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PROTEOGLYCANS SYNTHESIZED IN CALLUSES AT VARIOUS STAGES OF FRACTURE HEALING IN RATS

TAKESHI YOKOBORI [a], ATSUHIKO OOHIRA [b,*] and HIROSHI NOGAMI [b]

[a] *Tokyo Research Institute, Seikagaku Kogyo Co., Ltd., Tateno, Higashiyamato, Tokyo 189 and* [b] *Department of Embryology, Institute for Developmental Research, Kasugai, Aichi 480-03 (Japan)*

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Summary

The sequential cartilage and bone formation was observed in fracture calluses which were formed at fibula diaphysis of Sprague-Dawley rats. The proteoglycans synthesized in the calluses at different stages during fracture healing were labeled in vitro with [^{35}S]sulfate and then analyzed by sucrose density gradient centrifugation. A heavy proteoglycan, in which chondroitin 4-sulfate accounted for approx. 90% of total radioactivity, was predominantly synthesized in addition to light, dermatan sulfate-containing proteoglycans in day-10 and day-20 calluses, when cartilaginous areas were predominant in these calluses. The synthesis of the heavy proteoglycan started around day 7, when chondrogenesis started locally in the callus, and ceased by day 30, when cartilage had been replaced by newly formed bone. The heavy proteoglycan had chemical and physical properties similar to those of the major proteoglycan synthesized by bone matrix-induced cartilages, but different from those of the major one synthesized by the epiphyseal cartilage of neonatal rats. These findings suggest that the sequential molecular transitions observed in fracture healing differ from those in the endochondral ossification of embryonic skeletal tissues but resemble those in bone matrix-induced bone formation.

Introduction

The sequential cellular transitions observed in fracture healing [1] are similar to those in the endochondral bone formation of embryos and in bone matrix-

* To whom correspondence should be addressed.

induced bone formation. Many investigators have demonstrated the changes in both collagen and proteoglycan types during the endochondral bone formation of embryos [2—6]. The sequential molecular transitions in bone matrix-induced endochondral ossification differ somewhat from those in embyonic bone formation [7,8]. It is of interest to compare the pattern of the transitions in fracture healing with those observed in other types of endochondral ossification.

This paper shows the changes in proteoglycan types during the endochondral bone formation in the fracture callus of the rat fibula, based on sucrose density gradient separation.

Materials and Methods

Materials. The following commercial materials were used: carrier-free $H_2{}^{35}SO_4$ from Japn Radioisotope Association, Tokyo; chondroitinase-ABC and chondroitinase-AC II from Seikagaku Kogyo Co., Tokyo; Pronase-P from Kaken Kagaku Co., Tokyo; Sepharose CL-2B and Sepharose CL-6B from Pharmacia, Uppsala; tissue culture medium 199 and newborn calf serum from Nakarai Chemicals, Ltd., Kyoto.

Animal experiments. Male Sprague-Dawley rats (100 days old, weighing 377 ± 35.8 g) were anesthetized with inhaled ethyl ether. 1 mm of the fibula diaphysis in both legs was excised by a dental technician's micromotor drill under sterile conditions. Neither external nor internal immobilization was applied. In this way, a standard 'fracture' was provided in all animals, the tibia bone satisfactory immobilizing the fractured fibula bone. A few days after the operations, the rats were using their legs freely.

The animals thus operated upon were randomly divided into four groups of five animals each and used, providing calluses from 7-, 10-, 20- and 30-day-old fractures. Immediately after the animals had been killed on the designated day, all hind legs were dissected. Callus tissue at the site of the fracture was removed and put into ice-cold 0.9% NaCl. No fracture showed signs of infection. Callus tissues from eight fractures were cut into two pieces and replaced in 2 ml of 199 medium containing 15% newborn calf serum and 3.7 MBq of $Na_2{}^{35}SO_4$. The labeling incubation was carried out for 3 h at 37° C with gentle shaking. Under these conditions the incorporation of label was linear with time up to a 6 h exposure of label. Callus tissues from two fractures were used for the histological and roentgenographic examinations.

Preparation of proteoglycan samples. The labeled tissues were immediately placed in 2 ml of ice-cold 4 M guanidine-HCl/0.05 M Tris-HCl, pH 7.5/0.1 M 6-aminohexanoic acid/0.005 M benzamidine HCl/0.01 M EDTA in order to prevent proteolysis during extraction procedure [9]. The suspension was stirred at 4° C overnight, and the residue was removed by centrifugation at 20 000 × g for 30 min. The supernatant was used as a starting material for further analysis of proteoglycans.

The extracted residue was washed exhaustively with ice-cold, 10 mM Na_2SO_4, and then digested with pronase in the presence of EDTA [8]. The residual polysaccharides in the digest were precipitated with 95% ethanol/1.3% potassium acetate, and the precipitate was assayed for radioactivity and for

hexuronate by the method of Bitter and Muir [10].

Analysis of proteoglycans. Separation of proteoglycans with sucrose density gradient centrifugation was performed under dissociative conditions by the method of Kimata et al. [11], with slight modification as described previously [8].

Each proteoglycan fraction thus separated was pooled. The pooled fraction was diluted with 2 vols. of cold water, and proteoglycan was precipitated with 6 vols. of 95% ethanol/1.3% potassium acetate. The polysaccharide moiety of the proteoglycans was isolated after treatment with 0.3 M NaOH overnight at room temperature. The solution was neutralized by making to 5% (w/v) with trichloroacetic acid and after centrifugation the polysaccharides were precipitated with ethanol [12]. An aliquot (approx. 10 000 cpm of ^{35}S) was digested with chondroitinase-ABC or with chondroitinase-AC to determine the relative amounts of isomeric condroitin sulfates by the method of Saito et al. [13]. Another aliquot (about 30 000 cpm) was dissolved in 1 ml of 2 M guanidine-HCl/0.02 M Tris-HCl, pH 7.5, and subjected to gel filtration on a Sepharose CL-6B column (1.5 × 50 cm) in the buffer at room temperature. The column was eluted at a flow rate of 7 ml/h. About 2-ml fractions were collected, and analyzed for radioactivity.

Results

Light microscopy

On the seventh day after the operation, proliferation of large number of mesenchymal cells was observed around the affected site of the fibula diaphysis. Hyaline cartilage had already developed, but very partially. By day 10, the resected portion of the diaphysis had been occupied by the cartilage and resorption of the cartilage and replacement with newly formed bone had begun locally. On day 20, both cartilaginous and osseous areas predominated. By day 30, cartilaginous area had been replaced completely by osseous area. Osseous union was also confirmed at this stage of the fracture healing by the roentgenographic examination (Softex type-C-SM, Tokyo).

Incorporation of [^{35}S]sulfate

The amounts of ^{35}S-labeled materials in the extracts and in the residues are shown in Table I. Total radioactivities incorporated in day-10 and day-20 fracture calluses were more than four times as much as that in day-7 and day-30 fracture calluses. These results are consistent with the morphological observation, indicating that cartilaginous area is predominant in day-10 and day-20 calluses.

Approx. 60% of total labeled materials could be extracted in all cases with 4 M guanidine-HCl. The extracted yields of the unlabeled proteoglycans (hexuronate) nearly coincided with those of the ^{35}S-labeled. The residual polysaccharides solubilized by digestion of the residues with pronase were treated with chondroitinases. The relative amounts of isomeric chondroitin [^{35}S]sulfates were nearly equal to those of the polysaccharides in the extract of the corresponding fracture callus tissue (see below). The reason why the residual polysaccharides could not be brought into solution by the treatment

TABLE I

^{35}S-RADIOACTIVITY INCORPORATED INTO CALLUSES AT VARIOUS STAGES OF FRACTURE HEALING, AND EXTRACTION OF THE LABELED MATERIALS WITH 4 M GUANIDINE-HCl SOLUTION

Values expressed in cpm/fracture: in parentheses as the percentage of total radioactivity.

Callus	Day 7	Day 10	Day 20	Day 30
Total radioactivity	4164 (100)	22 633 (100)	24 364 (100)	4203 (100)
Extract	2223 (53)	15 085 (67)	14 965 (61)	2314 (55)
Residue	1941 (47)	7 548 (33)	9 399 (39)	1889 (45)

with 4 M guanidine-HCl remains unknown.

At most 10% of total labeling materials were released from the tissues to the medium during the labeling incubation.

Proteoglycans synthesized by fracture calluses

The labeled proteoglycans extracted with 4 M guanidine-HCl were separated by ultracentrifugation in a sucrose density gradient (Fig. 1). In all cases, the labeled materials were separated into two components; a heavy component and a light component. On day 7, small amounts of the labeled materials were yielded in the heavy fraction. The synthetic rate of the heavy component increased about 10 times between day 7 and day 10. By day 30, synthesis of the heavy component had almost ceased. The sedimentation velocity of the heavy component remained unchanged at all stages of the fracture healing.

The relative amounts of chondroitinase-digestion products of the ^{35}S-labeled polysaccharides of these components were shown in Table II. The major product formed by the digestion of the polysaccharides of the heavy components was the A-unit (approx. 90% of total radioactivity), which is 3-*O*-Δ^4-glucuronosyl-*N*-acetylgalactosamine-4-sulfate derived from condroitin 4-sulfate. Negligible small amounts (less than 1%) of B-unit, namely 3-*O*-Δ^4-glucuronosyl-*N*-acetylgalactosamine-4-sulfate derived from dermatan sulfate, were formed. When the polysaccharides of the light components were digested with the enzymes, substantial amounts of B-units were formed in addition to A-units. It has been proved that a light fraction of cartilage proteoglycan preparations contains some kinds of proteoglycans one of which has a dermatan sulfate-chondroitin sulfate copolymer chain [4]. Most of the B-units detected in the present work may also originate from the copolymer chains, although no further study has been performed on the light components due to the small amount available.

To estimate length of the polysaccharide side-chain of the heavy proteochondroitin sulfate (synthesized by 10-day-old callus), the polysaccharide obtained by alkaline β-elimination was chromatographed on a Sepharose CL-6B column (Fig. 2). K_{av} value of the labeled polysaccharide was 0.55 ± 0.02. The length of the polysaccharide of the corresponding molecule synthesized by 20-day-old callus was the same as that synthesized by 10-day-old callus.

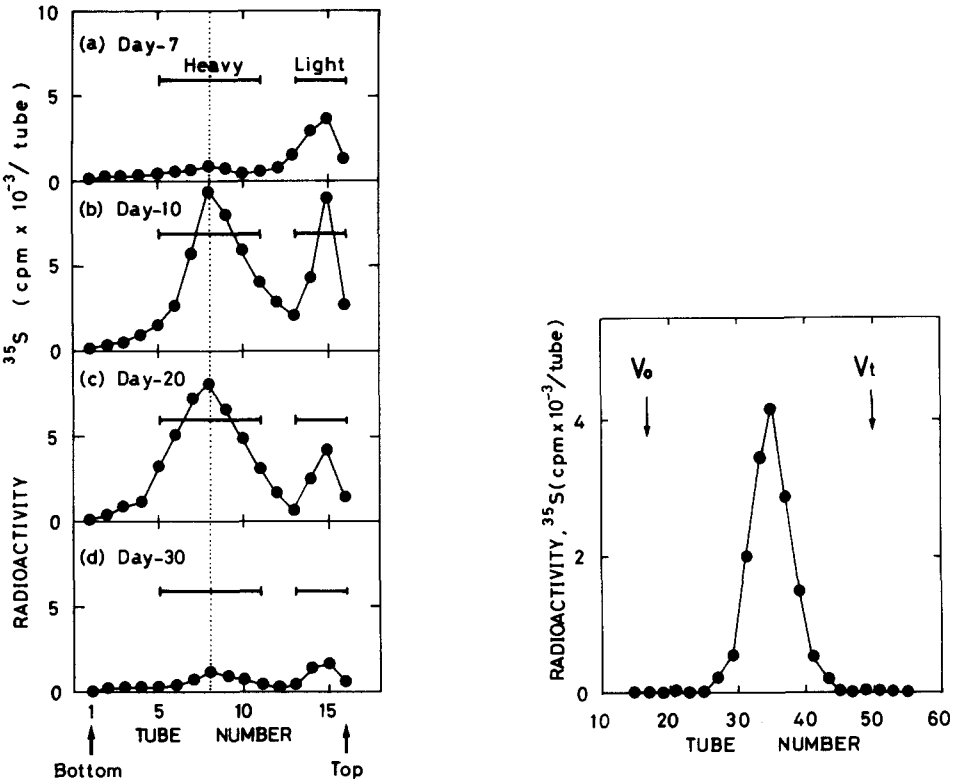

Fig. 1. Sucrose density gradient centrifugation of ^{35}S-labeled proteoglycans isolated from day-7 (a), day-10 (b), day-20 (c), and day-30 fracture calluses (d). A 1 ml portion of the extract was layered on a 14.5 ml linear sucrose gradient (5—20% in the protease inhibitor-containing 4 M guanidine-HCl buffer) in a cellulose nitrate tube, at the bottom of which 0.5 ml of sucrose/guanidine-HCl solution had been placed. The tube was centrifuged in a RPS-27 rotor in a Hitachi 65P ultracentrifuge at 25 000 rev./min for 30 h at 4°C. The tube was then punctured at the bottom, and 1-ml fractions were collected and assayed for radioactivity. Solid bars indicate fractions of a heavy component and a light component which were pooled separately for further analyses. The dotted line indicates Tube 8.

Fig. 2. Gel chromatography on Sepharose CL-6B of the ^{35}S-labeled polysaccharide chain of the heavy component obtained from day-10 callus. V_0, void volume. V_t, total volume.

Proteoglycans from other sources in rats

It has been shown that there are some kinds of proteochondroitin sulfate which differ in physical and chemical properties from each other; synthesized by various hyaline cartilages [4,5,8,11], by mesenchymal cells just prior to chondrogenesis [4,5], and by bone matrix-induced cartilages [8]. It is of interest to compare the properties of the heavy component synthesized by the cartilaginous fracture callus with those of these proteochondroitin sulfates.

Some properties of epiphyseal cartilage proteochondroitin sulfate from neonatal SD rats and that synthesized by bone matrix-induced cartilage formed in adult SD rat muscles are shown in Table III, together with those of this heavy component. The parameters of this heavy component shown in Table III differ from those of the component from epiphyseal cartilage, but coincide

TABLE II

CHONDROITINASE DIGESTION PRODUCTS OF ^{35}S-LABELED POLYSACCHARIDES IN PROTEOGLYCAN FRACTIONS ISOLATED FROM CALLUSES AT VARIOUS STAGES OF FRACTURE HEALING

The values are expressed as the percentage of total ^{35}S-radioactivity. The nomenclature used are: Resistant, part which is resistant against digestion with chrondroitinase-ABC; A-unit, 3-*O*-Δ^4-glucuronosyl-*N*-acetylgalactosamine-4-sulfate produced by digestion with only chrondroitinase-AC; B-unit, 3-*O*-Δ^4-glucuronosyl-*N*-acetylgalactosamine-4-sulfate produced by digestion with only chondroitinase-AC; B-unit, 3-*O*-Δ^4-glucuronosyl-*N*-acetylgalactosamine-4-sulfate which can not be produced by digestion with chondroitinase-AC, but can be produced with chondroitinase-ABC; C-unit, 3-*O*-Δ^4-glucuronosyl-*N*-acetylgalactosamine-6-sulfate produced by digestion with chondroitinase-ABC. Others are composed mainly of oversulfated, unsaturated disaccharides and *N*-acetylgalactosamine 4-sulfate.

Fraction *	Day 7 Light	Day 10		Day 20		Day 30 Light
		Heavy	Light	Heavy	Light	
Product						
Resistant	6	2	12	2	7	2
A-unit	45	88	57	91	64	77
B-unit	23	<1	22	<1	22	6
C-unit	19	5	3	4	2	10
Others	7	5	6	3	5	5

* See Fig. 1.

closely with those of that from bone-matrix induced cartilage. The relative amounts of isomeric condroitin [^{35}S]sulfates were nearly equal in all the cases. These results indicate that the heavy proteochondroitin sulfate synthesized by the cartilaginous fracture callus can not be distinguished from the bone-matrix induced catilage component but was smaller in both overall size and polysaccharide side chain size than that from epiphyseal cartilage.

Discussion

Antonopoulus et al. [15] have isolated and partly analyzed the glycosaminoglycans from rabbit-fracture callus of various ages. As we presently know that

TABLE III

SOME PROPERTIES OF VARIOUS PROTEOCHONDROITIN [^{35}S]SULFATES

PCS-H, proteochondriotin sulfate synthesized by epiphyseal cartilages of neonatal rats; PCS-BMG, by bone matrix-induced cartilages.

Proteochondroitin sulfate	Sedimentation velocity [a] (No. tube)	Overall size [b] (K_{av} on 2B)	Length of polysaccharide [c] (K_{av} on 6B)
Heavy component from day-10 callus	8	0.28 ± 0.03	0.55 ± 0.02
Rat PCS-H [d]	5	0.12 ± 0.04	0.46 ± 0.02
PCS-BMG [d]	8	0.29 ± 0.03	0.54 ± 0.02

[a] See Fig. 1.
[b] For detailed experimental procedures, see Ref. 8. The values represent the average of three determinations, with the standard deviation.
[c] See Fig. 2. The values represent the average of three determinations, with the standard deviation.
[d] Data taken from Ref. 8.

most of glycosaminoglycans occur in tissues in the form of protein-bound glycosaminoglycans so-called proteoglycans, it is of interest to analyze the proteoglycans isolated from fracture callus. This paper describes the results of isolation and partial characterization of proteoglycans synthesized by the fracture callus of rats at various stages of the fracture healing.

The process of appearance and disappearance of the heavy proteoglycan component in the callus, the polysaccharide moiety of which consists mainly of chondroitin 4-sulfate, bears a close resemblance to the morphological process of differentiation and resorption of hyaline cartilage in the callus. These data suggest that the proteochondroitin sulfate is synthesized by the cells in cartilaginous areas of the fracture callus. The proteochondroitin sulfate was smaller both in overall size and in chain size of its chondroitin sulfate than the major proteochondroitin sulfate synthesized by epiphyseal cartilages of neonatal rats, but had chemical and physical properties similar to those of proteochondroitin sulfate synthesized by bone matrix-induced cartilages formed in adult rat muscle (Table III). Since the size of cartilage proteoglycans decreases with increasing of age [16,17], the differences in properties between the proteochondroitin sulfate of cartilaginous fracture callus and from hyaline cartilages may be attributed to the age-related changes. Recent experiments [18], however, showed that various cartilages of rats with different ages, namely costal, epiphyseal, articular and bone matrix-induced cartilages, contain proteochondroitin sulfates with properties different one from another, even though these cartilages are excised from rats of the same age. Furthermore, the proteochondroitin sulfate of bone-matrix induced cartilages has been proved not to be subjected to the age-related changes. These results, together with the findings presented in this paper, suggest that the molecular and cellular transitions in fracture healing are similar, if not identical, to those observed in the sequential endochondral bone formation induced by implantation of bone matrix.

Embryonic mesenchymal cells, just prior to differentiation to chondroblasts, synthesize a peculiar proteochondroitin sulfate which can be easily separated from other types of proteochondroitin sulfate by the sucrose density gradient centrifugation [4,5]. Day-7 fracture callus, when mesenchymal cells were predominant in it, did not synthesize this mesenchymal proteochondroitin sulfate but synthesized a light proteoglycan with dermatan sulfate-containing polysaccharide (Fig. 1a and Table II). It was also proved that mesenchymal cells which appeared prior to chondrogenesis induced by bone matrix synthesized a light, dermatan sulfate-containing proteoglycan [8]. These findings also support the idea mentioned above.

Fracture callus is a highly heterogeneous tissue which consists of fibrous, cartilaginous and osseous tissues, and bone marrow cells. Additionally it is hard for most of biochemists to obtain large amounts of callus tissue under the clinically well-controlled conditions because of the difficulty of the operations to provide the fracture. On the contrary, the experimental system of bone matrix-induced endochondral bone formation circumvents most of these complications and difficulties [7,8,19]. Therefore, bone matrix-induced bone formation may be concluded to be a useful experimental model for biochemical analysis of the sequential process of fracture healing.

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