Isolation, Characterization, and Localization of Glycosaminoglycans in Rabbit Bone Marrow

By Kayoko Oguri, Eiko Okayama, Bruce Caterson, and Minoru Okayama

The glycosaminoglycans that exist in rabbit bone marrow were analyzed chemically, and their in situ localization was studied immunohistochemically. Femoral bone marrow of 3-month-old rabbits was defatted with organic solvents. Glycosaminoglycans were prepared from the defatted tissue after its digestion with pronase, treatment with mild alkali, and then digestion with DNase-I. The tissue contained glycosaminoglycans equivalent to 195 mg of hexosamine per femur, which accounted for 27.3% of the total hexosamine in the tissue. Studies with hyaluronidase from Streptomyces hyalurolyticus and chondroitinase ABC showed that the glycosaminoglycans were composed of hyaluronic acid (16% of the total glycosaminoglycan) and chondroitin 6-sulfate (79%). The chondroitin 6-sulfate was separated on Bio-Gel A-0.5m gel into two molecular species with mol wt of >12,000 (Kd = 0.2) and ~8,000 (Kd = 0.47). Bone marrow digested with chondroitinase ABC and then treated with three monoclonal antibodies 4/8/9-A-2, 5/6/3-B-3, and 5/6/1-B-5, which were specific for unsaturated 4-sulfated, 6-sulfated, and nonsulfated disaccharide structures, respectively, at the nonreducing end of chondroitin sulfate chains, reacted with only 5/6/3-B-3. This result indicated that the chondroitin sulfate isomer in the bone marrow is chondroitin 6-sulfate, consistent with the biochemical results. The chondroitin 6-sulfate was localized mainly in the extracellular compartment and was considered to be involved in construction of the hemopoietic microenvironment in the bone marrow.

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Preparation of samples for analyses of various components in bone marrow tissue. For preparation of samples for analyses of various components in bone marrow tissue, nine femora were processed separately as described in Fig 1. Both ends of the femora were cut off, and the bone marrow was washed out with phosphate-buffered saline (PBS). The bones were then washed and dried with acetone, and their length and the volume of their marrow cavity were measured. The marrow tissue was defatted by successive treatments twice each at -20°C with acetone, ether, chloroform/methanol (2:1, vol/vol) and acetone. The acetone powder was dried at room temperature and weighed. The defatted tissue was processed as described in Fig 1 and assayed for individual components as described below.

Enzymatic digestions. Pronase digestion was carried out with the enzyme (1/25 of dry weight or 1/40 of protein), which was preincu-
hated for 1 hour at 50°C to eliminate the activity of contaminating DNase, in 2 mmol/L of CaCl₂ and 0.1 mol/L of Tris-HCl, pH 8.0, at 50°C for 70 hours with two further additions of the same amount of the enzyme at 24-hour intervals. Digestions with chondroitinase ABC and chondro-6-sulfatase were carried out as described previously. Hyaluronidase digestion was performed as described elsewhere. Digestion with DNase-I (200 U/mg of DNA equivalent to dAMP) was carried out in 5 mmol/L of NaCl and 0.1 mol/L of sodium acetate, pH 5.4, at 37°C for 1 hour.

Analytical methods. Protein was measured by the method of Lowry and associates. Hexurionate was determined by the carba- zole method of Bitter and Muir. DNA was determined by the method of Hinegardner, with dAMP as a standard. Hexosamine was determined by the Elson-Morgan method as modified by Boas with galactosamine as a standard, after hydrolysis of the samples. In brief, the samples were hydrolyzed with 3 mol/L of HCl in evacuated tubes at 100°C for 7 hours. The hydrolysates were adjusted to a concentration of 0.5 mol/L of HCl, and adsorbed to a column (0.7 x 15 cm) of AG 50W-X4 (200-400 mesh, H⁺ form). The column was washed thoroughly with water, and then hexos- amine was eluted with 2 mol/L of HCl. An aliquot of the eluate with 2 mol/L of HCl was dried on NaOH and dissolved in water, and its hexosamine content was determined.

Histochernical and immunohistochemical methods. Small pieces of bone marrow were taken out and fixed in periodate-lysine-paraformaldehyde, pH 7.4, for 1 hour at 4°C. After dehydration by passage through an ascending alcohol series, the speci-mens were embedded in paraffin and sectioned at 3 μm. The sections were deparaffinized in xylene, hydrated in a descending alcohol series, stained with alcian blue, or treated with monoclonal antibodies.

Alican blue staining was carried out by the method of Lev and Spicer, before and after digestion of the preparations with chondroitinase. The sections were incubated with or without chondroitinase ABC (1 U/mL of 0.1 mol/L of Tris-HCl, pH 7.4, containing proteinase inhibitors) at room temperature for 10 hours. They were then washed with water and stained with 1% (wt/vol) alcian blue 8GX in 0.1 mol/L of HCl for 30 minutes at room temperature. Preparations were counterstained with Kernechtrot. Materials were embedded in paraffin and sectioned at 3 μm. The sections were deparaffinized in xylene, hydrated in a descending alcohol series, stained with alcian blue, or treated with monoclonal antibodies.

The distributions of chondroitin sulfate isomers were examined with monoclonal antibodies 9-A-2, 3-B-3, and 1-B-5 as follows: The sections were digested with chondroitinase ABC (0.05 U/mL of 0.1 mol/L of Tris-HCl, pH 7.3, containing proteinase inhibitors) for 5 minutes at room temperature, incubated with monoclonal antibod- ies for 1 hour, washed for 30 minutes in 0.15 mol/L of phosphate-buffered saline (PBS), and then incubated with a solution of horseradish peroxidase-conjugated second antibody. Sections were washed with 50 mmol/L of Tris-HCl, pH 7.4, and color was developed in 3,3-diaminobenzidine solution containing 0.3% H₂O₂. Sections were counterstained with Giemsa. Control sections were processed in the same manner, except that they were treated with buffer without the enzyme.

Other methods. Two-dimensional electrophoresis of glycosami- noglycan samples was carried out on a cellulose acetate membrane as described previously. Paper chromatography was carried out on Toyo No 51A paper in 1-butrycric acid/acetic acid/1 mol/L of NH₄OH (2/3/1).

RESULTS

Glycosaminoglycan content of bone marrow. To prepare glycosaminoglycans from bone marrow and to determine their contents and those of various other compounds in bone marrow, we processed nine femora of 3-month-old rabbits separately, as shown in Fig 1. The length of femur cut at both ends was 4.00 ± 0.11 cm, and the volume of the marrow cavity was 0.99 ± 0.10 cm³. Bone marrow tissue was defatted by extraction with organic solvents as described in the Materials and Methods section and dried. The dry weight of the defatted powder was 116.0 ± 20.0 mg/femur. Analysis of parts of defatted powder of individual samples showed that the total hexosamine content was 0.61 ± 0.73 mg/100 mg dry weight of tissue. The remaining portions of the samples were suspended in water, boiled for 5 minutes to denature proteins, adjusted to 0.1 mol/L of Tris-HCl, pH 8.0, and digested extensively with pronase. The preparations were then centrifuged to remove insoluble materials, and the protein contents of the aliquots of the supernatants were measured. The values obtained were corrected by subtracting the protein content of the control containing enzyme only. The protein content was 18.0 ± 1.9 mg/100 mg dry weight of tissue. This value was almost the same as that determined using aliquots of the suspensions before centrifugation, indicating that pronase digestion for 70 hours was sufficient to solubilize all the tissue protein. After dialysis, the dialyzates were treated with mild alkali with 1 mol/L of NaBH₄ added to release glycosaminoglycans and to degrade RNA in the samples. The DNA content of these samples was determined as described by Hinegardner. In this method, sialic acid from glycoproteins, which also shows fluorescence, was removed by the alkali treatment and dialysis step. The DNA content was 2.46 ± 0.44 mg equivalents of dAMP per 100 mg dry weight. DNA in the samples was digested with DNase-I; the enzyme was then degraded by pronase digestion, and resultant small molecules were removed by dialysis. The dialyzates were freeze-dried, dissolved in water, and used as glycosaminoglycan samples. The hexosamine content of these glycosaminoglycan samples was 0.168 ± 0.012 mg/100 mg dry weight of tissue. The hexosamine content of glycosaminoglycan fraction was ~27% of the total hexosamine. The remaining hexosamine (73%) was assumed to be derived from sugar moieties of glycoproteins in the defatted tissue. The hexuronate content of the glycosaminoglycan samples
was 0.189 ± 0.017 mg/100 mg dry weight of tissue. The molar ratio of hexuronate to hexosamine in the samples was ~1, indicating that the glycosaminoglycans in the samples were purified considerably. The concentration of hexuronate in the marrow cavity was 0.191 ± 0.017 mg/mL.

Isolation and characterization of glycosaminoglycans. The nine individual glycosaminoglycan samples were combined and subjected to ion-exchange chromatography on diethylaminoethanol (DEAE)-Sephacel (Fig 2). Hexuronate in the fractions was monitored. On elution with a linear concentration gradient of NaCl, materials containing hexuronate were eluted in two fractions: fraction DE-I eluted with 0.25 to 0.3 mol/L of NaCl as a sharp peak, and fraction DE-II eluted with 0.4 to 0.65 mol/L of NaCl as a rather broad peak. Recovery was ~90% as hexuronate. Aliquots of DE-I and DE-II were digested with hyaluronidase or chondroitinase ABC, respectively, and these digests were then chromatographed on Bio-Gel P-6 (Fig 3). Glycosaminoglycan in DE-I was completely degraded by hyaluronidase and was identified as hyaluronic acid (Fig 3A), and that in DE-II was depolymerized by chondroitinase ABC and identified as chondroitin sulfate isomers (Fig 3B). Thus, the glycosaminoglycans in rabbit bone marrow contain 79% chondroitin sulfate and 16% hyaluronic acid (Table 1). The remaining 5%, which was eluted between DE-I and DE-II from DEAE-Sephacel (Fig 2), was not studied further.

The chondroitin sulfates of the DE-II fraction were characterized further. After gel filtration on a Bio-Gel A-0.5 m column previously calibrated with heparins of known mol wt, the chondroitin sulfates were separated into two fractions (Fig 4A), fraction DE-II-A (18% of the applied hexuronate) and fraction DE-II-B (81%), respectively. The apparent mol wt of the former peak was estimated as >12,000, and that of the latter was estimated as ~8,000 from the calibration curve (inset in Fig 4A). On two-dimensional electrophoresis, the materials in DE-II-A migrated as one major spot with three other tiny spots (Fig 4B). All these spots disappeared after digestion of the sample with chondroitinase ABC (data not shown), indicating that the chondroitin sulfates in this fraction are heterogeneous in both chain size and charge density. The chondroitin sulfate in DE-II-B migrated as a single spot with a tail (Fig 4C). This spot also disappeared after digestion of the sample with chondroitinase ABC. These results indicate that the chondroitin sulfates in bone marrow consist of several molecular species differing in molecular size and charge density. To demonstrate the isomeric structures of these chondroitin sulfates in terms of the position of the sulfate residue, the samples were digested with chondroitinase ABC, chondroitinase AC-II, chondroitinase ABC plus chondro-6-sulfatase or chondroitinase ABC plus chondro-4-sulfatase, and the products were subjected to paper chromatography. As shown in Fig 5, after digestion with chondroitinase ABC, the chondroitin sulfates of the two fractions gave only one spot comigrating with the external reference ΔDi-6S, with no significant amounts of ΔDi-4S or ΔDi-OS (Fig 5, lanes a and c). The digests with chondroitinase ABC plus chondro-6-sulfatase comigrated with ΔDi-OS (Fig 5, lanes b and d). Digests with chondroitinase ABC plus chondro-4-sulfatase comigrated with ΔDi-6S, indicating that 4-sulfated disaccharides were not present (data not shown). These results indicated that all the chondroitin sulfates of the two fractions were 6-sulfated, and that the heterogeneity observed on two-dimensional electrophoresis was due solely to differences in their chain sizes. Similar results were obtained on digestion of the samples with chondroitinase AC-II, chondroitinase AC-II plus chondro-6-sulfatase, and chondroitinase AC-II plus chondro-4-sulfatase (data not shown). These results indicate that the bone marrow contained little, if any, dermatan sulfate. We demonstrated that the chondroitin 6-sulfates in bone marrow

![Bio-Gel P-6 gel chromatography of glycosaminoglycans treated with and without hyaluronidase or chondroitinase ABC.](image)

**Table 1. Molecular Species of Glycosaminoglycans Contained in Rabbit Bone Marrow Tissue**

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>Hexuronate/Femur (μg)</th>
<th>Ratio (%)</th>
</tr>
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<tbody>
<tr>
<td>Chondroitin sulfate</td>
<td>173.4</td>
<td>79.1</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>34.8</td>
<td>15.9</td>
</tr>
<tr>
<td>Unidentified glycosaminoglycans</td>
<td>11.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Fig 4. Bio-Gel A-0.5m gel chromatography and two-dimensional electrophoresis of the glycosaminoglycans of the DE-II pooled fraction from diethylaminoethanol (DEAE)-Sephacel. Fraction DE-II from DEAE-Sephacel was adjusted to 1 mol/L of NaCl in 50 mmol/L of Tris-HCl, pH 7.3, and chromatographed on a column (1 × 115 cm) of Bio-Gel A-0.5m (A), which was equilibrated and eluted with 1 mol/L of NaCl in 50 mmol/L Tris-HCl, pH 7.3, at a flow rate of 3.9 mL/h. Fractions of 1.4 mL were collected. Hexuronic acid in the eluate was monitored. Arrows show the void volume and total volume of the column. Fractions DE-II-A and DE-II-B, indicated with bars, were pooled for further study. The column was previously calibrated with heparins of known mol wt (22,000, 13,000, 9,400, 7,300, and 5,700). The inset in panel A shows the calibration curve obtained by plotting $K_D$ (abscissa) v mol wt (on a logarithmic scale). Arrowheads indicate the $K_D$ values of the two fractions DE-II-A and DE-II-B, respectively. Aliquots of DE-II-A (B) and DE-II-B (C) were subjected to two-dimensional electrophoresis on cellulose acetate membranes. The standard glycosaminoglycans used as external references were hyaluronic acid (HA), dermatan sulfate (DS), chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), and heparin (HP).

Distributions of glycosaminoglycans. The distributions of sulfated glycosaminoglycans in the bone marrow were first surveyed by alcian blue staining at pH 1.0, coupled with chondroitinase digestion. Under these conditions, strongly acidified glycosaminoglycans such as sulfated ones are stained with the dye, but weakly acidified glycosaminoglycans or glycoproteins such as hyaluronic acid and sialoglycoproteins are not stained. When specimens were
stained with alcian blue, the cytoplasms of megakaryocytes and granuloid cells stained strongly and the extracellular compartment stained moderately (Fig 6A). After extensive digestion of specimens with chondroitinase ABC, no stained material was apparent in megakaryocytes or the matrix, but that in granuloid cells remained, indicating that the former were chondroitin sulfate isomers, but the latter was not (Fig 6B). For these studies, rabbit tracheal cartilage was used as a positive control. Alcian blue strongly stained the cartilage matrix under these conditions, and almost all the stained materials disappeared on digestion with chondroitinase (data not shown). The stained material in granuloid cells was not studied further.

Monoclonal antibodies specific for the chondroitin sulfate isomers were used to determine whether the chondroitin sulfate isomers shown histochemically to be present in the cytoplasm of megakaryocytes and in the extracellular matrix were chondroitin 6-sulfate. The three monoclonal antibodies used were 3-B-3, 9-A-2 and 1-B-5, which are specific for unsaturated 6-sulfated, 4-sulfated, and nonsulfated disaccharide structures, respectively. These structures appear at the nonreducing end of chondroitin sulfate chains after digestion with chondroitinase ABC. As shown in Figs 8B, D, and F, specimens that had not been digested with chondroitinase ABC did not react with any of these antibodies. After digestion with chondroitinase ABC, specimens reacted with 3-B-3 (Fig 8A), but not with the other two antibodies (Figs 8C and E). Thus, the chondroitin sulfate isomer present in proteoglycans in the bone marrow is chondroitin 6-sulfate. However, because chains of chondroitin sulfates from other tissues consist of hybrid structures containing 6-sulfated, 4-sulfated, and nonsulfated disaccharides, the reactivities of the three antibodies were examined after the specimens had been digested with chondroitinase ABC to different degrees. The reactivities of these specimens with the three antibodies did not significantly change, using specimens treated with

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Fig 5. Paper chromatography of DE-II-A and DE-II-B glycosaminoglycan samples digested with chondroitinase ABC or chondroitinase ABC plus chondro-6-sulfatase. Aliquots of DE-II-A and DE-II-B were digested with the enzymes, and the products were chromatographed on Toyo No 51A paper. Lanes a and b are the digests of DE-II-A with chondroitinase ABC and chondroitinase ABC plus chondro-6-sulfatase, respectively, and lanes c and d are the digests of DE-II-B with chondroitinase ABC and chondroitinase ABC plus chondro-6-sulfatase, respectively. Lane R is a mixture of the unsaturated disaccharides ΔDi-0S, ΔDi-4S and ΔDi-6S, as external references.

Fig 6. Alcian blue staining of the bone marrow before and after digestion with chondroitinase ABC. The bone marrow specimens were stained with alcian blue at pH 1.0 before (A) and after (B) digestion with chondroitinase ABC. Counterstaining was performed with Kernechtrot. Bar = 20 μm. Me, megakaryocytes; G, granuloid cells; Ma, matrix.

Fig 7. Higher magnification of immunohistological staining of the bone marrow with monoclonal antibody 3-B-3, after digestion with chondroitinase ABC. The bone marrow specimen was treated as described in Fig 6. Bar = 20 μm.
Fig 8. Immunochemical staining of bone marrow with monoclonal antibodies 3-B-3, 9-A-2, and 1-B-5, specific for unsaturated 6-sulfated, 4-sulfated, and nonsulfated disaccharide, respectively. Bone marrow specimens were treated with monoclonal antibody 3-B-3, 9-A-2, or 1-B-5, after treatment with (A, C, and E, respectively) and without (B, D, and F, respectively) chondroitinase ABC, and then treated with horseradish peroxidase-conjugated second antibody. Color was developed with diaminobenzidine solution containing H2O2. Counterstaining was performed with Giemsa. Bar = 50 μm.
concentrations of chondroitinase ABC of 0.05 to 1 U/ml for 5 minutes, or for incubation times of 5 minutes to 10 hours with 0.05 and 1 U of the enzyme. This result indicates that the chondroitin sulfate chains of this tissue have unsaturated 6-sulfated disaccharides at their nonreducing end regardless of the different degrees of degradation of the chains. This is consistent with the biochemical result that the chondroitin sulfate isomer present in the bone marrow is fully sulfated chondroitin 6-sulfate and generates only unsaturated 6-sulfated disaccharides after digestion with chondroitinase ABC.

Higher magnification (Fig 7) showed that the materials reacting with 3-B-3 were localized in the matrix of the bone marrow and were somewhat concentrated in the periphery of certain hemopoietic cells. Although the cytoplasm of megakaryocytes contained chondroitinase-sensitive alcian blue-stainable materials (Fig 6A), megakaryocytes of chondroitinase-treated specimens did not react with any of the antibodies tested. The specimens were pretreated with several proteinases such as trypsin and pronase before chondroitinase digestion, because the chondroitinase-sensitive material in megakaryocytes was expected to be packed in granules with proteins that might prevent the interaction of antibodies with antigens. However, the three antibodies did not react with megakaryocytes under the conditions examined (data not shown).

Antibody 1-B-5, with specificity directed against the unsaturated nonsulfated disaccharide structure of chondroitin sulfate chains, cross-reacts with the unsaturated disaccharide units (unsaturated glucuronsyl N-acetylgalactosamine) at the nonreducing end of hyaluronic acid chains, which appear after digestion with chondroitinase or bacterial hyaluronidase. Hyaluronic acid, which accounted for ~16% of the total glycosaminoglycan in the bone marrow, as described above, should have been detectable using this monoclonal antibody. This antibody, however, did not react with specimens pretreated with various concentrations of chondroitinase ABC. At present, whether hyaluronic acid is covalently linked to core protein has not been determined, but hyaluronic acid produced by fibrosarcoma appears to occur as free chains. If this is also the case in bone marrow, hyaluronic acid oligosaccharides with the epitope produced by digestion with chondroitinase ABC probably were washed out from the specimens. In the case of chondroitin 6-sulfate, the epitope produced by digestion with chondroitinase ABC would have been retained in the specimens in molecular forms of oligosaccharide-core protein complexes.

DISCUSSION

In this study, we found that the bone marrow of 3-month-old rabbits contained glycosaminoglycans equivalent to hexurionate 0.189 mg/100 mg dry weight of defatted tissue. The glycosaminoglycan was composed mainly of chondroitin 6-sulfate, accounting for 79% (equivalent to 0.15 mg of hexurionate), and hyaluronic acid, accounting for 16% (0.03 mg hexurionate). The hexosamine of these glycosaminoglycans accounted for ~27% of the total hexosamine present in the defatted tissue. The remaining 73% of the hexosamine might be that of sugar moieties of glycoproteins in the defatted tissue. These values were consistent with those reported previously by other researchers for isolated rabbit bone marrow cells. Olsson reported that rabbit bone marrow cells, obtained by filtration through silk cloth of a bone marrow suspension in Krebs-Ringer's-phosphate solution without calcium or magnesium, contained chondroitin 6-sulfate equivalent to 0.9 mg of hexurionate/g of fat-free cells and a hyaluronic acid-like glycosaminoglycan equivalent to 0.3 mg of hexurionate. Taniguchi and colleagues estimated that rabbit bone marrow cells prepared by a method similar to that of Olsson contained glycosaminoglycans, mainly chondroitin 6-sulfate, equivalent to 1.5 mg hexurionate/g of fat-free cells. Olsson reported that >90% of the cells in her preparation were granuloid cells and that the glycosaminoglycans were tightly associated with these cells after dissociation into single cell suspension. The defatted samples obtained in the present study from the whole bone marrow were prepared in a similar way, and our values based on dry weight are comparable. A difference in the chondroitin 6-sulfate contents of whole bone marrow (1.5 mg/g of dry weight) and single cells (0.9 mg/g of dry weight) from marrow tissue might indicate that the chondroitin 6-sulfate is present in different forms in bone marrow. For instance, they could occur in a cell-associated form (60%) and an extracellular form (40%) that would be easily released on dissociation of the tissue into single cells. The amount of hyaluronic acid (0.3 mg/g dry weight) in the bone marrow was exactly the same as that of dissociated bone marrow cells reported by Olsson. Thus, although we could not determine the localization of the hyaluronic acid in the tissue, it is probably associated with cells in the marrow tissue.

Only one of the monoclonal antibodies demonstrated localization of the chondroitin 6-sulfate. The chondroitin 6-sulfates were present entirely in the extracellular compartment of the bone marrow, indicating that they are involved in construction of the hemopoietic microenvironment. Previous studies in this laboratory and elsewhere demonstrated that the glycosaminoglycan side chains of proteoglycans contained in and/or produced by peripheral blood constituents, ie, mature blood cells, and contained in the plasma, are all chondroitin 4-sulfates or low-sulfated chondroitin 4-sulfates with little, if any, chondroitin 6-sulfate. These results suggest that the chondroitin 6-sulfates present in bone marrow are not brought out into the peripheral blood by hemopoietic progeny cells and are not released into the plasma, but are involved in construction of the matrix of the bone marrow and may have an action on cells in their immediate vicinity.

The chondroitin sulfate chains described in this study are constituted purely of 6-sulfated repeating disaccharide and of no detectable amounts of 4-sulfated and nonsulfated disaccharides. This kind of chondroitin sulfate isomer has not been reported in adult tissues. Sampaio and Dietrich reported that several tissues of bovine fetus contained the chondroitin sulfate isomer constituted of only 6-sulfated repeating disaccharide and the concentration of that
increased up to the 50th day of bovine fetal development and then decreased progressively until its complete disappearance in those tissues. They proposed that the chondroitin 6-sulfate acts as a stimulant of cell division. Hart\textsuperscript{6} demonstrated that the major sulfated glycosaminoglycans synthesized by thymic lymphocytes were chondroitin 4-sulfate, but that the relative proportion of chondroitin 6-sulfate increased dramatically when the cells were stimulated with mitogens. Together with these data, the present finding that chondroitin 6-sulfate is present in bone marrow, a tissue that unlike most other tissues in adults maintains continuous cell proliferation, suggests that this compound plays an important role in regulation of proliferation of hemopoietic cells.

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