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Journal of Orthopaedic Research 19 (2001) 1140–1146

Journal of Orthopaedic Research

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Biosynthetic response and mechanical properties of articular cartilage after injurious compression

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Received 9 May 2000; accepted 31 January 2001

Abstract

Traumatic joint injury is known to produce osteoarthritic degeneration of articular cartilage. To study the effects of injurious compression on the degradation and repair of cartilage in vitro, we developed a model that allows strain and strain rate-controlled loading of cartilage explants. The influence of strain rate on both cartilage matrix biosynthesis and mechanical properties was assessed after single injurious compressions. Loading with a strain rate of 0.01 s^{-1} to a final strain of 50% resulted in no measured effect on the cells or on the extracellular matrix, although peak stresses reached levels of about 12 MPa. However, compression with strain rates of 0.1 and 1 s^{-1} caused peak stresses of approximately 18 and 24 MPa, respectively, and resulted in significant decreases in both proteoglycan and total protein biosynthesis. The mechanical properties of the explants (compressive and shear stiffness) were also reduced with increasing strain rate. Additionally, cell viability decreased with increasing strain rate, and the remaining viable cells lost their ability to exhibit an increase in biosynthesis in response to low-amplitude dynamic mechanical stimulation. This latter decrease in reparative response was most dramatic in the tissue compressed at the highest strain rates. We conclude that strain rate (like peak stress or strain) is an important parameter in defining mechanical injury, and that cartilage injuriously compressed at high strain rates can lose its characteristic anabolic response to low-amplitude cyclic mechanical loading. © 2001 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

Introduction

Traumatic joint injury has been demonstrated to be a risk factor for development of secondary osteoarthritis [4], but the precise mechanism by which this occurs is unknown. Studies of in vivo animal models have demonstrated that high-impact loads to the knee joint can induce cartilage degradation [16,19,21,22,28,29]. Attempts to model this process in vitro have led to the investigation of the effects of compressing cartilage tissue using loading conditions sufficient to produce acute injury [6,9,14,20,24,27,30].

To simulate a rapid impact injury having a known impact energy, one approach has been the use of a drop-tower apparatus [9,24]. However, such an approach does not allow one to study or control separately the displacement or stress waveform applied to the cartilage during compression. Thus, recent studies have used systems capable of controlling load or displacement during injury. In these experiments, the controlled variable in defining the injury has been either peak stress [6,30] or final strain [14,20]. Chen et al. [3] used a repetitive impact model in which a desired peak stress could be achieved using two different loading rates; they found that the level of injury depended on which loading rate was used.

Since the pathway leading from joint injury to OA is not well understood, it is important to quantify parameters of tissue damage as well as measures of cell metabolism and biosynthesis. Previous studies in vitro have therefore focused on tissue swelling, compressive strength [14] and, most recently, denatured collagen strand neoepitopes [3] to quantify specific damage to the collagen network during injury. Previous studies have also demonstrated that compression affects the chon-

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PII: S0736-0266(01)00033-X

drocytes themselves. Specifically, injurious compression may increase cell death and decrease biosynthesis [3,10,14,20,30].

In contrast, moderate (low-amplitude) dynamic compression of cartilage over a range of frequencies can increase chondrocyte biosynthesis of matrix macromolecules [12,18,26] and upregulate expression of aggrecan and type II collagen [23]. We hypothesized that a further consequence of injurious mechanical compression would be the inability of chondrocytes to respond in a stimulatory fashion to moderate dynamic compression, i.e., the failure of a potential reparative response associated with moderate loading.

Therefore, the objectives of this study were: (1) to quantify the effect of strain rate on certain biomechanical and biosynthetic measures of tissue injury, and (2) to examine the ability of chondrocytes to recover from an injury by quantifying the response of chondrocytes to subsequent low-amplitude dynamic compression.

Materials and methods

Articular cartilage explants

Articular cartilage disks were obtained from the femoropatellar groove of 1–2 week old calves as previously described [25]. In brief, cartilage-bone cylinders (9 mm in diameter) were drilled perpendicular to the cartilage surface and placed in a microtome holder. After creating a level surface by removal of the most superficial ~100 μm, the next 2 mm of articular cartilage were sliced by the microtome to produce two 1 mm thick slices. Four explant disks (3 mm in diameter × 1 mm thick) were punched out of each slice and equilibrated for 24 h in culture medium (low glucose DMEM supplemented with 10% FBS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/ml ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in a 37°C, 5% CO_2 environment. The four 3-mm diameter disks from each 9-mm diameter slice were distributed to four groups: 0.01, 0.1, and 1 s^{-1} strain rates, and controls.

Mechanical injury

Mechanical compression was applied to groups of four cartilage explants one day after dissection (Fig. 1). The explants were placed in chambers during (radially unconfined) compression by an incubator-housed loading device, as described previously [7,12]. The explants were initally held at their original cut thickness of 1 mm. Controlled displacement ramps to 50% final strain (500 μm) were then applied to the four disks simultaneously at rates of 0.01, 0.1, or 1 s^{-1} (corresponding to ramp velocities of 0.01, 0.1, and 1 mm/s, respectively), and held at the final strain such that the total time of compression was five minutes for each group. We henceforth refer to compression of the cartilage explants with this protocol at any of the three strain rates as "injury". After injury, the explants were maintained in culture for an additional 6 h or 3 days, and biochemical or mechanical measurements were made as described below.

Biochemical and biosynthesis studies

Wet weight measurements were taken before injury, 6 h after injury, and 3 days after injury. Radiolabel incorporation was measured 6 h and 3 days after injury by incubating explants in fresh culture medium containing 10 μCi/ml $^{35}SO_4^{-2}$ and 20 μCi/ml ^{3}H-proline for 6 h. After culture with radiolabel, the explants were washed three times over 60 min in 1 ml phosphate-buffered saline (PBS) supplemented with 0.8 mM sodium sulfate and 0.5 mM proline to remove free radiolabel, and digested in 1 ml protease K solution (100 μg/ml in 50 mM Tris-HCl and 1 mM $CaCl_2$ at pH 8) at 60°C for 12–18 h. Aliquots of the digest were analyzed for radiolabel incorporation in a liquid scintillation counter and corrected for spillover. Assuming equivalent rates of incorporation for labeled and unlabeled proline and sulfate, the fraction of radiolabel incorporated was multiplied by the concentration of sulfate and proline in the culture medium to calculate the incorporation of each substance. Incorporation was then expressed as pMol incorporation per hour per mg wet weight before injury, and normalized to the incorporation rate of uninjured control tissue. The glycosaminoglycan (GAG) content of the digested samples, as well as the GAG content of the conditioned medium, was measured using the dimethylmethylene blue (DMMB) dye-binding assay.

In separate experiments, the biosynthetic response of injured cartilage to low-amplitude dynamic mechanical stimulation was measured three days after injury. Explant disks from each of the four sample groups (0.01, 0.1, and 1 s^{-1} strain rates and uninjured controls) were subjected for 12 h to either dynamic compression (3% dynamic strain amplitude at 0.1 Hz superimposed on a 10% static offset strain) or static compression alone at the same static offset strain (10%). During the 12-h dynamic compression, the cartilage disks were incubated with fresh culture medium containing 10 μCi/ml $^{35}SO_4^{-2}$ and 20 μCi/ml ^{3}H-proline. The incorporation rates of the dynamically compressed explants were normalized to their corresponding static controls for each injury group.

Two additional experiments were performed to assess cell viability 3 days following injury. Viability was assessed by staining with a solution of 5 μg/ml ethidium bromide (EtBr) and 50 μg/ml fluorescein diacetate (FDA) in PBS. EtBr enters only cells which have lost the integrity of the cell membrane. Once inside the cell, EtBr forms a red

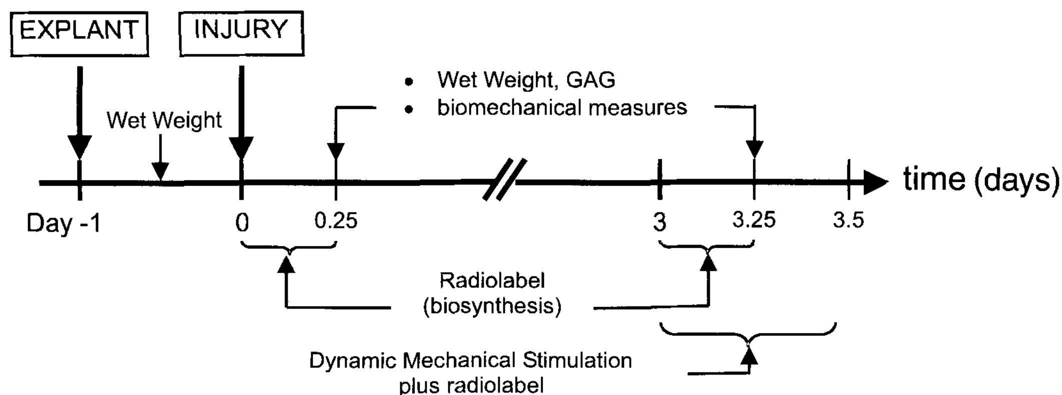

Fig. 1. Schematic timeline of the experimental design for study of the effects of injurious compression on biomechanical properties, biochemical changes, and subsequent response to moderate dynamic compression. Measurements at days 0 and 3 were made with separate groups of explants.

fluorescent complex inside the nucleus [5]. FDA readily crosses the cell membrane and is enzymatically converted by intracellular esterases into a green fluorescent, anionic product which can no longer leave the cell by crossing the cell membrane [2]. Following culture, cartilage explants were sectioned with a scalpel blade into slices approximately 200 μm thick. The slices were stained with 10 μl of the dye solution and incubated in the dark at room temperature for 2 min. Under a 10× microscope objective, red and green staining cells in a 400 × 300 μm^2 box were tabulated. As cells on the perimeter of the cut slice, as well as those cells immediately surrounding any gross fissure or disruption, are all nonviable, care was taken to ensure that the field of vision did not include these areas. Cell viability was then calculated as the number of green-staining cells over the total number of cells counted.

Measurement of mechanical properties

The mechanical properties of the explants were measured either 6 h or 3 days post-compression. The four explants in each injury or control group were simultaneously subjected to uniaxial unconfined compression in a polysulfone chamber as described above. Three sequential compression ramps (to 20%, 23%, and 26% strain) were applied, and after each ramp, the equilibrium stress attained after a 5-min hold was measured. The equilibrium compressive stiffness was calculated from linear regression of equilibrium stress vs. strain. Dynamic compressive stiffness was then measured during application of a sinusoidal compression (3% dynamic strain amplitude for three periods) superimposed on the 26% static offset strain, at frequencies ranging from 0.1 to 1 Hz. Similarly, the equilibrium shear modulus was measured by simultaneous application of simple shear strain to all four explants in each group, as described in detail elsewhere [7]. Disks were subjected to three sequential steps of 1% shear strain, each held for five minutes, at an axial compressive offset of 26%. Subsequent application of sinusoidal shear strain (3% dynamic shear amplitude at frequencies from 0.1 to 1 Hz) enabled measurement of the dynamic shear modulus.

Statistics

Differences among groups were tested with one-way analysis of variance (ANOVA) followed by a Dunnett test to compare loaded groups to the unloaded control group. To compare differences among treatment groups for the ratio (r) of the mean radiolabel incorporation (x) divided by the mean cell viability (y), we used a first-order Taylor series approximation of the deviations of estimates from their expected values, known as the delta method [13]. Since the estimates of x and y are independent, the asymptotic variance of r is approximated by $\mathrm{var}(r) \approx [\mathrm{var}(x) + r^2\mathrm{var}(y)]/y^2$. We assumed that the resulting estimated standard errors were approximately equal to the true standard errors and used a two-sample z test to compare ratios between different treatment groups.

Results

Single injurious compression

Representative stress–strain curves during injurious compression of cartilage disks at three different strain rates (to 50% final strain and held for a total of 5 min) are shown in Fig. 2. The peak stresses produced by compression ranged from ~12 MPa at a strain rate of 0.01 s^{-1} up to ~24 MPa at 1 s^{-1} (Fig. 3).

Biosynthetic activity and cell viability after injury

^{35}S-Sulfate and ^{3}H-proline incorporation in disks subjected to a strain rate of 0.01 s^{-1} was not significantly different from that in uncompressed control disks at 6 h and 3 days after injury (Fig. 4(A)). Cartilage disks subjected to strain rates higher than 0.01 s^{-1} showed decreased proline and sulfate incorporation in a manner dependent on strain rate. The decrease in biosynthetic activity compared to controls was significant for strain rates of both 0.1 and 1 s^{-1}. After injury at a strain rate of 1 s^{-1}, radiolabel incorporation was reduced to ~50% of control levels for proline and to ~35% of control levels for sulfate. No significant differences were observed between radiolabel incorporation 6 h and 3 days after injury at any applied strain rate.

Cell viability was measured three days after injury in two separate experiments, and the data pooled ($n = 5$–9 disks at each strain rate). Viability (mean ± SEM) was 99 ± 0.7% in control disks. This decreased to 98 ± 5% at 0.01 s^{-1}, 92 ± 3% at 0.1 s^{-1}, and 83 ± 6% at 1 s^{-1}. These results were used to recalculate the day-3 radiolabel in-

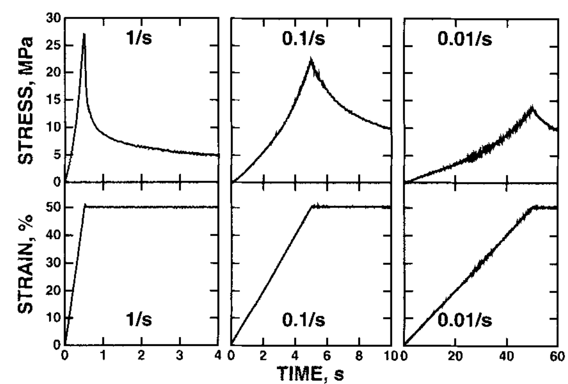

Fig. 2. Representative waveforms of applied strain and resulting stress versus time for the initial 4–60 s of a single 5-min injurious compression protocol. Four cylindrical explant disks (1 mm thick × 3 mm diameter) per group were compressed simultaneously at strain rates of 0.01, 0.1, and 1 s^{-1} until a final strain of 50% was achieved. Stress is computed as the total load divided by the initial uncompressed cross-sectional area of four disks.

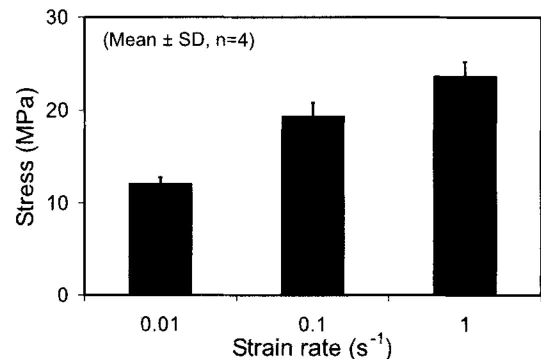

Fig. 3. Peak stress during simultaneous injurious compression of four cartilage explants to 50% final strain at three different strain rates (ANOVA: $P < 0.001$).

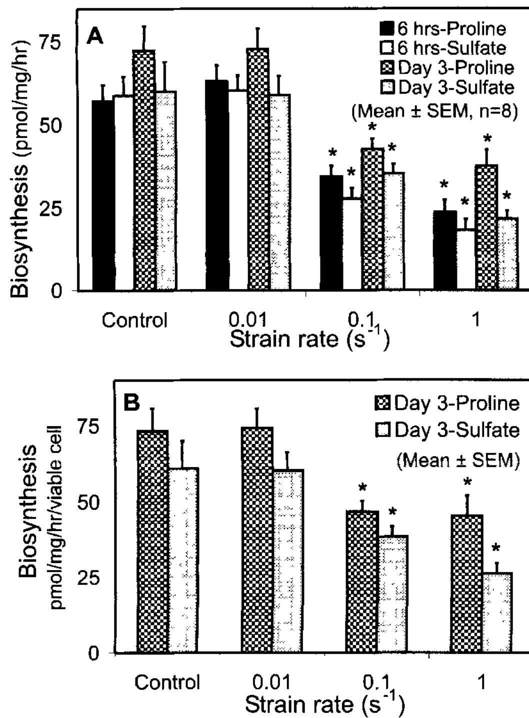

Fig. 4. (A) ^{35}S-sulfate and ^{3}H-proline biosynthesis per mg wet weight of cartilage explants at 6 hours and 3 days after injury. (ANOVA: all $P < 0.001$ for effect of strain rate; $^{*}P < 0.05$ compared to controls by Dunnett test). (B) Biosynthetic activity per viable cell 3 days after injury, calculated by dividing the mean radiolabel incorporation data of Fig. 4(A) by the mean cell viability of each group ($^{*}P < 0.05$ compared to controls by z-test).

corporation data of Fig. 4(A) to estimate the biosynthetic activity per viable cell. The proline and sulfate incorporation per viable cell three days after injury decreased with increasing strain rate, and remained significantly different from that of controls after injury at strain rates of 0.1 and 1 s^{-1} (Fig. 4(B)).

The biosynthetic activity of explants three days after injury was also assessed during a subsequent 12-h low-amplitude dynamic compression to examine the ability of the chondrocytes to respond to mechanical stimulation after injury. The data are normalized to cartilage disks which had received the same initial injurious compression, but then received only the 10% static offset compression, with no dynamic compression (Fig. 5). In uninjured controls, dynamic compression increased ^{3}H-proline incorporation by ~40% and ^{35}S-sulfate incorporation by ~25%, similar to that observed in previous studies [26]. In contrast, injuriously compressed disks showed reduced ability to respond to dynamic stimulation, and the response decreased with increasing strain rate. After injury at 1 s^{-1}, dynamic compression no longer stimulated radiolabel incorporation, and proline incorporation was significantly less than that of dynamically stimulated, uninjured control disks.

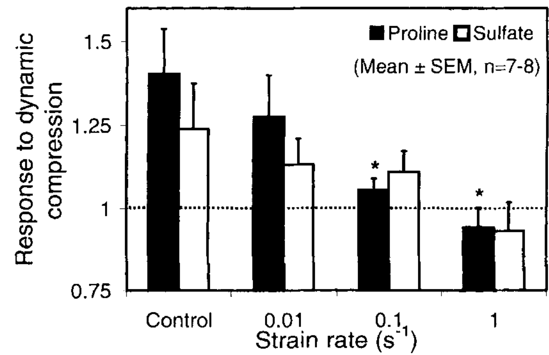

Fig. 5. ^{35}S-sulfate and ^{3}H-proline incorporation in cartilage disks during twelve hours of dynamic compression (3% dynamic strain amplitude at 0.1 Hz, superimposed on a 10% static offset compression), 3 days after injury. The data for each treatment are normalized to the radiolabel incorporation of explants which were subjected to injury at the same strain rate and then held at the 10% static offset without dynamic compression (dotted line). (ANOVA for proline: $P < 0.05$; ANOVA for sulfate: N.S.; $^{*}P < 0.05$ vs. dynamically stimulated, uninjured controls by Dunnett)

Tissue mechanical properties after a single injurious compression

After injury at 1 s^{-1}, the cartilage appeared damaged to gross visual inspection in approximately half of the disks. Damaged disks appeared elliptical in shape and were occasionally fissured. In contrast, no gross damage was seen after injury at 0.1 or 0.01 s^{-1}. The increase in tissue wet weight 6 h after injury increased with strain rate and was statistically significant after injury at 0.1 and 1 s^{-1} (Fig. 6). However, 3 days after injury, the increase was apparent only at 1 s^{-1}.

Compressive and shear stiffness of cartilage measured 6 h after injury at 0.01 s^{-1} was not significantly different than controls, but stiffness then decreased with increasing strain rate (Fig. 7). Equilibrium axial stiffness in unconfined compression (Fig. 7(A)) tended to decrease

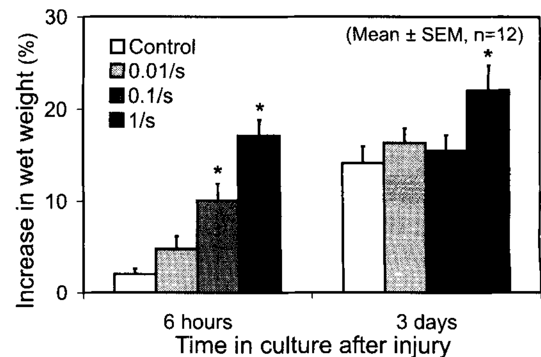

Fig. 6. Percentage increase of cartilage explant wet weight after injury. ANOVA showed a significant effect of strain rate at both 6 h ($P < 0.001$) and 3 days ($P < 0.05$) after injury ($^{*}P < 0.05$ vs. control by Dunnett). The mean wet weight before injury was ~8.1 mg.

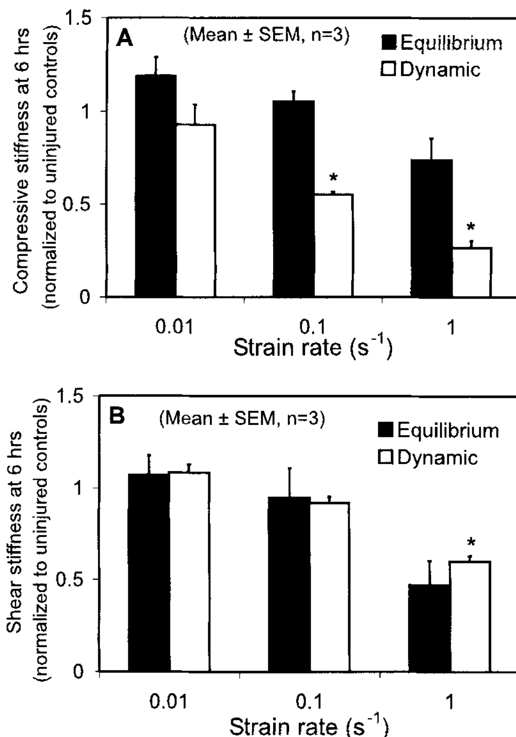

Fig. 7. (A) Equilibrium stiffness and dynamic stiffness (at 0.5 Hz) of groups of three cartilage explants measured in uniaxial unconfined compression 6 h after injury. The data are normalized to the mean stiffness of uninjured controls (equilibrium: 0.61 MPa; dynamic: 26 MPa). ANOVA showed a significant effect of strain rate (equilibrium: $P < 0.05$; dynamic: $P < 0.01$). (B) Equilibrium shear stiffness and dynamic shear stiffness (at 0.5 Hz) of groups of three cartilage explants 6 h after injury. The data are normalized to the mean stiffness of uninjured controls (equilibrium: 0.24 MPa; dynamic: 0.80 MPa). ANOVA showed a significant effect of strain rate for dynamic ($P < 0.01$) but not equilibrium stiffness ($P = 0.1$). * represents $P < 0.05$ vs. uninjured controls by Dunnett.

with increasing strain rate, although the effect was not significant by ANOVA. However, the dynamic stiffness in the 0.01 to 1 Hz frequency range (shown at 0.5 Hz in Fig. 7(A)) was significantly decreased after injury at 0.1 and 1 s^{-1}. Both the equilibrium and dynamic shear stiffness of the explants decreased with increasing strain rate, down by 50% after injury at 1 s^{-1} (Fig. 7(B)). There were no significant differences between compressive or shear stiffness values measured at 6 h and 3 days after injury (data not shown).

Glycosaminoglycan content of the tissue 3 days after injury tended to decrease with increasing strain rate, but ANOVA showed no significant effect. Uninjured controls contained 48 ± 4 μg GAG/mg wet weight, which decreased to 47 ± 2 μg/mg after injury at 0.01 s^{-1}, 45 ± 2 μg/mg at 0.1 s^{-1}, and 43 ± 2 μg/mg at 1 s^{-1} (mean ± SEM; $n = 8$). Cumulative GAG loss to the medium 3 days after injury showed the complementary trend, increasing with strain rate ($P < 0.01$ by ANOVA). After injury at 1 s^{-1}, cumulative GAG loss was 9.2 ± 0.8 μg/mg, compared to 6.9 ± 0.4 μg/mg in controls ($n = 12$).

Discussion

Our results demonstrated that a single injurious compression altered the mechanical properties of cartilage explants, chondrocyte biosynthesis, and chondrocyte response to dynamic compression in a manner that was dependent on the strain rate of the compression. In particular, this study demonstrated that injury affects not only the basal biosynthetic activity of chondrocytes, but also the ability of subsequent low-amplitude dynamic compression to upregulate biosynthetic activity.

A single injurious compression was found to have significant effects on the biosynthetic activity of the cartilage. Injury at strain rates higher than 0.01 s^{-1} produced a decrease in radiolabeled proline and sulfate incorporation, compared to free-swelling controls, at both 6 h and 3 days after compression. These results are consistent with past studies of the effects of single injurious compression on cartilage [10,30]. However, as demonstrated qualitatively by Torzilli et al. [30], a decrease in observed radiolabel incorporation (normalized to DNA content or wet weight) after injury could be due either to a decrease in cellular biosynthetic activity or to a reduction in the number of viable cells. We therefore quantified the cell viability of the tissue 3 days after loading in order to estimate the proteoglycan and total protein synthesis per viable cell. The results suggest that in addition to cell death after injury at high strain rates, the biosynthetic activity of the remaining viable cells may still be markedly reduced 3 days after injury. In contrast with these observations, Jeffrey et al. [10] reported that synthetic activity on a per cell basis recovered and exceeded control levels by three days for most impacts. These differences are most likely due to differences in the methods for quantification of cell viability after cell isolation in their study, which used enzymatic digestion of cartilage. Observations in our lab have led us to hypothesize that enzymatic digestion of tissue after injury may result in the loss of chondrocytes which, while initially viable, were too fragile to survive digestion.

A limitation of the FDA/ethidium bromide viability assay used here is that it may be an imperfect tool for assessment of cell death in such situations. Previous research has identified that injurious compression can cause apoptotic cell death [14], and it is unclear whether agents which assess membrane integrity (such as ethidium bromide and propidium iodide) will underestimate cell death due to apoptosis. In addition, the technique used here involving staining of tissue sections may introduce some bias in cell counting due to the difficulty in identifying red-staining nuclei and green-staining cells

corresponding to focal planes of identical thickness. Finally, some of the explants injured at the highest strain rate demonstrated a non-homogeneous pattern of cell death due to the presence of fissuring. Since cell viability was measured in regions of intact matrix, this could underestimate cell death in the explant as a whole. Nevertheless, we feel that these estimates are likely to be more accurate than those obtained by cell isolation.

In order to characterize further the effect of injurious compression on cellular biosynthetic response, we investigated the ability of injured chondrocytes to respond to dynamic compression. Normal chondrocytes respond to moderate or low-amplitude dynamic compression by upregulating biosynthetic activity [17,18,26], a property which may be an integral part of their ability to maintain a healthy tissue capable of withstanding compressive loads. We found that chondrocytes injured at the higher strain rates did not respond to dynamic mechanical stimulation, either because the cells had lost the ability to do so, or because damage to the extracellular matrix had disrupted the transduction of the physical signals which stimulate this response. It is important to note that biosynthesis after moderate dynamic compression in these injured plugs was compared to biosynthesis in control plugs which were injured using identical conditions, but not subjected to dynamic compression. Therefore, cell viability associated with injury is equivalent in the tested and control plugs. Furthermore, previous studies [12,26] have shown that the moderate dynamic compression protocol used in this experiment does not alter cell viability.

We also attempted to characterize further the effects of injurious compression on the cartilage tissue matrix itself. Many prior studies have noted that cartilage tissue swells in response to injury. Since the swelling of cartilage is essentially a result of the electrostatic repulsion forces of the charged proteoglycans, and opposed by the collagen network, swelling of the tissue after injury is thought to be due to damage to the collagen network [15]. As expected, our results show swelling of the tissue soon (6 h) after injury, and that swelling increased with increasing strain rate. In this study, unlike the studies of others [9,14], swelling of controls had increased to similar levels as injured plugs by three days after injury. It is known that after removal of these explants from native cartilage, culture in unconfined conditions leads to gradual but steady swelling in control tissue over a period of days to weeks [14]. In this experiment, while injury resulted in an immediate increase in swelling, by day three the swelling of the unconfined tissue appears to have obscured some effects of swelling due to injury.

In addition to the swelling of the tissue, we characterized the effects of injury on the mechanical properties of the tissue in both shear and unconfined compression. Stiffness measured in unconfined compression has been used in prior studies to characterize cartilage after injury [14,20] since the integrity of the collagen network plays an important role in this measurement. In this study, we also characterize the effect of injury on the shear stiffness of the cartilage, since the shear stiffness of the tissue is known to be very sensitive to the strength and integrity of the collagen network [31]. Although the proteoglycan content also contributes to shear strength [11,31], changes in proteoglycan content were relatively small among treatment groups in this experiment. Our results, demonstrating a decrease in mechanical properties before gross fissuring was visible (at a strain rate of $0.1\ s^{-1}$), suggests damage to the collagen network by injury at the higher strain rates used in this study. Further research with denatured collagen [3,8] and collagen II CTx neoepitopes [1] may clarify the molecular mechanisms behind this damage.

Different compression geometries, protocols, and specimen preparation (i.e., whether or not the cartilage is left on the subchondral bone) must all be kept in mind when comparing results among prior studies. In the presence of bone, several studies have demonstrated that much higher stresses are required to produce the same level of injury as in cartilage taken off the bone [9,24]. These studies may more accurately simulate certain conditions of the joint tissue in vivo. As a result, the precise strains, strain rates, and stresses reported in our study, performed with tissue removed from the bone and subjected to unconfined compression, should not be directly compared to the magnitudes of loading conditions in vivo. Our experimental design and related model systems used by others are rather intended to focus on mechanisms relating controlled mechanical parameters to observed changes in cells and matrix.

These results demonstrate the importance of reporting multiple parameters (i.e., strain, strain rate, and peak stress) when characterizing the injury to the cartilage of a single, controlled, compressive load. When cartilage was compressed to a final strain of 50% at three different strain rates varying over two orders of magnitude (0.01, 0.1, and $1\ s^{-1}$), the resulting peak stresses varied from approximately 12–23 MPa. There was little measured effect on the tissue when compressed at $0.01\ s^{-1}$ but significant injury to the tissue when compressed at $1\ s^{-1}$. Torzilli et al. [30] proposes a critical threshold for cell death and collagen damage in a single impact load at an applied stress of 15–20 MPa, and our data are consistent with this hypothesis. However, the study by Chen et al. [3] demonstrated that the rate at which stress is applied, in addition to peak stress, affects the amount of damage to the cartilage matrix, as quantified by swelling, in a cyclical loading model. One might hypothesize that this result could be expected if injury were related to peak power delivered to the tissue, or any suitable combination of either peak strain and strain rate or peak stress and stress rate. Thus, our results contribute the importance of specifying the rate at

which strain is applied, but further work, controlling two independent parameters, is required in order to investigate the full matrix of this factorial hypothesis for single impact injury.

Acknowledgements

The authors would like to thank Eliot H. Frank and Han-Hwa Hung for their support and technical assistance. Supported by NIH Grant AR45779, a grant from SmithKline, and the German Research Society (DFG Ku 1222/2-1).

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