Proteoglycan Synthesis and Deposition in Fetal Rat Bone†

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ABSTRACT: A pulse-labeling approach has been used to study proteoglycan metabolism in fetal rat bone. Pregnant rats were injected with [^{35}S]sulfate and sacrificed 6, 24, or 48 h later. Fetal calvaria were dissected and extracted sequentially with 4 M guanidine hydrochloride and 4 M guanidine hydrochloride/0.5 M (ethylenedinitrilo)tetraacetic acid (EDTA). With time after injection, the proportion of total incorporated radioactivity decreased in the guanidine pool (corresponding to nonmineralized bone and associated soft tissues) and increased in the guanidine/EDTA pool (mineralized bone). Chromatographic analysis of the proteoglycan species present in these pools after different labeling times indicated that three species of proteoglycan are synthesized in fetal rat calvaria. A large chondroitin sulfate (CS) proteoglycan and a smaller dermatan sulfate (DS) proteoglycan are located in the nonmineralized compartment. A CS proteoglycan similar in size to the DS proteoglycan is initially present in the nonmineralized bone but subsequently is located in the mineralized matrix. A fraction of the small CS proteoglycan is strongly associated with collagen.

Adult mammalian bone consists of an organic matrix upon which are deposited crystals of hydroxyapatite. The organic matrix consists of principally type I collagen, but several minor components have been described recently. These include the following: a small, γ-carboxyglutamate-containing polypeptide, bone gla protein or osteocalcin (Hauschka et al., 1975); a mineral-binding glycoprotein, osteonectin (Termine et al., 1981a); several other species of glycoprotein (Termine et al., 1981b; Linde et al., 1983). Although the presence of proteoglycan in bone was demonstrated first in 1968 (Herring, 1968), the proteoglycans of mineralized tissues remain only poorly characterized. In the 13-day-old fetal chick calvaria, a proteoglycan of M_r 70000 containing one to two chondroitin sulfate chains has been identified (Sugahara et al., 1981). This tissue also contains small amounts of heparan sulfate and keratan sulfate. The presence of keratan sulfate may be characteristic of avian bone, as the medullary bone of quail produces a small keratan sulfate proteoglycan in addition to a chondroitin sulfate proteoglycan (Hunter & Schraer, 1983). The ectopic bone formed by subcutaneous implantation of demineralized bone matrix in the rat contains a small proteoglycan with chondroitin sulfate chains of M_r 50000 (Reddi et al., 1978). In a recent study of fetal bovine diaphyseal bone, two distinct proteoglycan species were described: a large (M_r $\sim 10^6$) proteoglycan, which was extracted from fetal bone prior to demineralization, and a smaller (M_r $\sim 10^5$) species, most of which could be extracted from bone only with demineralizing agents (Fisher et al., 1983).

In the present study, synthesis and deposition of proteoglycans in fetal rat calvaria have been investigated. Since the bones of the calvaria form by intramembranous ossification rather than by the endochondral ossification of long bones, contamination by cartilaginous elements is minimized. Radiolabeling with [^{35}S]sulfate and extraction of proteoglycans in solubility pools corresponding to different compartments of bone have been combined to generate a dynamic picture of proteoglycan metabolism in developing bone.

Experimental Procedures

Materials

Ultrapure guanidine hydrochloride was obtained from Schwarz/Mann and used for extraction solutions. Sigma type I guanidine hydrochloride was used for chromatography. Ultrapure cesium chloride was obtained from Bethesda Research Laboratories. Sepharose CL-2B and Sepharose CL-6B were obtained from Pharmacia. Chondroitinase ABC (EC 4.2.2.4) and chondroitinase AC (EC 4.2.2.5) were obtained from Sigma. Purified bacterial collagenase (Worthington CLSPA, further purified by chromatography on Sephadex G-200) was kindly provided by Dr. J. Sodek.

Methods

Radiolabeling and Extraction of Proteoglycans from Fetal Rat Calvaria. Groups of three female Wistar rats of approximately 200 g (Charles River Canada, Montreal) were injected intraperitoneally on day 19, 20, or 21 of pregnancy with 1 mCi per animal [^{35}S]sulfate (25–40 Ci/mg; SJS-1, Amersham) contained in a volume of 0.5 mL of sterile water. Six hours after injection on day 21, all animals were sacrificed by cervical dislocation and the fetuses removed. Each litter was subsequently handled separately. Calvaria (frontal and parietal bones) were dissected out along the suture lines and immediately placed in 10 mL of cold sterile phosphate-buffered saline (PBS)[1] containing the proteolytic enzyme inhibitors benzamidine (5 mM) and 6-aminohexanoic acid (0.1 M). All remaining procedures were performed at 4 °C unless otherwise stated. After washing by vortexing 3 times in PBS plus inhibitors, the tissues were transferred to 10 mL of 4 M Gdn·HCl/50 mM sodium acetate, pH 6.8, containing proteinase inhibitors as above. Calvaria were disrupted by brief homogenization (10 s, setting 4) using a Polytron homogenizer and agitated gently for 24 h. The extract was then centrifuged (30000*g*, 20 min) and the supernatant removed. A second cycle of guanidine extraction was performed, and the supernatants were pooled (guanidine extract). The pellet was reextracted with 4 M Gdn·HCl/0.5 M EDTA (Termine et al., 1981b), as follows. Tissue residue was resuspended by vortexing in 10 mL of 4 M Gdn·HCl/0.5 M EDTA/50 mM sodium acetate, pH 6.8, plus inhibitors and agitated gently for 48 h. The samples were then centrifuged and the supernatant removed. A second cycle of guanidine/EDTA extraction was performed, and the supernatants were pooled (guanidine/EDTA extract).

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[1] Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; EDTA (ethylenedinitrilo)tetraacetic acid; PBS, phosphate-buffered saline; V_0, void volume; V_t, total volume; CPC, cetylpyridinium chloride; Gdn·HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Calvaria radiolabeled for 48 h prior to sacrifice were then digested with collagenase. Demineralized tissue residue was washed with 50 mM Tris-HCl, pH 7.4, and added to 5 mL of 0.36 mM $CaCl_2$/2 mM *N*-ethylmaleimide/50 mM Tris-HCl, pH 7.4, containing 400 units (approximately 1 mg) of collagenase. This was incubated at 37 °C for 16 h. Samples were then centrifuged, and the supernatant was removed (collagenase extract).

All samples were made 70% (v/v) ethanol and incubated for 24 h. Precipitates were then collected by centrifugation at 10000*g* for 10 min and washed once with 70% ethanol. Final pellets were redissolved in 4 M Gdn·HCl/50 mM sodium acetate, pH 6.8, plus inhibitors.

Demineralized bone residues were dissolved in 2 mL of 1 M NaOH at 60 °C for 24 h. Aliquots of ethanol-precipitated guanidine, guanidine/EDTA, and collagenase extracts, and NaOH extracts were removed and counted in an LKB 81000 liquid scintillation spectrometer to determine the distribution of ^{35}S radioactivity among these solubility pools at different labeling times.

Cesium Chloride Density Gradient Centrifugation. Triplicate samples at each time point were pooled, and solid CsCl was added (0.55 g/mL) to give an initial density of 1.43 g/mL. Samples (4 mL volume) were centrifuged at 135000*g* for 72 h in a Beckman L5-65 ultracentrifuge using an SW 50.1 rotor. Gradients were harvested by syringe from the top in 0.125-mL aliquots. Density was determined gravimetrically, and 10-μL aliquots of each fraction were removed for scintillation counting.

Sepharose CL-2B Chromatography. Pooled fractions from CsCl density gradients were dialyzed against distilled water, lyophilized, and redissolved in 4 M Gdn·HCl. Samples were then chromatographed on Sepharose CL-2B in the presence of 4 M Gdn·HCl; 100 × 1.5 cm columns were preequilibrated and eluted with 4 M Gdn·HCl/50 mM sodium acetate, pH 6.8, at a flow rate of 7 mL/h. Fractions of 1.5 mL were collected, and aliquots of 0.2 mL were added to 0.3 mL of distilled water and 6 mL of Aquasol (New England Nuclear) for scintillation counting. Void volume (V_0) and total volume (V_t) were determined by using Dextran Blue and [^{3}H]-hydroxyproline, respectively.

Glycosaminoglycan Analysis. GAG compositions of proteoglycan fractions from fetal rat calvaria were determined as previously described (Hunter et al., 1983). In brief, GAG chains were released by treatment with alkaline borohydride reagent and analyzed by specific enzymatic or chemical degradation. Heparan sulfate (HS) was quantitated by nitrous acid hydrolysis. Chondroitin sulfate (CS) and dermatan sulfate (DS) were quantitated by digestion with chondroitinase ABC and chondroitinase AC. In all experiments, nondegraded GAGs were recovered by precipitation with cetylpyridinium chloride (CPC), redissolved in methanol, and counted.

Chromatography of GAG Chains on Sepharose CL-6B. Chromatography on Sepharose CL-6B of free GAG chains from fetal rat calvaria proteoglycans was performed as described (Hunter et al., 1983). In brief, 50 × 1.5 cm columns were eluted with 0.2 M pyridinium acetate, pH 5.0, at a flow rate of 7 mL/h. Fractions of 1.1 mL were collected and aliquots of 0.5 mL used for scintillation counting.

Results

Extraction of Proteoglycans from Fetal Rat Calvaria. Fetal calvaria isolated 6, 24, or 48 h after radiolabeling with [^{35}S]sulfate were extracted sequentially with 4 M Gdn·HCl and 4 M Gdn·HCl/0.5 M EDTA, and the insoluble residue was dissolved in 1 M NaOH. The percentage of total incorporated ^{35}S radioactivity in each solubility pool is plotted as a function of labeling time in Figure 1. The guanidine pool, which represents 85% of total radioactivity 6 h after injection, diminishes to 69% at 24-h postinjection and to 62% at 48 h. There is a corresponding alteration in the guanidine/EDTA pool, which increases from 10% of the total at 6 h to 24% at 24 h and 29% at 48 h. The proportion of radioactivity in the "insoluble" fraction shows a gradual increase with labeling time, reaching 10% of the total by 48-h postinjection.

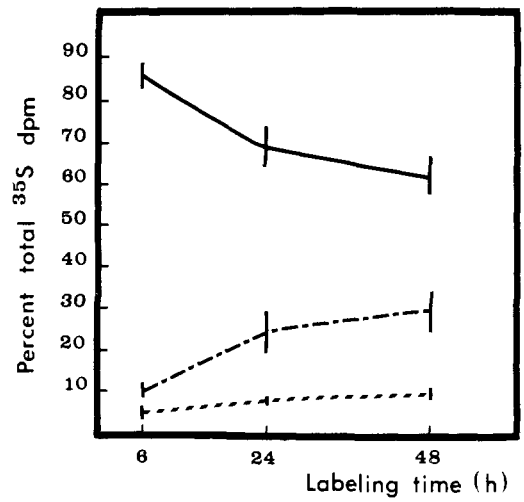

FIGURE 1: Distribution of ^{35}S activity in different solubility pools of fetal rat calvaria as a function of labeling time. Percentage of total incorporated ^{35}S in guanidine pool (—), guanidine/EDTA pool (– – –), and insoluble pool (---). Vertical bars represent standard deviations.

The total ^{35}S incorporated into calvaria decreases as a function of time after injection. Corrected for litter size, the values decrease from 1.42 × 10^5 dpm per fetus at 6-h postinjection to 1.05 × 10^5 dpm at 24 h and 2.31 × 10^4 dpm at 48 h.

Purification and Characterization of Proteoglycans from Guanidine and Guanidine/EDTA Pools. The guanidine and guanidine/EDTA extracts were purified by CsCl density grandient centrifugation. Proteoglycans were recovered from the lower three to four fractions of the gradients, corresponding to a buoyant density of approximately 1.7 g/mL (Figure 2). This density is similar to that of cartilage proteoglycan (Hascall & Sajdera, 1970) and indicates that the major calvarial proteoglycans have a high carbohydrate/protein ratio. The low density portions of the gradients in most cases contained only small amounts of ^{35}S activity, and this was not sufficient for separate analysis. The density gradient purified extracts were then characterized by chromatography on Sepharose CL-2B.

The profiles of these proteoglycans 6, 24, and 48 h after radiolabeling are shown in Figure 3. At 6 h, the guanidine extract exhibits a biphasic profile (Figure 3A). This probably represents the partial superimposition of two peaks, one at a K_{av} value of approximately 0.4 (termed G 0.4) and the other at a K_{av} value of approximately 0.7 (G 0.7). After 24 and 48 h, the profiles are characteristically different, with the G 0.7 peak decreased relative to the G 0.4 peak (Figure 3C,E). The guanidine/EDTA extract at 6-h labeling exhibits a major peak at $K_{av} \sim 0.7$ (E 0.7) and a minor peak at the void volume of the column (Figure 3B). At 24- and 48-h postinjection, the E 0.7 species remains the major peak, with minor peaks at apparently higher molecular weights (Figure 3D,F).

Glycosaminoglycan Composition of Proteoglycan Fractions from Guanidine and Guanidine/EDTA Pools. Proteoglycan fractions G 0.4, G 0.7, and E 0.7 were hydrolyzed with alkaline borohydride to produce free GAG chains. The compositions

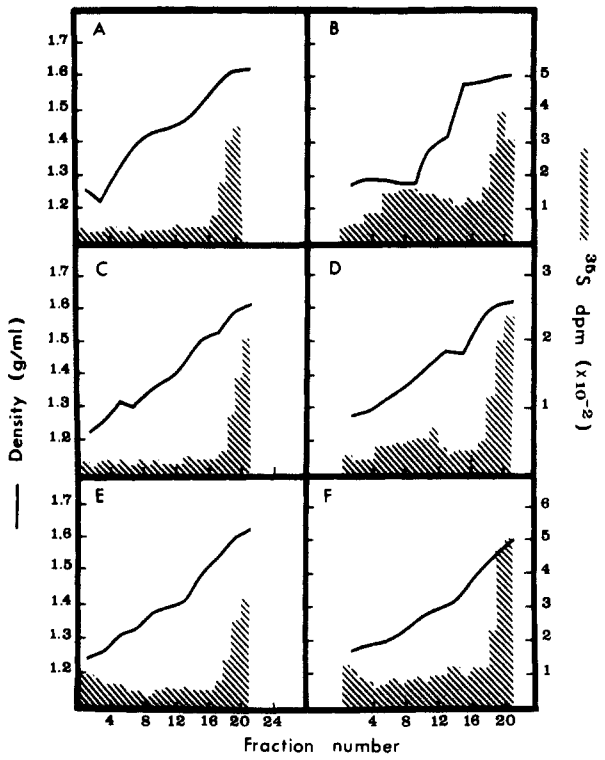

FIGURE 2: Density gradient centrifugation in CsCl of proteoglycans from fetal rat calvaria. Guanidine and guanidine/EDTA extracts were centrifuged in CsCl gradients in the presence of 4 M Gdn·HCl. (A, C, and E) Guanidine extracts; (B, D, and F) guanidine/EDTA extracts; (A and B) 48 h after labeling; (C and D) 24 h after labeling; (E and F) 6 h after labeling.

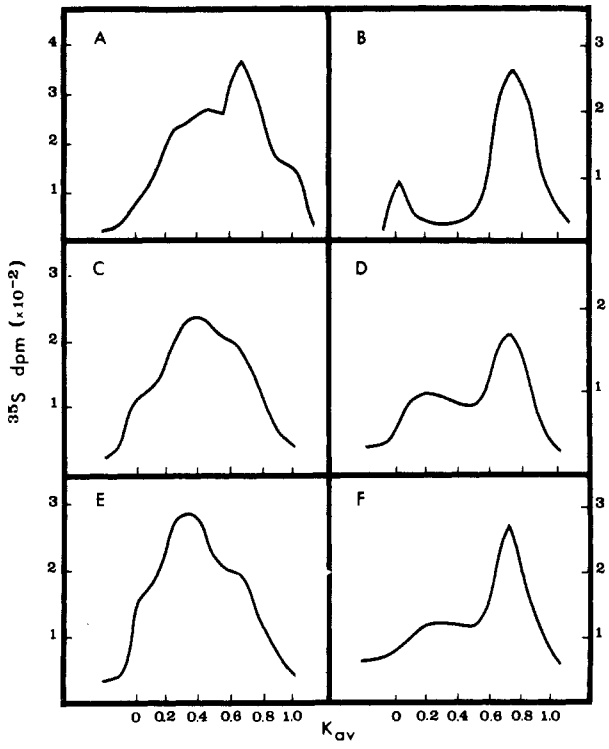

FIGURE 3: Chromatography on Sepharose CL-2B of proteoglycans from fetal rat calvaria. Proteoglycan fractions from calvaria radiolabeled for various times were chromatographed on Sepharose CL-2B in the presence of 4 M Gdn·HCl. (A, C, and E) Guanidine extracts; (B, D, and F) guanidine/EDTA extracts; (A and B) 6 h labeling time; (C and D) 24 h labeling time; (E and F) 48 h labeling time.

of these fractions were determined by degradation with specific chemical or enzymatic treatments (Table I). None of these fractions contained detectable quantities of heparan sulfate.

Table I: GAG Compositions of Proteoglycan Fractions from Guanidine Extracts of Fetal Rat Calvaria[a]

	labeling time (h)					
	6		24		48	
	CS	DS	CS	DS	CS	DS
G 0.4	89	8	93	6	96	3
G 0.7	47	48	31	66	22	74

[a] GAG chains from proteoglycan fractions were subjected to specific degradative treatments as described (see Experimental Procedures). Control samples contained only GAGs and buffer. Percentage degradation was determined as 1 - (experimental dpm/control dpm) × 100. Values represent the mean of three determinations. G 0.4: $K_{av} \sim 0.4$ peak from guanidine extracts. G 0.7: $K_{av} \sim 0.7$ peak from guanidine extracts.

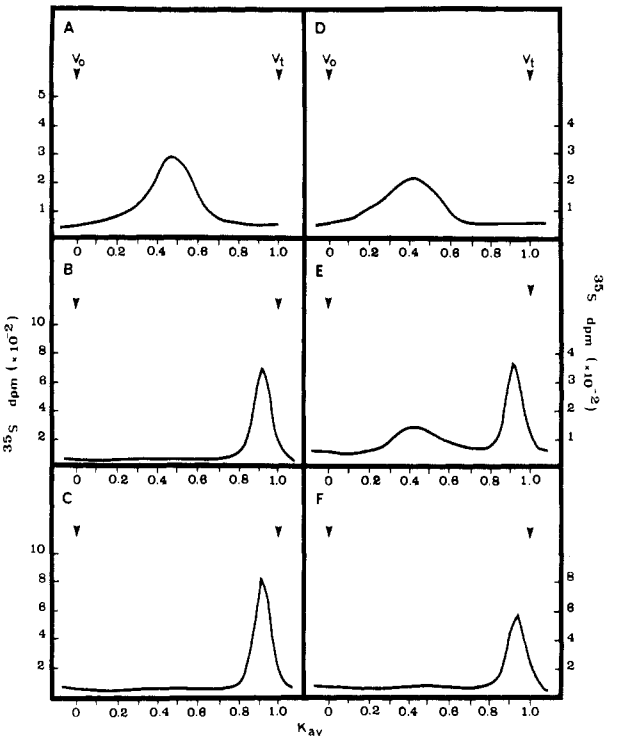

FIGURE 4: Chromatography on Sepharose CL-6B of GAG chains from guanidine-extracted proteoglycans of fetal rat calvaria. (A) GAG chains from proteoglycan fraction G 0.4; (B) GAG chains from (A) treated with chondroitinase AC; (C) GAG chains from (A) treated with chondroitinase ABC; (D) GAG chains from proteoglycans fraction G 0.7; (E) GAG chains from (D) treated with chondroitinase AC; (F) GAG chains from (D) treated with chondroitinase ABC.

Fraction G 0.4 consisted almost entirely of CS, at all labeling times. Fraction G 0.7 contained approximately equal amounts of CS and DS at 6-h labeling, but the proportion of DS increased with time after labeling, to 66% at 24 h and 74% at 48 h. The E 0.7 fraction in the guanidine/EDTA pool consisted almost entirely of CS at all labeling times.

Chromatography on Sepharose CL-6B of GAG Chains from Guanidine-Extracted Proteoglycan Fractions. Free GAG chains from the G 0.4 and G 0.7 proteoglycan fractions from guanidine-extracted calvaria were chromatographed on Sepharose CL-6B (Figure 4). In both cases, a single peak was found at a K_{av} value of approximately 0.45 (Figure 4A,D). By the criteria of Wasteson (1971), this corresponds to a molecular weight of 25 000. Treatment of these fractions with chondroitinase ABC (which degrades CS and DS) resulted in the loss of this peak, with the appearance of a peak of degradation products near the V_t of the column (Figure 4C,F). Treatment with chondroitinase AC (which degrades CS) digested the G 0.4 GAGs totally (Figure 4B), as expected from

the previous observation that this fraction contains only CS (Table I). However, the chondroitinase AC treated G 0.7 GAGs gave a reduced peak at $K_{av} \sim 0.45$ and a peak near the V_t. This indicates that the CS chains have been fully degraded, whereas the DS chains are still intact. Thus, the CS and DS in this proteoglycan fraction are contained on separate GAG chains. Since proteoglycans containing both CS and DS normally consist of hybrid CS/DS chains (Fransson et al., 1970), this observation may indicate that the G 0.6 fraction of fetal rat calvaria contains two proteoglycan species, a CS proteoglycan and a DS proteoglycan.

Extraction of Demineralized Bone Residue with Bacterial Collagenase. To determine whether the ^{35}S radioactivity in pulse-labeled calvaria which was resistant to guanidine/EDTA extraction was associated with collagen, demineralized residues of calvaria radiolabeled for 48 h were digested with highly purified bacterial collagenase. This resulted in the release of approximately 70% of the ^{35}S activity. Chromatography of the collagenase-release material on Sepharose CL-2B resulted in a peak at the same elution position as the major proteoglycan of the guanidine/EDTA pool (not shown). GAG analysis of this fraction showed that it consisted of almost entirely CS.

Discussion

Synthesis and deposition of proteoglycans in fetal bone have been studied by using a pulse-labeling approach. Pregnant rats were injected with [^{35}S]sulfate and sacrificed after various time periods. This approximates to a pulse–chase experiment, since sulfate is metabolized very rapidly in vivo (Mulder & Scholtens, 1978). In agreement with this, the serum ^{35}S level in this study decreased by 48-h postinjection to approximately 2% of the level at 6-h postinjection (not shown). Study of proteoglycans in various solubility pools which correspond to different compartments of bone should therefore permit analysis of the metabolism of newly synthesized proteoglycans in developing bone.

Using this approach, we have shown that with increased time after injection, the proportion of total incorporated radioactivity in the guanidine-soluble pool (nonmineralized bone) decreases, with a corresponding increase in the proportion of activity found in the guanidine/EDTA-soluble pool (mineralized bone). This suggests that proteoglycans are being "chased" from the nonmineralized matrix into the mineral phase over this time period. A small proportion of total ^{35}S activity remains in the demineralized bone residue after guanidine/EDTA treatment. This material, however, is largely removed by collagenase digestion and is apparently identical in size and GAG composition with the major proteoglycan of the guanidine/EDTA pool. This result indicates that a fraction of the proteoglycan in the mineralized matrix (or osteoid) is strongly (possibly covalently) associated with collagen. In agreement with this, it has been reported that approximately 10% of total fetal bovine bone uronic acid was resistant to guanidine/EDTA extraction (Fisher et al., 1983). In addition, it has been shown that proteoglycans released from human cortical and metaphyseal bone by hydroxylamine treatment contained hydroxyproline (Campo, 1981). Covalent cross-linking of extracellular matrix components by transglutaminase has been reported (Mosher et al., 1979).

Chromatographic analysis on Sepharose CL-2B of proteoglycans in the guanidine and guanidine/EDTA extracts of calvaria 6, 24, and 48 h after radiolabeling revealed that three species of proteoglycan are synthesized by this tissue. A large CS proteoglycan (G 0.4) is present in the guanidine pool at all time points. The other proteoglycan peak in the guanidine pool, G 0.7, contains two species of proteoglycan, one containing mostly (or entirely) CS and the other containing mostly (or entirely) DS. With increased labeling times, the proportion of DS in this fraction increases, indicating that the CS proteoglycan is selectivity lost from this pool.

Since the G 0.7 proteoglycan is lost from the guanidine pool and the major proteoglycan of the guanidine/EDTA pool is a CS proteoglycan of similar size to the G 0.7 proteoglycan(s), it seems probable that the change in the relative amounts of the two peaks in the guanidine pool represents preferential incorporation of a small CS proteoglycan into the mineralized matrix. An alternative possibility is that the small CS proteoglycan is degraded more rapidly than the other species in the guanidine pool. It was considered impractical in this study to calculate turnover rates of bone proteoglycans using absolute values of ^{35}S incorporation because of the large biological variation observed between animals. In part, this variation may be due to the absence of a large intracellular pool of sulfate to dilute the exogenous radioisotope. It is clear, however, from the decrease in total calvarial ^{35}S over time that there is a rapid turnover of bone proteoglycans at this stage of development. Similarly, in the 17-day-old chick embryo, bone is completely remodeled in 48–72 h (Tanzer & Hunt, 1963). In the absence of information on the relative turnover rates of bone proteoglycan fractions, it is considered probable that the increase in the guanidine/EDTA pool over time represents translocation of the small CS proteoglycan into the mineralized matrix.

The appearance of the E 0.7 proteoglycan in the mineral phase after only 6 h of labeling indicates that there is rapid transfer of this species from the osteoblasts to the mineralized matrix, probably faster than the rate of mineralization front movement. Preferential incorporation of phosphoproteins into mineral has previously been reported (Maier et al., 1983). At 24 and 48 h of labeling, minor peaks of higher molecular weight appear in the guanidine/EDTA extracts. These may represent the G 0.4 proteoglycan present in the nonmineralized matrix, which may become incorporated into the mineral phase as the osteoid calcifies. As the $K_{av} \sim 0.7$ proteoglycan is the only species found in the guanidine/EDTA extract of nonradiolabeled fetal bone (Fisher et al., 1983), the minor proteoglycans of the guanidine/EDTA extract may eventually be degraded.

The data discussed above are in general agreement with those of Fisher et al. (1983). These workers found that a large, guanidine-extractable proteoglycan was located in the mesenchymal tissue between nascent bone trabeculae and a smaller guanidine/EDTA-extractable proteoglycan was located within the trabeculae. In the present study, it was not possible to determine whether all the progeoglycan species described are the products of osteoblasts. In this context, it may be relevant that we have shown previously that clonal bone cell cultures synthesize two proteoglycan species ($K_{av} \sim 0.7$–0.8 on Sepharose CL-2B) which have GAG chains of M_r 25 000 and differ in CS/DS ratio (Hunter et al., 1983). Although proteoglycan expression may be altered in culture, these two proteoglycan species may be analogous to the two small proteoglycans described above. Thus, the small CS and small DS proteoglycans would be the products of osteoblasts, while the large CS proteoglycan, on the evidence of Fisher et al. (1983), is likely to be the product of mesenchymal cells present in fetal bone. The calvarial bones used in the present study contain small amounts of fibrocartilage associated with the coronal and sagittal sutures. Since no cartilage-type proteoglycan was detected, nor any keratan sulfate found in any proteoglycan

fraction, contamination by cartilage matrix molecules was probably insignificant.

The results of the present study may be summarized as a model for proteoglycan synthesis and deposition in fetal mammalian bone. Three species of proteoglycan are synthesized: a large CS and a small DS proteoglycan, which are located in the nonmineralized matrix, and a small CS proteoglycan, which is initially present in the nonmineralized matrix but subsequently becomes incorporated into the mineralized matrix. Some of the small CS proteoglycan undergoes a strong interaction with collagen which renders it resistant to extraction with denaturing solvents and demineralizing agents.

Acknowledgments

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Two-Stage Maturation Process for Newly Replicated Chromatin†

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ABSTRACT: HTC cells have been labeled by short exposures to [^{3}H]thymidine in order to identify newly synthesized DNA. By either isolating nuclei directly or isolating them after an extensive fixation with formaldehyde, we have been able to identify two phases in the maturation process of newly replicated chromatin. The first phase which is relatively brief (<5 min) is reflected in a diffuse, irregular organization of nucleosomes on new DNA immediately postreplicatively. The second phase which lasts from 5 to 30 min postreplication is characterized by a normal repeat length for the nucleosomes which are nonetheless more weakly bound than bulk nucleosomes. This is reflected in increased sliding during nuclease digestion as well as increased nuclease sensitivity and the presence of easily dissociated histones which has been described by other workers.

Studies investigating the structure of eucaryotic chromatin in the vicinity of the replication fork have shown that the nature of nucleosomes associated with this region of DNA differs from that seen for nucleosomes associated with nonreplicating DNA. Within 5–25 min of DNA synthesis, these immature nucleosomes undergo a rearrangement in which they attain the structural properties of bulk or mature chromatin (DePamphilis & Wassarman, 1980).

Some of the structural characteristics associated with immature chromatin include a lower buoyant density, an increased sensitivity to digestion by nucleases, and a decreased affinity between new H3 and H_4 and new DNA (Jackson & Chalkley, 1981; Seale, 1975, 1976, 1978; Walters & Hildebrand, 1976; Klempnauer et al., 1980; Levy & Jakob, 1978; Jackson et al., 1981). In addition, several investigators have reported that the nucleosomal repeat length for nascent chromatin is 15–50 base pairs (bp) shorter than that calculated for bulk chromatin (Murphy et al., 1978, 1980; Levy & Jakob, 1978; Annunziato et al., 1982). The difference in repeat lengths is thought to be due to a shorter linker region, but there has been no adequate explanation for the mechanism by which immature nucleosomes can rearrange to acquire the longer nucleosomal repeat length of bulk chromatin. Data obtained in this laboratory indicate that the shorter nucleosomal repeat may be generated during the micrococcal nuclease digestion process itself, perhaps as a consequence of the decreased affinity between newly replicated DNA and new H3 and H4 (Jackson et al., 1981).

The decreased affinity of new H3 and H4 for newly rep-

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