Biochemical and Biomechanical Properties of Lesion and Adjacent Articular Cartilage After Chondral Defect Repair in an Equine Model

Eric J. Strauss,† MD, Laurie R. Goodrich,‡ DVM, PhD, Chih-Tung Chen,† PhD, Chisa Hidaka,† MD, and Alan J. Nixon,‡ BVSc, MS
From the †Laboratory for Soft Tissue Research, Hospital for Special Surgery, New York, New York, and the ‡Comparative Orthopaedics Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, New York

Background: Chondral defects may lead to degradative changes in the surrounding cartilage, predisposing patients to developing osteoarthritis.

Purpose: To quantify changes in the biomechanical and biochemical properties of the articular cartilage adjacent to chondral defects after experimental defect repair.

Study Design: Controlled laboratory study.

Methods: Specimens were harvested from tissue within (lesion), immediately adjacent to, and at a distance from (remote area) a full-thickness cartilage defect 8 months after cartilage repair with genetically modified chondrocytes expressing insulin-like growth factor–I or unmodified, control chondrocytes. Biomechanical properties, including instantaneous Young’s and equilibrium aggregate moduli, were determined by confined compression testing. Biochemical properties, such as water and proteoglycan content, were also measured.

Results: The instantaneous Young’s modulus, equilibrium modulus, and proteoglycan content increased, whereas water content decreased with increasing distance from the repaired lesion. The instantaneous Young’s and equilibrium moduli of the adjacent articular cartilage were 80% and 50% that of remote area samples, respectively, whereas water content increased 0.9% and proteoglycan content was decreased by 35%. No significant changes in biomechanical and biochemical properties were found either in the lesion tissue or in adjacent cartilage with genetic modification of the chondrocytes.

Conclusion: Articular cartilage adjacent to repaired chondral defects showed significant remodeling 8 months after chondral defect repair, regardless of whether genetically modified or unmodified cells were implanted.

Clinical Relevance: Changes in the biochemical and biomechanical properties of articular cartilage adjacent to repaired chondral defects may represent remodeling as part of an adaptive process or degeneration secondary to an altered distribution of joint forces. Quantification of these changes could provide important parameters for assessing progress after operative chondral defect repair.

Keywords: chondral defect repair; biochemical and biomechanical properties; articular cartilage; confined compression testing
tissue.\textsuperscript{17} Chondrocyte death adjacent to areas of experimental cartilage wounding has been reported, as well as histologic evidence of cartilage degeneration.\textsuperscript{20} These cellular changes may be expected to be accompanied by biomechanical changes; however, such sequelae of cartilage injury are not well described. Quantification of biomechanical changes to the cartilage adjacent to chondral defects could provide important parameters for assessing the success or failure of cartilage repair. Regardless of how “cartilage-like” repair tissue in a defect may appear to be, if alterations in the surrounding cartilage are not addressed, degenerative changes in the affected joint may not be prevented.

The purpose of this study was to quantify the biochemical and biomechanical properties of articular cartilage adjacent to experimental chondral defects repaired with a chondrocyte-based technique in an equine model.\textsuperscript{18} In this model, chondrocytes were implanted into extensive (15-mm-diameter) full-thickness cartilage defects in the patellofemoral joints of adult horses. We hypothesized that implantation of chondrocytes genetically modified to overexpress insulin-like growth factor–I (IGF-I) would improve biomechanical and biochemical properties not only within the repaired defect but also in the cartilage adjacent to and remote from the defect.

MATERIALS AND METHODS

Graft Preparation

Chondrocyte grafts were prepared as previously described.\textsuperscript{18,19} Briefly, chondrocytes were isolated by overnight collagenase digestion of articular cartilage from the distal femur and proximal tibia of donor foals (younger than 3 months old). One day before surgery, cryopreserved cells were thawed and plated at a density of $4 \times 10^6$ cells per T75 flask in Ham’s F12 medium with 10% fetal bovine serum, 30 \text{µg/mL} of \textalpha-ketoglutarate, 300 \text{µg/mL} of \textit{L}-glutamine, penicillin, and streptomycin, and 25 mM HEPES (all from Gibco, Gaithersburg, Md). Just before surgery, chondrocytes were incubated with 20,000 particle units/cell of an adenovirus (Ad) vector-encoding equine IGF-I (AdeIGF-I).\textsuperscript{34} This dosage of Ad vectors correlates to approximately 200 infectious units of Ad per cell and has previously been established in our laboratory as an optimal dose of Ad for therapeutic gene transfer to chondrocytes.\textsuperscript{5,19} The control group of chondrocytes was uninfected (no virus). After incubation, chondrocytes were washed, trypsinized, pelleted, and resuspended in fibrinogen that was prepared by cryoprecipitation of plasma from the horse undergoing experimental cartilage repair surgery. The chondrocyte-fibrinogen mixture was polymerized in situ in the cartilage defect using calcium-activated bovine thrombin (500 \text{U/mL}, Sigma, St Louis, Mo) at the time of surgery (Figure 1).

Operative Procedure

Eight normal adult horses ranging in age from 2 to 6 years underwent experimental cartilage repair using a previously described arthroscopic-assisted technique that was approved by the institutional animal care and use committee.\textsuperscript{18} Briefly, horses were anesthetized and placed in dorsal recumbency, and the area over both patellofemoral joints was prepared aseptically for arthroscopic surgery. A video arthroscope was inserted through a standard arthroscopic approach between the lateral and middle patellar ligaments, and the articulations of the patellofemoral joint were explored to rule out any preexisting cartilage lesions. A guarded spade bit cutter 15 mm in diameter, premeasured to drill 3 mm deep, was inserted into the joint through a second portal. The cutter was used to create a single 15-mm-diameter circular cartilage defect in the lateral trochlear ridge extending down to but not through the subchondral plate. All calcified cartilage was removed, and an awl was used to roughen the exposed subchondral bone for adequate attachment of the fibrin graft. The joint was lavaged with lactated Ringer’s solution to remove debris and then was distended with sterile helium gas to allow the subchondral bed of the cartilage lesion to be dried by several lint-free sponges. The fibrinogen-chondrocyte mixture (see above) and calcium-activated bovine thrombin were placed into separate chambers of a double-barrel syringe and co.injected into the defect (Figure 1). Approximately $2 \times 10^7$ cells in 0.6 mL were injected into the defect until the fibrin graft completely filled the defect to the level of the surrounding normal articular cartilage. The fibrin graft was allowed to polymerize in situ for 5 minutes. The joints were gently lavaged with sterile saline, and the skin incisions were closed. In each horse, the defect in one joint (right vs left determined at random)
The experimental protocol consisted of increasing intervals of weeks, confining the horses to the stall for 6 weeks, and withholding the stall for 6 weeks. After surgery, the horses were confined to a stall for 6 weeks. Subsequently, the rehabilitation protocol consisted of increasing intervals of walking for 5 weeks, followed by unlimited pasture exercise.

Tissue Retrieval

Eight months after surgery, the horses were euthanized by an overdose of barbiturate. For biomechanical and biochemical analysis, a 5-mm-diameter skin biopsy punch was used to harvest cylindrical samples from the center of the repaired defect (lesion, see Figure 2). Care was taken to include the full thickness of the repair tissue but to exclude any subchondral bone. Similar samples were also harvested from the surrounding cartilage immediately outside the repaired defect (adjacent articular cartilage) and from grossly normal-appearing cartilage on the medial trochlear ridge (remote area). The harvested lesion, adjacent articular specimens, and remote area specimens were placed in phosphate-buffered saline (PBS) solution, pH 7.4, containing protease inhibitor cocktail (Sigma) and stored at −80°C until biomechanical or biochemical testing.

Biomechanical Analysis

Compressive strength of the lesion tissue, adjacent articular cartilage, and normal-appearing remote area cartilage was assessed by confined compression testing using a custom-designed computer-controlled soft tissue test apparatus. Briefly, specimens were placed with articular surface facing up into a 5-mm-diameter loading chamber and bathed in PBS with protease inhibitors (Sigma) to prevent tissue degradation during testing. The thickness of the specimen was determined before each test by lowering down the flat-ended porous indenter to contact with the surface of the specimen. To determine the approximate maximum load for the load-displacement tests, the samples were subjected to 5 steps of compression, each comprising a distance of 3% of the original specimen thickness, for a total compression of 15%. The predetermined maximum load was then applied to the sample at a rate of 0.05 mm/s, followed by a complete unloading of the specimen. All displacements and loads were recorded, and a stress-strain curve (as determined from load and deformation) was generated from the data. The instantaneous Young's modulus of each test was curve fitted from the linear region of the stress-strain plot using the least squares method (Excel 97, Microsoft, Redmond, Wash). Stress-relaxation testing was then performed to determine the equilibrium aggregate modulus. Five steps of displacement, 4% of strain each, were applied with the indenter held still between each step to allow the specimen to reach equilibrium state. The equilibrium stress at the end of each step was recorded. The equilibrium aggregate modulus of each test was determined from the slope of the equilibrium stress-strain curve.

Biochemical Analysis

Water content (expressed as percentage water) was calculated by subtracting the dry weight of the sample from its wet weight and dividing the difference by the wet weight. The wet weight of the sample was measured using an ultrasensitive microbalance (Cahn 25, Cahn, Madison, Wis) after being immersed in the PBS solution for 60 minutes after biomechanical tests. Then, the specimen was lyophilized at −50°C for 12 to 15 hours, and the dry weight was measured. Proteoglycan content (milligrams glycosaminoglycan/milligrams dry weight) of each specimen was measured using the dimethylmethylene blue dye binding (DMMB) assay. Briefly, lyophilized samples were digested in 0.5 mg/mL papain (Sigma) at 65°C for 4 hours. A 20-mL aliquot of the digest was mixed with DMMB solution, and the absorbance at 525 nm was read immediately in a spectrophotometer. Known concentrations of chondroitin-4-sulfate were used to establish a standard curve against which the glycosaminoglycan content of the sample was calculated.

Statistical Analysis

Results are expressed as the mean for 8 samples per group ± standard error of the mean. As the contralateral knee in each animal served as its own control, a 2-tailed paired t test was used to compare AdelGF-I-treated versus control groups. Data from each test subset were compared by a 1-way analysis of variance (ANOVA), with Tukey post hoc test used where required. Statistical significance was set at \( P < .05 \).
Figure 3. Instantaneous Young’s modulus of lesion, adjacent articular cartilage, and remote area specimens (mean ± standard error of the mean). Young’s modulus in repair tissue, adjacent articular cartilage, and remote area specimens for 8 specimens per group. One-way analysis of variance showed that the Young’s modulus increased with increasing distance from the lesion (P < .01). *Demonstrates a statistically significant difference between lesion and remote area specimens. **Demonstrates a statistically significant difference between lesion and adjacent articular cartilage specimens. ***Demonstrates a statistically significant difference between adjacent articular cartilage and remote area specimens.

Figure 4. Equilibrium aggregate modulus of lesion, adjacent articular cartilage, and remote area specimens (mean ± standard error of the mean). Equilibrium aggregate modulus in repair tissue, adjacent articular cartilage, and remote area specimens for 8 specimens per group. One-way analysis of variance showed that the equilibrium modulus increased with increasing distance from the lesion (P < .01). *Demonstrates a statistically significant difference between lesion and remote area specimens. **Demonstrates a statistically significant difference between lesion and adjacent articular cartilage specimens. ***Demonstrates a statistically significant difference between adjacent articular cartilage and remote area specimens.

RESULTS

Biomechanical Properties

The instantaneous Young’s modulus was lowest in the lesion tissue, intermediate in the adjacent tissue, and highest in the remote area cartilage (Figure 3). The instantaneous Young’s modulus of the lesion tissue was approximately 50% that of the samples taken from the remote area and 66% that of adjacent articular cartilage specimens (P < .02 for both comparisons). The instantaneous Young’s modulus of the adjacent articular cartilage tissue was approximately 80% that of remote specimens (P < .02). One-way ANOVA demonstrated that the instantaneous Young’s modulus of the cartilage samples increased with increasing distance from the lesion (P < .01).

The equilibrium aggregate modulus also increased with increasing distance from the lesion (P < .01) (Figure 4). The mean equilibrium aggregate modulus of the repair tissue was approximately 12% that of the samples taken from the remote area and 25% that of adjacent articular cartilage specimens (P < .01 for both comparisons). The equilibrium aggregate modulus of adjacent articular cartilage specimens was approximately 49% that of remote cartilage samples (P < .01). Whereas both the instantaneous Young’s modulus and equilibrium aggregate modulus varied with distance from the repair site, neither was affected by whether the implanted cells had been genetically modified (P > .05, all comparisons).

Biochemical Properties

Water and proteoglycan content also varied significantly with distance from the lesion (Table 1). The mean water content of repair tissue in both the treated and control groups was 2% to 3% higher than that found in the adjacent articular cartilage and remote area specimens (P < .01 for...
both comparisons). Water content was also found to be increased in adjacent articular cartilage specimens, with a mean value 0.9% higher than that from remote area samples (P < .01). The mean proteoglycan content of the repair tissue was approximately 40% that of samples taken from the remote area and 63% that of adjacent articular cartilage specimens (P < .02 for both comparisons). The proteoglycan content of adjacent articular cartilage samples was approximately 65% that of the remote area cartilage specimens (P < .01). One-way ANOVA demonstrated that the percentage proteoglycan content of the cartilage specimens increased and the water content decreased with increasing distance from the lesion (P < .01 for both analyses). As with the biomechanical parameters and proteoglycan and water content measurements, no variation based on genetic modification of the chondrocytes could be detected (P > .05, all comparisons).

**DISCUSSION**

In this study, we compared the biomechanical and biochemical properties of lesion tissue, adjacent articular cartilage, and remote area specimens harvested from equine joints 8 months after cartilage repair with genetically modified chondrocytes. Although we did not detect any differences between samples from joints treated with genetically modified versus unmodified cells, we did show significant differences among lesion tissue, adjacent articular cartilage, and remote area specimens. Consistent with previous studies by this and other groups, our data showed that lesion tissue from within the repaired defect is significantly weaker, with higher water and lower proteoglycan content than that of grossly normal-appearing cartilage from a remote, unaffected part of the joint. Lesion samples had a mean instantaneous Young's modulus that was approximately 2-fold less than their normal remote area counterparts. This reduction in the instantaneous Young's modulus indicates a decreased ability of the lesion cartilage to bear load in a dynamic environment, such as walking or running. Lesion samples also had a mean equilibrium aggregate modulus that was approximately 8.5-fold lower than their normal remote area counterparts. This reduction in equilibrium indicates a decrease in the elastic strength and integrity of the proteoglycan-collagen network under a static load as occurs when standing. Lesion specimens also had a reduced proteoglycan content (60% less than that of samples from the remote area), as well as increased water content (up to 3% higher than remote areas).

The most salient finding in our study was that the articular cartilage adjacent to the repaired defect had biomechanical and biochemical properties that were intermediate between the lesion and remote tissues. Although changes in the joint surfaces after focal cartilage defects have often been described, few studies have previously quantified the changes that occur in the cartilage immediately surrounding such defects. Our study showed that 8 months after chondrocyte-based cartilage repair, the adjacent articular cartilage had an instantaneous Young's modulus that was reduced by 20% and an equilibrium aggregate modulus that was reduced by 51% versus remote cartilage. Proteoglycan content decreased by 35%, and water content increased by 0.9%. These quantitative changes correlated well to histologic findings similar to those previously reported, such as the presence of chondrocyte death and chondrocyte cloning in specimens harvested from areas adjacent to chondral defect repair. We acknowledge that measuring the biomechanical properties of tissue samples including the subchondral bone would have been closer to physiologic conditions, but this procedure would have required unconfined compression testing. In this investigation, we harvested samples of tissue that were as uniform as possible to perform a confined compression analysis, which allowed for more controlled, albeit less physiologic, loading conditions. This method was chosen to carefully characterize the properties of the repair tissue, adjacent cartilage, and remote cartilage samples themselves. Although examination of the integration of the repair tissue with the underlying subchondral bone would be an important outcome to measure, this analysis was not the aim of the current investigation. Future studies correlating findings such as ours with clinical measures of joint function may lead to the development of truly quantitative and functionally relevant methods for assessing the success or failure of cartilage defect repair in protecting the rest of the joint.

**Biochemical and Biomechanical Properties of Cartilage Repair Tissues**

Our study used the measurements of the instantaneous Young's modulus and the equilibrium aggregate modulus as parameters for assessing the outcomes of chondrocyte-based cartilage repair, as previous investigations have shown that these measures can serve as a quantitative and functional index for structural changes in cartilage tissue healing after injury or during the progression of osteoarthritis. The biomechanical properties of cartilage are dependent on the integrity of its extracellular matrix, which is composed primarily of large aggregating proteoglycans such as aggrecan, type II collagen, and water. Because increased water content is an indicator for the breakdown of the collagen network and because decreased proteoglycan greatly affects the biomechanical properties of the cartilage tissue, we also measured these components. Increased water content and decreased proteoglycan content have also been found in early osteoarthritic cartilage. Our study confirmed that increased water content and decreased proteoglycan content correlate with decreased biomechanical properties.

Our findings were in agreement with other studies using cell-based strategies for cartilage repair that have also found that the biochemical and biomechanical properties of repair tissue were inferior to those of normal articular cartilage. In a porcine full-thickness defect model, Liu et al. reported that at 8 weeks after surgery, repair tissue had approximately 50% the biomechanical properties of normal articular cartilage. Lee et al. reported similar results in a study examining the transplantation of autologous chondrocyte-seeded type II collagen scaffold into canine cartilage defects. At 15 weeks, confined compres-
sion testing of repair tissue resulted in an equilibrium aggregate modulus that was 6-fold lower and an instantaneous Young’s modulus that was 20-fold lower than that of normal articular cartilage controls.

The tendency of cartilage repair tissue to degenerate in the long term has been reported by several investigators. Although the instantaneous Young’s modulus of repair tissue was higher in our study than that of Lee et al., the equilibrium aggregate modulus and biochemical composition in our 8-month study were similar to those in the above-mentioned short-term studies, suggesting that the lesion tissue, although perhaps not degenerating, may also not have improved over time. Furthermore, previous studies on long-term results of chondrocyte-based cartilage repair have shown results similar to ours. Using indentation testing, Hale et al found that repair tissue showed substantial decreases in aggregate modulus and Poisson ratio, as well as increases in tissue permeability. Defect size was an important factor in this assessment, with the equilibrium modulus of repair tissues from 3- and 5-mm defects being significantly lower than that of repair tissue from 1-mm defects. In the largest defect tested by Hale et al (5-mm diameter), the equilibrium aggregate modulus of the repair tissue was 8-fold lower than that of the control. Although this difference is very similar to that which we found between lesion tissue and remote cartilage, the Hale et al study used adjacent articular cartilage as their “normal” control. In light of our findings that adjacent articular cartilage is significantly weaker than is normal remote cartilage, the differences reported in the Hale et al study may have underestimated the difference between lesion and remote cartilage.

As with the Hale et al study, however, our study did present a limitation based on the locations from which our samples were taken. Although our remote area specimens were harvested from an area on the medial trochlear groove—an area likely to be loaded very similarly to the lateral trochlear groove in which the defect was made—it was nonetheless not harvested from the exact same anatomical location as were the lesions and adjacent articular cartilage. There was no information to this date about anatomical variation in equine stifle cartilage. A recent study of bovine knee cartilage by Laasanen et al, however, suggested that there was only about 5% to 20% variation in thickness, instantaneous Young’s modulus, equilibrium modulus, and Poisson ratio between medial and lateral sides of the trochlear groove. We believe that the anatomical variation of equine stifle cartilage is similar to bovine, ranging from 5% to 20%.

A lack of any detectable difference in the properties of lesion tissue repaired with genetically modified versus unmodified chondrocytes was a surprising finding in our study. Previously, we found that use of recombinant IGF in the same experimental chondral defect repair model increased the amount of type II collagen in the repair tissue, but mechanical properties were not measured in this study. Delivery of IGF-I to the defect in our current gene therapy model was confirmed (Goodrich et al, manuscript in preparation); however, this factor did not appear to affect the biomechanical properties of the repair tissue. As type II collagen does not primarily contribute to the compressive strength of the tissue, it may be that other studies, such as those of tensile strength, may have revealed significant differences. The similarity in the 2 treatment groups showed that although IGF gene transfer does not improve biomechanical properties in the repair tissue, Ad-mediated genetic modification of the chondrocytes does not negatively affect the outcome.

Effect on Adjacent Cartilage

Immediately after chondral injury, a phase of chondrocyte necrosis begins, demonstrated histologically by the presence of hollow lacunae with no chondrocytes adjacent to the margins of the wound. Subsequently, increased mitotic activity with the appearance of “cloning” or chondrocyte clusters in the tissue adjacent to the chondral defect as well as increased rates of both matrix synthesis and matrix degradation have been reported. The predominance of catabolic changes in the adjacent articular cartilage has been shown by Shapiro et al in a study of healing 3-mm-diameter full-thickness cartilage defects in rabbits. As we have quantified the biomechanical properties, water content, and proteoglycan content of the adjacent articular cartilage at one time point, 8 months after repair, we cannot conclude from our studies whether the decrease in biomechanical properties and proteoglycan content and increase in water content are adaptive or degenerative. Although the persistence at 8 months would certainly indicate a slow remodeling process, a demonstration of stabilization or improvement over time could suggest that the changes may be adaptive.

In a study using a sheep model of intra-articular step-off fractures, Trumble et al reported evidence of a tendency for articular congruency to improve after fracture. This is of interest as excessive mechanical loading, such as that which develops on the high side of an intra-articular step-off, may also occur in adjacent articular cartilage around a defect. Although biomechanical properties of the cartilage were not tested in the study of Trumble et al, the collagen fiber bending and cartilage thinning they found suggest that cartilage on the higher side may have become more compressible and less stiff. Excessive mechanical load has been shown to induce chondrocytes to express and/or activate matrix metalloproteinases, potentially leading to matrix remodeling. Although resultant changes such as proteoglycan loss are usually interpreted as indicating cartilage tissue degeneration, the study of Trumble et al suggested that at least in some cases, these changes may reflect structural adaptation in the tissue. Intermediate biomechanical properties in the adjacent articular tissue, such as we have reported here, would improve the stress distribution and/or reduce stress concentration between the highly compliant lesion tissue and the remote, unaffected cartilage. Regardless of whether the changes observed in our study represent adaptation or degeneration, in measuring the changes in the articular cartilage adjacent to a repaired chondral defect, our study

References 3, 10, 11, 14, 17, 24, 28-30, 33, 37.
provides important quantitative parameters by which future strategies for cartilage repair may be evaluated. Clinically, the prevention of degeneration in the affected joint is an important rationale for repairing focal cartilage defects. Whereas most cartilage repair studies focus on the properties of the repair tissue itself, measuring the biomechanical and biochemical properties of adjacent articular cartilage may offer a more relevant method for quantifying the potential benefit of any given cartilage repair strategy.

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