Osteoarthritis and Cartilage (2008) **16,** 1–11

doi:10.1016/j.joca.2007.05.019

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Cartilage growth and remodeling: modulation of balance between proteoglycan and collagen network *in vitro* with β-aminopropionitrile[1]

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Summary

Objective: To examine the effect of β-aminopropionitrile (BAPN), an inhibitor of lysyl oxidase, on growth and remodeling of immature articular cartilage *in vitro*.

Design: Immature bovine articular cartilage explants from the superficial and middle layers were cultured for 13 days in serum-containing medium with or without BAPN. Variations in tissue size, accumulation of proteoglycan and collagen (COL), and tensile mechanical properties were assessed.

Results: The inclusion of serum resulted in expansive tissue growth, stimulation of proteoglycan and COL deposition, and a diminution of tensile integrity. Supplementation of medium with BAPN accentuated this phenotype in terms of a further increase in tissue size in explants from the superficial layer and further diminution of tensile integrity, without affecting the contents of proteoglycan and COL in explants from both the superficial and middle layers.

Conclusion: COL crosslinking is a major factor in modulating the phenotype of cartilage growth and the associated balance between proteoglycan content and integrity of the COL network.

Key words: Articular cartilage, Growth and remodeling, Cartilage matrix, Glycosaminoglycan, Collagen crosslinks, β-Aminopropionitrile.

Introduction

Articular cartilage is a layer of connective tissue located on the ends of long bones[1] that normally functions as a low friction, wear-resistant, load-bearing material, facilitating joint motion[2,3]. The ability of cartilage to withstand compressive, tensile, and shear forces depends on the composition and structure of the extracellular matrix[2–4]. The proteoglycan constituent of the extracellular matrix provides the tissue with a fixed negative charge that increases the tissue's propensity to swell and to resist compressive loading[5,6]. The crosslinked collagen (COL) network resists the swelling tendency of the proteoglycan molecules and provides the tissue with tensile and shear stiffness and strength[3,7,8]. Chondrocytes in cartilage normally maintain a functional matrix by modulating synthesis and degradation of the matrix components.

Growth and remodeling are biological processes that, together, transform cartilage tissue *in vivo* from an immature to a mature state. Tissue growth is generally defined as an increase in tissue size due to accretion of one or more solid tissue components similar to those already present, while tissue remodeling is defined as a change in tissue composition and/or structure of tissue components[9,10]. Many tissues, including articular cartilage, can expand due to accretion of fluid; however, this process is not generally considered growth, but rather tissue swelling. Two distinct mechanisms of tissue growth have been recognized: appositional growth, or growth at a tissue surface, and interstitial growth, or growth within tissue volume[11]. While it is possible that tissues can grow appositionally in the absence of remodeling, interstitial tissue growth must involve both growth and remodeling since accretion of a single tissue component will change the overall tissue structure and mechanical properties. Tissues can exhibit growth and remodeling in the form of hyperplasia (increase in the number of cells), hypertrophy (addition of cellular components), deposition of the extracellular matrix components or any number of these processes occurring concomitantly. In articular cartilage, the incidence of cell division is low and matrix deposition is the major contributor to the increase in size (i.e., growth) and changes in biochemical composition

[1]Supported by the NIH and NSF.
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Received 6 May 2007; revision accepted 26 May 2007.

(i.e., remodeling) of this tissue *in vivo*[12–14]. Since articular cartilage tissue may undergo both appositional and interstitial growth and the major contributor to cartilage growth is matrix deposition, the term *growth* is used subsequently in this paper to refer, collectively, to both growth and remodeling, which can occur in presence or absence of cellular proliferation.

Alterations of cartilage function, structure, and composition during growth *in vivo* and during serum-supplemented culture *in vitro* appear to depend on the metabolic balance between proteoglycan molecules and the components of the COL network. Fetal and postnatal growth of articular cartilage normally involves a net deposition of COL that is greater than that of proteoglycan, as well as an increase in mechanical integrity. During maturation of bovine articular cartilage, from the fetal stage, through the newborn calf, and to the skeletally mature adult, there is an increase in the COL and pyridinoline (PYR) crosslink densities, but little or no change in the content of glycosaminoglycan (GAG)[13–17]. These biochemical changes are accompanied by an increase in the tensile modulus and strength, and each of these biomechanical properties is positively correlated with the COL and PYR crosslink densities[14]. In contrast to this type of *in vivo* growth, growth of immature cartilage tissue *in vitro* in the serum-supplemented medium results in a net deposition of proteoglycan that is greater than that of COL and a decrease in mechanical integrity. For cartilage explants from bovine fetus and calf, and neonatal rat, incubation in the serum-supplemented medium results in an increase in tissue size, maintenance of proteoglycan concentration and a decrease in the concentrations of COL and PYR crosslinks[18–20]. These changes in composition are associated with a decrease in tensile modulus and strength[20].

Cartilage hydration and the load-bearing biomechanical function are influenced by the balance between the swelling propensity of proteoglycan molecules and the restraining function of the COL network. This idea was proposed by Maroudas[21] and has been supported by theoretical models[5,6,22,23]. The increased hydration and loss of mechanical integrity of osteoarthritic cartilage, compared with normal cartilage, is due to a weakening of the COL network and an associated swelling of the tissue[24–26]. Analogously, in the context of growth, a low or reduced restraining function of the COL network, due to either variations in network composition or structure or due to excessive swelling pressure imposed by the newly synthesized proteoglycan, is predicted to allow tissue swelling and growth[10]. Thus, we hypothesize that cartilage growth results from a dynamic imbalance between the swelling pressure of endogenous (that present at the time of explant) and newly synthesized GAG and the restraining function of the COL network. Previously[27], we compared the growth of calf cartilage explants, some of which were depleted of proteoglycan with chondroitinase ABC before subsequent culture, and hence should have a reduced propensity to grow and/or to swell. Removal of proteoglycan caused a functional tissue stiffening, and, when such explants were subsequently incubated in the serum-supplemented medium, the volumetric tissue growth was restricted, matrix GAG restored, the enhanced tensile properties maintained, and the resultant tissue features were characteristic of a more mature tissue state.

Another way to test this hypothesis is to examine the effect of blocking the metabolic pathway for the formation of enzyme-mediated COL crosslinks on growth of cartilage explants. COL crosslinks are a major element in stabilizing the COL network, hence explants where crosslinking is blocked should have an enhanced propensity to grow and/or to swell. Lysyl oxidase mediates covalent crosslinking among COL II, IX and XI fibrils[28,29] by oxidizing hydroxylysine residues to hydroxylysyl aldehydes which then, through several reactions, lead first to immature dehydroxylysinorleucine (DHLNL) crosslinks, then to stable PYR crosslinks[30]. Lysyl oxidase-mediated COL crosslink formation can be inhibited with a lathyrogen such as β-aminopropionitrile (BAPN), which binds covalently and irreversibly to the active site of the enzyme[31–33]. BAPN has been found to cause a 50% inhibition of lysyl oxidase at a concentration of 3–5 μM[34], and at even higher concentrations (e.g., 0.1–0.25 mM), BAPN has little or no discernible effect on matrix synthesis by chondrocytes[32,35]. Specific inhibition of COL crosslink formation without an effect on general biosynthesis makes BAPN a useful tool to study the effect of inhibition of functional assembly of the COL network on the integrity of the COL network and on cartilage growth. Thus, to address the hypothesis of this study, the objectives were to examine the effect of BAPN on culture-associated variations in (1) tissue size, (2) accumulation of GAG and COL network components, and (3) tensile mechanical properties.

Methods

SAMPLE PREPARATION AND CULTURE

Articular cartilage was harvested from the patellofemoral groove of five newborn (1–3 weeks) bovine calves, as described previously[20]. Blocks, 9×3 mm^2 in area, were prepared using a sledge microtome to either include the intact articular surface (superficial layer, ~0.4 mm thick, S) or to include the middle zone, starting at a distance of ~0.6 mm from the articular surface (middle layer, 0.25 mm thick, M). The term layer rather than zone is used to avoid associations with classical zonal classification of articular cartilage. The long axes of the blocks were in the anterior–posterior direction and, thus, approximately perpendicular to the split line direction. Blocks were weighed wet (WW_i) under sterile condition.

Some blocks were (1) analyzed immediately. Other blocks were incubated in (2) medium (Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum (FBS) and 100 μg/mL of ascorbate) or (3) medium with 0.1 mM BAPN (Sigma, St. Louis, MO). Medium was changed every other day, and during the first 12 days of culture supplemented with 4.5 μCi/mL [^{3}H]proline and 1.8 μCi/mL [^{35}S]sulfate. To remove unincorporated isotopes, blocks were washed, transferred to a new culture plate, and incubated for an additional day in medium without radiolabel. Spent medium was collected throughout the culture duration. At termination, blocks were weighed wet (WW_f) and punched to form a tapered tensile test specimen and residual cartilage.

BIOCHEMICAL ANALYSIS

Residual cartilage and the failed portions of the corresponding tensile strip (see below) were analyzed together to quantify the biochemical composition of the fresh and cultured samples. Samples were lyophilized, weighed dry, and solubilized with proteinase K[20]. Portions of the tissue digest were analyzed to quantify the content of DNA[36], GAG[37], and hydroxyproline (HYPRO)[38]. Digest portions from each animal/layer/experimental condition were pooled for analysis of PYR[39]. DNA was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell[40]. HYPRO content was converted to COL content by assuming a mass

ratio of COL to HYPRO equal to 7.25[16,41]. The molar ratio of PYR per COL molecule was calculated, assuming the molecular weight of COL triple helix of 300,000. Because initial tissue wet weight (WW_i) varied among samples on day 0, mass of each biochemical constituents on day 13 was normalized to WW_i, to provide information about the content of biochemical constituents in the tissue. To represent biochemical constituent concentration, mass of each biochemical constituents on day 13 was normalized to the final wet weight of the tissue (WW_f).

ANALYSIS OF MATRIX METABOLISM

Other portions of the solubilized tissue and portions of the medium were analyzed for the incorporated radioactivity to assess matrix metabolism. The contents of [^{35}S] and [^{3}H] radioactivity were determined in the tissue digest and used to estimate the absolute rates of sulfate and proline incorporation as indices of 12-day average sulfated-GAG and protein synthesis, respectively. To more specifically assess COL synthesis, portions of tissue digests and medium were pooled for each animal/layer/experimental condition and analyzed for [^{3}H]HYPRO residues using Dowex columns[18,42]. The content of [^{3}H]HYPRO was used to estimate the absolute rate of HYPRO formation. To estimate the absolute rate of sulfate release into the medium, a portion of spent medium was analyzed for the content of [^{35}S]GAG using the Alcian Blue precipitation method[43].

BIOMECHANICAL ANALYSIS

Tapered tensile specimens were analyzed to determine mechanical properties as described previously[44]. From each cartilage block, a tapered strip[45] with a gage region of 4 mm $\times$ 0.80 mm was prepared using a punch. The thickness of each tensile strip was measured at three locations in the gage region, using a contact-sensing micrometer, and the average was used for cross-sectional area calculations. Tapered specimens were then secured in clamps (4.0 mm apart) of a mechanical tester and elongated at a constant extension rate (5 mm/min) until failure. Structural tensile parameters were obtained from the load–displacement curves. Structural tensile strength was determined as the maximum load sustained at failure. Ramp stiffness was calculated as the slope of the linear regression of the load–displacement curve from 25 to 75% of the maximum load. Load and displacement were converted to stress (defined as load normalized to the cross-sectional area of the gage region) and strain (defined as the elongation distance normalized to the initial clamp to clamp distance) to obtain material tensile parameters of tensile strength, strain at failure (the strain at which maximum stress was attained), and ramp modulus. The failed portions of each tensile strip, resulting from the tensile test, were saved for biochemical analysis (described above) in addition to the adjacent cartilage samples obtained during preparation of the tensile strips.

STATISTICAL ANALYSIS

For each layer (S and M), the effects of experimental conditions (d0, d13, d13 + BAPN) were assessed by analysis of variance (ANOVA) with experimental condition as fixed factor and donor animal as a random factor. For dependent variables that involved PYR and HYPRO, which were determined from a pool of samples, the effects of experimental conditions were assessed by ANOVA with experimental condition and layer as fixed factors and donor animal as a random factor. Tukey's *post hoc* testing was performed to compare groups. To analyze the effect of day in culture on wet weight, repeated measures ANOVA was performed for each layer and experimental condition with wet weight (WW_i, WW_f) as a repeated factor. The average load–displacement and stress–strain curves were plotted to demonstrate overall trends but not analyzed statistically at each point since statistical analysis was performed on the structural and material biomechanical properties derived from such curves for individual samples. Data are expressed as mean $\pm$ S.E.M., and significance level was set to 0.05. Statistical analysis was performed using Systat 10.2 (Systat Software, Inc., Richmond, CA).

Results

The extent of *in vitro* volumetric growth of articular cartilage blocks was markedly affected by experimental conditions as assessed by changes in thickness ($P < 0.05$) [Fig. 1(A)], wet weight ($P < 0.001$) [Fig. 1(B)], and water content [Fig. 1(C)]. Both the thickness and wet weight increased during culture in medium (42–60% and 38–73%, respectively; $P < 0.05$) and medium with BAPN (44–80% and 44–98%, respectively; $P < 0.05$). The extent of change in wet weight during culture with BAPN exceeded that of explants cultured without BAPN, with the largest difference detected in the S layer (25%, $P < 0.05$). The volumetric growth of all explants appeared to be predominantly in the direction perpendicular to the articular surface, as changes in wet weight can mostly be accounted for by changes in thickness alone. The content of water did not vary with experimental conditions in the S layer ($P = 0.30$) but varied in the M layer ($P < 0.01$), decreasing slightly during culture in medium without BAPN (~3.6%, $P < 0.01$). Since the content of water did not change during culture with BAPN ($P > 0.44$), it was higher than that of explants cultured in medium for explants from the M (~2.7%, $P < 0.05$) and the S (~1.7%) layers, however, in the S layer this trend did not reach statistical significance ($P = 0.28$). Since water content varied only slightly on an absolute basis, changes in wet weight were largely due to changes in tissue volume and not density and, thus, along with changes in thickness, represent volumetric growth of tissue samples.

The content of cells [Cells/WW_i, Fig. 2(A)] did not vary with experimental conditions ($P > 0.25$), and, when normalized to WW_f to give a measure of concentration of cells [Cells/WW_f, Fig. 2(E)], reflected the changes in wet weight ($P < 0.001$). Cells/WW_f decreased in explants cultured in medium (~39%, $P < 0.001$), and medium with BAPN (~43%, $P < 0.001$).

The extent of volumetric growth was generally paralleled by variations in the tissue content of GAG, COL and PYR. GAG content [GAG/WW_i, Fig. 2(B)] varied with experimental conditions ($P < 0.001$), while the content of COL [COL/WW_i, Fig. 2(C)] varied only in the S layer ($P < 0.05$). While GAG/WW_i increased markedly during culture in medium (~48%, $P < 0.001$) and medium with BAPN (~43%, $P < 0.001$), COL/WW_i remained unchanged (during culture with BAPN and in the M layer during culture in medium $P > 0.87$), or increased slightly (~14% during culture in medium, $P < 0.05$ in S). The variations in COL/WW_i were small, so that the extents of deposition of both GAG/WW_i and COL/WW_i were similar in explants incubated with and without BAPN ($P > 0.15$). The content of PYR [PYR/WW_i, Fig. 2(D)] varied with experimental conditions ($P < 0.05$),

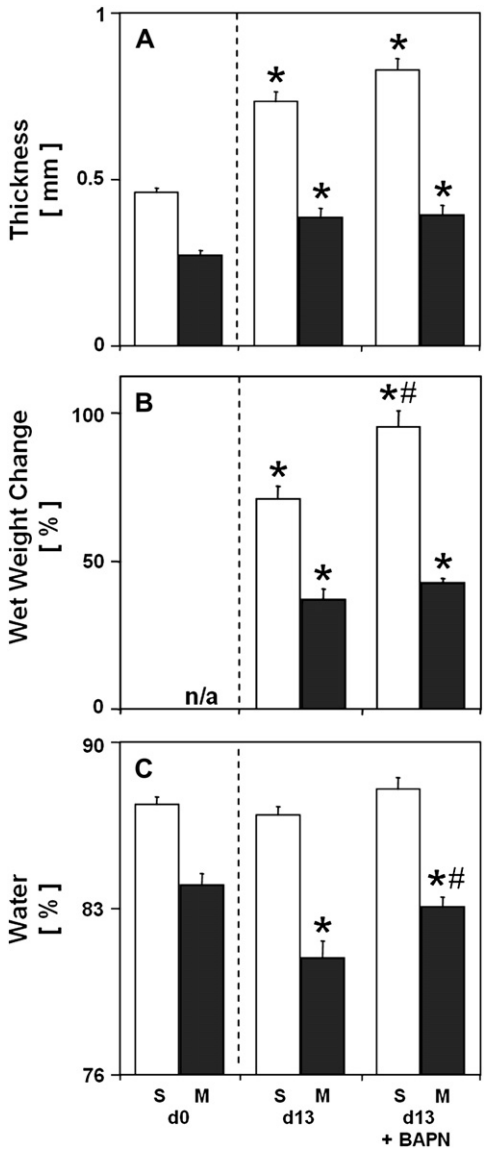

Fig. 1. Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S) and middle (M) layers. Blocks were analyzed on day 0 (d0), or incubated for 13 days (d13) in medium (20% FBS) or medium supplemented with 0.1 mM BAPN. (A) Thickness, (B) change in wet weight, and (C) percent water of cartilage blocks. The dotted line separates explants analyzed on day 0. * Indicates $P < 0.05$ vs d0, # indicates $P < 0.05$ vs d13 for a corresponding layer. Data are mean $\pm$ S.E.M., $n = 10-20$ blocks from five animals.

such that PYR/WW_i increased during culture in medium (37%, $P = 0.07$), but remained unchanged during incubation in medium with BAPN ($P = 0.83$). As a result PYR/WW_i was higher in explants incubated in medium than in explants incubated in medium with BAPN (33%, $P < 0.05$).

When normalized to WW_f to give an index of concentration in the tissue, the concentrations of extracellular matrix components reflected changes in the content of the components and in the wet weight of the tissue during culture. The concentration of GAG [GAG/WW_f, Fig. 2(F)] varied with experimental conditions in the S layer ($P < 0.001$) but did not change during culture of explants from the M layer ($P > 0.20$), while the concentration of COL [COL/WW_f, Fig. 2(G)] varied in both layers ($P < 0.001$). GAG/WW_f decreased slightly during incubation in medium with BAPN (18%, $P < 0.001$), but not during incubation in medium ($P = 0.33$), while COL/WW_f decreased during incubation in medium ($\sim$24%, $P < 0.05$) and the medium with BAPN ($\sim$37%, $P < 0.001$). As a result, both GAG/WW_f and COL/WW_f were similar among explants incubated with and without BAPN, except explants from the S layer where GAG/WW_f and COL/WW_f were slightly lower after incubation with BAPN than after incubation without BAPN (17% and 22%, respectively; $P < 0.05$). The concentration of PYR [PYR/WW_f, Fig. 2(H)] varied with experimental conditions ($P < 0.01$). PYR/WW_f did not change during incubation in medium ($P = 0.78$), and decreased ($\sim$44%, $P < 0.01$) during incubation in medium with BAPN, so that it was lower than PYR/WW_f in explants cultured without BAPN ($\sim$37%, $P < 0.05$). As a result of variations in PYR/WW_f the ratio of PYR to COL [Fig. 2(I)] varied with experimental conditions ($P < 0.05$). While the ratio did not change during culture with and without BAPN ($P > 0.20$), it was lower in explants incubated with BAPN than those incubated without BAPN ($\sim$24%, $P < 0.05$).

The changes in GAG content [Fig. 2(B)] during culture were generally paralleled by similar variations in sulfate incorporation [Fig. 3(A)], as well as proline incorporation [Fig. 3(B)] and HYPRO formation [Fig. 3(C)]. The addition of BAPN to the culture medium did not affect sulfate incorporation into the tissue ($P > 0.28$), while the incorporation of proline into the tissue decreased slightly ($\sim$20%, $P \leq 0.05$). The percent of HYPRO residues in the tissue did not vary and reached 36%. As a result, the content of HYPRO in the tissue paralleled variations in proline incorporation with a slightly lower HYPRO content ($\sim$20%, $P < 0.05$) in explants incubated with BAPN. While the release of both sulfate and HYPRO into the medium was not detectably affected by BAPN ($P > 0.24$), the release of HYPRO into the medium tended to be slightly higher in explants ($\sim$15% in M) that were cultured with BAPN. BAPN did not affect the overall COL synthesis, as the total HYPRO formation (in tissue and medium) was similar ($P = 0.64$) for explants incubated with and without BAPN.

Experimental condition also affected the tensile biomechanical behavior of the cartilage explants as demonstrated by the average load–displacement and stress–strain curves and the mechanical properties derived from these curves (Figs. 4 and 5). Incubation of cartilage explants in medium and medium with BAPN reduced the strength at failure and the slopes of the average load–displacement and stress–strain curves (Fig. 4) with a greater reduction of tensile parameters in explants incubated with BAPN than those incubated without BAPN.

Certain structural biomechanical properties of cartilage explants varied with experimental condition ($P < 0.001$). Both ramp stiffness [Fig. 5(A)] and strength [Fig. 5(B)] decreased during culture in medium ($\sim$36%, $P < 0.01$; $\sim$31% in S, $P < 0.05$ and $\sim$17% in M, respectively) and sharply decreased during incubation in medium with BAPN ($\sim$65% and $\sim$68%, respectively; $P < 0.001$). As a result, the decrease in ramp stiffness and strength of explants incubated in medium with BAPN was more marked than that of explants incubated in medium without BAPN ($\sim$49% and $\sim$59%, respectively; $P \leq 0.05$).

The variations in the material biomechanical properties of cartilage explants paralleled the statistical variations and accentuated the trends in the structural biomechanical properties. Both ramp modulus [Fig. 5(C)] and strength [Fig. 5(D)] decreased during incubation in medium ($\sim$53%

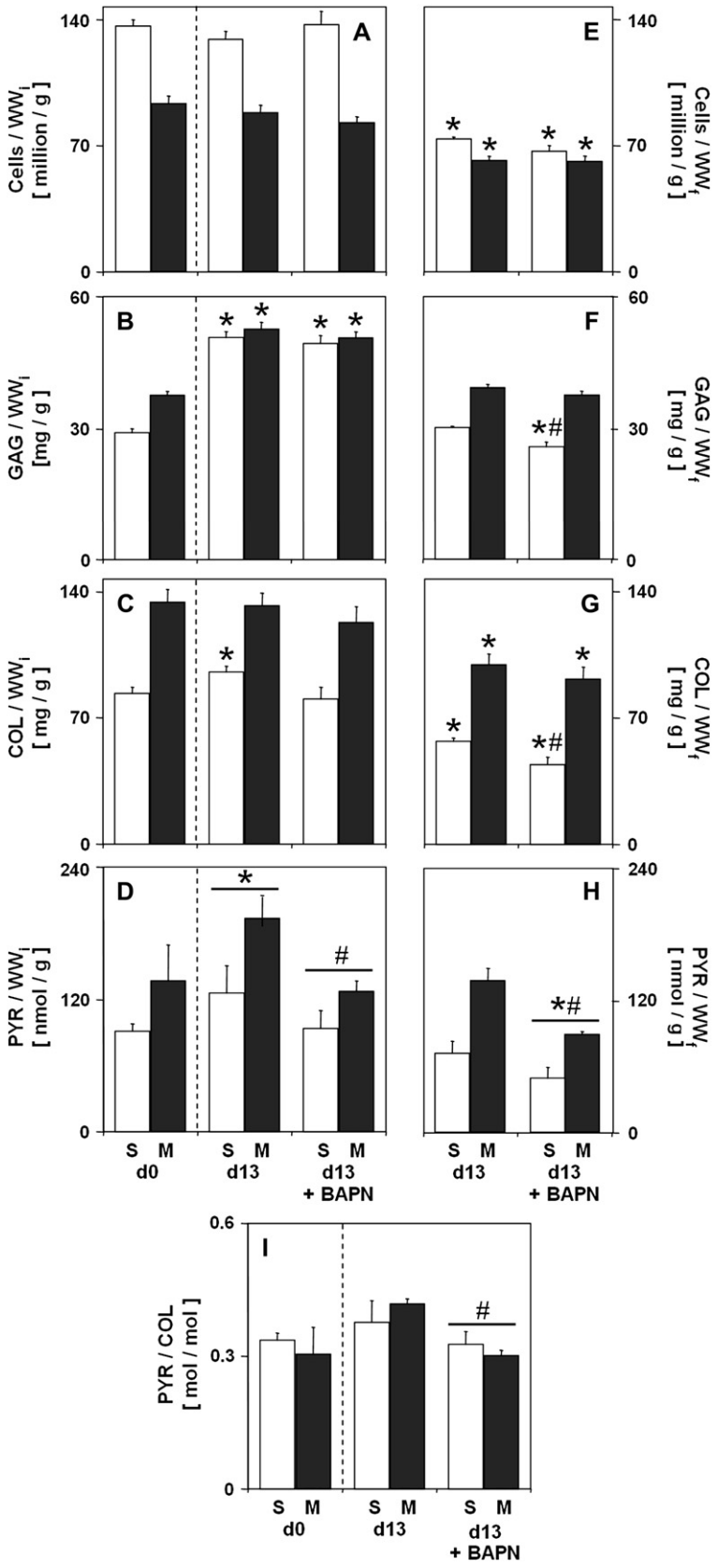

Fig. 2. Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the S and M layers. (A, E) Cells, (B, F) GAG, (C, G) COL, and PYR crosslink (D, H) normalized to initial wet weight (WW_i) to represent constituent content (A−D) and to final wet weight (WW_f) to represent constituent concentration (E−H). (I) Molar ratio of PYR to COL. The dotted line separates explants analyzed on day 0, where $WW_f = WW_i$. * Indicates $P < 0.05$ vs d0, # indicates $P < 0.05$ vs d13 for a corresponding layer, while ——— indicates where statistical analysis was performed on both layers together. Data are mean ± S.E.M., $n = 10$−20 blocks from five animals.

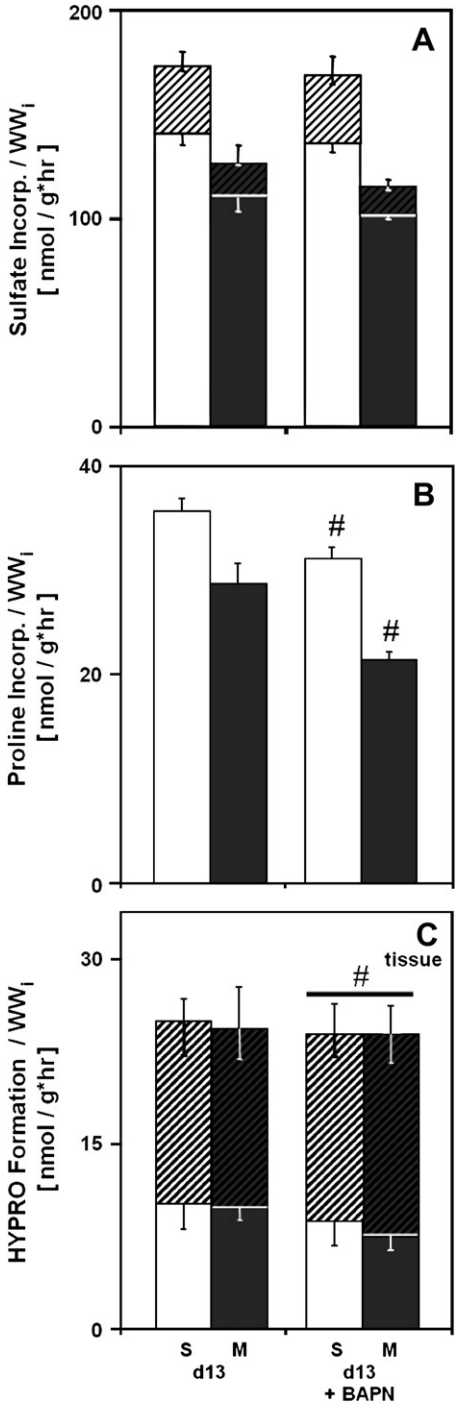

Fig. 3. Effect of experimental conditions on sulfate and proline incorporation and HYPRO formation of calf articular cartilage explants from the S and M layers. Content of (A) sulfate and (B) proline incorporation, and (C) HYPRO formation in the tissue (solid) and released into the medium, where applicable (striped). Total sulfate incorporation and HYPRO formation is represented by the overall bar height and upward error bar. # Indicates $P < 0.05$ vs d13 for a corresponding layer, while ——— indicates where statistical analysis was performed on both layers together. Data are mean ± S.E.M., $n = 10–20$ blocks from five animals.

and ~47%, respectively; $P < 0.01$) and sharply decreased during incubation in medium with BAPN (~76% and ~79%, respectively; $P < 0.001$). As a result, the decrease in ramp modulus and strength of explants incubated in medium with BAPN was more marked than those of explants incubated in medium without BAPN (~46% and ~60%, respectively, $P < 0.05$). Failure strain [Fig. 5(E)] did not vary with experimental conditions in the S layer ($P > 0.22$) but varied in the M layer ($P < 0.01$). Failure strain increased slightly (17%, $P < 0.01$) in explants incubated in medium so that it exceeded the failure strain of explants incubated in medium with BAPN (~10%, $P < 0.01$).

Discussion

The data presented here demonstrate that manipulation of COL network assembly can distinctly alter the growth phenotype in explants of immature bovine articular cartilage, as indicated by changes in tissue size, content of matrix components, and the integrity of the COL network. (1) Incubation with 20% FBS resulted in *expansive cartilage growth* [Fig. 6(B)] characterized by a marked increase in tissue volume [Fig. 1(A,B)] and the content of GAG [Fig. 2(B)], and a slight increase in the contents of COL and PYR [Fig. 2(C,D)]. The result was a maintenance of the concentrations of GAG and PYR [Fig. 2(F,H)], reduction in the concentration of COL [Fig. 2(G)], and a concomitant diminution in tensile mechanical integrity (Figs. 4 and 5). (2) Addition of BAPN induced *accelerated cartilage growth* [Fig. 6(C)] by accentuating the *expansive cartilage growth* phenotype in terms of a further increase in tissue size in explants from the superficial layer [Fig. 1(A,B)], and a further reduction in the concentration of GAG and COL [Fig. 2(F,G)] and of mechanical integrity (Figs. 4 and 5) in explants from both the superficial and middle layers. Consistent with the specific effect of BAPN on COL crosslinking, the increase in PYR content during culture was blocked by inclusion of BAPN [Fig. 2(D)], without affecting the contents of GAG and COL [Fig. 2(B,C)].

The use of immature cartilage explants for studying mechanisms of articular cartilage growth required consideration of a number of issues. The tissue was harvested in layers, using the top ~1 mm of articular cartilage [Fig. 1(A)]. The superficial 0.4 mm layer included the articular surface and was prepared to include the cells that are situated to become those present in mature articular cartilage. Because the various zones of normal cartilage exhibit differences in biochemical composition and mechanical properties, a middle layer was also analyzed, and displayed an initial state different than that of the superficial layer. The patellofemoral groove was used as the source of tissue, similar to tissue used in previous studies[14,18,20]. Consequently, the biochemical and biomechanical properties of tissue samples at the time of explant were similar to those reported previously[14,20].

While BAPN at doses effective at blocking crosslink formation may not effect matrix synthesis in adult bovine cartilage, with levels being similar to samples incubated in medium without BAPN[32,35], the slight inhibitory effect observed here is consistent with previous studies on calf cartilage[35]. The inhibitory effect on protein synthesis [Fig. 3(B)] is combined with a slight increase in secretion of HYPRO into the culture medium [Fig. 3(C)][35], suggesting that BAPN does not affect the overall synthesis of COL, as is also indicated by similar rates of total HYPRO formation (in tissue and medium) [Fig. 3(C)], but reduces the retention of newly synthesized molecules in the tissue. The increased

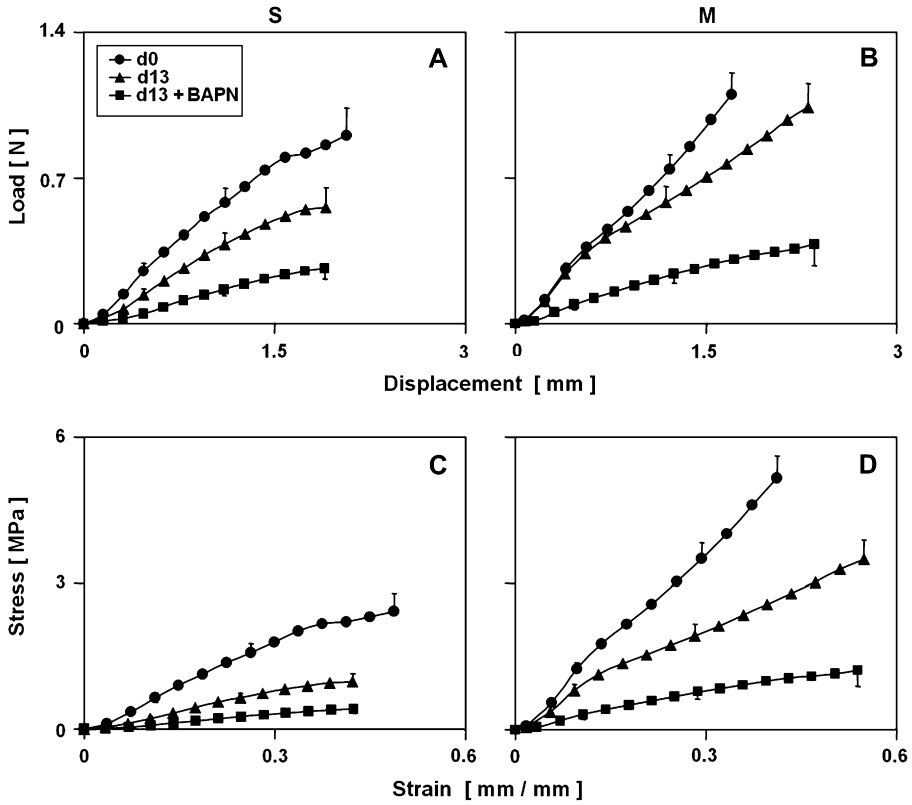

Fig. 4. Effect of experimental conditions on growth associated changes in structural and material tensile mechanical behavior of calf articular cartilage explants from the S (A and C) and M (B and D) layers. Average load–displacement (A–B) and stress–strain (C–D) profiles preceding the failure stress of the weakest sample within a group are displayed for blocks that were analyzed on day 0 (d0) (●) and on day 13 (d13) after incubation in medium (▲) or medium supplemented with 0.1 mM BAPN (■). Values are the mean ± S.E.M. at selected points, $n = 10–20$ blocks from five animals.

extractability of newly synthesized COL after culture with BAPN[32,35] may facilitate the release of a small number of these molecules into the medium. While the retention of the newly synthesized COL may be lower in explants incubated with BAPN than those without BAPN, it is not likely to be the major factor in determining the phenotype of growth, as the overall COL deposition is not affected [Fig. 2(C)].

It appears that free-swelling growth of immature cartilage explants *in vitro* in the presence of serum involves a shift in the balance between the swelling pressure of the proteoglycan molecules and the restraining ability of the COL network, in favor of the swelling pressure. Factors that contribute to this imbalance, and consequent volumetric tissue growth, involve both the additional swelling pressure associated with the newly synthesized GAG [Fig. 2(B)] as well as remodeling and reorganization of the COL network that render it loose and weak (Figs. 4 and 5). During *expansive cartilage growth*, the swelling pressure of intrinsic and newly synthesized GAG was sufficient to not only expand the weakened endogenous COL network, but also prevent its functional reinforcement with the newly deposited COL. This was accentuated further during *accelerated cartilage growth*, when the formation of COL crosslinks, a major element in stabilizing the COL network, was inhibited. Similar levels of the swelling pressure resulted in even further volumetric expansion of the tissue. Such types of tissue growth appear to result from active metabolic processes that are mediated by chondrocytes and not from a passive process of tissue swelling since inhibition of biosynthesis at 4°C (data not shown) resulted in maintenance of cartilage explant geometry (±2% of initial wet weight) for up to 13 days in the serum-supplemented culture. The relative insensitivity of absolute water content [Fig. 1(C)], e.g., especially in the S layer, to *in vitro* growth or growth in the presence of BAPN treatment may be due to effects of molecules that are deposited and remodeled during the growth period as the tissue expands [Fig. 1(B)]. Thus, COL crosslinking is a major element in modulating the phenotype of cartilage growth and the associated balance between proteoglycan content and integrity of the COL network.

The effect of BAPN on the development of a distinct cartilage growth phenotype is consistent with the specific inhibitory effect of BAPN on COL crosslink formation[31] and kinetics of COL crosslinking in articular cartilage[33]. During culture of adult bovine articular cartilage, PYR crosslinks increase gradually, with a characteristic time constant of formation of 7–30 days[33], and thus can form during a 13-day incubation. While other forms of crosslinks that are not blocked by BAPN can also form during culture, they are not likely to contribute to the growth of immature cartilage tissue during a relatively short, 13-day incubation *in vitro*. Maillard crosslinks formed through non-enzymatic glycation accumulate slowly after skeletal maturity[46,47], but not during growth[48], while COL network crosslinking mediated by transglutaminase is generally associated with cartilage regions undergoing terminal differentiation[49]. The conversion of endogenous DHLNL into mature PYR crosslinks can still occur during culture with BAPN since lysyl

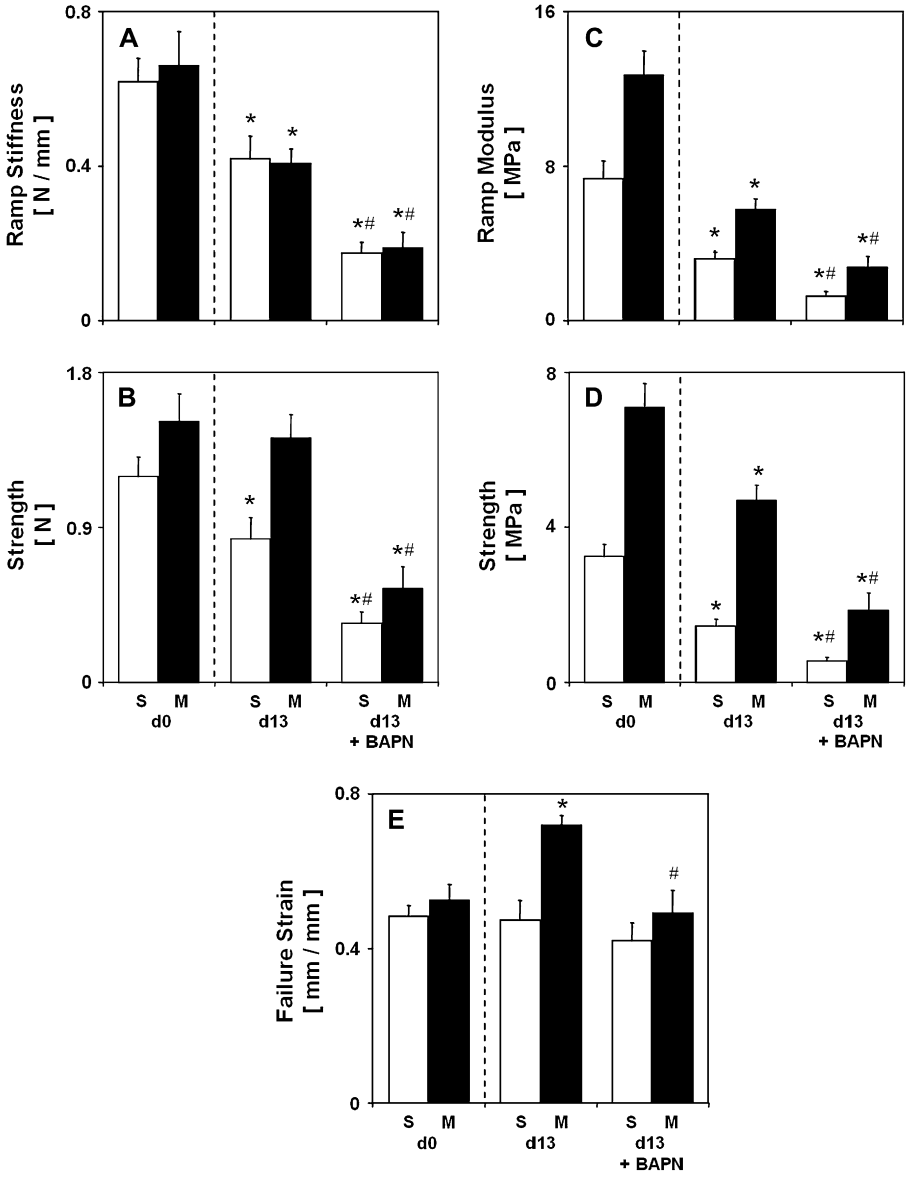

Fig. 5. Effect of experimental conditions on structural and material tensile mechanical properties of calf articular cartilage explants from the S and M layers. Structural properties: (A) Ramp modulus, and (B) strength. Material properties: (C) ramp modulus, (D) strength, and (E) failure strain. The dotted line separates explants analyzed on day 0. * Indicates $P < 0.05$ vs d0, # indicates $P < 0.05$ vs d13 for a corresponding layer. Data are mean $\pm$ S.E.M., $n = 10-20$ blocks from five animals.

oxidase acts upstream of DHLNL formation. However, this conversion is estimated to be <4% of DHLNL that mature into PYR in the absence of BAPN, based on the kinetics of maturation of the newly formed DHLNL in bovine cartilage during culture with and without BAPN[33,50].

The assembly of a functional COL network through synthesis of COL fibrils and the formation of COL crosslinks is critical to the integrity and tensile properties of articular cartilage as well as other connective tissues. In animals, where enzyme-mediated COL crosslinking has been inhibited with administration of BAPN, the mechanical properties[51,52] and wound healing response[53–55] of various tissues greatly diminished. *In vitro*, incubation of cartilage explants with BAPN blocked the development of strength between apposing cartilage surfaces in a model of integrative cartilage repair[35].

Growth of all explants appeared to be inhomogeneous with respect to the depth from the articular surface of the source tissue and also anisotropic. Inhomogeneity of growth, as demonstrated by a large extent of volumetric growth in the superficial layer [~80%, Fig. 1(B)] and only modest volumetric growth in the middle layer [~45%, Fig. 1(B)], could be due to depth-associated variations in the content and structural organization of matrix components and the associated mechanical integrity of cartilage tissue [Figs. 2(B–D), 4 and 5][2,56,57]. It is possible that the parallel orientation of COL fibrils of the superficial zone of cartilage[56] has a larger propensity to expand upon application of the swelling pressure associated with the newly synthesized GAG than the COL network in the middle layer where COL fibrils do not exhibit a preferred orientation. Alternatively, such inhomogeneous growth could be due

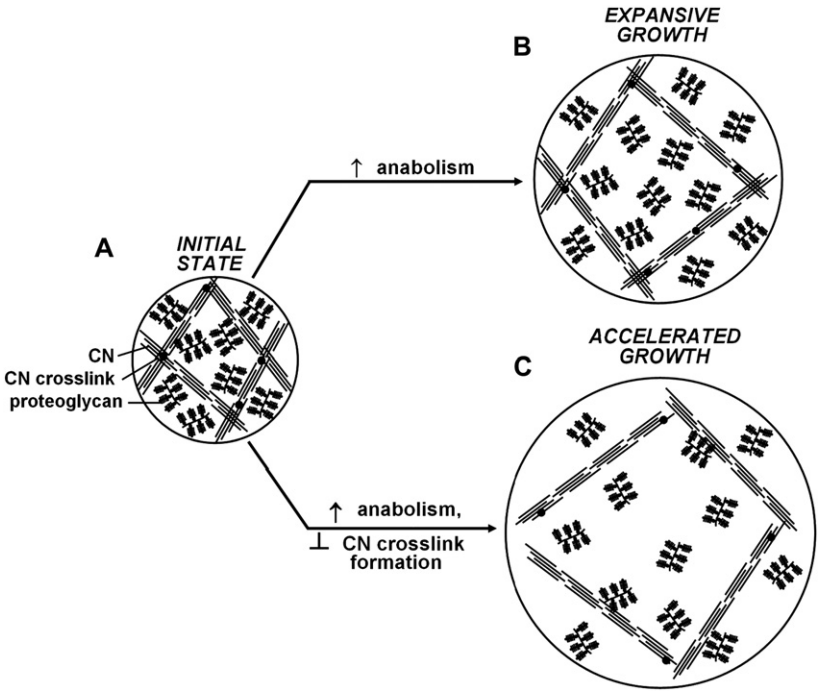

Fig. 6. Schematic of metabolism of proteoglycan and COL network components, leading to different types of cartilage growth. Initial state (A) with the major components of the solid extracellular matrix. Stimulation of anabolism with 20% FBS leads to (B) *expansive growth* or (C) *accelerated growth* when COL crosslink formation is inhibited with BAPN.

to a number of resident chondrocytes, which also varies with depth from the articular surface [Fig. 2(A)][58]. Although different extents of volumetric growth occurred in the superficial and middle layers, in each layer the volumetric growth appeared to be mostly in the direction perpendicular to the articular surface, as >80% of the change in wet weight can be accounted for by the change in thickness. While it is possible that tissue dimensions may determine the preferential axis of growth, these patterns suggest that cartilage tissue may grow appositionally or that appositional growth mechanisms are dominant[59].

While the relationship between manipulations *in vitro* and growth and maturation control mechanisms *in vivo* remain to be defined, the findings of the current study may have practical utility for tissue engineering and cartilage repair. The *expansive growth* phenotypes (1) may be beneficial for volumetric tissue growth and used to create cartilage tissue constructs *in vitro* or fill cartilage defects directly *in vivo*. The *accelerated growth* phenotypes (2) may be useful for promoting large extents of volumetric tissue growth and, after generating a cartilage tissue construct of a desired volume, used to fill cartilage defects *in vivo*. In addition to being able to fill large defect spaces, cartilage tissue constructs formed in the presence of BAPN can potentially accelerate and enhance integration of this tissue with the host tissue[60] by providing a source of the non-crosslinked COLs that build up during culture with BAPN[35].

Acknowledgments

This work was supported by the National Institute of Health and National Science Foundation.

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