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Effects of Proteoglycan Modification on Mineral Formation in a Differentiating Chick Limb-Bud Mesenchymal Cell Culture System

Adele L. Boskey,* Dalina Stiner, Itzhak Binderman, and Stephen B. Doty

Hospital for Special Surgery, New York, New York

Abstract In the presence of 4 mM inorganic phosphate, differentiating chick limb-bud mesenchymal cells plated in micromass cultures form a mineralized matrix resembling that of chick calcified cartilage. To test the hypothesis that cartilage proteoglycans are inhibitors of cell mediated mineralization, the synthesis, content, and turnover of proteoglycans were altered in this system, and the extent of mineralization and properties of the mineral crystals examined. In all cases where the proteoglycan synthesis or proteoglycans present were modified to provide fewer or smaller molecules, mineralization was enhanced. Specifically, when proteoglycan synthesis was blocked by treatment with 10^{-10} M retinoic acid, extensive mineral deposition occurred on a matrix devoid of both proteoglycans and cartilage nodules. The crystals, which formed rapidly, were relatively large in size based on analysis by X-ray diffraction or FT-IR microspectroscopy, and were more abundant than in controls. When 2.5 or 5 mM xylosides were used to cause the synthesis of smaller proteoglycans, the extent of mineral accretion was also increased relative to controls; however, the matrix was less affected, and the extent of mineral deposition and the size of the crystals were not as markedly altered as in the case of retinoic acid. Modification of existing proteoglycans by either chondroinase ABC or hyaluronidase treatment similarly resulted in increased mineral accretion (based on ^{45}Ca uptake or total Ca uptake) relative to cultures in which the proteoglycan content was not manipulated. Crystals were more abundant and larger than in control mineralizing cultures. In contrast, when proteoglycan degradation by metalloproteases was inhibited by metal chelation with o-phenanthroline, the Ca accretion at early time points was increased, but as mineralization progressed, Ca accumulation decreased. These data provide evidence that in this culture system, proteoglycans are inhibitors of mineralization. J. Cell. Biochem. 64:632–643.

Key words: calcification; proteoglycans; chondrocyte culture; micro-mass culture; cartilage calcification

Within the epiphyseal growth plate, the large aggregating chondroitin sulfate proteoglycans of cartilage (aggrecan) change in physical properties as calcification commences [1,2]. Whether this change is essential for growth plate calcification or coincident to this process is in debate. Some solution studies indicate that aggrecan modification (decreases in size, desulfation) facilitates cell-free in vitro calcification [3–7], while others suggest that it is the change in local phosphate concentration, in the absence of proteoglycan modification, which facilitates calcification [8,9].

Mesenchymal cells, plated in a micromass culture [10], differentiate into chondroblasts and chondrocytes [11,12], and can form a mineralized matrix when the media is supplemented with inorganic or organic phosphate [12–15]. The mineralized matrix resembles that of the calcified cartilage of 17-day-old chicks [15]. Aggrecan core protein expression, along with the synthesis of types II and X collagen, occurs in a time-dependent and reproducible fashion [13]. This mineralizing system is therefore appropriate for studying the effect of proteoglycan modification on the calcification process. This report describes the effects of five conditions known to alter aggrecan synthesis and turnover on the amount and properties of the mineral formed in the micromass cultures. Retinoic acid was used to block mesenchymal cell differentiation and chondrocyte maturation [16], and to prevent glycosaminoglycan synthesis while having no detectable effect on total protein synthesis [17–19]. Treatment with β-D-xylosides was used to

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*Correspondence to: Adele L. Boskey, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021.

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assess the effect of the synthesis of smaller aggrecan molecules [20–22]. The proteoglycan matrix was disrupted using hyaluronidase, which degrades proteoglycan aggregates leaving the aggrecan monomers intact [23], and treatment with chondroitinase ABC, which removes chondroitin sulfate from the aggrecan protein core [24]. Finally, the activity of matrix metalloproteases implicated in proteoglycan turnover during endochondral ossification [25–26] was inhibited by addition of the zinc chelator ortho-phenanthroline [27]. Results indicate that alteration of aggrecan production and loss of aggrecan structural integrity have significant effects on the amount and properties of the apatite mineral deposited in culture.

MATERIALS AND METHODS

Cell Culture

Chick mesenchymal cells were isolated from stage 21–24 [28] fertilized White Leg Horn eggs (Truslow Farms, Chestertown, MD) as described in detail elsewhere [13]. In brief, the eggs were maintained in a humidified incubator at 37°C. At 4.5 days, embryos were sterilely withdrawn from the eggs and their limb buds removed into 0.9% USP grade saline (Abbott Laboratories, North Chicago, IL). Cells, released from the limb buds by digestion with 5 ml 0.25 wt % trypsin-0.53 mM EDTA (GIBCO, Grand Island, NY), were separated from debris by passage through two layers of 20 μm Nitex membrane (Tetko Inc., Ardsley, NY). Cells were counted with a hemocytometer, checked for viability by trypan blue dye exclusion, and pelleted in the cold at 2,300 rpm. Cells were resuspended in medium containing 1.1 mM Ca and plated using the micro-mass technique [10] at a density of 0.5 million cells per 20 μl drop in 35 × 10 mm Falcon (Lincoln Park, NJ) dishes and allowed to attach for 2 h in a humidified atmosphere of 5% CO_2 at 37°C. After 2 h, Dulbecco's Modified Essential Medium (DMEM; GIBCO Formula 80-0303A), containing 1.1 mM calcium chloride, 1,000 mg/l glucose, and supplemented with 50 U/ml penicillin and 25 μg/ml streptomycin, and 10% Fetal calf serum (GIBCO), was added. Medium was changed every 48 h until the termination of the experiment, along with addition of 25 μg/ml ascorbic acid, and 0.3 mg/ml glutamine after day 2. Mineralizing cultures were also supplemented with 3 mM inorganic phosphate from day 2 onward, making the total inorganic phosphate content of the medium 4 mM; non-mineralizing cultures received no additional inorganic phosphate, their inorganic phosphate content was 1 mM. The mineralizing conditions were previously shown to be optimal for cell maturation and mineral deposition in the absence of dystrophic or artifactual mineralization [13].

Modification of Aggrecan Synthesis and Content

Disruption of aggrecan synthesis. To alter cell differentiation, and prevent aggrecan synthesis, 10^{-8}–10^{-10} M retinoic acid (Sigma Chemicals, St. Louis, MO) was added to mineralizing and non-mineralizing cultures either on days 2, 5, or 7, or from day 5 until the conclusion of the experiment. At these initial times mesenchymal differentiation into chondrocytes had commenced, but hypertrophic chondrocytes had not formed [13]. Control mineralizing cultures for these experiments received ethanol, the carrier for retinoic acid. Non-mineralizing cultures were similarly treated. Ethanol concentrations in all cultures were less than 0.1%. Based on preliminary observations that the higher concentrations were toxic to the cells, most studies were conducted with 10^{-10} M retinoic acid.

Alteration of aggrecan size. To prepare aggrecan with shorter glycosaminoglycan chains, 4-methyl umbelliferyl β-D-xyloside (Sigma Chemicals), an artificial initiator, was added to mineralizing and non-mineralizing cultures such that the final concentrations were 0, 2.5, or 5 mM. Addition was on either days 5, 7, or 9 or from day 5 onward.

Modification of existing proteoglycans. To digest any proteoglycan aggregates present, hyaluronidase (final concentration 1 U/ml; streptomyces, Sigma Chemical) was added to mineralizing and non-mineralizing cultures on either days 7 or 12, or from day 12 onward. The enzyme was kept in the cultures until the next change of medium. Control cultures received no enzyme treatment.

To remove the chondroitin sulfate from proteoglycan aggregates, aggrecan, and other CS-proteoglycans, chondroitinase ABC (0.1 U/ml; Sigma Chemicals) was added to mineralizing and non-mineralizing cultures either on days 7 or 12, or from day 7 onward with each change of medium. Control cultures, as above, received no enzyme treatment.

Alterations of aggrecan turnover. Orthophenanthroline (o-phe) (Sigma Chemicals) was

added to mineralizing and non-mineralizing cultures to block the activity of the aggrecan degrading metalloproteases. Cultures were supplemented with o-phe such that the final concentration was 10 μM either from day 9 onward or once on day 9. Control mineralizing cultures received no o-phe, while non-mineralizing cultures were treated the same ways as mineralizing cultures (±o-phe).

Analytical Methods

Mineral analysis. The nature and the quality of the mineral formed was determined by X-ray diffraction and FT-IR microspectroscopic analyses. For X-ray diffraction, three micromass spot cultures, washed with pH 8 ammonium hydroxide to remove salts, were lyophilized, ground in a mortar and pestle, placed in a 0.7-mm diameter capillary, and subjected to wide angle X-ray diffraction analysis using a Debye-Scherrer camera and Ni filtered Cu K-α radiation. The characteristics of mineral were also determined by FT-IR microspectroscopy [15]. For these studies, cultures, fixed with absolute ethanol and sectioned to 5 μ on a microtome, were placed on barium fluoride windows and examined using a FT-IR microscope (Bio-Rad UMA500, Boston, MA) at 20 μ resolution. Mineral content was estimated from the relative ratios of the integrated areas of the phosphate ν_1, ν_3 and amide I absorbances at 900–1,200 cm^{-1} and 1,680–1,720 cm^{-1}, respectively [29]. At least 5 spectra were recorded for each spot culture at different positions within the culture (edge; center of nodule, and 20, 40, and 60 μ from the center of the chondrocyte nodule). A minimum of three different cultures were examined. Relative crystal size and perfection were estimated from the relative subband distribution of the ν_1, ν_3 phosphate band as determined by second derivative and curvefit analysis [30]. The percent areas of these bands have been related to the quality of the mineral crystals: crystal size, perfection, and composition including relative amounts of acid phosphate and non-stoichiometric apatite [30].

Mineral accumulation was monitored by measuring ^{45}Ca uptake or total calcium content of the cultures. For ^{45}Ca uptake experiments, cultures were labelled with 0.1 μCi/ml ^{45}Ca (Amersham Corp, Arlington Heights, IL) from day 5 onward with every change of medium. ^{45}Ca uptake was monitored 2 days after the last addition of label. At the indicated time points, the medium was removed, cultures washed with fresh, cold medium, and the matrix scraped into scintillation vials, and hydrolyzed (1 h, 60°C) in 0.2 ml 4N HCl. Scintillation fluid (5 ml Aquasol, Amersham) was added, and the ^{45}Ca content of the cultures determined by scintillation counting. ^{45}Ca incorporation was expressed per micromass spot culture or as the differences between comparably treated mineralizing and control cultures (differential uptake). For total Ca analyses, after washing with saline, the individual unlabelled micromass spot cultures were dried to constant weight at 60°C, weighed on a micro-gram balance, hydrolyzed in 1N HNO_3, and analyzed by flameless atomic absorption using a Perkin Elmer (Norwalk, CT) 5100 graphite atomic absorption spectrometer. Results were expressed as μg Ca per mg dry weight of micromass culture or as differential Ca uptake (μg) as previously described.

Proteoglycan Content

Glycosaminoglycan content of the culture was estimated based on alcian blue dye binding [31]. In brief, cultures were fixed with ethanol, stained for 12 h in the cold with 0.5% alcian blue GX (Sigma Chemicals) in 0.1N HCl, the aggrecan-dye mixture extracted into 4N guanidine hydrochloride for 48 h at 0°C, and the absorbance of the extract at 595 nm recorded. Results were expressed as fraction of controls analyzed concurrently.

Immunohistochemistry and Ultrastructural Studies

Keratan sulphate and chondroitin sulfate were localized in micromass cartilage cultures with monoclonal antibodies (ICN Biomedicals, Costa Messa, CA) using the streptavidin-biotin-DAB (diaminobenzidine) indirect method. The micromass spot was collected following different time periods in culture, rinsed in PBS, and fixed for 5 min in ice-cold absolute alcohol. Each spot was treated to inhibit endogenous peroxidase, incubated in the primary antibody (diluted 1:100) overnight at 4°C, and the DAB-peroxidase reaction used to localize the antibody binding. The DAB reaction product was photographed using the entire microspot. Control reactions consisted of omitting the primary antibody or pre-treatment of the cartilage with chondroitinase ABC.

Ultrastructural studies included transmission electron microscope analyses of cells and matrix in the different treatment groups. Samples for electron microscopy were washed

with 0.05 M cacodylate buffer, placed in EM fixative (0.5% glutaraldehyde, 2% paraformaldehyde, pH 7.2, 0.05 M cacodylate buffer) for 12–18 h at 4°C. Samples were stored at 4°C in 0.05 M cacodylate buffer containing 7% sucrose. Fixed cultures were removed from the dish, post-fixed with 2% aqueous osmium, dehydrated in a graded series of alcohols, and embedded in Spurr's resin. Thin sections were collected on water containing bromthymol blue as an indicator for pH above 8.0 to prevent dissolution of mineral. Sections stained with lead citrate and alcoholic uranyl acetate were examined on a Philips (Mahwah, NJ) CM12 electron microscope. Samples were embedded in Epon, and 1–2-μ thick sections collected. These sections were stained with toluidine blue and used for orientation. Thin sections (75–80 nm) for EM were collected and examined by transmission electron microscopy. Unstained micrographs were photographed to show cell characteristics, localization and orientation of collagen, mineral, and proteoglycans. In some cases to facilitate viewing of the matrix, cultures developed in 0.8 mM Ca, rather than 1.2 mM Ca, were used.

Data Analyses

Numerical data for each replicate group of experiments were compared by ANOVA, and the Bonferroni multiple comparisons test, taking a Bonferroni P of ≤ 0.05 as significant.

RESULTS

Time-Dependent Changes in Proteoglycan Content

Figure 1 demonstrates the time-dependent changes in distribution of the chondroitin sulfate epitope as the mineralizing micromass cultures matured. At day 7 (day 0 = day of plating), there were fewer nodules in the culture, but each nodule stained positively (Fig. 1a). At day 16, there were more nodules present, and staining per nodule was visibly increased (Fig. 1b). Figure 1c shows the anti chondroitin-sulfate antibody stain distribution in a single nodule in a day-16 culture. The antigen appeared to be localized around hypertrophic chondrocytes, with decreased staining at a distance from the nodule. In contrast, the keratan sulfate was found in the matrix between nodules (Fig. 1d).

Alteration of Proteoglycan Synthesis and Turnover

Treatment with reagents which modulate aggrecan synthesis had distinct effects on the appearance of the matrix at both the light and electron microscopy levels. Although these changes could not be quantitated from the light micrographs, some general characteristics were apparent. Mineralizing control and treated cultures stained with the chondroitin-sulfate antibody at day 16, were compared. Retinoic acid–treated cultures were so heavily mineralized that they could not be analyzed. Cultures treated with xylosides from day 5 showed a chondroitin-sulfate distribution similar to untreated controls (Fig. 1b,c) but the staining was less intense. All the chondroitinase-treated cultures showed markedly decreased staining. The ortho-phenanthroline treated cultures stained diffusively for chondroitin-sulfate such that there was little distinction between the nodules and the matrix. Figure 2 illustrates the effects of these treatments on the matrices within these cultures at the EM level. The typical appearance of the unmineralized collagen matrix in the untreated cultures at day 16 is seen in Figure 2a. When treated with 10^{-10} M retinoic acid from day 5 (Fig. 2b) or on day 2 (not shown) there was a good deal of cell debris (not shown) and the collagen fibrils appeared banded, more clumped, and unorganized. There was also a greater abundance of fibroblast type cells throughout the matrix and few chondrocyte nodules were present. In the xyloside-treated cultures (Fig. 2c), the matrix did not look that distinct from the control cultures. Hyaluronidase treatment, like chondroitinase abc treatment, caused cultures to appear dense (not shown). Proteoglycan distribution, visualized as condensed spherules upon the collagen, also appeared altered in the treated cultures. In cultures to which o-phenanthroline was added once on day 9, the proteoglycans were found adjacent to and between the collagen fibrils. The collagen fibrils also appeared thicker (Fig. 2d).

The changes were confirmed based on the total amount of alcian blue positive complexes extractable as a function of time (Fig. 3). The alcian-blue-glycosaminoglycan (GAG) dye content is expressed in this figure as the ratio of absorbances in treated/control (d16) cultures. Values are mean ± SD for $n \geq 3$ experiments. In general, in the untreated cultures, the GAG content increased gradually with time reaching the maximal value at d16, and

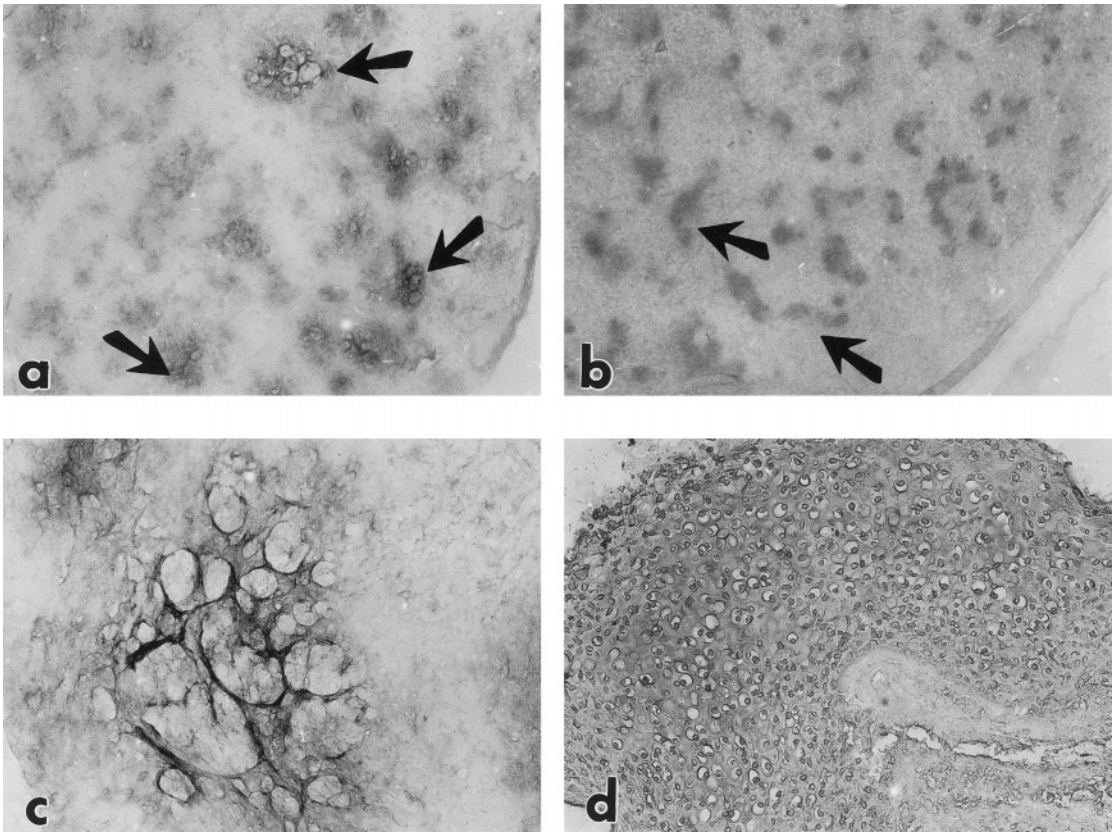

Fig. 1. Immunohistochemical localization of proteoglycan epitopes in differentiating cartilage cells, cultured in media containing 4 mM inorganic phosphate. The DAB reaction product is black; there is no counterstain. **a:** A survey photograph of chondroitin sulphate localization in cartilage nodules (arrows) scattered through a 7-day-old culture. **b:** Localization of chondroitin sulfate in a 16-day-old culture. The number of cartilage nodules (arrows) has increased compared to the 7-day culture and there is intense staining for proteoglycan within each nodule. **c:** This demonstrates the presence of chondroitin sulfate within a single large nodule. Note that the extracellular distribution is especially heavy around these hypertrophic/degenerating cartilage cells. **d:** Keratin sulphate antibody is shown by dark staining throughout the cartilage nodule and surrounding matrix. This is a frozen section through a 16-day-old culture so there is cellular detail which is not seen in the cultures described in a–c. Magnification: a, b = ×9; c, d = ×44.

decreasing after that time (data not shown). In cultures treated continuously with 5 mM xyloside from day 5, the GAG content was always less than control cultures at the same time, and the content did not increase significantly. Cultures treated with 10^{-10} M retinoic acid continuously from day 5 showed reduced GAG content relative to control at d14 and 16. Cultures treated with chondroitinase ABC continuously from d5 had significantly reduced GAG contents, especially at the earliest time points. Hyaluronidase treatment also reduced GAG content of the cultures (not shown). Addition of o-phenanthroline resulted in a slight, but not significant increase in extractable alcian blue–proteoglycan complexes (not shown).

Effects of Proteoglycan Modification on Mineralization

Each of the treatments that disrupted proteoglycan synthesis or content increased the ^{45}Ca uptake, total Ca content, and mineral to matrix ratio as determined by FT-IR microspectroscopy. The data in Figure 4 are presented as the difference between the ^{45}Ca uptake or total Ca content of the mineralizing and non-mineralizing cultures, since even the proteoglycan-rich matrices in the non-mineralizing cultures bind appreciable amounts of calcium [13]. Disrupting proteoglycan synthesis with 10^{-10} M retinoic acid caused a significant increase in ^{45}Ca uptake relative to control (Fig. 4a) at all time points. When 10^{-8} M retinoic acid was used, ^{45}Ca uptake at day 14 was $>50\times$ that of the untreated controls, but most cultures could not be maintained beyond day 11. Addition of 5 mM xyloside after the mesenchymal cells had differentiated into chondrocytes and the chondrocytes formed nodules (from day 5 onward) caused an increase in ^{45}Ca uptake relative to untreated cultures (Fig. 4b). The effect was less

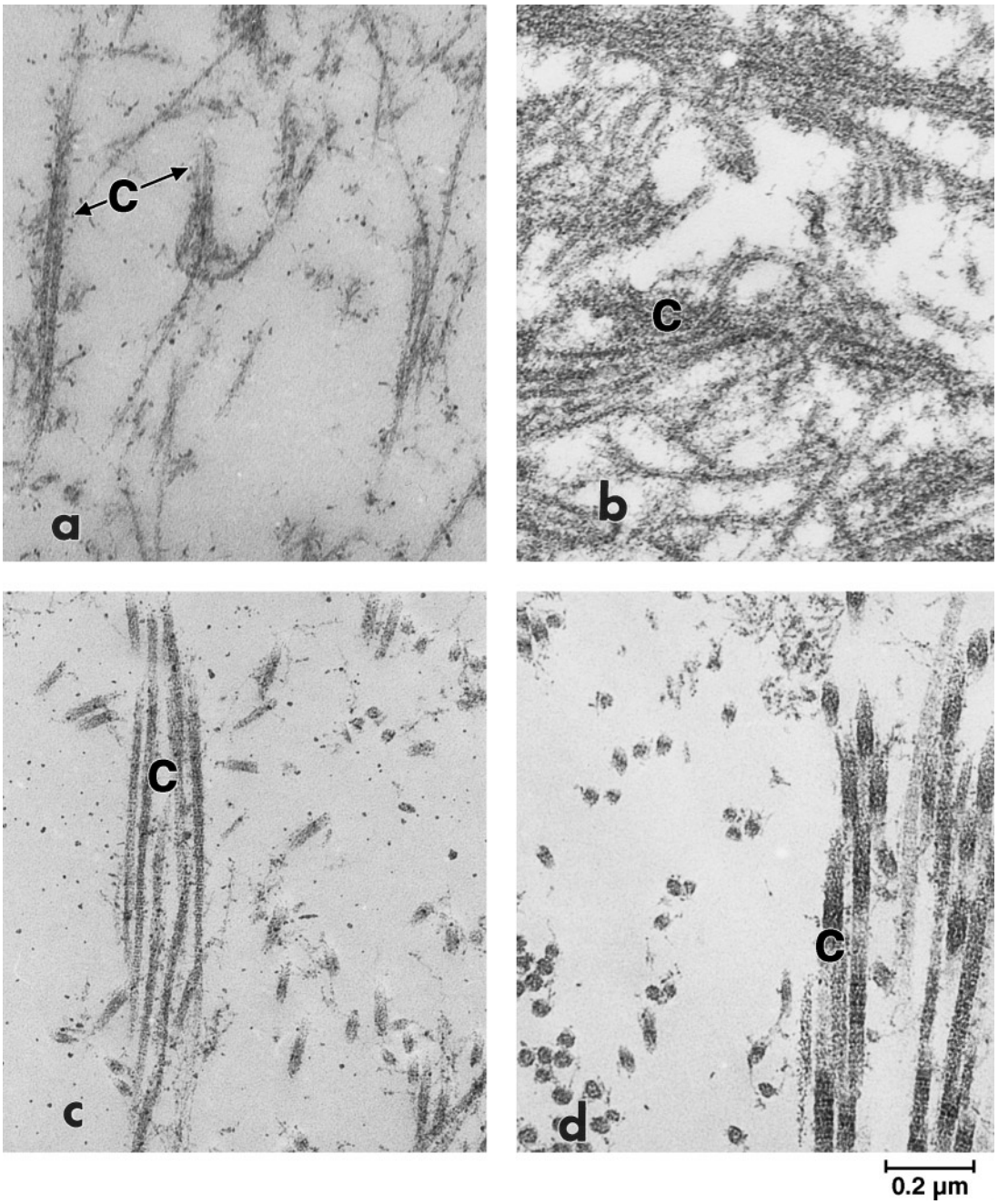

Fig. 2. Electron micrographs of differentiating mesenchymal cell cultures at d16 under different culture conditions. Thin sections were stained with lead citrate and uranyl acetate. All photos presented at 57,200 magnification. Bar = 0.2 μ. The basic culture conditions included 0.8 mM Ca and 4 mM Pi to minimize mineral deposition. **a:** This micrograph demonstrates the collagen fibrils (C) and condensed proteoglycan complexes (small dark spots scattered around collagen) found in control cultures. **b:** This micrograph was obtained from cultures with 4 mM Pi and 10^{-10} M retinoic acid added continuously from d5. The collagen fibrils are thicker than in control a and there are no obvious condensed proteoglycans visible. **c:** This micrograph was taken from cultures with 4 mM Pi and 5 mM Xyloside added continuously from d5. The collagen fibrils appear similar to controls, and the condensed proteoglycans are scattered throughout the matrix. **d:** This micrograph was taken from cultures with 4 mM Pi and 10 mM o-phenanthroline added on d9 only. The collagen fibrils are larger than control fibrils. The proteoglycans are not easily seen in the matrix, but can be found adjacent to and between the fibrils.

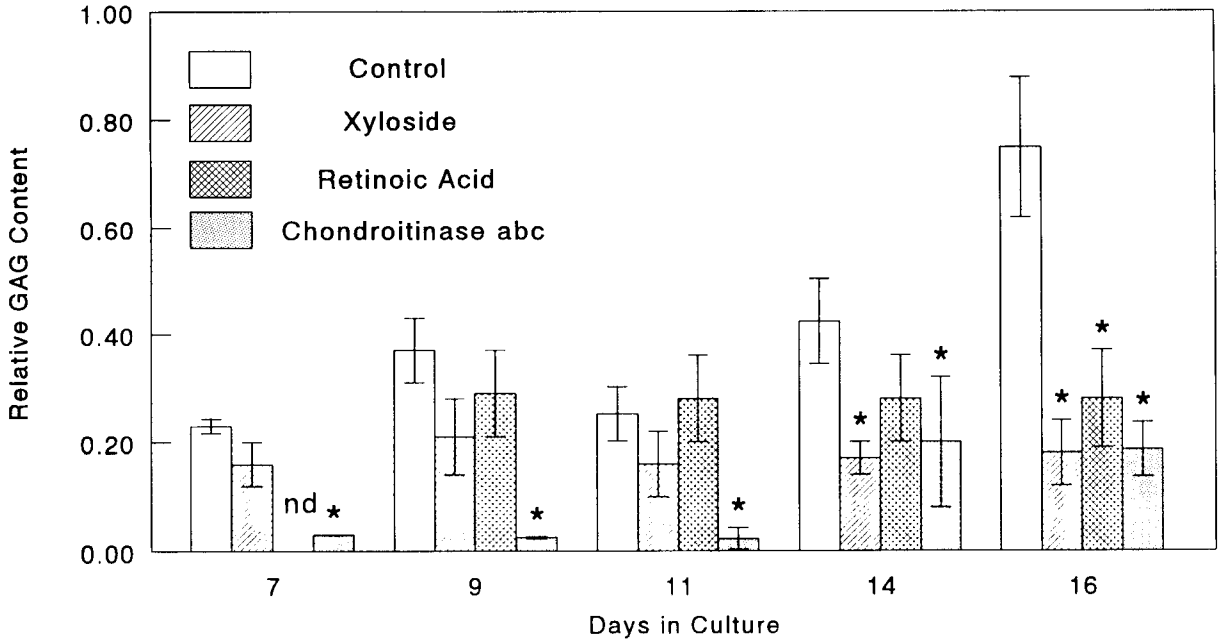

Fig. 3. Effect of agents which modulate proteoglycan properties on proteoglycan GAG content assessed by the alcian blue dye binding. Absorbances at 595 were normalized to the d16 value for mineralizing control cultures run concurrently. Xyloside-treated cultures received 5 mM xyloside continuously from d5. Cultures received 10^{-10} M retinoic acid were treated from day 5 onward. Cultures treated with chondroitinase ABC received the enzyme with each change of medium from day 5. All values mean ± SD for 3 separate experiments; *significantly different from control $P \leq .05$.

pronounced with 2.5 mM xyloside (not shown). Data in Figure 4 come from five independent experiments, and were normalized to the d21 value for mineralizing cultures. Hyaluronidase treatment (Fig. 4c), either once on day 7, or continuously with each change of medium, increased the total calcium content of the cultures. Chondroitinase abc had a similar effect (not shown). In contrast, ortho-phenanthroline, added on day 9 to inhibit neutral proteinase activity, initially had little effect, appeared to increase Ca content by day 14, but then significantly decreased Ca uptake as mineralization progressed in untreated mineralizing controls (Fig. 4d).

The mineral formed when proteoglycans were disrupted also differed in structural characteristics from controls. X-ray diffraction analysis of the mineralizing retinoic acid–treated cultures, the xyloside-treated cultures, and the chondroitinase abc–treated cultures had sharper apatite diffraction patterns than the untreated mineralizing control cultures, suggesting the crystals were larger (not shown). To quantitate the changes in mineral characteristics, representative cultures were analyzed by FT-IR microspectroscopy. Figure 5 shows a spectrum of a chondroitinase abc treated culture 20 μ from the center of a chondrocyte nodule. The peaks whose integrated areas were used to calculate mineral (phosphate) to matrix (amide I) ratios are indicated. These ratios calculated after spectral subtraction are presented in Table I along with an estimate of the relative crystallite size. Data shown for estimated crystal sizes in untreated cultures were taken from an earlier study in which a single sub-band was used to predict size [15]. The data for the proteoglycan modified cultures are presented as relative percent areas. In general, the % area of the 1,076 cm^{-1} band, which is directly related to crystal size in the a, b, and c axis directions [30] was greater than that in mineralizing controls in the cultures treated from d5–21 with chondroitinase, not significantly different from controls in the cultures treated with chondroitinase only one time, and significantly greater than controls in the retinoic acid treated cultures. Retinoic acid–treated cultures could only be examined up to d16, since they became too brittle to section after this time.

DISCUSSION

This study has demonstrated that disrupting proteoglycan aggrecan formation, causing smaller glycosaminoglycan chains to form or disrupting the already formed proteoglycans, increases the extent of calcification in differentiating mesenchymal cell micromass cultures. The initial report describing calcification in this

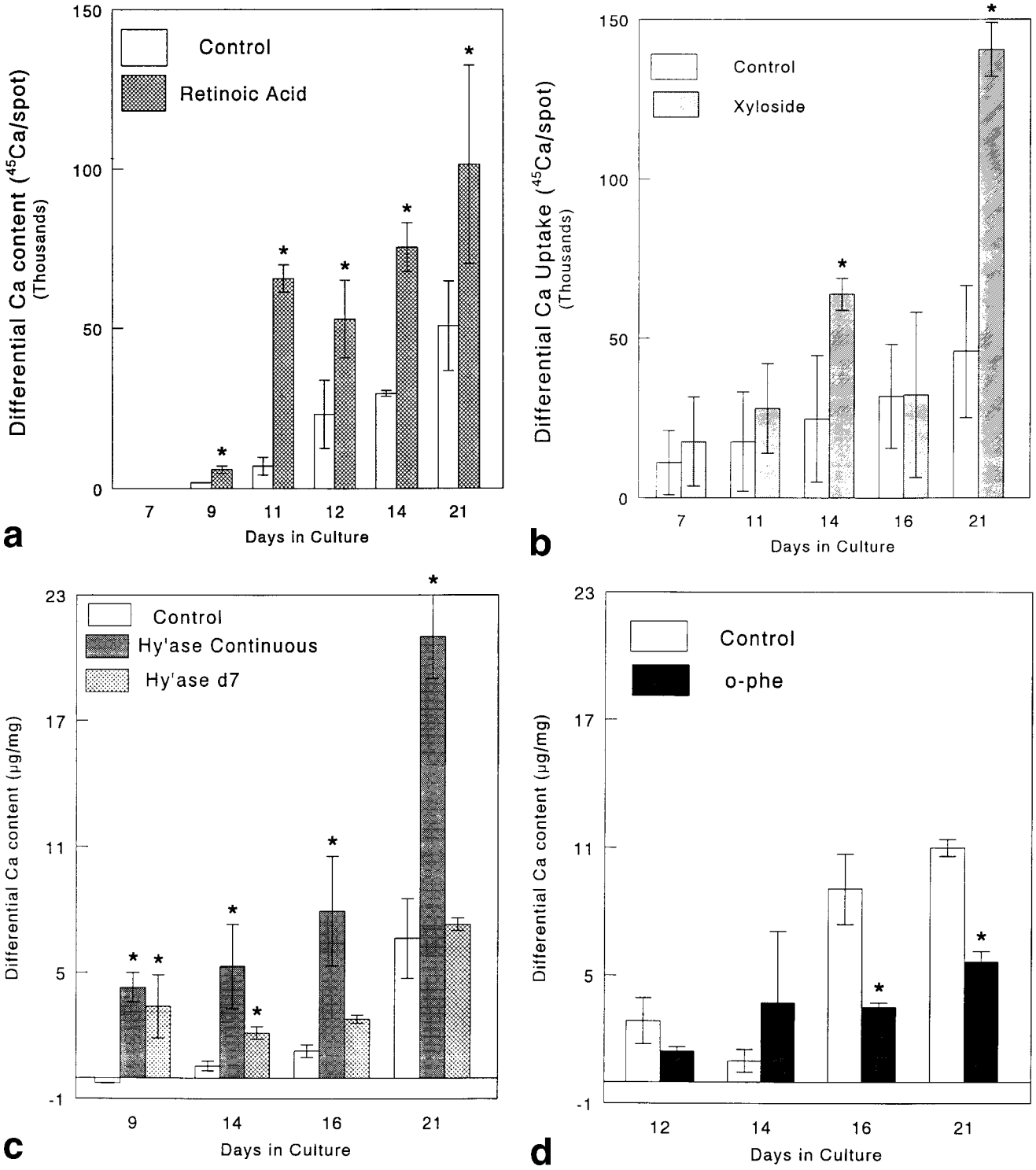

Fig. 4. **a:** ^{45}Ca or total Ca uptake in mineralizing (with 4 mM Pi) cultures as a function of time, with day 0 = day of plating. Incremental difference between mineralizing and non-mineralizing cultures treated with the same agents is shown. **a:** Cultures treated from d5 onward with 10^{-10} M retinoic acid. **b:** Cultures treated from d5 onward with 5 mM xyloside. **c:** Cultures to which hyaluronidase was added from d7 with each change of medium (continuous), or once on d7. **d:** Ortho-phenanthroline (o-phe) was added once on day 9. All values are mean ± SD; *$P \leq 0.05$ relative to untreated culture at same time point.

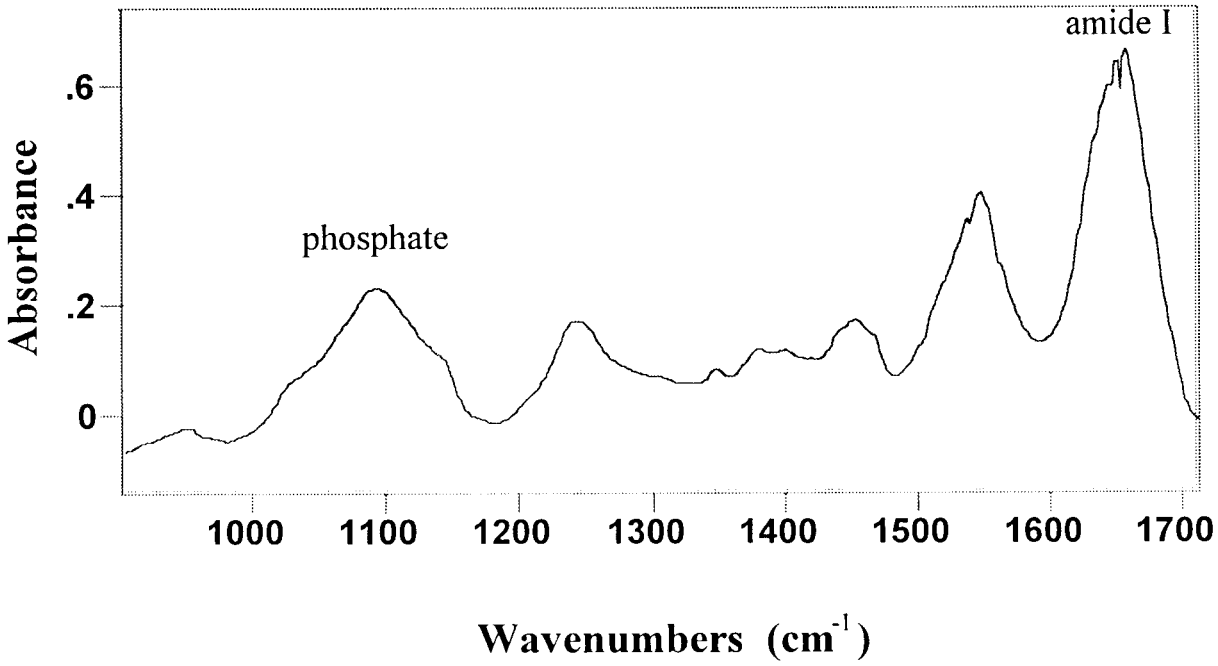

Fig. 5. Typical raw FT-IR spectra of a site in a culture treated with chondroitinase abc, 20 μ from the center of a cartilage nodule. Regions of interest are labelled. The phosphate band contains contributions from the glycosaminoglycan sulfate chains which accumulate in the matrix. These contributions are spectrally subtracted using spectra from non-mineralizing control cultures.

culture system had commented on the increase in von Kossa staining seen in retinoic acid and hyaluronidase treated cultures [12]. The present study extends those observations in more quantitative terms and provides some explanation for what may be happening during cartilage calcification in situ.

Within the epiphyseal growth plate, there is a modification of proteoglycan structure [2], presumably brought about by both alterations in synthetic activity and expression of degradative enzymes [25,26,32]. The chondroitin sulfate epitopes decrease while keratan sulfate epitopes increase [33]. The smaller proteoglycans, decorin and biglycan, components of both cartilage (where they contain dermatan sulfate) and bone (where they can contain chondroitin sulfate [34]), persist [31]. A similar pattern of expression is seen in these differentiating limb-bud mesenchymal cell cultures long established as a model of cartilage differentiation and endochondral ossification [11,12].

By manipulating the structure of the proteoglycans in this culture system, the extent of mineralization and the properties of the apatitic mineral deposited in the extracellular matrix were modulated. Retinoic acid has previously been shown to block proteoglycan synthesis when added to micromass cultures of differentiating mesenchymal cells at 10^{-8}–10^{-10} M [16,31,35]. Collagen synthesis and cell differentiation may also be affected [35]. When such cultures are maintained in a mineralizing solution (1.1 mM Ca × 4 mM Pi) mineral deposits over the entire culture, the crystals are large, and the mineral to matrix ratio is extremely high. Proteoglycans function to keep the cartilage matrix hydrated, thus in their absence the matrix is exposed to higher concentrations of calcium and phosphate ions. In the absence of sulfated proteoglycans, which bind calcium ions [36], and in the absence of aggregates, which may shield crystal nuclei limiting or preventing crystal growth, as suggested by solution studies [4], a large number of large crystals may deposit at random sites in the matrix.

Retinoic acid effects on cellular differentiation are dependent both on concentration and the developmental status of the cells. Retinoic acid in the concentrations used in this study accelerates the differentiation of mature (hypertrophic) chondrocytes and the synthesis of a mineralized matrix [37–43], yet when added to less mature chondrocytes causes cessation of mesenchyme differentiation [31], as was seen in this study. Since retinoic acid effects are complicated, emphasis was placed on addi-

TABLE I. FT-IR Microspectroscopic Analysis of Mineral Properties in Experimental Cultures at Day 21

Experimental Conditions	Site in Culture	Mineral to Matrix Ratio	Relative Size*
No Treatment	nodule center	0.22	nd
	20 μ from center	0.25	147Å
	40 μ from center	0.29	149Å
	60 μ from center	0.26	139Å
	edge	0.51	160Å
Chondroitinase ABC d4–21	matrix (no nodules visible)	3.2 ± 1.6	1.51×
	edge	7 ± 3	1.1×
Chondroitinase ABC d7, 1×	nodule center	nd	nd
	20 μ from center	0.5 ± 0.2	0.8×
	40 μ from center	0.7 ± 0.2	1.1×
	60 μ from center	0.7 ± 0.3	1.1×
	edge	0.7	0.7×
Vitamin A 10^{-10} M d2–5	matrix (no visible nodules)	0.5 ± 0.2	1.8×
	edge	3.2 ± 0.8	4.3×

*Values above the double line are based on comparison to % area of 1060 cm^{-1} subband [ref 13]; values below the double line are based on the ratios of the 1076 cm^{1} subband in the experimental to comparable sites in controls; nd = not detectable.

tional means of modulating the aggrecan content of the cultures.

When aggregates smaller in size are synthesized, as in the case in the presence of β-D-xyloside which presents increased numbers of start sites on the core protein [20,22], excessive mineralization again occurs, albeit to a lesser extent. In solution, the ability of proteoglycans to inhibit apatite crystal growth is a function of proteoglycan size, with larger aggregates being more effective than smaller aggregates, and aggregate monomers being less effective than small aggregates [4]. Thus, in these cultures, although apatite crystal nuclei may form at normal sites in collagen and matrix vesicles [13], the regulation of proliferation of these crystals is decreased in the presence of the smaller proteoglycans, and crystals grow to a larger size than normal.

The same mechanism is likely to occur in the case of hyaluronidase treatment, which would leave proteoglycan monomers intact, and chondroitinase abc treatment, which would alter the size and shape of aggregate and monomers. Both of these treatments would also increase the proteoglycan solubility and could decrease proteoglycan retention within the matrix. Thus with each medium change, the total amount or proteoglycan would be depleted, as was seen. Alterations in rates of chondroitin sulfate and aggrecan synthesis may occur when proteoglycan degradation products are present [44]. This may account for variations in proteoglycan content with time and treatment. It is important to note that others have also reported a decrease in proteoglycan core protein expression and proteoglycan synthesis relative to DNA in chondrocyte cultures prior to the onset of mineralization [45,46]. In the control mineralizing cultures studied here, the GAG content reached its maximum value at d16, while cell proliferation continued beyond this time. Thus a similar decrease in GAG/DNA was occurring.

Inhibiting some of the enzymes responsible for the modification of proteoglycans [25–27,32] led to increased Ca uptake in the matrix, but decreased mineral accumulation. It has been suggested [1] that proteoglycan modification is required for growth plate mineralization and that removal of the proteoglycans facilitates the growth and proliferation of mineral crystals. Thus the failure to produce a temporal increase in mineral accretion when stromelysin and other metalloproteases were inhibited, supports this hypothesis. It is important to note, however, that total GAG content increased progressively in mineralizing controls levelling off at d16, implying the extent of such modification is limited.

Each of the treatments used in this study may also have affected the less abundant small proteoglycans. Decorin, through its interaction with collagen [47,48], is known to regulate matrix structure. Since retinoic acid treated cultures and xyloside treated cultures showed EM alterations in collagen organization, it is possible that decorin synthesis may also have been impaired. Merker et al. [49] also found increases in collagen fibril diameter in mouse limb buds cultured with xylosides for only 6 days. Decorin in solution has no direct effect on apatite formation and growth [50] and, other than changing matrix organization, probably had little effects in these studies. Biglycan, in contrast, promotes apatite formation at low concentrations, and inhibits it at higher concentra-

tions [50]. Perhaps this is one of the protein factors promoting mineralization in the culture, but this remains to be proven.

The results of this study are not in agreement with those of Hunter et al. [51]. Adding similar concentrations of xylosides to chondrocyte cultures, he found an inhibition rather than a stimulation of mineral ion accumulation. Although the differences may be due to the systems studied (micro-mass cultures of differentiating mesenchyme vs. differentiating monolayer chondrocyte cultures) wherein the xylosides blocked differentiation [51] and the use of β-glycerophosphate (which requires alkaline phosphatase activity) rather than inorganic phosphate, there may be another reason for the apparently divergent results. In the studies presented in the current paper, ^{45}Ca and total Ca accumulation are presented as the difference between mineralizing and non-mineralizing control cultures (4 mM Pi minus 1 mM Pi) treated with the same proteoglycan modifiers. Because proteoglycans bind calcium, there is calcium uptake in non-mineralizing controls [13]. Thus although the total Ca uptake may not be enhanced, the incremental uptake is. Further, mineral analyses by FT-IR and X-ray diffraction confirmed the incremental values, and demonstrated, as suggested by cell-free in vitro studies [2–5,52], that in this culture system, proteoglycans were inhibitors of physiologic calcification.

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