. Conditioned media was assayed for sGAG by the DMMB dye method and for NO by the Griess reaction (Serum-free DMEM without phenol red was used in the experiment for which NO was assayed). Residual biosynthetic activity was measured via a 20 hour radiolabel with 6 μCi/ml ^{35}S-sulfate and 20 μCi/ml ^{3}H-proline beginning 5 days after compression (n=6). ***Mechanical Properties:*** Initial mechanical changes were assessed for 4 conditions (n=4-20) ranging from severe (55% strain; 24 MPa) to relatively mild (30% strain; 2-4 MPa). Matched disks were transferred to a protease inhibitor PBS solution and tested in uniaxial confined and unconfined compression modes using a Dynastat mechanical spectrometer. A series of ramp/relaxation compression steps were applied and dynamic stiffness (.01-1 Hz) measured at 0.92 mm thickness. Equilibrium moduli were computed using the relaxed stress values at the end of each step. ***Statistics:*** Control and experimental groups were compared using paired t-tests with a significance level of $p<0.05$.

Results: Relatively mild injurious compression (30%; 4 MPa) of bovine cartilage explants resulted in a significant increase in apoptotic nuclei (Fig. 1). In both experimental and unloaded control disks, numerous cells near the cut edge stained positively for apoptosis as previously reported [7]. In contrast, a dramatic increase in apoptotic nuclei was observed in the central region of the majority of sections from these loaded disks.

Under similiar conditions, viability as measured with vital dyes was generally greater then 95%, with similar artifacts near the cut edges. Neither sGAG release nor wet weight significantly differed from control values at any time point (Fig. 2), although ^{3}H-proline and ^{35}S-sulfate incorporation were reduced by 12% ($p<0.05$) and 13% ($p=0.1$), respectively. For the more severe injurious compressions, viability rarely exceeded 25%, sGAG loss rates increased significantly for the first three days following compression, and wet weights were higher significantly than controls for all days following compression (Fig. 2). To the limits of our assay, NO release rates had a marginally significant increase ($p=0.1$) for only the first day after the severe compression. Incorporation of ^{3}H-proline and ^{35}S-sulfate were significantly reduced by 49 and 60%, respectively after the severe compression.

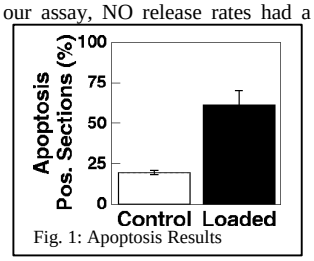

Fig. 1: Apoptosis Results

No significant changes were found in either the equilibrium or dynamic *confined* compression moduli for any loading condition, although a nonsignificant trend toward degraded stiffness with increasing loading severity was observed (Fig. 3). In contrast, significant reductions were noted in the equilibrium *unconfined* compression modulus for peak injurious compression stresses at and above 12 MPa and in the dynamic unconfined compression modulus for peak compression stresses at and above 7 MPa. No significant differences from control disks were noted at the mild stress level comparable to that for which the apoptosis assay was done (n=20, $p>0.5$).

Discussion: The key finding of this study was a marked increase in the number of apoptotic cells in injuriously compressed cartilage. In particular, we found apoptotic cell death at loading levels that produced no changes in sGAG release, NO release, vital dye staining, wet weight or mechanical properties. The observation that apoptosis precedes these changes suggests that this could be one of the earliest events in response to injury. It has been proposed that reduced cellularity in OA tissue is achieved through apoptotic cell death [1]. Our findings are consistent with this concept, and suggest a possible paradigm for the development of traumatically induced cartilage degradation. Even low levels of cartilage injury may induce chondrocyte apoptosis without producing any immediate extracellular matrix damage or functional impairment. Ongoing studies are examining apoptosis under a wider range of injurious loading conditions.

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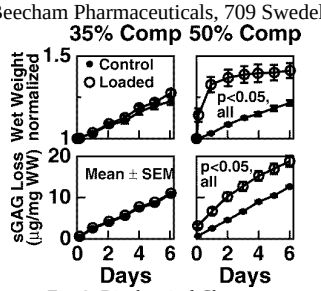

Fig. 2: Biochemical Changes

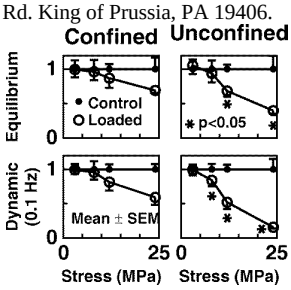

Fig. 3: Mechanical Changes

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