

MECHANISMS OF ACUTE TISSUE DEGRADATION FOLLOWING IN VITRO CARTILAGE INJURY

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INTRODUCTION: Injury and Cartilage Degeneration

Degenerative joint diseases, such as osteoarthritis (OA), result in degradation of articular cartilage, characterized by release of structural components from the tissue and subsequent loss of tissue integrity and mechanical properties¹. While the precise etiology of OA is unknown, its prevalence increases with age. Consistent with observations that the mechanical environment of cartilage influences its cellular biosynthesis^{2,3}, abnormalities in joint loading, such as those due to obesity, joint laxity, or altered joint geometries (e.g., dysplasia⁴), have also been recognized as predictors of OA⁵.

Traumatic joint injury has also been postulated to be a precursor of OA development, though the long-term evolution from joint injury to a disease state is poorly understood. In the hours following injury, the level of degradative enzymes such as stromelysin (matrix metalloproteinase-3; MMP-3) in the synovial fluid increases up to 40-fold over normal levels, and elevated levels of MMP-3 persist for up to 20 years following injury⁶⁻⁸. Further analysis of post-injury synovial fluid reveals fragments of cartilage proteoglycans that appear to be cleaved by MMPs and aggrecanases and are similar in structure to the proteolytic fragments observed in joint fluids of patients with OA⁹⁻¹¹.

Because joint injury is a complex phenomenon, involving high amplitudes and complex modes of loading, and potentially, multiple joint tissues, *in vitro* models of acute cartilage injury have been developed by several groups to allow more precise control of the tissue geometry, loading patterns, and incubation conditions. The parameters used in "injurious compression" experiments vary, but *in vitro* cartilage injury generally results in cell death (necrosis, apoptosis, or both), release of cartilage proteoglycans, increased tissue water content and

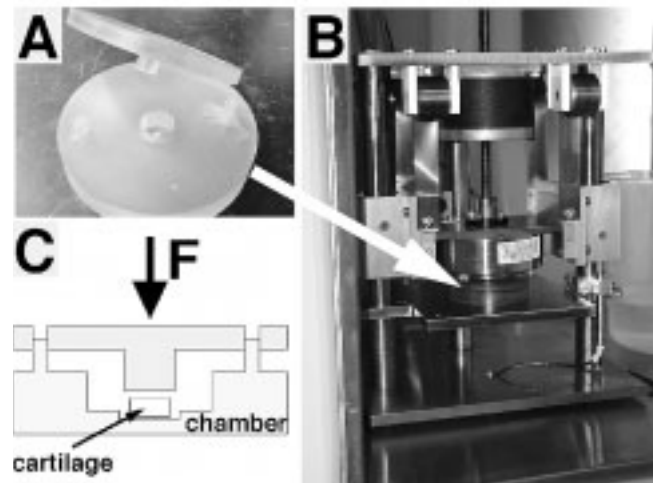


Figure 1: Injurious compression system. Cartilage samples are loaded into a poly-sulfone chamber (A), which is inserted into an incubator-housed loading apparatus (B). When a force is applied to the top of the chamber (C), the cartilage inside is compressed.

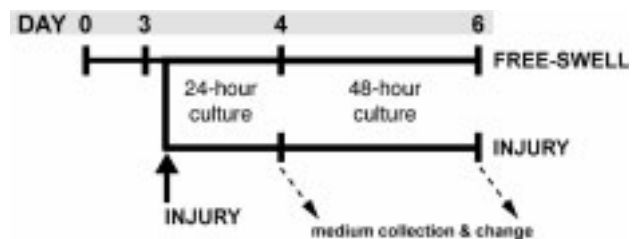


Figure 2: Experiment Timeline

swelling, decreasing mechanical functionality, and increased sensitivity to cytokines¹²⁻¹⁷, characteristics reminiscent of early stages of degenerative joint disease¹. Although OA progression *in vivo* occurs over a period of years, these injury-induced changes can appear on a relatively short (hours to days) time scale following *in vitro* injury. It is possible that the short-term effects of applied loading may involve direct damage to the cartilage extracellular matrix, initiation of cell-mediated tissue destruction, and accelerated transport of degraded matrix from the tissue, any or all of which may be important in long-term tissue changes.

The objective of our studies is to determine whether acute release of cartilage matrix molecules during the first hours and days after injury is mediated by cellular biosynthesis (of matrix molecules and/or degradative enzymes), instigation of proteo-

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lytic activity, mechanical disruption of the cartilage extracellular matrix, or a combination of these. For this approach, we have utilized an *in vitro* cartilage injury model, in which bovine cartilage explants are subjected to compressive stress using an incubator-housed loading apparatus¹⁸, then returned to free-swelling culture (Figure 1). The condition of the cartilage is then followed for several days post-injury, and the release of matrix molecules to the culture medium is monitored by a series of biochemical assays (Figure 2). Determination of the injury-induced changes in cartilage composition and metabolism could elucidate the progression from joint injury to joint-scale tissue degradation.

METHODS

Cylindrical cartilage samples (3 mm diameter x 1 mm thick) were prepared from the patellofemoral grooves of immature bovine knee joints. Location-matched samples were distributed among experimental groups, and cultured for 2-3 days in medium (DMEM + 20 µg/mL ascorbate and antibiotics) with 10% fetal bovine serum. On Day 3, medium was changed to be serum-free (medium with 1% ITS-A). For inhibitor experiments, culture medium was also supplemented at this point with inhibitors of protein translation (cycloheximide) or MMP activity (one of two broad-spectrum hydroxamate MMP inhibitors: GM6001¹⁹ or CGS 27023A²⁰), and the efficacy of these inhibitors was tested in parallel experiments in free-swelling culture. In injury studies, cartilage samples were allowed to equilibrate in medium with or without inhibitors for 6 hours before being subjected to either *in vitro* injury (1 uniaxial unconfined compression to 50% thickness, at a strain rate of 1 mm/s, followed by immediate release of load) or maintained in a free-swelling state. Following compression, injured tissue was returned to culture in fresh serum-free medium with or without inhibitors. Culture media were collected and replaced at 24 and 72 hours post-injury, and collected media were frozen before biochemical analysis. To determine the effect of injury on protein and glycosaminoglycan biosynthesis, media were supplemented at various timepoints with radiolabeled precursors to these species (³H]proline and [³⁵S]sulfate, respectively).

On termination of cultures, tissue samples were processed for analysis of radiolabel incorporation. Media samples were assayed for content of sulfated glycosaminoglycan (sGAG), as an indicator of matrix release.

RESULTS

Application of this *in vitro* injurious compression protocol generated peak stresses around 20 MPa within the tissue. Gross inspection of the tissue samples immediately following compression showed that ~50% of the samples had assumed an ellipsoidal geometry, and this shape change persisted through the remainder of the culture period. However, no sample was observed to have macroscopic tissue fissuring after loading. Staining of tissue with cell viability dyes indicated that qualitatively, cell death in injured samples was localized predominantly to the peripheral regions of the samples, where the unconfined compression protocol induces high tissue tensile strain and high fluid flow during compression²¹. Overall, sGAG

and protein biosynthesis was reduced in injured samples compared to uninjured samples, which can be partially explained by the observed decrease in cell viability.

Inspection of conditioned culture medium revealed that sGAG was released from both free-swelling and injured cartilage, with much higher rates of release from injured samples. The appearance of sGAG in the culture medium of noninjured samples is thought to be reflective of normal matrix turnover mediated by the cells within the tissue. During the first 24 hours after injury, the amount of sGAG released to the culture medium was approximately twice that released from uninjured samples over the same time period, and amounted to approximately 5% of the total tissue content. Closer inspection revealed that the rate of sGAG release was higher during the first 4 hours than during the next 24 hours. The nearly-immediate nature of this matrix release suggested the possibility that injury-induced mechanical damage may be a mediator of short-term cartilage matrix degradation. Furthermore, the presence of inhibitors of protein translation and MMP activity were unable to reduce the matrix release during this time period.

During the subsequent 48 hours, the amount of sulfated GAG released to the culture medium from injured cartilage approximated that from uninjured samples. However, unlike in the most acute case, sGAG release from injured cartilage during this period was sensitive to the inclusion of inhibitors in the culture medium. Inhibition of protein translation using cycloheximide had no effect, but one of the two MMP inhibitors (CGS 27023A) reduced sGAG release by 20% compared to injured but untreated controls, while having no discernible effect on the magnitudes of protein or glycosaminoglycan biosynthesis.

DISCUSSION AND ONGOING WORK

Taken together, the preliminary results of this work suggest that in the hours after cartilage injury, the release of structural matrix components from cartilage to the surroundings may be predominantly an effect of mechanical damage to the cartilage, rather than to cell-mediated processes. Measurements at early timepoints after injury indicate a burst of sGAG release, followed by slower matrix degradation. The lack of effect of inhibitors of protein translation and matrix metalloproteinase activity in the acute post-injury phase further supports a mechanism based on mechanical damage.

However, the effect of the matrix metalloproteinase inhibitor CGS 27023A at later timepoints suggests that the activity of catabolic enzymes may be important in the longer-term response to injury. It is interesting to note that inhibitors of protein translation did not reduce sGAG release during this time period, which implies that the catabolic activity inhibited by CGS 27023A may be due to a population of latent MMPs present in the matrix prior to injury, rather than proteolytic enzymes synthesized in response to injury. Experiments in which p-aminophenylmercuric acetate (APMA), a chemical activator of MMPs, is added to cartilage show a dramatic increase in cartilage matrix degradation, even in the absence of protein biosynthesis, illustrating the possibility of such a latent

enzyme population²². Ongoing studies are investigating this possibility further.

Since it is known that the sGAG chains on aggregated proteoglycans within cartilage represent the main barrier to molecular diffusion, it is likely that the removal of a portion of these molecules from the tissue by the initial injury could facilitate access of degradative enzymes to their substrates within the tissue, allowing a progression of cartilage degradation.

It is not yet known whether the released sGAG molecules represent proteolytic fragments, or rather full-length proteoglycan molecules that could be released by damage to the collagen network. Even though no macroscopic tissue fissures were observed, it is likely that molecular damage of the collagen network is responsible for the observed shape changes in injured samples. Studies are planned to determine the size distribution of sulfated GAG-containing species released to culture medium, and proteolytic fragments will be identified using antibodies generated against cleavage sites of specific degradative enzymes (MMPs and aggrecanases).

It is clear that “joint injury” *in vivo* involves more than the isolated cartilage samples as studied in this idealized *in vitro* system, but important insights into the progression from trauma to degenerative joint disease may be gained from this type of work. It is well-known that cartilage does not heal well following injury, and the presence of a population of nonviable cells in the current model system suggests that injury induces cell death, which may reduce the capacity of the tissue for repair. Also, it has been previously observed that increased levels of degradative enzymes exist in the synovial fluid of injured joints, and that proteolytic matrix fragments also appear in the synovial fluid following trauma, and it is possible that inhibition of these catabolic activities could slow matrix degradation as was observed in the present system. By adding layers of complexity to this model system (such as inclusion of other joint tissues, cytokines, more complicated loading during and after injury), further understanding of the progression of post-traumatic secondary osteoarthritis may be achieved.

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