Kinetics of Proteoglycans and Cells in Growth Plate of Normal, Diabetic, and Malnourished Rats

I. AXELSSON, J. C. PITA, D. S. HOWELL, R. LORENTZON, I. BERMAN, AND L. BOQUIST

University of Miami School of Medicine, Miami, Florida [I.A., J.C.P., D.S.H., R.L., I.B.]; and University of Umeå, Umeå, Sweden [R.L., L.B.]

ABSTRACT. The metabolism of proteoglycans in normal growth plate and the changes in growth plate morphology induced by diabetes and malnutrition were studied in rats. The proteoglycans had a significantly faster turnover (half-life measured with [35 S]sulfate labeling: 25–30 h) than the cells in the growth plate. Morphometric studies showed significant reductions of cell number, zone height, and [3 H] thymidine incorporation in growth plates from rats with untreated streptozotocin-induced diabetes compared to normal rats. Similar, although less pronounced alterations were observed in malnourished, nondiabetic rats. Disaggregation and degradation of proteoglycans are probably necessary prerequisites for calcification. Our data indicate that the proteoglycans are in a dynamic state of rapid biosynthesis and degradation throughout the growth plate with a shift in the balance at the calcification front toward less synthesis and more degradation. (*Pediatr Res* 27: 41–44, 1990)

Longitudinal growth of tubular bones takes place by cell divisions and synthesis of extracellular matrix by the chondrocytes of the growth plate. This process is regulated by several hormones, including growth hormone, insulin, and somatomedins (1–7). The stimulation of growth by insulin (4–7) is clinically demonstrated by the retarded growth that may occur in insulinopenic diabetic children (8).

Bone formation is preceded by provisional calcification of the cartilage matrix. Most investigators have found that the concentration of proteoglycans decreases during calcification, which may suggest that calcification is inhibited by proteoglycans (9–12). The transformation of the large, dense proteoglycan aggregates in the proliferative and upper hypertrophic zone to the relatively small, star-like proteoglycan aggregates in preparations from rabbit growth plate (13) has been suggested to allow the movement of calcium and phosphate ions and the growth of mineral clusters (14). Shepard and Mitchell (15) did not find any decrease in proteoglycan concentration during calcification but described that mineralization starts with star-shaped proteoglycan aggregates as templates.

We have described profound changes of proteoglycan metabolism in the growth plate of diabetic rats, namely a complete absence of large proteoglycan aggregates and a sharp reduction of the synthesis of sulfated glycosaminoglycans, the chains of

which were shorter than in normal rats (5). Insulin-treated diabetic rats and malnourished nondiabetic rats showed changes in proteoglycan metabolism that were intermediate between normal and diabetic rats.

Our study was designed to investigate the turnover of growth plate proteoglycans in normal rats and the morphologic changes of growth plate in diabetes and malnutrition. A preliminary report has been published (16).

MATERIALS AND METHODS

Studies of [35 S]-labeled proteoglycans. Twenty 25-d-old male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were injected with carrier-free $\text{Na}_2[^{35}\text{S}]\text{SO}_4$ (3 $\mu\text{Ci/g}$ body wt; 3 μCi = 111 kBq) into the tail vein and killed 2, 24, 48, 72, or 96 h later by intracardial injection of sodium pentobarbital. The growth plate and adjacent calcified metaphyseal tissue were dissected from the proximal end of each tibia and pieces were used for autoradiography or extraction of proteoglycans.

Autoradiography was performed as described by Campo and Dziewiatkowski (17). Extraction and characterization of proteoglycans were performed in the following way: Pieces of cartilage were immediately after dissection placed in ice-cold 4 M guanidine-HCl in 0.25 M sodium acetate, pH 5.8 (about 1 mL/20 mg wet tissue) containing protease inhibitors (5). The suspension was dialyzed against 20 vol of the extraction solution with magnetic stirring at -12°C for 24 h and then centrifuged at 8°C in a Beckman SW 50.1 rotor at 15 000 rpm for 10 min. The insoluble residue was digested with 1 mL 88% formic acid at 90°C for 15 min.

A portion of the supernatant, which contained dissociatively extracted proteoglycans, was applied to a dissociative Sepharose CL-2B column (elution buffer = extraction buffer), or brought to associative conditions by dialysis at 4°C for 6–8 h against 10 vol of associative solution (extraction solution minus guanidine-HCl) and then immediately applied to an associative Sepharose CL-2B column (elution buffer = 0.5 M guanidine-HCl-0.05 M sodium acetate (pH 5.8) with protease inhibitors) (5). The partition coefficient k_d and the ratio of elution volume (V_1) to total included volume (V_i) were computed for various elution volumes. k_d is defined by the equation

$$k_d = (V_e - V_o)/(V_i - V_o); V_o = \text{void vol}$$

Another portion of the dialyzed extract was kept at -20°C overnight and subjected to transport centrifugation (18) the next day. Portions from the digested extraction residues and the clarified extracts, and the fractions from gel chromatography and ultracentrifugation, were diluted with Aquasol and the radioactivity was measured by liquid scintillation spectrometry (5).

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Correspondence I. Axelsson, M.D., Department of Pediatrics, County Hospital, S-831 83 Östersund, Sweden.

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Extraction yield of proteoglycans was computed from the radioactivity measurements on extract and residue.

Quantitative morphologic studies. Thirty male Sprague-Dawley rats were 5 wk old when the experiments started (day 0); they were killed 8 d later (d 8). Diabetes was induced in 15 rats on d 0 by a single intravenous dose of streptozotocin (Sigma Chemical Co., St. Louis, MO) (19, 20) as described previously (5). Ten rats served as controls. They were fasted, anesthetized, and injected with the buffer without streptozotocin.

Five diabetic rats were treated with daily subcutaneous injections of NPH-insulin (protamine insulin; 1 IU/100 g body wt) (5). Malnutrition was induced in another five rats by feeding them water *ad libitum* but a reduced number of pellets (Purina Laboratory Chow, Purina, St. Louis, MO) during d 0–8. The number of pellets was adjusted so that these rats showed the same weight development as the diabetic rats, *i.e.* a constant or slightly decreasing weight. All other rats were fed pellets and water *ad libitum*.

On d 8, all animals were killed by intracardial injection of sodium pentobarbital. The proximal growth plate of each tibia was cut out in its full thickness with a small rim of bone on the epiphyseal and metaphyseal sides. The tissue was immediately fixed at room temperature by immersion into 2.5% glutaraldehyde in Sørensen's 0.1 M sodium phosphate buffer at pH 7.4 for 3 h and then rinsed thoroughly in the buffer. The specimens were stored in the buffer overnight at 4°C, and postfixed the following day in 1% OsO₄ in 0.1 M sodium phosphate, pH 7.4 for 2 h. The tissue blocks were dehydrated through graded concentrations of alcohol and propylene oxide and embedded in Epon 812.

Each growth plate was cut into several semithin (1 µm) sagittal sections for light microscopy. The sections exactly perpendicular to the growth plate were stained with toluidine blue. Micrographs were taken at a primary magnification of 40× and printed at a final magnification of 250×.

In each growth plate, three to five cell columns with the plane of section parallel to the columns were selected for measurement of zone width and cell number. The width of the proliferative zone was measured from the first chondrocyte of the cell column to the level where the cells began to enlarge in size and the width of the hypertrophic zone from the level of enlarging cells to the last hypertrophic chondrocyte at the zone of provisional calcification.

On d 8, five diabetic and five control rats were injected intraperitoneally with 1 µCi/g body wt (1 µCi = 37 kBq) of tritiated thymidine (5 Ci/mmol = 185 GBq/mmol; Amersham International, Bucks., U.K.). These animals were killed 1 h after injection and the growth cartilage (whole width) of the proximal tibia was rapidly dissected out. Incorporation of [³H]thymidine in growth plate chondrocytes and DNA concentrations were determined as described by Weiss and Reddi (21). For comparison of data, Student's *t* test for unpaired observations was used (22). Data are expressed as mean ± SD.

RESULTS

Proteoglycan turnover in normal rats. The [³⁵S]-sulfate concentration in the growth plate reached its peak value within 24 h after sulfate injection (Fig. 1). The elimination of sulfate followed first-order kinetics with a *t*_{1/2} of 25–30 h.

Agarose gel chromatography and transport ultracentrifugation of dissociatively extracted proteoglycans (extraction yield 86–94% of total tissue [³⁵S]sulfate) did not reveal any differences in molecular size of monomers at various times after [³⁵S]sulfate injection (Fig. 2, *broken line*; ultracentrifugal data not shown), but the proportion of labeled proteoglycans with low *k*_d values and high apparent sedimentation coefficients in the dialyzed extracts tended to decrease with time (Fig. 3), suggesting a decreasing ratio of proteoglycan aggregates to monomers. In

Fig. 1. ³⁵S radioactivity in growth plates of rats (*n* = 20) at various time intervals after ³⁵S-sulfate injection. The vertical scale is logarithmic.

Fig. 2. Chromatography of proteoglycan extracts on associative (*solid line*) and dissociative (*broken line*) Sepharose CL-2B columns. The time from injection of ³⁵S-sulfate to death of the rat is shown. The *hatched areas* show the material eluted in the void volume (*k*_d < 0.1) of the associative column.

repeated experiments, similar elution and centrifugation patterns were consistently observed.

Cell kinetics in normal, diabetic, and malnourished rats. The growth plates of the various experimental groups did not differ from the control group as to general microscopic architecture, *i.e.* all showed chondrocytes arranged in cell columns that could be divided into resting, proliferative, and hypertrophic zones. In some growth plates of all groups proliferative cell columns occasionally showed focal discontinuity. The resting zone was very narrow in all groups.

The zone width and cell numbers of the proliferative and hypertrophic zones in the various experimental groups are shown in Figure 4, and significant deviations from the control groups are indicated. There was also one significant difference within the experimental groups: Diabetic rats with no insulin treatment had significantly fewer proliferative cells than insulin-treated diabetic rats (*p* < 0.05).

The incorporation of [³H]thymidine in control and diabetic growth plates was 996 ± 45 (*n* = 5) and 727 ± 201 (*n* = 5) cpm/µg DNA, respectively (*p* < 0.01). An even distribution of [³⁵S]

sulfate throughout the growth plate was demonstrated by autoradiography (autoradiograms not shown).

DISCUSSION

Normal growth plate. Boström (23), Bélanger (24), and Campo and Dziewiatkowski (17, 25) pioneered studies of proteoglycan metabolism using labeling with [35 S]sulfate. The present data on the kinetics of rat growth plate proteoglycans (Fig. 1) may be compared with previous data on rat costal cartilage proteoglycans (23). In both cases, the half peak value and peak value for sulfate concentration in cartilage were reached after 2 and 24 h, respectively, although we do not know the sulfate incorporation *e.g.* 12 h after injection. The elimination of sulfate was, however, about 14 times faster in growth plate than in costal cartilage. Approximately 95% of the sulfate in cartilage is linked to proteoglycans (5, 25). We conclude that the proteoglycans in growth plate are in a dynamic state of rapid turnover with a $t_{1/2}$ of about 25–30 h. Proteoglycans in the costochondral junctions of immature rabbits also have a $t_{1/2}$ of 1–2 d (12).

Walker and Kember (26) described the cell kinetics in growth plates of proximal tibia from rats of different ages. We have compared their data with ours and conclude that the cells in the upper part of the proliferative zone will reach the zone of provisional calcification after 3–4 d.

Fig. 3. Proportions of aggregates among [35 S]labeled proteoglycans at various time intervals after [35 S]sulfate injection, measured as percentage of [35 S]sulfate migrating 1) with $k_d < 0.1$ in associative Sepharose CL-2B chromatography (corresponding to hatched area in Fig. 2) (closed circles), and 2) with apparent sedimentation coefficient above 40 S at transport centrifugation (squares).

Fig. 4. Morphometry of growth plates: width of (A) proliferative and (B) hypertrophic zones; number of cells per cell column in (C) proliferative and (D) hypertrophic zones. Co, control group; Di, diabetic rats with no insulin treatment; IDi, insulin-treated diabetic rats; Ma, malnourished rats. Mean values $\pm$ SD (vertical bars) are shown; each mean value is based on 12 or 15 observations. Statistical significance of the difference between each experimental group and the control group: n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. E, correlation between total width and total number of cells per cell column for proliferative zone + hypertrophic zone in the four groups of rats. The regression line (best least squares) and r are shown.

The proteoglycan turnover in normal growth plate thus seems to be significantly faster than the cell turnover. Any segment of the growth plate hence metabolizes its proteoglycans several times before it is reached by the calcification front. This conclusion is strengthened by the even distribution of [35 S]sulfate and thus proteoglycan biosynthesis throughout the growth plate in previous (24) and present investigations and by the relatively small differences in gel chromatographic and ultracentrifugal profiles at various time intervals after sulfate injection (Figs. 2 and 3). The straight line in Figure 1 suggests that there is only one major pool of proteoglycans in the growth plate.

Changes in the concentration, tissue distribution, and aggregation of proteoglycans are probably necessary prerequisites for calcification (9–12, 14, 15). Our data indicate that the breakdown of growth plate proteoglycans is not exclusively localized to the zone of calcification. We believe that the proteoglycans are in a dynamic state of rapid biosynthesis and degradation throughout the growth plate, and that the decrease in proteoglycan concentration at the calcification front is the result of a shift in balance toward less synthesis and more degradation.

Diabetes and malnutrition. A severe disturbance of the proliferative ability of the chondrocytes in diabetic rats is suggested by the decreased uptake of [3 H]thymidine, the decreased number of cells in the proliferative zone, and the reduction of zone width. Insulin-treated diabetic rats exhibited morphometric parameters qualitatively similar to those of untreated diabetic rats but the changes were less severe.

The appearance and arrangement of the cells within the different zones did not differ between the control group and the experimental groups. This is demonstrated by the good correlation between cell number per cell column and width of the growth plate (Fig. 4E).

Only small changes of the size of the proliferative zone have previously been observed after interventions that disturb bone growth, such as hypophysectomy, or administration of growth hormone or cortisol (27). Weiss *et al.* (28) suggest that streptozotocin-induced diabetes of 14 d duration is associated with only minor morphologic alterations. It is, however, evident from their illustrations that the width of the proximal growth plate of tibia is reduced by approximately 40% and both the proliferative and hypertrophic zones are thinned. Marked thinning of the proximal growth plate of rat tibia and focal discontinuity of the cell columns were observed in long-term (7 wk) streptozotocin-induced diabetes by Hough *et al.* (29). Such discontinuity was sometimes observed in the proliferative zone of both diabetic and control rats in the present study. The biologic significance of this finding, if any, is not clear. It may well be an artifact in tissue sections not perfectly parallel to the cell columns.

Insulin treatment for 3–5 wk completely normalized the growth plate in diabetic rats in the study by Hough *et al.* (29). This was not the case in our study. This difference may be explained by different ages of the animals, different doses of insulin, and different duration of treatment.

The decrease of chondrocyte proliferation reported here and the decrease of proteoglycan biosynthesis reported previously (5) are probably caused by a reduction of the activity of somatomedins. Decreased intracellular concentration of the precursor sugar nucleotides involved in glycosaminoglycan biosynthesis may also play a role for the decrease of proteoglycan biosynthesis (30). Reduction of somatomedin activity is found in experimental diabetes and may be caused by the occurrence of somatomedin inhibitors (31) or lack of stimulation of somatomedin synthesis (7). Decreased stimulation of cartilage by somatomedins due to imbalance between somatomedins and inhibitors of somatomedins is probably a common feature of protein-energy malnutrition and diabetes in children and animals (32). Malnutrition is also associated with low levels of insulin (33).

Despite a similar weight development in our diabetic and malnourished rats, all morphologic changes were significantly more pronounced in diabetic rats in our investigation as was the

decrease in proteoglycan synthesis in our previous investigation (5). It may be concluded from these quantitative differences that the two conditions affect body weight and cartilage metabolism by a different mechanism.

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