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Effects of Bone CS-Proteoglycans, DS-Decorin, and DS-Biglycan on Hydroxyapatite Formation in a Gelatin Gel

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Abstract. The small leucine-rich bone proteoglycans, biglycan and decorin, can be purified by chromatography on hydroxyapatite columns, demonstrating their potential affinities for bone apatite. To determine their effects on *in vitro* apatite formation and growth, a mixture of the chondroitin-sulfate (CS) bone proteoglycans, or purified fractions of the dermatan sulfate (DS) containing proteoglycans, DS-decorin and DS-biglycan obtained from skin and articular cartilage, respectively, were analyzed in a gelatin gel diffusion system in which apatite formation occurs in the absence of proteins in a 3.5 day period. Low concentrations of the bone CS-proteoglycan mixture and low DS-biglycan concentrations (5–25 μg/ml) increased apatite formation relative to proteoglycan-free controls at 3.5 days. The CS-proteoglycan mixture was less effective at 50 μg/ml than at 10 μg/ml. DS-biglycan was similarly most effective at 5–25 μg/ml. At 5 days, when apatite growth and proliferation were assessed, 10 and 50 μg/ml of both CS-bone proteoglycan and DS-biglycan increased mineral yields. DS-decorin, in contrast, had no significant effect on mineral accumulation at any of these concentrations. In seeded growth experiments, 1 and 10 μg/ml CS-proteoglycan and 10 and 50 μg/ml DS-biglycan were significant effective inhibitors of mineral accretion, whereas DS-decorin showed no tendency to inhibit seeded growth. Using molar extinction coefficients to determine concentrations, the binding of DS-biglycan and DS-decorin to apatite (specific surface 54 m^2/g) was determined using a Langmuir adsorption isotherm model. DS-biglycan had a greater affinity for apatite than DS-decorin (0.285 ml/μmol versus 0.0098 ml/μmol). DS-biglycan binding was more specific with fewer binding sites (3.5 $\mu mol/m^2$ compared with 18.2 $\mu mol/m^2$ for DS-decorin). Data suggest that of the small proteoglycans, biglycan may play a more significant role than decorin in the regulation of mineralization.

Key words: Mineralization — Biglycan — Decorin — Hydroxyapatite — Bone proteoglycan.

The matrix proteins of bone and cartilage play a variety of roles: regulating matrix organization, cell adhesion, matrix turnover, and mineral deposition, to name a few. Among the better characterized of these bone matrix proteins are the small leucine-rich proteoglycans, biglycan and decorin [1, 2]. The chondroitin sulfate (CS) containing decorin of bone, like the small dermatan sulfate (DS) proteoglycan of cartilage and skin, is bound to the surfaces of type I collagen fibrils [3, 4], and regulates collagen fibrillogenesis *in vitro* [5]. The functions of biglycan in bone, cartilage, and other soft tissues are less well understood, although recently biglycan has also been reported to bind to type I collagen fibrils [6]. Biglycan also binds to a number of other connective tissue constituents, e.g., fibronectin [7] and heparin co-factor II [8], as well as certain growth factors [9]. The small proteoglycans have an appreciable affinity for hydroxyapatite, as indicated by their retention on apatite columns [10] and in the mineralized tissues [1, 11–15]. Based on their affinity for apatite, and their distribution within mineralized tissues, the small bone proteoglycans had been postulated to play a role in mineralization [13].

The large cartilage proteoglycans (aggrecan and the aggregates it forms) had been shown to inhibit hydroxyapatite formation and growth in solution [16–18] and in a gelatin gel [19]. There is some debate, however, as to whether this inhibition is caused by the reduction in solution calcium content associated with calcium chelation by the highly anionic proteoglycans [20–22]. It has been suggested that Ca-chelation by glycosaminoglycans enables the large proteoglycans to act as mineralization promoters [20–22], whereas others argue that the large aggregating proteoglycans exert much of their inhibitory effect by steric hindrance of growth sites on the apatite crystals [16–18]. The bone proteoglycans which contain one (decorin) or two (biglycan) glycosaminoglycan (GAG) chains are appreciably smaller in molecular size and hydrodynamic volume than aggrecan, and thus, even if the GAG chains were longer than those in aggrecan, would have fewer binding sites for calcium. Therefore, due to Ca-chelation and/or steric hindrance, the small proteoglycans were predicted to have a lesser effect on mineralization than the larger, more anionic, proteoglycans.

Data on tissue distribution of the bone proteoglycans also implied that bone proteoglycans were associated with mineralization. Several studies [13, 23] suggested these small proteoglycans disappeared from the initial sites of mineralization. However, Sauren et al. [12] reported that this disappearance does not occur, and that although larger proteoglycans are found predominately in the unmineralized osteoid, the small proteoglycans are equally distributed over the lamellae and osteons. Bianco et al. [3], studying the distribution and expression of biglycan and decorin in mineralized tissues, suggested that the localization of decorin confirmed its function in collagen fibrillogenesis, whereas the distribution of biglycan implied more diverse functions. Studies of bone formation in osteoblast-like culture systems also provided conflicting views of the functions of these proteoglycans, indicating in one instance that the bone pro-

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teoglycans had to be modified before mineralization started [15], and in another that an increase in the content of chondroitin sulfate glycosaminoglycans occurred coincident with the start of mineralization [14].

The purpose of the present study was to test the hypothesis that the small bone proteoglycans inhibited mineralization, albeit to a lesser extent than aggrecan, using an *in vitro* gel diffusion system previously employed to demonstrate the multifunctional effects of several matrix proteins on *in vitro* mineralization [19, 24]. Because sufficient pure bone CS-decorin and CS-biglycan were not available for this study, a mixture of bone CS-proteoglycans was tested, and the effects were compared with purified articular cartilage DS-biglycan or DS-skin decorin.

Materials and Methods

A mixture of the small bovine bone CS-proteoglycans was kindly provided by Dr. L. Fisher of the National Institute of Dental Research. Details of isolation, purification, and characterization appear elsewhere [23]. The mixture was approximately 95% pure proteoglycan of which, based on staining of an SDS-polyacryl amide gel, was about 80% CS-decorin and 20% CS-biglycan (L. Fisher, personal communication).

DS-biglycan and DS-decorin were isolated from bovine articular cartilage and fetal bovine skin, respectively, as described in detail elsewhere [25, 26], purified on an octyl-sepharose column, and stored lyophilized. Details of the physical properties and deduced primary sequences of these small proteoglycans are published elsewhere [27, 29]. DS-biglycan was resuspended in 4 M guanidinium chloride, and dialyzed against the starting buffer for each experiment, whereas DS-decorin and the CS-bone proteoglycans were readily dissolved directly in buffer.

To minimize the amount of proteoglycan required for *in vitro* assay, hydroxyapatite formation and growth in a gelatin gel was monitored using 100, 50, 25, 10, 1, or 0 μg/ml proteoglycan in 0.05 M pH7.4 tris-hydroxymethylamino-methane (Tris) buffer. The proteins were placed in a 10% gelatin gel (Bloom gelatin, Fisher Chemical, Springfield, NJ). The gels, in 6 cm-long segments cut from disposable polystyrene pipets (Fisher Chemical) were mounted on an apparatus that allowed the circulation of 100 mM $CaCl_2$ and 100 mM $(NH_4)_2HPO_4$ from 2 liter containers on opposite sides of the gel [19, 30]. Experiments were conducted at room temperature (25 ± 2°). Nitrogen gas (~1 atm) was used to maintain continuous circulation of the fluids. The flow of the solutions was maintained at a constant rate by maintaining the nitrogen propellant at constant pressure with a valve and flow meter system. The circulating calcium and phosphate solutions were prepared in 0.05 M pH 7.4 Tris buffer containing 0.02% sodium azide to prevent bacterial growth. Concentrations of the bulk circulating solutions were monitored at both the start and conclusion of each experiment to insure that there were no detectable changes. The gels were analyzed at 3.5 days (when the first precipitant band was detectable and the CaxP concentration adjacent to the precipitant band was 4.5×4 mM^2) and at 5 days (to analyze for apatite growth and proliferation).

To prepare the gels, equal volumes of the proteoglycan solutions in 0.05 M Tris buffer or Tris buffer alone (controls) were rapidly mixed with 20% gelatin (45°C) and 200 μl aliquots of the resulting gelatin solution placed at a distance corresponding to the location in the gel where mineral deposition first occurs in controls (1.35 ml from the calcium-entrance side of the 10% gelatin gel). After the proteoglycan gel mixture set, the remainder of the tube was filled with 10% gelatin [19]. Each individual experiment involved 18 gels: 3 experimental and 3 control double diffusion gels, and 6 experimental and 6 control single diffusion gels. The site of initial precipitant band formation in the double-diffusion gels was consistently at the position where the proteoglycan had been placed. The single diffusion gels, through which either only calcium or only phosphate was allowed to circulate, were used to monitor the accumulation of individual ions. In some experiments, additional double-diffusion gels were used to provide material for mineral analyses (X-ray diffraction and electron microscopy).

After 3.5 or 5.0 days, gels were sliced into 0.3 cc volume slices, with the precipitant band as the central slice. All slices, other than those saved for X-ray diffraction or electron microscopy, were hydrolyzed in 2 N HCl and the calcium [31] and phosphate (Pi) [32] concentrations of the hydrolysates were determined. The amount of Ca or Pi in the precipitant band was determined by subtracting single-diffusion values from the double-diffusion values. Concentrations in slices numbered 3 and 5, where slice 4 contained the precipitant band, were compared to ensure proper alignment of single- and double-diffusion gels.

Calculated data in the experimental gels was compared with the controls run at the same time to avoid variation due to alterations in temperature or rate of circulation of ions. Analysis of variance (ANOVA) was used to determine variations from experiment to experiment, and when no variation in experiments using the same concentrations of proteoglycans was noted, results from all similar experiments were combined. N in each case was the number of independent (triplicate) experiments. All comparisons were made to the control using the Dunnett's *t*-test with $P \leq 0.05$ accepted as significant.

For X-ray diffraction analyses of the mineral formed in the precipitant bands, the gelatin was melted at 60°, and the precipitate centrifuged (Beckman microfuge) for 2 minutes. Following aspiration of the melted gelatin solution, the pellet was dried and examined by wide angle X-ray diffraction using Cu K-α radiation. The only phase present in these experiments was a poorly crystalline apatite.

To determine the affinity of the DS-proteoglycans for hydroxyapatite, solutions containing approximately 2–4 mg/ml protein, and serial dilutions thereof, were incubated with a previously characterized synthetic hydroxyapatite preparation [33] of specific surface 54 m^2/g. The DS-proteoglycan solutions were prepared in 0.15 M pH 7.4 Tris-buffer containing 1 mM calcium chloride. Two hundred and fifty microliters of solution were incubated at 37°C for 24 hours under constant agitation with 0, 0.5, 1, 2, 5, 10, or 15 mg hydroxyapatite. The dispersions were then centrifuged in an Eppendorf microfuge (5 minutes, room temperature) and the protein concentration of the supernate was determined based on its absorbance at 280 nm, using a molar extinction coefficient of 0.225 mg/ml for DS-biglycan and 0.252 mg/ml for DS-decorin, as determined by sedimentation equilibrium (L. Rosenberg, unpublished results). Molar concentrations were calculated using molecular weights (as determined by sedimentation equilibrium) of 93,000 for DS-biglycan [25] and 65,000 for DS-decorin (unpublished). Spectrophotometric readings were made using a micro-cell (Beckman, Fullerton, CA) which required only 100 μl of the solution. After measurement of absorbance, the solutions were recovered, those with comparable absorbance pooled, the new initial concentration determined, dilutions performed where necessary, and the experiment repeated with decreasing lower proteoglycan concentrations. The extent of DS-biglycan or DS-decorin binding calculated for each experimental point was expressed as mg or μmole protein bound per hydroxyapatite specific surface (Q). This was plotted as a function of the equilibrium concentration of proteoglycan (Ce). To linearize the results, it was assumed that the data could be fit to a Langmuir adsorption isotherm [34]. Ce/Q was plotted as a function of Ce, giving a slope equal to the reciprocal of the number of binding sites (N), and an intercept equal to the reciprocal of the product of the number of binding sites times the affinity constant (K).

To determine whether the presence of proteoglycans affected the morphology or shape of the apatite crystals formed in the gel, precipitant bands from gels with 0, 10, 25, 50, or 100 μg/ml DS-biglycan or 0, 25, 50, and 100 μg/ml DS-decorin were fixed in EM fixative for 18 hours at 40°C. The EM fixative consisted of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M pH 7.4 cacodylate buffer. Opaque regions in the center of the gel were sliced into ~1 mm^3 cubes, dehydrated in alcohol, and embedded in Spurr's resin. Thin sections were collected on pH 8–10 water containing bromthymol blue. Sections were not poststained. Pho-

Table 1. Ca binding[a] by small proteoglycans

	(mmole Ca/μg PG)	(μmole Ca/μmole PG)
Bone CS-PG	0.0163 ± .007	—
DS-biglycan	0.0121 ± .027	1.5×10^3
DS-decorin	0.0014 ± .008[b]	91

[a] Mean ± SD based on three concentrations of each PG at both day 3.5 and 5
[b] $P \leq 0.005$ relative to DS-biglycan and bone CS-PG

tographs of 15 randomly selected fields were taken on a Phillips CM-12 transmission electron microscope. Because of clumping of crystals, measurement of individual (single) crystallite lengths and widths was not possible.

Results

Each of the proteoglycan preparations studied bound small amounts of calcium, as approximated by the difference between the single diffusion data for proteoglycan-free controls and gels with proteoglycans (Table 1). Values in this table are means for at least three concentrations of proteoglycans at both 3.5 and 5 days. Data presents both per μg of proteoglycan and per mole, where molecular weights were known. DS-biglycan bound 1.5 mole Ca per μmole PG, whereas DS-decorin bound ~91 μmole Ca per μmole PG. Based on a glycosaminoglycan chain weight of 20–22,000 [27], assuming DS-biglycan has ~400 μmoles SO_4 per μmole of proteoglycan, and DS-decorin ~200 μmole of SO_4, the Ca to SO_4 molar ratio for biglycan would be ~3.8: 1; that for decorin would be ~0.5:1. There were no differences between experimental and control single diffusion gels when phosphate concentrations were compared, indicating the absence of any phosphate binding to the proteoglycan, and the lack of an effect of the proteoglycan present on phosphate diffusion into the gels.

None of the small proteoglycans inhibited the *de novo* formation of apatite in the gel diffusion system. In contrast, the bone CS-proteoglycans increased the accumulation of calcium and phosphate ions (Table 2). At 3.5 days, 10 and 25 μg/ml bone CS-proteoglycan was most effective: at 5 days, significant increases in Ca and Pi accumulation were noted, with yields at 10 μg/ml exceeding those at 50 μg/ml. To study these effects in more detail, highly purified fractions of DS-biglycan and DS-decorin were used in similar assays. DS-biglycan's actions paralleled those of the bone proteoglycans (Table 2). At 3.5 days, with concentrations of 5–25 μg/ml, mineral accretion was significantly enhanced; at 5 days, maximum yields were seen with 10 μg/ml DS-biglycan. DS-decorin had no effect at any of these concentrations, although there was a slight but not statistically significant decrease when the DS-decorin concentration was 100 μg/ml.

In the seeded growth experiments, both bone CS-proteoglycan and DS-biglycan reduced the mineral yield in a dose-dependent manner (Table 3). DS-decorin, again, had no significant effect.

Figure 1 describes the binding of DS-decorin and DS-biglycan to hydroxyapatite using a Langmuir adsorption isotherm model. DS-biglycan bound with greater affinity (K = 440 ml/μg or 0.285/μmole and more specificity (1/N); N = 575 μg/m^2 or 3.5 μmol/m^2) than DS-decorin (K = 298 ml/μg or 0.0098 ml/μmole; N = 50 μg/m^2 or 18.2 μmol/m^2).

Figure 2 depicts the effects of these proteoglycans on the morphology and size of the crystals formed in the gel. The crystals formed in the presence of 1, 5, and 10 μg/ml DS-biglycan showed an apparent dose-dependent increase in length relative to DS-biglycan-free controls, whereas those formed in the presence of 50 and 100 μg/ml were smaller and less abundant. Figure 2A–C shows typical crystals found in the gelatin gel in the presence of 5 (Fig. 2B) and 50 (Fig. 2C) μg/ml DS-biglycan, as contrasted to the proteoglycan-free control (Fig. 2A). Crystals in the DS-decorin containing gels (Fig. 2D) did not appear to change in size as the decorin concentration increased, however, at all concentrations of decorin the crystals tended to agglomerate in a different pattern than those in the control (Fig. 2A) and DS-biglycan-containing gels (Fig. 2B, 2C). X-ray diffraction analysis (not shown) confirmed the apatitic nature of all these precipitates.

Discussion

This study has shown that the small leucine-rich CS-proteoglycans of bone, and the DS-proteoglycan, biglycan, from articular cartilage have similar effects on *in vitro* hydroxyapatite formation and proliferation. Further, these effects are distinct from those of aggrecan and DS-decorin. The bone CS-proteoglycan mixture promoted mineralization at low concentrations, but was less effective at higher concentrations than purified DS-biglycan. Both the CS-proteoglycan mixture and DS-biglycan inhibited growth of preformed apatite seed crystals. Skin DS-decorin had little or no effect on mineralization, assayed in terms of the amount of calcium and phosphate ions accumulated.

Comparing the results of the present study with studies of the effects of proteoglycan aggregates on mineralization in the same system [30] reveals significant differences between the large proteoglycan aggregates of cartilage, and the smaller proteoglycans of bone and cartilage. At a concentration of 100 μg/ml proteoglycan, at 5 days, Ca and Pi uptake in the presence of the larger proteoglycans was inhibited 11% and 20%, respectively, and at no concentration was an increase in mineral accumulation detected [30]. In contrast, the small CS-proteoglycans from bone and DS-biglycan promoted apatite formation, and these proteoglycans were only shown to inhibit apatite proliferation and growth in the experiments which included preformed apatite crystals. Thus, as predicted, the large proteoglycans are the better inhibitors. The surprising finding was the ability of the small proteoglycans, and DS-biglycan in particular, to facilitate apatite formation. The small proteoglycans of the mineralized/mineralizing tissues of bone and dentin [36], as well as those produced in bone and dentin cell cultures [14, 15, 37], are chondroitin sulfate proteoglycans. In contrast, the small proteoglycans produced in the tissues that do not normally mineralize, i.e., articular cartilage and skin [25, 26, 29] as well as periodontal ligaments [38] and tendons [39], are predominantly dermatan sulfate proteoglycans. Since earlier studies demonstrated that dermatan sulfate chains and chondroitin sulfate chains have similar effects on solution-mediated apatite growth [17], these differences were not expected to have a major impact on the studies reported here. However, since mixtures of DS-proteoglycan with a weight ratio matching that of the bone CS-

Table 2. Effect of bone CS-proteoglycan, DS-biglycan, and DS-decorin on *de novo* apatite formation in a gelatin gel[a]

	Bone CS-proteoglycan		DS-biglycan		DS-decorin	
	3.5 days					
μg/ml	*Ca*	*Pi*	*Ca*	*Pi*	*Ca*	*Pi*
0	0.37 ± 0.02	0.22 ± 0.03	0.32 ± 0.05	0.21 ± 0.03	0.32 ± 0.03	0.20 ± 0.03
1	0.38 ± 0.04	0.35 ± 0.03	0.41 ± 0.15	0.23 ± 0.06	0.31 ± 0.06	0.21 ± 0.03
5	—	—	0.42 ± 0.01[b]	0.23 ± 0.01	—	—
10	0.51 ± 0.01[b]	0.45 ± 0.02[b]	0.45 ± 0.01[b]	0.24 ± 0.01	0.31 ± 0.04	0.23 ± 0.04
25	0.47 ± 0.01[b]	0.35 ± 0.02[b]	0.47 ± 0.02[b]	0.35 ± 0.02[b]	0.29 ± 0.03	0.20 ± 0.04
50	0.35 ± 0.05	0.24 ± 0.02	0.35 ± 0.04	0.24 ± 0.02	0.35 ± 0.02	0.19 ± 0.03
100	—	—	—	—	0.30 ± 0.03	0.22 ± 0.01
	5.0 days					
0	0.59 ± 0.01	0.33 ± 0.05	0.59 ± 0.01	0.33 ± 0.05	0.59 ± 0.01	0.33 ± 0.05
1	—	—	0.65 ± 0.09	0.32 ± 0.01	0.60 ± 0.05	0.32 ± 0.03
5	—	—	0.67 ± 0.02[b]	0.38 ± 0.02[b]	—	—
10	1.17 ± 0.01[b]	0.74 ± 0.02[b]	0.93 ± 0.3[b]	0.52 ± 0.17[b]	0.56 ± 0.05	0.33 ± 0.05
25	—	—	0.49 ± 0.06	0.34 ± 0.04	0.60 ± 0.05	0.30 ± 0.02
50	0.79 ± 0.08[b]	0.54 ± 0.03[b]	0.77 ± 0.06[b]	0.55 ± 0.03[b]	0.55 ± 0.09	0.36 ± 0.05
100	—	—	—	—	0.56 ± 0.05	0.34 ± 0.03

Mean ± SD for n = 3 sets
[a] Values represent yield of Ca or Pi calculated as the difference between double diffusion and single diffusion gels for each experimental set
[b] Significantly different from 0 μg/ml same type of proteoglycan

Table 3. Effect of bone CS-proteoglycan, DS-biglycan, and DS-decorin on seeded apatite growth in a gelatin gel[a]

	Bone CS-proteoglycan		DS-biglycan		DS-decorin	
	3.5 Days					
μg/ml	*Ca*	*Pi*	*Ca*	*Pi*	*Ca*	*Pi*
0	0.11 ± 0.02	0.070 ± 0.006	—	—	0.11 ± 0.02	0.070 ± 0.006
1	0.11 ± 0.02	0.076 ± 0.005	—	—	0.131 ± 0.04	0.10 ± 0.03
10	0.056 ± 0.04[b]	0.038 ± 0.005[b]	—	—	0.13 ± 0.03	0.10 ± 0.01
	5.0 days					
0	0.46 ± 0.008	0.26 ± 0.04	0.46 ± 0.008	0.26 ± 0.04	0.41 ± 0.03	0.33 ± 0.07
1	0.39 ± 0.02[b]	0.22 ± 0.03	—	—	—	—
10	0.26 ± 0.05[b]	0.20 ± 0.01[b]	0.33 ± 0.04[b]	0.19 ± 0.04[b]	—	—
50	—	—	0.22 ± 0.02[b]	0.15 ± 0.03[b]	0.44 ± 0.02	0.36 ± 0.07

Mean ± SD for n = 3 sets
[a] Values represent yield of Ca or Pi calculated as the difference between double diffusion and single diffusion gels for each experimental set. Each experiment included 0.5 mg/ml apatite seed crystals along with the indicated concentration of proteoglycan
[b] Significantly different from 0 μg/ml proteoglycan

proteoglycan mixture did not behave the same way as the CS-proteoglycan mixture (unpublished data), there must be differences between the CS- and DS-proteoglycans that impact the mineralization process.

For initial crystal formation (nucleation) to occur, the calcium, phosphate, and hydroxyl ions of apatite must come together with the correct orientation, and with sufficient energy to form the first crystal nidus, or nucleus. A macromolecule, such as DS-biglycan, which has a high and specific affinity for apatite, could stabilize that nucleus. Crystals proliferate as ions are added to the nucleus causing crystals to get larger (growth) or to branch off forming additional nucleii (secondary nucleation). Although DS-biglycan or other macromolecules could initially stabilize the nucleii, coating of crystals with macromolecule might block their proliferation and growth thereby preventing further proliferation of mineral. This mechanism is supported by the solution data and by EM observations which showed an apparent increase in crystal length as DS-biglycan concentrations increased from 1 to 10 μg/ml, and an apparent decreased size at 50 μg/ml.

There may be several explanations for the differences in behavior of the DS- and CS-proteoglycans. First, while DS- and CS-chains of the same molecular size interact comparably with preformed apatite crystals [17], the CS-chains of the fetal bone proteoglycans and the DS-chains of adult articular cartilage biglycan and skin decorin are different in size (20–22,000 kDa in DS-biglycan [25] ~40,000 kDa in

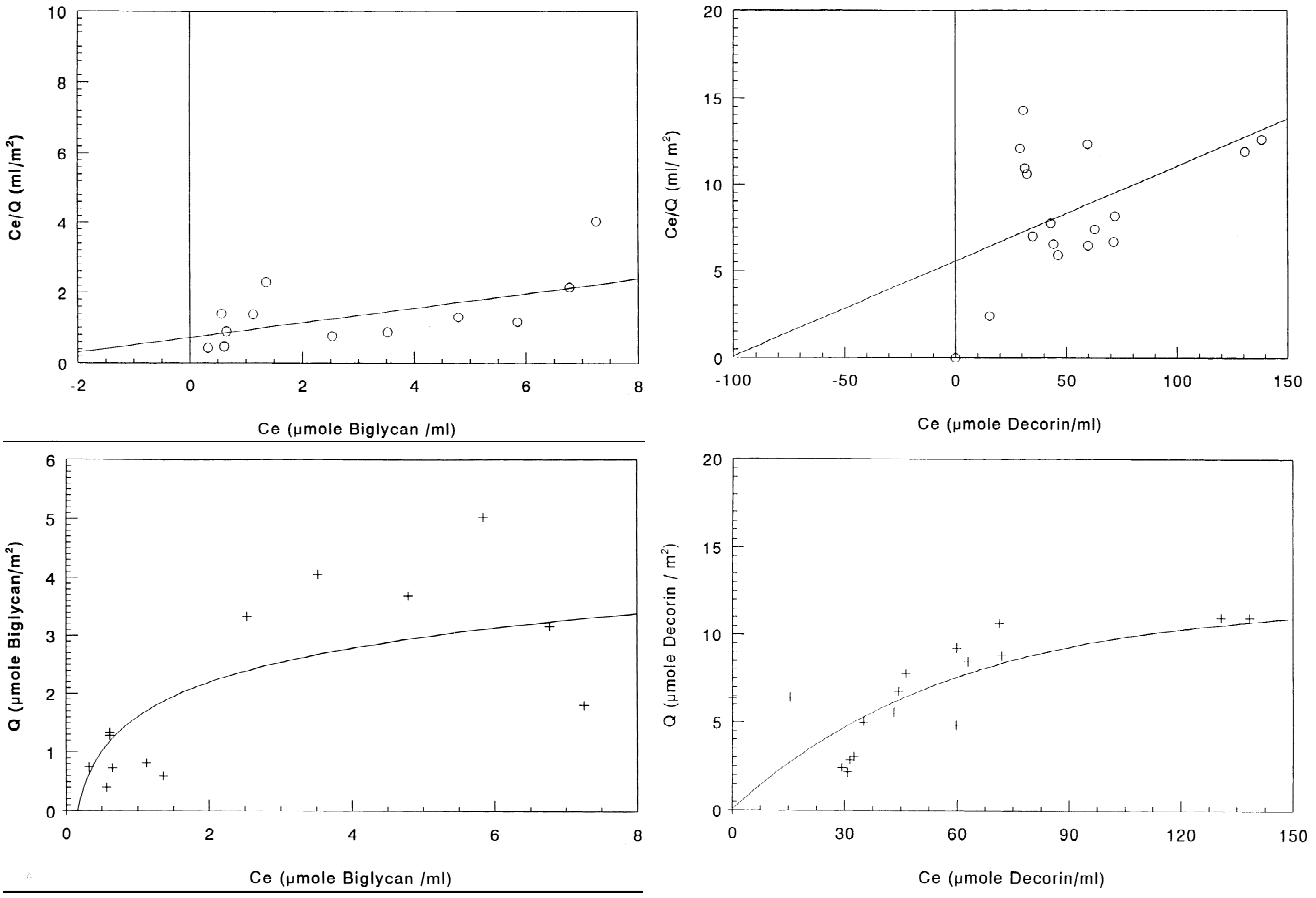

Fig. 1. Langmuir adsorption isotherms illustrating the binding of (**A**) DS-biglycan and (**B**) DS-decorin to synthetic apatite of specific surface 54 m^2/g. The top figure shows the amount of proteoglycan-bound per apatite surface. The lower curves represent the linearized version of this data. Ce = the equilibrium concentration of proteoglycan, Q = the amount of proteoglycan bound per apatite (normalized for specific surface). The slope of the linearized data (Ce/Q versus Ce) is 1/N, where N is the number of binding sites, the intercept is K/N where K is the affinity constant. The R for the biglycan data was 0.86, for decorin 0.76.

CS-bone proteoglycans [40]). This could explain the increased Ca affinity of the bone CS-proteoglycans compared with the DS-proteoglycans, and would suggest that lesser weights of CS-proteoglycans would be effective in stabilizing apatite nuclei and crystals. Second, interactions between apatite mineral nuclei and crystals with CS- and DS- proteoglycans core proteins might vary. Though the protein cores of decorin, biglycan, and aggrecan are distinct [27–29, 41, 42], the core protein of bovine bone CS-decorin is completely homologous to that of cultured bovine fibroblasts [41] and skin DS-decorin [42]. It was for this reason that DS-decorin from skin was chosen for these studies. Since DS-decorin binds poorly to apatite, it is unlikely that interactions with the decorin core proteins explains variations between the CS- and DS- proteoglycan mixtures. Although the N-terminal amino acids of skin, cartilage, and bone CS-biglycan are identical, the primary sequences of the core protein chains, and the relative proportion of iduronate and glucuronate epimers varies [29]. Thus, differences in CS- and DS-biglycan-core protein-mineral interactions may exist. The possibility that these core proteins interact specifically with apatite was not considered experimentally in these studies because of the difficulties in completely separating chondroitinase ABC and other enzymes from proteoglycan digests.

The DS-proteoglycan data suggest that at least in cartilage, it is biglycan rather than decorin that promotes mineralization. Biglycan differs from decorin in several ways. It is more hydrophobic [25, 28, 29], it contains two rather than one glycosaminoglycan chains [28], and as seen for DS-proteoglycans from this study, it has a greater affinity for apatite and calcium. The affinity of DS-biglycan for apatite (K = 440 μg) on a weight basis exceeds that of DS-decorin (298 ml/μg), and the previously reported [33] values for aggrecan (K = 9 ml/μg) and hyaluronan (K = 7 ml/μg). These properties are probably interrelated, and the last two properties explain how DS-biglycan can both promote apatite formation and retard apatite crystal growth. Whether CS-biglycan has a similar effect remains unproven. Clinically, decreased DS-biglycan expression is associated with Turner's syndrome, a disease in which, *inter alia*, growth plate closure is delayed [43]. Results of the present *in vitro* study indicate that this could be associated with improper mineralization—a suggestion that will require additional analyses for verification.

In conclusion, the observation that different proteoglycans have distinct effects on mineralization may explain some of the conflicting reports in the literature. Much of the debate concerning proteoglycans in the calcification process arises from the question of whether proteoglycans must be modified before mineralization occurs, as appears to be the case in cartilage [30]. Microscopic studies of bone have

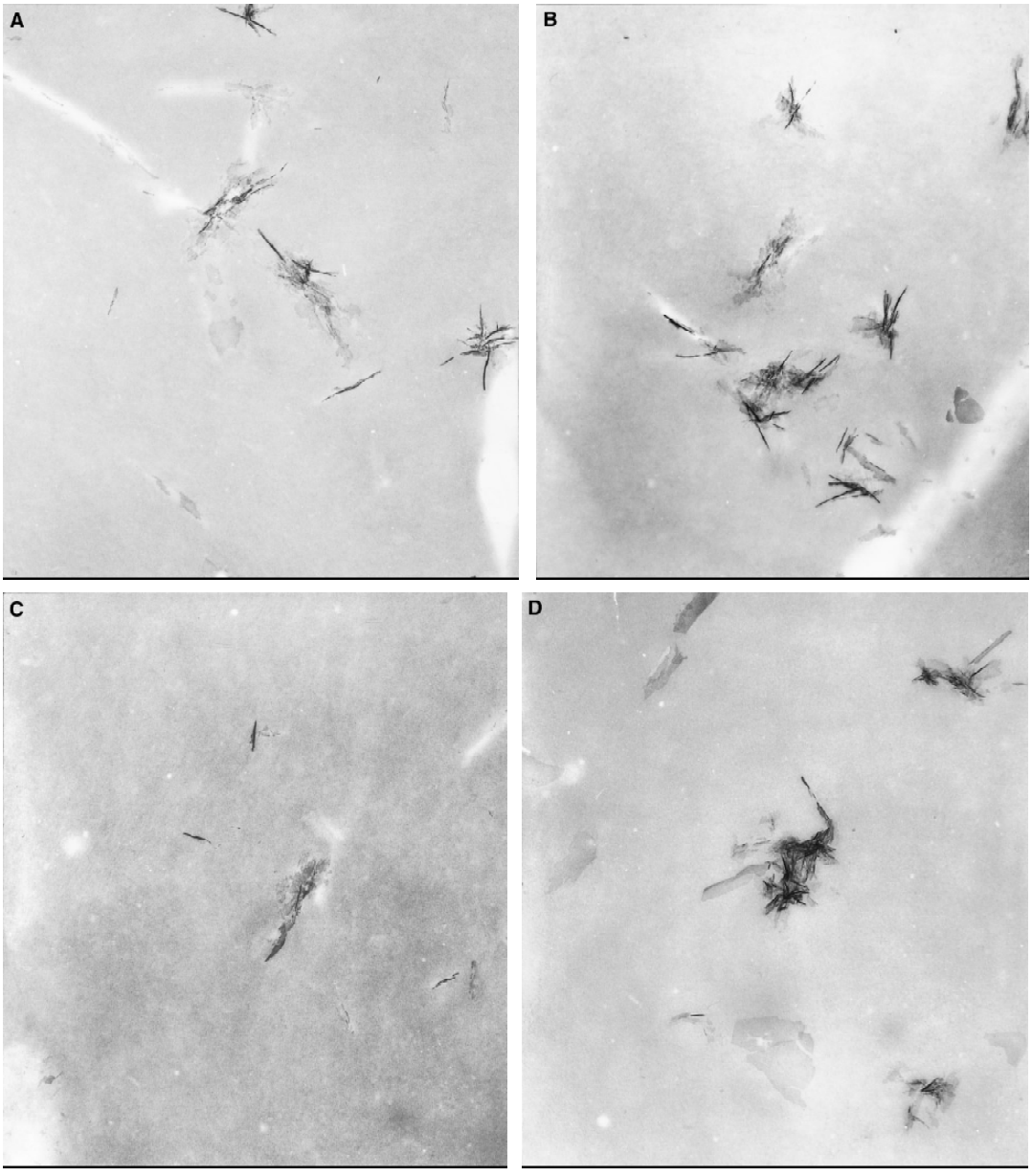

Fig. 2. Typical electron micrographs of the mineral in the center of the precipitant band in the presence of O (A), 5 (B) and 50 (C) μg/ml biglycan, and 50 μg/ml decorin. Original magnification ×45,000.

demonstrated the persistence of small proteoglycans in the mineralized matrix [3], and have suggested that these proteoglycans do not have to be removed prior to mineralization [12]. However, in mouse osteoblast cell culture, Takeuchi et al. [15] and Takagi et al. [44], found an alteration in the profile of proteoglycans formed before and after mineralization suggesting that proteoglycan modification did precede mineral deposition. Such partial degradation was said to involve loss of hydrophobicity [15]. Takagi et al. [44], studying the distribution of proteoglycans in rat bone, found a preferential staining in the lacunocanalicular spaces, and suggested that the stainable proteoglycans were inhibiting expansion of matrix mineralization. Whether these were large or small proteoglycans was not stated, but based on *in vitro* data, one might postulate that aggrecan was involved. Bianco et al.'s histochemical data [3] suggest that CS-biglycan may be involved, since this antigen was detected within lacunae and canalicular spaces. Recently, small bone proteoglycans have been identified in osteoclasts [45, 46] implying that these proteoglycans are subject to remodeling. Combining the histochemical and cell culture data, it appears that there is some proteoglycan turnover during bone mineral deposition. Further, the data presented in this paper suggests that it may be a specific proteoglycan, biglycan, that interacts with the mineral.

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