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Increased Turnover of Small Proteoglycans Synthesized by Human Osteoblasts During Cultivation with Ascorbate and β-Glycerophosphate

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Abstract. The small proteoglycan decorin had been localized previously at the d-band in the gap zone of collagen fibrils in nonmineralizing tissues. In bone matrix this zone is proteoglycan free and is at least in some species the place where mineralization along collagen fibrils starts. To study the metabolism of the small proteoglycans decorin and biglycan under mineralizing conditions, osteoblasts from human nasal bone were cultured for several weeks in the presence or absence of β-glycerophosphate and ascorbate. An immediate consequence of the treatment was a reduced expression of decorin, as judged by immune precipitation, whereas the biosynthesis of biglycan was not affected. Pulse-chase experiments were performed with osteoblasts embedded in floating type I collagen gels. In the presence of β-glycerophosphate and ascorbate, a more rapid turnover of both proteoglycans was noted; the one of biglycan reached statistical significance. Indirect evidence for an enhanced rate of proteoglycan endocytosis was obtained. This effect was not seen in cultured skin fibroblasts. Thus, osteoblasts respond rapidly to mineralizing conditions with alterations of small proteoglycan biosynthesis and turnover.

Key words: Decorin — Biglycan — Collagen gel — Osteoblasts — Vomer.

About 90% of the organic matrix of mammalian bone consists of collagen which is almost exclusively represented by type I collagen. Noncollagenous proteins, however, are considered to play an important role in the assembly and maintenance of the bone collagen matrix as well as in the formation and growth of hydroxyapatite crystals in spite of their rather low quantity [see Refs. 1–3 for review]. Among these noncollagenous proteins, heparan sulfate proteoglycans, the large interstitial chondroitin/dermatan sulfate proteoglycan versican, and the two small chondroitin/dermatan sulfate proteoglycans, biglycan and decorin, have been identified in bone and/or as products of cultured osteoblasts [4–9]. Of the latter two proteoglycans, decorin is considered to play a special role in the mineralization process since it is found specifically at the d-band in the gap zone of collagen fibrils in nonmineralizing tissues [10]. In the mineralized bone, however, this zone is proteoglycan free, and is the place where the first apatite crystals along collagen fibrils are localized, at least in some species [11]. Immunohistochemical investigations indicated the presence of decorin in pre-bone and bone cells [12] and in the unmineralized matrix. It is not known whether decorin occupies its place in the gap zone before the onset of mineralization or whether this site already is blocked for binding decorin before mineral deposition. Biglycan has recently been shown to compete with decorin for collagen binding [14]. In the bone it was localized in the walls of osteocyte lacunae and on bone cell surfaces [12]. It was tentatively considered as a proteoglycan that is associated with the mineral phase [13]. Interestingly, high biglycan expression was noted during osteoblast differentiation [12, 15] and in human bone cells from fetal and pubescent donors, i.e., from donors with rapid bone growth [16].

The potential biological roles of decorin and biglycan in bone attracted additional attention by the proposal that both proteoglycans form inactive complexes with transforming growth factor (TGF)-β [17, 18], although in cultured bone cells, TGF-β became more active when added together with decorin [19]. Whereas the active cytokine initiates endochondral ossification [20], other studies demonstrated that TGF-β suppresses mineral deposition [21], and that only selected functions of TGF-β can be blocked by complex formation with decorin [22].

In light of the proposed importance of the small proteoglycans decorin and biglycan for matrix biomineralization, and considering the controversies about their roles as modulators of growth factor activities, we studied the metabolism of both macromolecules in human bone cell cultures in the presence and absence of ascorbate and β-glycerophosphate, which are widely used to achieve the deposition of calcium phosphate by bone cells. The results of this study are presented here.

Materials and Methods

Cell Culture

Osteoblast cultures from nasal bone of metabolically normal individuals 13–26 years of age were established as described previously [23, 24]. Briefly, pieces of vomer bone which forms a part of the nasal septum, were obtained during plastic nasal surgery by approval of the local Ethical Committee. Periost-free bone pieces were incubated for 4 hours at 37°C with 400 U/ml of collagenase (type IV, Sigma, Deisenhofen, Germany) in Ca^{2+}-free DMEM and

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10% fetal calf serum (FCS). The pieces were then transferred to enzyme-free medium containing additionally ascorbate (50μg/ml), penicillin (400 U/ml), and netilmycin (50μg/ml). Outgrowing cells were maintained in DMEM/F12 medium (1:1, by vol.) containing 5 mg insulin/liter, 10% FCS, and penicillin (1 U/ml). They were used usually between the third and sixth passage, but occasionally up to the 10th passage. At the beginning of all experiments they were in a proliferative state and stained positive for alkaline phosphatase. Mineralization was initiated by supplementing the medium with ascorbate (50 μg/ml) and β-glycerophosphate (10 mM; Sigma) according to previous protocols [24]. Medium was changed three times a week.

Floating cell-populated collagen lattices were prepared as described [25] in hydrophobic Petri dishes (Greiner, Nürtingen, Germany, article number 627102) using acid soluble type I collagen from calf skin (Sigma) by adding the following components per dish: 675 μl 1.78-fold concentrated culture medium, 150 μl fetal bovine serum, 450 μl type I collagen (1.5 mg, dissolved in 17 mM acetic acid), 75 μl 0.1 M NaOH, and 300,000 cells in 150 μl medium.

Metabolic Labeling and Proteoglycan Preparation

Incubations of monolayer cultures of osteoblasts with [^{35}S]sulfate were performed as described before [25]. Briefly, cultures that had usually been passaged three to six times and had just reached confluency were trypsinized, and about 350,000 cells were seeded into a 25 cm^2 Falcon plastic flask. After 24 hours medium was changed, the new medium was supplemented either with 50 μg/ml ascorbate and 10 mM β-glycerophosphate or with an equal volume of phosphate-buffered saline (PBS). After appropriate times, individual cultures were incubated for 24 hours with 2 ml of medium containing 1.48 MBq of [^{35}S]sulfate. For subsequent immune precipitation, monospecific rabbit antisera were used. The respective antigens were the chemically deglycosylated decorin core protein from human fibroblast secretions and a neoantigen containing amino acids 11–24 of the secreted form of biglycan linked with bovine serum albumin. The production and characterization of the antisera have previously been described [26]. For the quantification of secreted proteoglycans, culture media (2 ml/25 cm^2 Falcon culture flask) were made 70% saturated with $(NH_4)_2SO_4$, and after centrifugation, the proteoglycan-containing pellet was dissolved in 0.9 ml of 0.1 M Tris/HCl, pH 7.4, containing 1 M NaCl, 0.5% Triton-X-100, 0.5% sodium deoxycholate, and protease inhibitors [25]. Cells were extracted with this buffer for 1 hour at 4°C. An aliquot of the extract was used for protein determination after precipitation with trichloroacetic acid (10%, final concentration). Immune precipitation was performed using immune globulin that had been immobilized on protein A-Sepharose (Sigma). Protein A-Sepharose (3 mg) was used for each precipitation step. Immune precipitation of decorin was followed by precipitation of biglycan, and this sequence of precipitations was repeated two more times. In the last step, only traces of labeled proteoglycans could be recovered. The sum of the immune precipitations was taken for quantitation and statistical analysis by Student's *t*-test.

When indicated, the immune complexes were digested with chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) for the removal of chondroitin/dermatan sulfate chains prior to SDS-PAGE [25].

Pulse chase experiments were performed with osteoblast-populated collagen lattices. Labeling was done in the absence of ascorbate and β-glycerophosphate because of the influence of these reagents on small proteoglycan synthesis described above. Sulfate (1.85 MBq of [^{35}S]) was added per dish at the time of initiation of the culture. After 4 days of incubation, the radioactive medium was removed and replaced by the same volume of non-radioactive medium containing 5 mM Na_2SO_4. Three additional medium changes were performed at 6-hour intervals. Six hours after the last change, i. e., 24 hours after the end of the labeling period, follow-up of the release of [^{35}S]sulfate into the medium and of the radioactivity remaining in the lattice started, and this time point was considered as zero time. The chase medium contained 1 mM Na_2SO_4 and was mixed with ascorbate (final concentration 50 μg/ml) and β-glycerophosphate (final concentration 10 mM). For the control cultures, an equivalent volume of PBS, pH 7.4 was added.

^{35}S-radioactivity released into the culture medium was determined before and after $BaCl_2$ precipitation to remove inorganic sulfate. Media were also subjected to DEAE-Trisacryl chromatography followed by polyacrylamide gel electrophoresis in the presence of SDS as described below.

Collagen lattices were digested by treatment with 50 bovine tendon collagen units of collagenase (Advanced Biofacture, Lynbrook, NY) in 100 μl of 25 mM Tris/HCl, 0.15 M NaCl, 10 mM $CaCl_2$ for 15 minutes at 45°C. Neither biglycan nor decorin core proteins were degraded by the enzyme. Cells were collected by centrifugation, the supernatant was removed, and the cells were washed with 100 μl of enzyme-free buffer. Both supernatants were combined and subjected to DEAE-Trisacryl chromatography. The cell pellet was dissolved in 100 μl 0.1 M NaOH for radioactivity determination.

A partially purified proteoglycan fraction was obtained by chromatography on DEAE-Trisacryl M (Serva, Heidelberg, Germany) as described [27]. High molecular weight proteoglycans such as versican are partially excluded from this resin and, hence, the proteoglycan fraction is relatively enriched in small proteoglycans.

Separation of decorin and biglycan was performed by SDS-PAGE on 3–12.5% acrylamide gradient slab gels. A high ionic strength separation gel buffer (0.375 M Tris, 0.1% SDS, pH 8.8) makes it possible to separate these proteoglycans in case they are carrying either one or two glycosaminoglycan chains [28]. SDS-PAGE was followed by fluorography. Quantification was performed by densitometric scanning of the autoradiographs using an LKB 2202 Ultroscan laser densitometer and Packard 604 data processor.

Other Methods

Protein was quantitated with bicinchoninic acid [29]. Ca^{2+} depositions were detected by a modified Kossa stain [30].

Results

Characterization of Osteoblasts

Osteoblasts can be obtained from nasal bone under the same conditions as osteoblasts from trabecular bone and from calvaria. Confluent cultures stained positive for alkaline phosphatase, and they synthesized almost no other fibrillar collagen than type I collagen. At the beginning of all experiments described below, the cells were still proliferative and did not contain a mineralized matrix. The deposition of calcium phosphate could be achieved within 4 weeks by treatment with ascorbate and β-glycerophosphate (data not shown). Mineralization was considerably stimulated by glucocorticoids. However, they were omitted in the investigations described below since dexamethasone alone leads to dramatic alterations of decorin and biglycan metabolism in osteoblasts and fibroblasts [31, 32].

Decorin and Biglycan Biosynthesis

The immunoprecipitation procedure with monospecific polyclonal antibodies was well suited to quantify decorin (Fig. 1) and biglycan (Fig. 2), respectively. Compared with classical chromatographic and electrophoretic techniques, the immunological method has the advantage of recognizing simultaneously biglycan containing two glycosaminoglycan chains and biglycan that is substituted with one chain only.

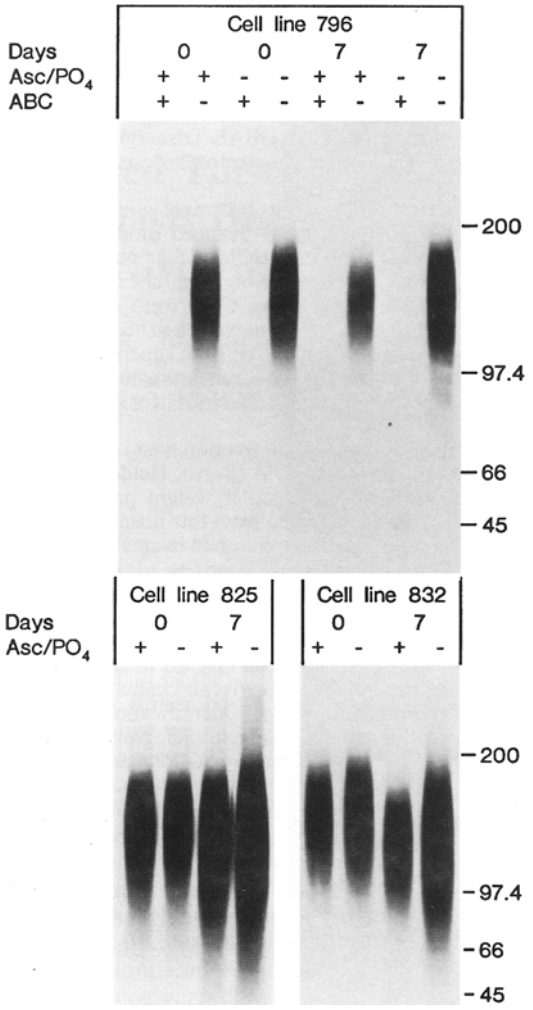

Fig. 1. Immunoprecipitation and SDS-PAGE of decorin. About 350,000 osteoblasts were seeded into a 25 cm^2 Falcon plastic flask and allowed to attach for 24 hours. Thereafter, medium was changed and the new medium was supplemented either with 50 μg/ml ascorbate and 10 mM β-glycerophosphate (Asc/PO_4) or with an equal volume of PBS. This time point was considered as zero time. Incubations with [^{35}S]sulfate (0.74 MBq/ml) started at the time points indicated and were for 24 hours. Decorin was isolated by immunoprecipitation, subjected to degradation by chondroitin ABC lyase (ABC) when indicated, and subsequently separated by SDS-PAGE under reducing conditions. The migration distance of reference proteins in kDa is indicated in the right margin.

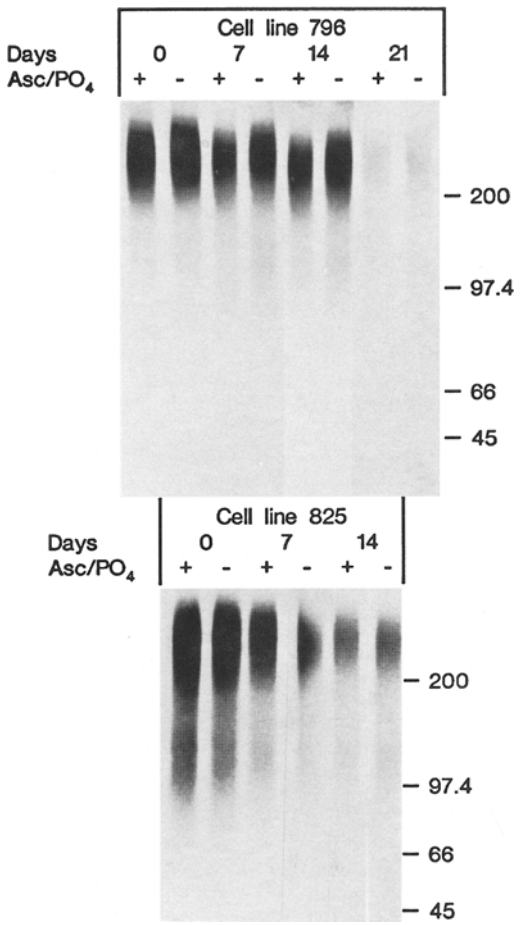

Fig. 2. Immunoprecipitation and SDS-PAGE of biglycan. Experimental details are as indicated in the legend of Figure 1. Biglycan carrying two glycosaminoglycan chains exhibits a slower mobility than the reference protein of 200 kDa (myosin) whereas the species with only one chain has a slower mobility than the 97.4 kDa reference protein (phosphorylase b) but migrates faster than myosin.

Such biglycan species have been found to be secreted by human skin fibroblasts [33]. Figure 2 shows that there are some differences in the relative proportion of monoglycanated biglycan on the total proteoglycan quantity when osteoblast cell lines from different donors are compared. Cell line 825 was exceptionally rich in the monoglycanated species which usually represented only about 10% of the biglycanated form. It is also evident that the antisera did not immunoprecipitate other [^{35}S]sulfate-labeled materials, and that therefore the immunoprecipitation protocol could reliably be used for quantification of decorin and biglycan.

Figures 1 and 2 provide evidence for the specificity of the antisera against decorin and biglycan, but they also indicate some variability between different cell lines regarding the effects of ascorbate and β-glycerophosphate on the biosynthesis of the two proteoglycans. Cell line 825 was exceptional among all cell lines studied because, in contrast to later time points, it did not show an immediate response (day 0) with respect to the down regulation of decorin under the influence of these agents. The reason for this delay is not known.

Under standard culture conditions, i.e., in the absence of ascorbate and β-glycerophosphate, proliferating osteoblasts regularly synthesized more decorin than biglycan, at least during the first week after trypsinization (Fig. 3). Both proteoglycans were rapidly secreted into the culture medium. After a labeling period of 24 hours, at the most 7% of the total radioactivity incorporated into either proteoglycan remained associated with the cell layer. In cultures from seven different individuals, incorporation of [^{35}S]sulfate into decorin was 1.5–2.8 times higher than into biglycan. On an average, a 2.1-fold higher incorporation was obtained. On a molar basis, this biosynthetic ratio should almost be doubled because of the different numbers of glycosaminoglycan chains per molecule. It had previously been found that, at least in fibroblasts and osteosarcoma cells, the glycosaminoglycan chains of biglycan and decorin are of similar size and composition [34, 35].

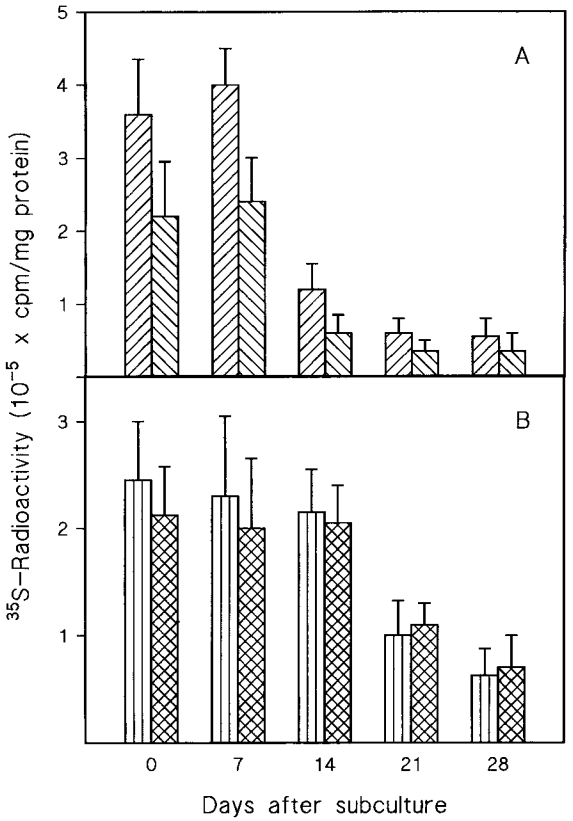

Fig. 3. Time-dependent changes of biglycan and decorin biosynthesis by osteoblasts cultured in the presence or absence of 50 μg/ml ascorbate and 10 mM β-glycerophosphate. Experimental details are as indicated in Material and Methods and in the Figure 1 legend. The data for cell-associated and secreted proteoglycans are combined for each of the separately analyzed five different cell lines. **(A)** decorin biosynthesis **(B)** biglycan biosynthesis are shown, measured in the absence (▨, ▥) or presence (▧, ▩) of ascorbate and β-glycerophosphate. Bars represent standard error measurements. Significant differences are found for decorin between treated and untreated cultures at days 0, 7, and 14 ($P < 0.05$) and for the decline in decorin production under both conditions between days 7 and 14 ($P < 0.01$). The reduction of biglycan biosynthesis between days 14 and 21 is also significant ($P < 0.05$).

When osteoblasts are maintained in monolayer culture without subculturing there is a small increase in decorin biosynthesis when the cultures become confluent, i.e., after 1 week (Figs. 1 and 3). This increase was 30% at the most and could not be statistically verified. The dramatic induction of decorin biosynthesis in fibroblasts upon confluency [36] could not be reproduced. On the contrary, in very dense cultures the production of decorin declined analogously as in fibroblasts embedded in a collagen lattice [25].

The biosynthesis of biglycan did not significantly differ between subconfluent and moderately confluent cultures. During prolonged culture, however, biglycan production declined, too (Figs. 2 and 3).

In the presence of ascorbate and β-glycerophosphate, decorin biosynthesis was reproducibly diminished. This was evident immediately after the cells had been exposed to mineralizing conditions, i.e., before any calcium phosphate crystals were deposited. This down-regulation of decorin production was observable throughout the experimental period of 4 weeks, but the absolute differences were smaller at later time points. The decreased quantity of decorin in the culture medium was not compensated by a higher amount of cell-associated proteoglycans since, similar to control cultures, only 5–8% of labeled decorin was obtained from the cell extract. In four different osteoblasts cell lines this inhibition of decorin production was reproducibly observed. On days 0 and 7, [^{35}S]sulfate incorporation into decorin was between 40% and 70% of that of the control culture (Figs. 1 and 3). No significant difference of biglycan production was observed in the presence or absence of ascorbate/β-glycerophosphate (Figs. 2 and 3).

In some, but not all, cell lines there were small differences in the electrophoretic mobility of decorin synthesized under the two experimental conditions. The faster mobility of decorin from cell line 832 produced under mineralizing conditions is an indication of shorter glycosaminoglycan chains (Fig. 1). Since this phenomenon was not observed in all cell lines it was not further investigated.

Turnover of Decorin and Biglycan

Monolayer cultures are unsuited for studying the turnover of secretory products since these molecules are removed with the culture medium at regular intervals. We therefore took advantage of the possibility of culturing osteoblasts within a collagen lattice. This culture technique is considered to be a better method to demonstrate differentiated osteoblast functions [21], and it had been shown previously that decorin and biglycan are preferentially retained within the collagen gel [14]. To study the turnover of biglycan and decorin, osteoblasts, which had been maintained in monolayer culture under nonmineralizing conditions, were embedded in a collagen lattice and at the same time exposed to [^{35}S]sulfate for 4 days. Since cell-associated proteoglycans were removed by trypsin prior to gel entrapment, almost all of the proteoglycan molecules present after these 4 days of incubation should be uniformly labeled. After a carefully performed washing out protocol of the radioactive precursor, proteoglycan turnover was followed for more than 2 weeks (Fig. 4). It is evident that β-glycerophosphate and ascorbate caused a significant increase in the quantity of ^{35}S-radioactivity released into the medium. Between 8% and 15% of the released radioactivity could not be precipitated by barium ions, but there was no significant difference between glycerophosphate/ascorbate-treated and control cultures. Thus, the radioactivity in the culture medium represents predominantly inorganic [^{35}S]sulfate. Small quantities of decorin, biglycan, and high-molecular-weight proteoglycans could be recovered from the medium and visualized by SDS-PAGE and fluorography. The quantity of decorin and biglycan released into the culture medium in a form allowing immune precipitation was always less than 3% of that remaining in the collagen lattice indicating that diffusion of intact small proteoglycans out of the gel is of negligible importance for their turnover. Furthermore, there was no indication of differences that could be related to the different culture conditions (results not shown).

As expected from the differences between the quantities of inorganic radiosulfate released into the culture medium with or without ascorbate and glycerophosphate, greater quantities of [^{35}S]sulfate-labeled proteoglycans remained within the collagen gels in the absence than in the presence of these reagents (Fig. 4). An SDS-PAGE analysis indicated that during chase, especially the quantity of biglycan declined more rapidly in the presence of ascorbate and β-glyc-

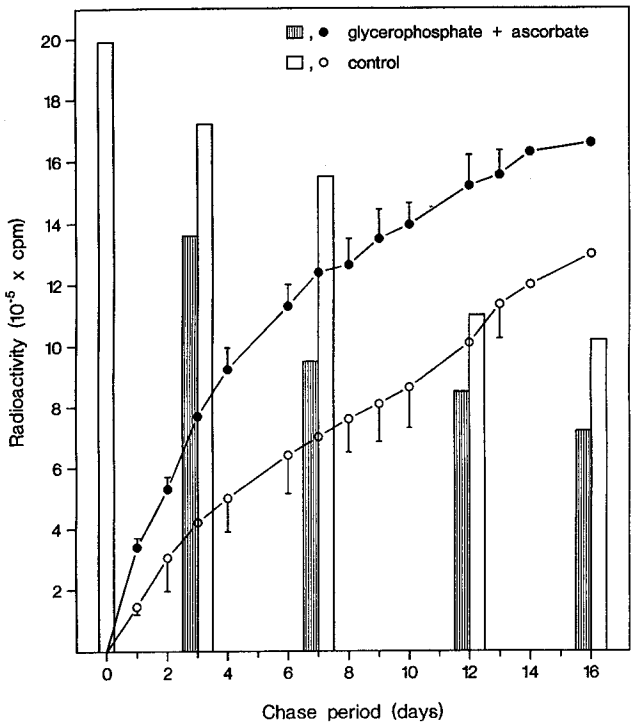

Fig. 4. Pulse-chase experiment of osteoblasts embedded in floating collagen gels. Nine osteoblast cultures (300,000 cells/dish) were maintained in a collagen gel and labeled with [^{35}S]sulfate (1.85 MBq/dish) for 4 days as described in Materials and Methods. After a washout period of 24 hours, one of the gels was harvested (zero time) and the other cultures were kept in the presence or absence of 50 μg/ml ascorbate and 10 mM β-glycerophosphate. Radioactivity was determined in the liquid medium (○ and ●) and in the collagen lattices after collagenase digestion (□ and ▨). Cumulative data are given for the radioactivity in the medium. The bars indicate the range of data.

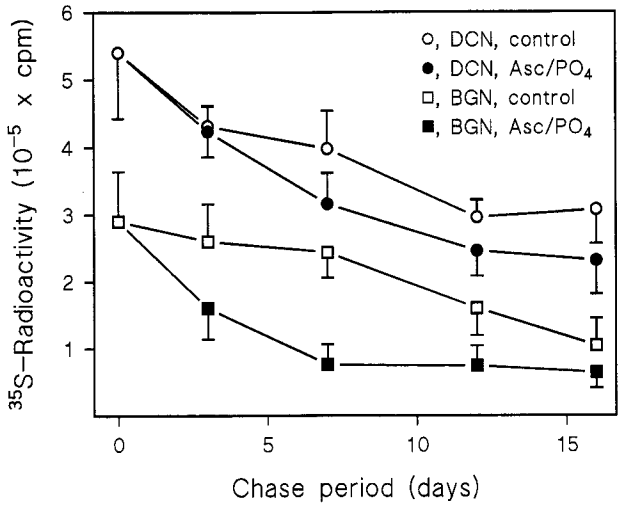

Fig. 5. Turnover of decorin (DCN) and biglycan (BGN) in osteoblast-populated collagen lattices. Cultures were pulse-labeled and chased as described in Materials and Methods. Small proteoglycans were recovered from the lattices by collagenase digestion, DEAE-chromatography, and SDS-PAGE. Quantification was performed by densitometric scanning, normalizing the data to the radioactivities recovered from the gels. Bars represent standard error measurements obtained from the analysis of three separate osteoblast cell lines. Significant differences for biglycan were obtained for cultures maintained in the presence or absence of ascorbate and β-glycerophosphate at days 3 ($P < 0.05$), 7 ($P < 0.01$), and 12 ($P < 0.05$) whereas the differences for decorin were not significant ($P < 0.1$ on days 7, 12, and 16).

erophosphate. The normal half-life time of about 2 weeks became reduced to about 4 days. The turnover of decorin was also accelerated under mineralizing conditions, but the differences were apparently smaller than in the case of biglycan and did not reach statistical significance. The half-life of decorin under mineralizing conditions was 11 days and more than 16 days in control cultures (Fig. 5).

Further experiments with lattice-embedded cells indicated the following. Treatment with ascorbate alone was insufficient to enhance the turnover of proteoglycans (Fig. 6, left panel). More important was the observation that there was almost no response of cultured skin fibroblasts to the treatment with ascorbate and β-glycerophosphate, indicating the cell specificity of the effects (Fig. 6, right panel).

Discussion

There are conflicting data on the relative rates of synthesis of the two small proteoglycans biglycan and decorin in cultured osteoblasts. Some investigators find that trabecular bone cells have reproducibly about twofold greater quantities of biglycan than of decorin [8, 16, 31]. The data of other authors give evidence that mouse osteoblasts produce similar quantities of both proteoglycans [5]. MC3T3-E1 cells from newborn mouse calvaria predominantly synthesize decorin, with little biglycan [9], and in the rat osteoblast-like cell line UMR 106-01, probably only decorin was found [37]. In seven separate cultures of osteoblasts from vomer bone, i.e., from a nontrabecular bone, we found, without exception, a higher production of decorin than of biglycan when the cultures were analyzed within 3 weeks after plating. Though an effect of tissue culture conditions on the relative expression of biglycan and decorin cannot be excluded we have previously found that under our culture conditions the osteosarcoma cell line MG-63 expresses much more biglycan than decorin [35]. Therefore, it seems likely that the favored synthesis of decorin is an inherent property of osteoblasts from nasal bone obtained from individuals of 13–26 years of age.

Conflicting data also become evident when the results on the changes of glycosaminoglycans during biomineralization are compared (see [1] for a review). An increase of chondroitin sulfate was observed in β-glycerophosphate-treated human fetal osteoblasts [38]. In a culture system where bovine fetal osteoblasts were forced to deposit a mineralized matrix within 5–6 days, there was a transient increase of biglycan mRNA with a maximum at day 6 and a continuous rise of decorin mRNA at least up to day 20 [39]. However, the synthesis of these small proteoglycans has not been followed at the protein level, and the tissue culture system was clearly different from the one used in the present study. We found reproducibly that specifically the biosynthesis of decorin is down-regulated when the cells are exposed to β-glycerophosphate and ascorbate. With one exception, this effect occurred immediately and was, therefore, independent of the deposition of mineral crystals. The effect could not be explained simply by a change in the rate of sulfation and/or desulfation. Degradation experiments

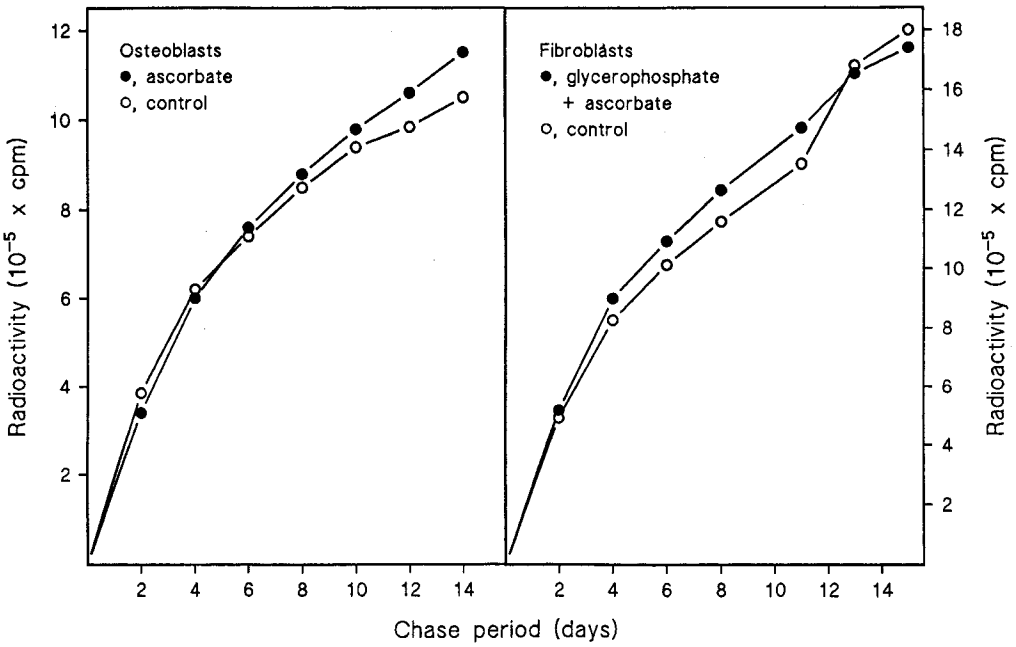

Fig. 6. Pulse-chase experiment of osteoblasts and fibroblasts embedded in floating collagen gels. Experiments were performed as described in Figure 4 except that osteoblasts were exposed to 50 μg/ml ascorbate and not additionally to β-glycerophosphate. Cumulative data of the ^{35}S-radioactivities released into the liquid media are given.

with bacterial eliminases yielded exclusively monosulfated disaccharide units as building blocks of the glycosaminoglycan chains. Disulfated disaccharides were below the limit of detection, and small proteoglycans are known to contain unsulfated disaccharide units only near the carbohydrate protein linkage region [34]. Desulfation is exclusively an intralysosomal event and therefore does not confuse the data interpretation.

An important result of this study is the finding that ascorbate/β-glycerophosphate treatment caused an accelerated turnover of proteoglycans. Among the small proteoglycans, biglycan was more strongly affected than decorin. Previous studies in monolayer cultures of osteoblasts had already mentioned the short half-life of biglycan [8], and it is known that virtually all noncollagenous proteins in bone eventually become degraded [40]. In contrast to the possibility of extracellular protein degradation, the sulfate ester groups of decorin and biglycan are exclusively liberated intralysosomally. The core proteins of both proteoglycans bind to a specific small proteoglycan endocytosis receptor [26]. Binding is followed by internalization as a prerequisite for subsequent degradation. Thus, under the influence of β-glycerophosphate and ascorbate there must be an increased rate of endocytosis. In this regard it is of interest to note that collagen-bound decorin is still recognized by the endocytosis receptor [22, and unpublished observation], which is probably also true for collagen-bound biglycan. Since biglycan binds less tightly to type I collagen fibrils than decorin [14] biglycan may perhaps reach the receptor by diffusion within the extracellular matrix whereas decorin interacts with the receptor while it is still bound to the collagen fibril.

In summary, the present data suggest a hypothesis for the absence of small proteoglycans at the d-band in the gap zone of collagen fibrils of bone. Under cultivation with ascorbate and β-glycerophosphate, decorin synthesis is down-regulated even before the onset of mineral deposition, and the turnover of small proteoglycans, especially of biglycan, is augmented. It is therefore suggested that before and possibly during mineralization, small proteoglycans are removed from the gap zone, thereby providing the necessary space for the first apatite crystals along the collagen fibrils. This hypothesis, however, has to be supported by electron microscopical investigations.

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