In Vitro Modulation of Cartilage Shape Plasticity by Biochemical Regulation of Matrix Remodeling

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With consideration of the need for cartilage grafts of specific sizes and shapes in orthopedics and other fields, immature cartilage explants and grafts have recently been molded *in vitro* and *in vivo*. Nonsurgical correction of cartilage deformities and malformations often uses mechanical stimuli and further demonstrates the plasticity of cartilage shape. Cartilage shape plasticity appears to diminish with maturation, coincident with changes in matrix composition. This study's objectives were to characterize shape plasticity of articular cartilage from immature and mature bovines and test whether altering proteoglycan and collagen (COL) remodeling modulates shape plasticity *in vitro*. Cartilage explants were analyzed fresh on day 0 or after 14 days of culture in the presence of β -D-xyloside to suppress glycosaminoglycan accumulation or β -aminopropionitrile (BAPN) to inhibit lysyl oxidase-mediated COL crosslinking. Culture with β -D-xyloside and BAPN differentially regulated cartilage size, composition, and shape plasticity, with an inverse association between shape plasticity and the ratio of tissue COL to glycosaminoglycan. Retention of a mechanically imposed contour was increased by culture with BAPN compared to day 0 calf cartilage (90% vs. 69%), and BAPN-treated samples had higher shape retention than β -D-xyloside-treated samples for both calf (90% vs. 74%) and adult cartilage (54% vs. 31%). The findings provide quantitative measures of cartilage shape plasticity at immature and mature stages and are consistent with the concept of diminishing shape plasticity with maturation. The ability to modulate cartilage shape plasticity by varying *in vitro* biochemical conditions may be a useful tool for the formation of contoured chondral grafts.

Introduction

CHONDRAL GRAFTS OF desired shapes and sizes are therapeutically useful for replacing or augmenting cartilage compromised by injury, disease, or malformation. Focal articular cartilage lesions may be treated by autologous or allogeneic osteochondral grafts with careful attention to matching the normal joint contours and to filling the defect.^{1,2} Likewise for surgical reconstruction of the ear or nose, grafts of costal, auricular, or septal cartilage are routinely shaped through carving, suturing, and scoring.³ Emerging technologies in cartilage tissue engineering may address some of biomedical need for chondral grafts, but tools and techniques are needed to create cartilage constructs with desired shapes.⁴ Recently, the concept that cartilage grafts can be contoured *in vitro* through the application of mechanical stimuli was demonstrated using a static flexion bioreactor and immature articular cartilage.⁵ Extension of this concept to an *in vivo* application was accomplished using a resorbable template to guide the reshaping of subcutaneously implanted costal cartilage grafts in rabbits.⁶ These studies demonstrate the plasticity

of cartilage shape, defined as the ability to change free-swelling conformation through mechanically guided remodeling.

Altering the forms of developing cartilaginous structures through the application of mechanical stimuli is a technique that has long been used for nonsurgical correction of malformations and deformities. Examples include the treatment of hip dysplasia through external harnessing,⁷ of club foot by manipulation and casting,⁸ of cleft palate by nasoalveolar molding,⁹ and of ear deformity by splinting.¹⁰ Among these procedures, a commonly practiced philosophy is that outcomes are better when the corrections are attempted early during neonatal life. However, the mechanisms by which these procedures correct cartilage shape and their dependence on maturation remain speculative. In a study of club foot correction, changes in the shape of cartilage anlagen were observed immediately after manipulation and were maintained upon cast removal a week later, leading the authors to hypothesize that cartilage matrix collagens (COL) and proteoglycans (PG) remodel to accommodate the structural changes.⁸ Similarly, the high shape plasticity of auricular and nasal cartilage in early neonates has been

hypothesized to result from an abundance of hyaluronic acid and PG in the tissues, which facilitates remodeling during mechanical correction.^{9–11}

During *in vivo* growth and maturation, changes occur in the predominant components of articular cartilage, including an increase in COL concentration and a maintenance or slight decrease in PG concentration.^{12,13} Functional properties are altered concurrently with composition as indicated by increasing compressive and tensile stiffness with maturation.^{12,13} *In vitro* studies of immature articular cartilage have also shown that regulation of cartilage matrix metabolism and remodeling can modulate matrix composition and functional properties. The results of such studies suggest that a balance of PG and COL remodeling is a key determinant of *in vitro* volumetric expansion, composition, and tensile integrity of cartilage explants.^{14,15} However, it is undetermined how cartilage shape plasticity may be affected by similar *in vitro* modulation of matrix composition.

Specific biochemical agents can be used in cartilage explant culture to selectively alter PG and COL remodeling. β -D-xyloside acts as an exogenous substrate for the initiation of free chondroitin sulfate synthesis, the major glycosaminoglycan (GAG) of cartilage aggregating PG.¹⁶ When added to cultures of chondrogenic cells, β -D-xyloside competes with PG core protein for chondroitin sulfate synthesis.¹⁷ Consequently, the newly synthesized PG incorporated into the matrix is significantly depleted of GAG, while soluble GAG chains synthesized on β -D-xyloside readily diffuse from the tissue.¹⁸ In addition, COL remodeling can be perturbed using β -aminopropionitrile (BAPN), an inhibitor of lysyl oxidase-mediated COL crosslinking.¹⁹ In cartilage explant cultures, BAPN inhibits the formation of difunctional COL crosslinks and consequently impairs the chemical stabilization of the COL network.^{20,21}

This study was conducted to further the development of techniques for manipulating the shape of cartilage grafts and to help elucidate the relationship between composition and shape plasticity. This study tested the hypothesis that shape plasticity of articular cartilage may be modulated from an initial state by altering the balance of PG and COL remodeling *in vitro*.

Materials and Methods

Experimental design

Articular cartilage explants from calf and young adult bovines were analyzed fresh on day 0 or after being cultured for 14 days in the presence of BAPN or a β -D-xyloside, *p*-nitrophenyl- β -D-xylopyranoside (PNPX). Some explants were analyzed for changes in tissue size and matrix composition. Other explants were assessed for shape plasticity by application of flexural deformation during additional culture.

Sample preparation and culture

Samples were prepared from bovine stifle joints obtained from an abattoir as previously described.⁵ Articular cartilage blocks were harvested from the patellofemoral grooves of two calves (1–3 weeks old) and two young adults (1–2 years old). The superficial ~ 0.3 mm of cartilage including the curvilinear articular surface was removed using a vibrating microtome and discarded. An adjacent flat slice of cartilage

was then removed and punched into strips measuring $\sim 10 \times 2$ mm. Cartilage strips, ~ 1 mm thick, were divided at intervals along the length to produce three site-matched explants for analysis of growth and matrix composition. The different growth rates among conditions (see Results section) necessitated that cartilage strips intended for shape plasticity analysis be cut to different initial thicknesses (~ 0.6 – 1 mm for calf and ~ 1 mm for adult) to target a uniform ~ 1 mm thickness among all conditions at the time flexure was applied. Each explant was weighed and measured for thickness using a noncontacting laser micrometer before and after culture.

Cartilage explants were cultured, free-swelling, for 14 days in nontissue culture treated plates. Explants were kept in $\sim 70 \times$ tissue volume of the basal medium with 20% fetal bovine serum (FBS) and either 0.2 mM BAPN or 1 mM PNPX. The basal medium consisted of Dulbecco's modified Eagle's medium supplemented with 100 μ g/mL ascorbate, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B. Cultures were placed in standard incubators with 5% CO₂ atmosphere at 37°C, and the medium was changed every other day.

Biochemical analysis

The site-matched explants, consisting of one sample from each of the day 0 control, days 14 BAPN, and days 14 PNPX groups, were lyophilized and weighed dry to determine water content as a percent of final wet weight. Samples were then digested with a solution of proteinase K for analysis of cartilage matrix content. Alternatively, some samples were extracted with a solution of 4 M guanidine, 50 mM sodium acetate, 10 mM dithiothreitol, and protease inhibitors (PIs) at $20 \times$ tissue volume for 24 h at 4°C. Samples were rinsed once in phosphate-buffered saline+PIs for 1 h and the rinse solution was added to the extract. Extracts were dialyzed against water (molecular weight cutoff = 2000) and then the extracts and residual tissue were digested with proteinase K. Digests were assayed for sulfated GAG²² and hydroxyproline.²³ COL content was determined from hydroxyproline using a ratio of 7.25 g COL/g hydroxyproline.^{24,25} Data were normalized to initial tissue wet weight (WW_i) to indicate matrix constituent content and to the final wet weight (WW_f) to indicate constituent concentration at the end of culture. Additionally, the spent medium was pooled for each sample and assayed for GAG released by the explants during culture.

Shape plasticity analysis

Assessment of cartilage shape plasticity was based on a previous study examining mechanically induced changes in cartilage shape via culture of cartilage strips subjected to static flexure.⁵ Before shape analysis, cartilage strips cultured in the medium with BAPN or PNPX were thoroughly washed (6 times over 2 h) in the basal medium to remove residual treatment agents. Using custom bioreactors, fresh and cultured cartilage strips were subjected to static flexural deformations and further cultured with the basal medium and 2% FBS for 4 days (calf) or 8 days (adult). These loading durations were determined from preliminary studies and differed to provide better sensitivity to changes in cartilage shape plasticity.

Specimen shape was photographed in the free-swelling state before the application of flexure (initial shape) and at culture termination while loaded (imposed shape) and after 2 h of stress relaxation (relaxed shape) by free-swelling in phosphate-buffered saline with PIs at 4°C (Fig. 1). Specimen shape was quantified as an opening angle (γ) using Image J (NIH, Bethesda, MD), and, with this analysis, the average imposed opening angle was $98 \pm 7^\circ$ for all specimens. Shape retention [%], a measure of cartilage shape plasticity, was calculated as $[(\gamma_{\text{initial}} - \gamma_{\text{relaxed}}) / (\gamma_{\text{initial}} - \gamma_{\text{imposed}})] \times 100$.⁵

Statistical analysis

Data are presented as means $\pm$ standard error. For each tissue maturity level (calf and adult), data were analyzed by analysis of variance for the effect of culture condition on biochemical composition, shape retention, and changes in size. For composition and size data, site of matched explants was treated as a random factor; whereas, animal was treated as a random factor for shape data. Tukey *post-hoc* analysis was used to make individual comparisons between day 0, day 14 BAPN, and day 14 PNPX groups. The significance criterion, α , was set at 0.05 for all tests.

Results

Expansion of cultured cartilage explants

Volumetric expansion of calf articular cartilage occurred during 14 days of culture and was dependent on culture condition, while the size of adult cartilage changed little irrespective of condition (Fig. 2). Culture with BAPN induced larger increases in calf cartilage wet weight (+70% vs. +35%, $p < 0.001$) and thickness (+67% vs. +39%, $p < 0.001$) than PNPX. In the adult cartilage cultures, PNPX treatment did not produce significant changes in either wet weight or thickness ($p = 0.09$ and 0.43 , respectively), whereas BAPN treatment produced small but significant increases in wet weight (+7%, $p < 0.001$) and thickness (+3%, $p < 0.05$).

FIG. 1. Shape plasticity was determined as the retention of a mechanically imposed deformation by cartilage explants. Shape was quantified as an opening angle (indicated in red) at three stages: initial (γ_{initial}) measured before the deformation, imposed (γ_{imposed}) measured at culture termination, and relaxed (γ_{relaxed}) measured after unloading and stress relaxation. Color images available online at www.liebertonline.com/ten.

FIG. 2. Change in (A) wet weight and (B) thickness for calf and adult articular cartilage explants cultured for 14 days with PNPX or BAPN. For each maturity, (*) $p < 0.05$ versus zero (i.e., no change), and (♦) $p < 0.05$ for BAPN versus PNPX. Mean $\pm$ SE; $n = 10$. BAPN, β -aminopropionitrile; PNPX, p -nitrophenyl- β -D-xylopyranoside; SE, standard error.

To account for different magnitudes of expansion between treatments, cartilage samples used for shape plasticity analysis were cut to slightly different initial thicknesses (~ 0.6 – 1.00 mm). At the onset of flexure, the mean thicknesses of cartilage strips from each condition were similar. This helped ensure that samples received similar mechanical deformations, since sample thickness is an important determinant of flexure strains.²⁶

Effects of PNPX and BAPN on cartilage composition

The remodeling of cartilage matrix during culture was differentially regulated during culture with PNPX and BAPN. The water content of calf cartilage was increased during culture with PNPX (+1.7%, $p < 0.01$) and with BAPN (+3.6%, $p < 0.001$) and was significantly higher in the BAPN group compared to PNPX ($p < 0.005$) (Fig. 3A). Total COL content [mg/g WW_i] was increased in calf cartilage during culture with PNPX (+14%, $p < 0.001$) and with BAPN (+16%, $p < 0.001$) but did not differ between the two treatments (Fig. 3B). Accounting for increasing tissue wet weight during culture, final COL concentration [mg/g WW_f] decreased in the calf cartilage during culture with PNPX (−16%, $p < 0.01$) and with BAPN (−31%, $p < 0.001$) and was lower in the BAPN treatment group ($p < 0.05$). GAG content in the calf tissue was also increased during culture with PNPX (+43%, $p < 0.001$) and with BAPN (+80%, $p < 0.001$) and was higher in the BAPN-treated samples ($p < 0.001$) (Fig. 3C). The final

GAG concentration in calf explants was not affected by culture condition ($p = 0.49$). Reflecting the changes in cartilage matrix constituents, the ratio of COL to GAG in calf cartilage was lowered from day 0 with PNPX treatment

FIG. 3. Effects of culture condition on cartilage composition: (A) water, (B) total tissue COL, (C) total tissue GAG, and (D) COL:GAG ratio. COL and GAG are normalized to WW_i (gray bars) to indicate content or to WW_f (black bars) to indicate final concentration (except for day 0 [d0], where WW_i = WW_f). For each maturity, (*) $p < 0.05$ versus d0, and (♦) $p < 0.05$ for BAPN versus PNPX. Mean $\pm$ SE; $n = 10$. COL, collagen; GAG, glycosaminoglycan; WW_i, initial tissue wet weight; WW_f, final wet weight.

(1.6 ± 0.2 vs. 1.3 ± 0.1 , $p < 0.01$) and reduced even further with BAPN treatment (1.1 ± 0.1 , $p < 0.001$ vs. day 0, $p < 0.05$ vs. PNPX) (Fig. 3D).

In contrast to the effects on calf cartilage, culture condition had no effect on the hydration ($p = 0.32$), COL content ($p = 0.22$), or COL concentration ($p = 0.05$) of adult cartilage (Fig. 3A, B). However, GAG content of adult cartilage was decreased during culture with PNPX (-20% , $p < 0.01$) but increased with BAPN ($+14\%$, $p < 0.05$) (Fig. 3C). The final concentration of tissue GAG was also decreased with PNPX (-22% , $p < 0.01$) but not significantly altered with BAPN ($p = 0.33$). Both GAG content and concentration were higher in the adult BAPN-treated samples than in those cultured with PNPX ($p < 0.001$). Consequently, the ratio of COL to GAG was lower in the BAPN condition versus PNPX (4.4 ± 0.3 vs. 6.7 ± 1.1 , $p < 0.05$), but did not significantly deviate from that of day 0 adult samples with either culture condition (5.3 ± 0.6 , $p > 0.24$) (Fig. 3D).

To confirm BAPN and PNPX treatment efficacy, COL and GAG metabolism during culture were analyzed. For both calf and adult cartilage, the percent of tissue COL that was extractable under dissociative conditions was higher in the BAPN-treated samples than in either PNPX-treated or day 0 samples ($p < 0.05$, for all) (Fig. 4A). Additionally, the spent medium was examined for GAG released from cartilage explants (Fig. 4B). Calf and adult cartilage cultured with PNPX released greater amounts of GAG into the medium than with BAPN treatment ($p < 0.001$, for all). When

FIG. 4. Effects of culture condition on matrix metabolism: (A) COL extracted under dissociative conditions (% of total tissue COL) and (B) total GAG released into the medium by cartilage explants. GAG data are normalized to WW_i (gray bars) or to WW_f (black bars). For each maturity, (*) $p < 0.05$ versus d0, and (♦) $p < 0.05$ for BAPN versus PNPX. Mean $\pm$ SE; (A) $n = 4-5$, (B) $n = 10$.

FIG. 5. A measure of cartilage shape plasticity was determined as the percent of the mechanically imposed shape retained after unloading. For each maturity, (*) $p < 0.05$ versus d0, and (♦) $p < 0.05$ for BAPN versus PNPX. Mean $\pm$ SE; $n = 9-10$.

normalized to the initial wet weight of explants and culture duration, calf cartilage released GAG at rates of $2.8 \text{ mg}/[\text{g WW}_i \cdot \text{day}]$ with PNPX and $1.0 \text{ mg}/[\text{g WW}_i \cdot \text{day}]$ with BAPN. Similarly, adult cartilage released GAG at rates of $1.7 \text{ mg}/[\text{g WW}_i \cdot \text{day}]$ with PNPX and $1.0 \text{ mg}/[\text{g WW}_i \cdot \text{day}]$ with BAPN.

Effects on cartilage shape plasticity

Shape plasticity, measured as the retention of a mechanically imposed bending deformation by cartilage explants, differed with culture condition (Fig. 5). The shape retention of calf cartilage was increased with culture with BAPN relative to day 0 samples ($90\% \pm 2\%$ vs. $69\% \pm 2\%$, $p < 0.001$), but was maintained with culture with PNPX ($74\% \pm 2\%$, $p = 0.37$). Samples cultured with BAPN had higher shape retention than those cultured with PNPX for both calf ($p < 0.001$) and adult cartilage ($54\% \pm 5\%$ vs. $31\% \pm 8\%$, $p < 0.01$). Shape retention of adult cartilage tended to decrease with culture with PNPX relative to day 0 ($42\% \pm 5\%$, $p = 0.11$), and tended to increase with culture with BAPN ($p = 0.15$).

Discussion

This study provides a quantitative characterization of the shape plasticity of calf and adult bovine articular cartilage, based on the *in vitro* application of mechanical deformations. In addition, alteration of PG or COL remodeling differentially modulated the size, matrix composition, and shape plasticity of cartilage explants (Fig. 6). Changes in the concentration of COL relative to GAG were inversely associated with trends in shape plasticity. This finding appears consistent with recent experimental and theoretical work, indicating that COL-GAG interactions may modulate cartilage mechanical properties in a manner dependent upon the maturational dynamics of matrix composition.^{27,28} It has been postulated that a highly compliant matrix in immature cartilage may allow for tissue expansion during periods of growth.²⁸ Extending this concept, the results of this study suggest that the maturation-dependent shape plasticity of cartilage may facilitate tissue conformational changes earlier in development.

Formulation of the experimental design required consideration of a number of factors. A dose of 0.2 mM BAPN was a choice supported by evidence that $0.1-0.25 \text{ mM}$ BAPN in cultures of bovine articular cartilage inhibits crosslink formation with minimal cytotoxicity or alteration of PG synthesis and incorporation.^{15,20,21,29} In this and previous studies, a marked increase in extractable tissue COL with BAPN treatment indicated the efficacy of COL crosslink inhibition and accumulation of uncrosslinked COL within the matrix.^{20,29} A dose of 1 mM β -D-xyloside has been previously shown to produce near maximal stimulation of chondroitin sulfate synthesis in chondrogenic cell cultures.^{18,30} Chondrogenic cells chronically cultured with 1 mM β -D-xyloside also produce a matrix greatly depleted of GAG but only slightly depleted of COL, consistent with a small decrease in total protein synthesis.¹⁸ In this study, lower tissue GAG contents and higher rates of GAG release in PNPX-treated cultures are consistent with the formation of soluble, β -D-xyloside-initiated GAG chains at the expense of GAG chain synthesis on PG, but also may partially reflect an increased loss of PG aggregates with increasing cartilage permeability. Distinguishing the effects of inhibiting glycosylation of large aggregating PG (i.e., aggrecan) versus smaller PGs (e.g., fibromodulin and biglycan) on cartilage shape plasticity remains a challenge for future studies.

FIG. 6. Summary of findings. Shape plasticity and the ratio of COL:GAG of immature and mature cartilage were inversely associated and differentially modulated by culture with biochemical agents (BAPN and PNPX). For each maturation stage, *in vitro* expansion (e.g., change in wet weight or thickness) is indicated by relative changes in height. Color images available online at www.liebertonline.com/ten.

Immature articular cartilage was observed to have a higher degree of shape plasticity than mature cartilage, which is consistent with the clinical wisdom of correcting cartilage deformities at an early age.^{7,9,10} Flexural deformations were applied over 4 days for calf cartilage and 8 days for adult cartilage to improve the sensitivity of the assessment for each group. Previously, it was found that shape retention increased with loading time for calf cartilage explants,⁵ and this trend was also found with adult cartilage in preliminary tests. This suggests that shape retention results would be even more disparate between calf and adult cartilage if the assays had been conducted for the same duration.

In all free-swelling cultures, the medium was supplemented with 20% FBS to stimulate PG and COL anabolism above basal levels.^{14,31} However, the effects of FBS on the composition of calf and adult cartilage have been shown to differ. Culture with 20% FBS stimulates calf cartilage to grow by accumulating GAG in excess of COL and also results in a reduction of compressive stiffness and tensile integrity.^{14,31,32} On the other hand, 20% FBS is more homeostatic for adult cartilage with GAG, COL, and compressive stiffness being maintained.^{31,33} This maturation-dependent response to FBS could explain some of the different trends observed in cartilage matrix composition between calf and adult cultures. For example, the reduced accumulation of GAG in PNPX-treated calf cartilage and loss of GAG in the corresponding adult samples are consistent with the differential effects of FBS stimulation combined with suppression of GAG accumulation by β -D-xyloside. It may also explain why COL content increased in calf cartilage cultures but was maintained in adult cartilage cultures.

Although the primary goal of this study was to determine whether cartilage shape plasticity could be modulated relative to an initial state (i.e., day 0), culture control samples were used in preliminary experiments to help distinguish the roles of treatment agents. Culture control samples, explants cultured in the medium with 20% FBS and no treatment agent, behaved similarly in terms of volumetric expansion and matrix composition (e.g., GAG and COL) to BAPN-treated samples, consistent with previous findings using sub-surface zone cartilage.^{15,29} Changes in shape plasticity were also nearly identical between culture control and BAPN-treated samples (Supplementary Materials, available online at www.liebertonline.com), despite previously identified differences in mechanical function with calf cartilage (i.e., accelerated reduction of tensile stiffness and strength with BAPN treatment).¹⁵

An additional experiment was conducted to test whether BAPN increases shape plasticity of calf cartilage through a build-up of COL crosslinking precursors during the free-swelling culture that subsequently stabilize the imposed deformation when no BAPN is present. However, this hypothesis was not substantiated, since samples that continued to receive BAPN during the deformation did not differ in shape plasticity compared to BAPN washout or culture control samples. Together, the findings of these additional studies indicate that the increase of shape plasticity in the day 14 BAPN calf samples relative to day 0 is not solely due to the effect of BAPN on COL network stability, but more likely attributable to a culture condition which promotes accumulation of PG in excess of COL. Additional biochem-

ical agents including the crosslink inhibitor/disruptor penicillamine or COL-specific proteases may facilitate further investigation of the effects of altered COL network stability in the absence of appreciable changes in PG content on cartilage shape plasticity.

The use of mechanical stimuli to alter cartilage shape is a potential tool for customizing cartilage grafts with applications to tissue engineering for articular joint repair or craniofacial reconstruction.^{5,6} While articular cartilage explants served as the model system here, investigating the shape plasticity of other types of cartilage (e.g., nasal, auricular, or costal) that serve as graft sources and may benefit from shaping is of further interest. This study expands the understanding of mechanically induced cartilage shape change and provides additional options for manipulating the process. While increasing cartilage shape plasticity may facilitate changing the shape of a graft, reducing shape plasticity may be desirable to set a shape before implantation. Changes in other functional properties, such as load-bearing ability, may be related to shape plasticity through the underlying dynamics of cartilage matrix composition. Since shape and biomechanical maturity may be among a number of important clinical requirements for a cartilage graft, these properties may need to be considered in a coordinated manner.

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Disclosure Statement

No competing financial interests exist.

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