Proteoglycans in Human Long-Term Bone Marrow Cultures: Biochemical and Ultrastructural Analyses

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Proteoglycans within the extracellular matrix of human bone marrow have been implicated in the process of hematopoiesis, but little is known about the structure and composition of these macromolecules in this tissue. Hematopoietically active human long-term bone marrow cultures were incubated with medium containing 35S-sulfate and 3H-glucosamine as labeling precursors. Proteoglycans present in the medium and cell layer were extracted with 4 mol/L guanidine HCl and purified by diethylaminoethyl (DEAE)-Sephacel ion exchange and molecular sieve chromatography. Both culture compartments contain a large chondroitin sulfate proteoglycan (MI, CI) that eluted in the void volume of a Sepharose CL-4B column and contained glycosaminoglycan chains of molecular weight (mol wt) $\sim$38,000. A second population of sulfate-labeled material was identified as a broad heterogeneous peak (MII, CII) that was included peak eluting at $K_v = 0.31$. This material when chromatographed on Sepharose CL-6B could be further separated into a void peak (MIIa, CIIa) and an included peak eluting at $K_v = 0.39$ (MIIb, CIIb). The void peaks (MIIa, CIIa) were susceptible to chondroitinase ABC digestion (99%) but slightly less susceptible to chondroitinase AC digestion (90%). Papain digestion of these peaks revealed them to be proteoglycans with glycosaminoglycan chains of mol wt $\sim$38,000. The included peaks on Sepharose CL-6B (MIIb, CIIb) from both medium and cell layer compartments resisted digestion with papain, indicating the presence of glycosaminoglycan chains of mol wt $\sim$38,000 either free or attached to a small peptide. Although this material was susceptible to chondroitinase ABC (98%), it was considerably less susceptible to chondroitinase AC (60%), indicating that it contained dermatan sulfate. A small amount of heparan sulfate proteoglycan was also identified but constituted only $\sim$10% of the total sulfated proteoglycan extracted from these cultures. Additionally, approximately 40% of the incorporated 3H-activity radioactivity was present as hyaluronic acid. Electron microscopy revealed a layer of adherent cells covered by a mat containing ruthenium red–positive granules that were connected by thin filaments. The extracellular matrix layer above the adherent cells contained a mixture of hematopoietic cells. Chondroitinase ABC treatment of the cultures completely removed the ruthenium red–positive granules overlying the cells and resulted in a loss of $\sim$70% of the 35S-sulfate–labeled material from the cell layer. This culture system is offered as a model to investigate the role of proteoglycans in hematopoiesis.

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attachment of early hematopoietic cells to an adherent cell layer in vitro can be blocked by removing cell surface glycosaminoglycans from the hematopoietic cells and/or adherent cells by specific glycosidases. Thus, proteoglycans appear to regulate stromal cell–hematopoietic cell interaction.

To date, the majority of studies implicating proteoglycans or their constituent glycosaminoglycans in hematopoiesis have been indirect (ie, examining glycosaminoglycan content in systems exhibiting defective hematopoiesis). Recently, a more direct approach has been taken by Dexter and his colleagues in experiments in which proteoglycan synthesis in long-term mouse bone marrow cultures has been perturbed by treatment with β-D-xyloside. Modification of proteoglycan synthesis in these cultures was associated with an increased production of stem cells. Since xylosides increase free-chain glycosaminoglycan synthesis but decrease overall proteoglycan synthesis, the nature of the proteoglycan effect responsible for the elevated level of hematopoiesis in this culture system is not yet clear.

Little is known about the types of proteoglycans present in hematopoietic systems since previous studies have analyzed only glycosaminoglycans of hematopoietic tissues and have failed to consider the nature of the entire proteoglycan molecule in which the glycosaminoglycans are found. Since glycosaminoglycans are synthesized within the tissue, not as free chains but as proteoglycan monomer, knowledge of the molecular structure of proteoglycans within hematopoietic tissue would appear essential if their role in hematopoiesis is to be clarified. Recent studies by Thompson and Spooner demonstrated that inhibition of branching morphogenesis in mouse salivary glands was correlated with decreased proteoglycan synthesis rather than with increased free-chain glycosaminoglycan synthesis. These studies highlight the importance of native proteoglycan structure in events associated with morphogenesis.

The objective of this study was to characterize proteoglycans synthesized and secreted by hematopoietically active long-term human bone marrow cultures and to determine whether these macromolecules are localized to specific extracellular domains within these cultures. These studies will serve as a basis for future studies designed to test the hypothesis that proteoglycans within hematopoietic organs regulate hematopoiesis.

MATERIALS AND METHODS

Materials

Guandine HCl, cesium chloride, Tris-HCl, and papain were all purchased from Sigma Chemical Co, St Louis; 6-aminohexanoic acid and benzamidine HCl from Eastman Kodak Co, Rochester, NY; chondroitinase AC-II (Arthrobacter aurescens), chondroitinase ABC (Proteus vulgaris) from Seikagaku, Kogyo, Japan, through Miles Laboratories, Inc, Naperville, III; Streptomyces hyaluronidase from Calbiochem-Behring Corp, San Diego; Sephadex, Sepharose, and diethylaminoethyl (DEAE)-Sephacel were from Pharmacia Fine Chemicals, Inc, Piscataway, NJ; Na235SO4 (10 mCi/mL:379 mCi/mmol); D-[6-3H]-glucosamine (1.0 mCi/mL:31 Cl/mmol) from New England Nuclear, Boston; AquaMix from WestChem Products, San Diego; urea from MCB, Cincinnati; McCoy’s complete medium and horse serum from GIBCO, Grand Island, NY; fetal bovine serum and hydrocortisone from Reheis Chemical Co, Kankakee, Ill; Falcon tissue culture dishes from Falcon Labware, Oxnard, Calif.

Cell Culture

Marrow aspirates, drawn into 10 U/mL of preservative-free heparin were obtained from five normal marrow transplant donors. Marrow buffy coat cells were prepared, and long-term cultures were generated from the marrow of each donor using a modification of the Gartner and Kaplan method. Briefly, 20 x 106 marrow buffy coat cells were seeded into 25-cm2 tissue culture flasks containing McCoy’s modified complete medium with 12.5% fetal bovine serum, 12.5% horse serum, and 1.0 mol/L hydrocortisone at 2 x 104 cells/mL. The cultures were incubated at 37°C in 5% CO2 for 1 week at 37°C and then transferred to 33°C. Half the medium was removed weekly after gentle agitation of the flasks and replaced with fresh medium. The nonadherent cells removed with the medium were assayed for committed hematopoietic progenitors. Long-term marrow cultures were studied for proteoglycan biosynthesis at 4 to 5 weeks when all cultures had confluent stromal cell layers and were producing committed hematopoietic progenitor cells at optimal levels.

Radioisotopic Labeling of Cultures and Extraction of Proteoglycans

Cell cultures were labeled by the addition of fresh medium containing Na235SO4 (50 μCi/ml) and 3H-glucosamine (20 μCi/mL) for times ranging from eight to 72 hours. After labeling, the medium fraction was removed and frozen at −70°C after the addition of solid guanidine-HCl (GuHCl) (0.5 M) to bring the solution to approximately 4 mol/L. The cell layer of each culture was extracted with 4 mol/L GuHCl (dissociative solvent) containing 0.1 mmol/L 6-aminohexanoic acid, 5 mmol/L benzamidine HCl, 50 mol/L sodium EDTA, and 50 mmol/L sodium acetate, pH 5.8. This results in removal of 95% of the macromolecular 35S activity from the cell layer, with 5% of the total 35S activity associated with an insoluble residue (as determined by NaOH extraction of the insoluble cell residue). The cell layers were disrupted by scraping with a rubber policeman and immediately frozen at −70°C until further analysis.

Purification and Characterization of Proteoglycans

DEAE-Sephacel ion-exchange chromatography. Aliquots (5 mL) of 4 mol/L GuHCl extracts of the medium and cell layer were eluted on a Sephadex G-50 column (0.9 x 45 cm) equilibrated with 8 mol/L urea, 0.10 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.5, 2 mol/L EDTA, and 0.5% Triton X-100. Portions of the excluded peaks were counted for radioactivity to determine recoveries and distributions of labeled macromolecules in the medium and cell layer. The remainder of the excluded fractions were pooled (18.0 mL) and applied to a 3.0-mL column of DEAE-Sephacel equilibrated with the same solvent used for elution on Sephadex G-50. After the sample was applied and washed with 10 mL of solvent, a continuous 0.1 to 0.7 mol/L NaCl gradient was applied in the same solvent using a total of 60 mL. Fractions of 0.9 mL were collected at a flow rate of approximately 3.6 mL/h, and aliquots were counted for radioactivity. For both medium and cell layer, a major 3H-labeled peak (A) and two 35S-labeled peaks (B and C) were identified.

Enzymatic and chemical treatment. An aliquot of each of the labeled peaks (A, B, and C) was dialyzed against distilled water, lyophilized, and subjected to one of the following treatments:
Streptomyces hyaluronidase (50 turbidity reducing units [TRU]/ml in 0.5 mol/L sodium acetate buffer, pH 5.2, for four hours at 22 °C) to identify hyaluronic acid; nitrous acid degradation at a low pH to identify heparan sulfate; and chondroitinase ABC or chondroitin AC (25 U/ml in enriched Tris buffer, pH 8.0, for three hours at 37 °C) to identify chondroitin sulfate and/or dermatan sulfate. 28 The extent of digestion was assessed by separating labeled digestion products from undigested material using Sephadex G-50 columns (0.7 x 30 cm) with 0.1 mol/L ammonium acetate in 20% ethanol as the eluting buffer.

Isopycnic CsCl centrifugation. A 300-μL aliquot of peak C was added to a 4 mol/L GuHCl solution (~4.0 ml), and the mixture was brought to an initial starting density of 1.46 g/ml by adding solid CsCl (0.5 g/g). 22 Dispersive density gradients were formed by centrifuging at 37,000 rpm at 10 °C for 65 hours in an SW 50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif). Gradients were divided into four equal fractions (D1, D2, D3, D4) using a Beckman tube slicer, and an aliquot from each fraction was counted for radioactivity.

Molecular sieve chromatography. For both medium and cell layer, portions of the major 35S-labeled peak (peak C, see Fig 2) were first concentrated by dialyzing pooled fractions (~20 ml) with an equal volume of 8 mol/L urea buffer that did not contain NaCl to reduce NaCl concentration. This mixture was then reapplied to a DEAE-Sephacel column (bed volume, 0.6 ml). After sample application, the column was washed with a small volume (0.6 to 0.8 ml) of 4 mol/L GuHCl buffer containing 0.5% Triton X-100. 27 Recovery for the concentration step was between 80% and 90%. The concentrated sample was applied to a preparative Sepharose CL-4B column (0.9 cm x 58 cm) and eluted with 4 mol/L GuHCl, 0.1 mol/L Tris-HCl, 0.1 mol/L Na2SO4, and 2.5 mmol/L EDTA, pH 7.0. Individual peaks were identified by counting small aliquots of each fraction, and fractions from each peak were then pooled and concentrated by contact desiccation. Proteoglycan carrier (100 μL of 10 mg/mL prepared from rat chondrosarcoma) 27 was added to each concentrated peak obtained from the Sepharose CL-4B column and rechromatographed on a preparative Sepharose CL-6B column (0.9 cm x 58 cm) eluted with 4 mol/L GuHCl as previously described. Peak fractions were identified by scintillation counting, pooled, and concentrated by contact desiccation. The concentrated peaks were desalted on Sephadex G-50 (bed volume, 10 ml) and proteoglycans present in each precipitant peak were precipitated with 3 vol of ethanol containing 1.3% potassium acetate. Each precipitated peak was digested with either chondroitinase ABC or AC as previously described and digestion products separated by chromatography on Sephadex G-25 (PD-10) chromatography by scintillation counting.

RESULTS

Accumulation of 35S-Sulfate-Labeled Macromolecules

The amount of 35S-sulfate radioactivity incorporated into macromolecules released into the medium and present in the 4 mol/L GuHCl extracts of the cell layer of human bone marrow cultures are shown in Fig 1. The accumulation of 35S-sulfate–labeled macromolecules in the medium was nearly linear throughout a 72-hour labeling period. On the other hand, the cell layer accumulated 35S-sulfate radioactivity linearly for the first 24 hours and then reached a plateau, staying fairly constant up to 72 hours. By 72 hours, approximately 70% of the macromolecular 35S-sulfate activity was present in the medium, with the remainder deposited in the cell layer. The amount of DNA in these cultures did not increase during the 72-hour labeling period (data not shown).

Proteoglycan Isolation and Characterization

Portions of 4 mol/L GuHCl extracts of the cell layers and media were desalted and equilibrated in an 8 mol/L urea buffer by Sephadex G-50 chromatography. 23 The excluded volume fractions were applied to DEAE-Sephacel in the same solvent and eluted with a continuous NaCl gradient. The profiles of radioactivity eluting from the columns were similar for the medium and cell layer fractions (Fig 2). Approximately 40% to 45% of the applied 3H radioactivity in
the medium and cell layer did not bind to the ion-exchange column, eluting ahead of the salt gradient in the wash as a glycoprotein fraction. Less than 3% macromolecular 35S-sulfate activity eluted in this fraction. In both medium and cell layer samples a large peak (A) containing only 3H activity eluted early (~0.26 mol/L NaCl) and was followed by two closely spaced peaks containing 3H and 35S-sulfate activity (peaks B and C). Peak A was the predominant 3H-labeled peak for both medium and cell layer (~38% of the total counts). Peak C was the predominant 35S-sulfate-labeled peak in both the medium and cell layer and contained approximately 89% to 94% and 83% to 91% of the total 35S-sulfate activity, respectively. The remainder of the 35S-sulfate activity was present in peak B for both culture compartments.

Peaks A, B, and C from the DEAE columns were further pooled, dialyzed, and subjected to Streptomyces hyaluronidase digestion to identify hyaluronic acid, chondroitinase ABC or AC,26 chondroitin sulfate and dermatan sulfate or nitrous acid,25 and the presence of heparan sulfate. Digestion products were separated on Sephadex G-50 columns (Fig 3). Peak A from both the medium and cell layer was >95%

![Fig 1. Accumulation of 35S-sulfate-labeled macromolecules in the culture medium (solid circles) and cell layer (open circles) for a 72-hour period in 3- to 4-week bone marrow cultures.](image1)

![Fig 2. DEAE-Sephacel ion-exchange chromatographic profiles of 35S-sulfate- and 3H-glucosamine-labeled macromolecules present in 4 mol/L GuHCl extracts of medium (A) and cell layer (B). The 4 mol/L GuHCl extracts were desalted on Sephadex G-50 columns into 8 mol/L urea in Tris-HCl, pH 7.5, with 0.5% Triton X-100 and 0.1 mol/L NaCl and applied to DEAE in the same buffer. The radioactivity was eluted during a continuous (0.1 to 0.7 mol/L) NaCl gradient. Peaks a and c were pooled as indicated for further study.](image2)

![Fig 3. Sephadex G-50 profiles of pooled DEAE peaks with and without enzymatic or nitrous acid degradation. (A) Peak A, treated with Streptomyces hyaluronidase (open circles) and control aliquot, similarly treated in buffer without enzyme (closed circles). (B) Peak B, treated with nitrous acid (open circles) and control aliquot (closed circles). (C) Peak C, treated with either chondroitinase ABC (open circles), chondroitinase AC (squares), or buffer alone (closed circles).](image3)

![Fig 4. Distribution of 35S-sulfate-labeled peak C obtained from DEAE-Sephacel ion-exchange chromatography following separation in a dissociative CsCl density gradient centrifugation. Each gradient was separated into four equal fractions from top (D4) to bottom (D1). (A), medium compartment; (B), cell layer compartment. The specific gravity of each fraction is given in parentheses.](image4)
susceptible to *Streptomyces* hyaluronidase, indicating that this peak contained hyaluronic acid (Fig 3A). Peak B from the cell layer and medium was partially susceptible to nitrous acid (30% to 40%) (Fig 3B), with the remainder of activity sensitive to chondroitinase ABC (not shown). The major sulfate peak from both the medium and cell layer (peak C) was almost totally susceptible to chondroitinase ABC (~86% to 93%) and partially sensitive to chondroitinase AC II (~47%) (Fig 3), indicating the presence of both chondroitin sulfate and dermatan sulfate. Aliquots of the major sulfate-labeled peak (C) from the medium and cell layer were centrifuged in a 4 mol/L GuHCl CsCl density gradient (Fig 4). The bulk of the radioactivity (>75%) was recovered in the high-buoyant density fractions of D1 (ρ = 1.550) and D2 (ρ = 1.473), indicating that proteoglycans in peak C contain a high carbohydrate-to-protein ratio.

Peak C from both the medium and cell layer extracts was further analyzed by chromatography on Sepharose CL-4B in 4 mol/L GuHCl. Both culture compartments gave similar chromatographic profiles (Fig 5). A single, well-defined peak (M1; C1) eluted in the void volume of the column, followed by a broad second peak that eluted at a position of Kav = 0.3 (MII; CII). Major peak fractions (MI, MII, CI, CII) were pooled as indicated in Fig 5 and further analyzed by chromatography on Sepharose CL-6B using 4 mol/L GuHCl as the eluting buffer. M1 and C1 eluted as well-defined peaks in the void volume (Fig 6), while MII and CII eluted as two peaks on Sepharose 6B, one eluting in the void volume of the column (MIIa; CIIa) and the other eluting at a Kav = 0.39 (MIIb; CIIb). Each of the identified peaks were further analyzed as to their susceptibility to papain and chondroitinase AC digestion. Papain treatment of M1, MIIa,
CI, and CIIa shifted these peaks from the void position on Sepharose CL-6B to an elution position of $K_{av} = 0.38$ to $0.40$ (Figs 7 and 8). Similar results were obtained when chains were released by alkaline borohydride. These results indicate that each of these peaks represent an intact proteoglycan with glycosaminoglycan chains of nearly equal size ($\approx 38,000$). On the other hand, papain treatment of MIIb and CIIb did not shift the elution position of these peaks, indicating that these peaks contain either free glycosaminoglycan chains or a single chain attached to a small peptide.

Digestion of MII and CII with chondroitinase AC resulted in greater than 98% degradation of each peak of disaccharides as determined by Sepharose CL-6B chromatography (Figs 7D and 8D). Peak MIIa was also nearly totally (97%) susceptible to chondroitinase AC digestion, whereas CIIa was ~86% susceptible (Figs 7E and 8E). Peaks MIIb and CIIb contained a much higher percentage (~40% of the total label) of partially degraded material following chondroitinase AC treatment that eluted ahead of the disaccharide peak (Figs 7F and 8F). Since this material was totally deglycosaminioglycanated, it was concluded that the partially degraded glycoproteins were the result of chondroitinase AC digestion of side chains attached to a single chain proteoglycan.

Fig 9. (A) Low-magnification scanning electron micrograph of a long-term bone marrow culture (4 weeks) fixed and processed in the presence of ruthenium red. A mat of fluffy material covers a layer of flattened stromal cells. Bar = 10 µm (original magnification ×300); current magnification ×207. (B) A higher magnification of this matted material reveals a fibrillar meshwork extending a considerable distance above the adherent cells. (arrow) Original magnification ×1890; current magnification ×1138. Bar = 10 µm. (C) This scanning electron micrograph was taken of a comparable long-term bone marrow culture that was prepared for electron microscopy in the absence of ruthenium red. No matlike substance is seen. Numerous hematopoietic cells can be seen associated with the stromal layer of adherent cells (arrow). Original magnification ×1290; current magnification ×890. Bar = 10 µm. (D) This scanning electron micrograph was taken from a long-term bone marrow culture that had been pretreated with chondroitinase ABC prior to processing for electron microscopy in the presence of ruthenium red. The enzyme treatment completely eliminated the mat of surface material lying above the adherent cells. Original magnification ×360; current magnification ×248. Bar = 10 µm. (E) A high magnification of this flocculent material reveals a number of rounded cells enmeshed in the material preserved with ruthenium red (arrow). Original magnification ×1,500; current magnification ×1035. Bar = 10 µm. (F) Transmission electron microscopy of SEM preparations reveals that the round cells are early hematopoietic cells. Dark staining at the left of the micrograph is the gold coating on the surface of the mat. Cells on the right side of the micrograph are those present within the adherent layer (b. culture dish). Original magnification ×3,750; current magnification ×2,587. Bar = 5 µm.
degraded to disaccharide by chondroitinase ABC treatment (inset, Figs 7F and 8F), peaks MIIb and CHIb must be enriched in iduronic acid and considered to contain dermatan sulfate.

Electron Microscopy

Cultures fixed in the presence of ruthenium red and processed for scanning electron microscopy revealed a mat of flocculent material lying upon portions of layered and flattened adherent cells (Figs 9A and B). This material was absent if ruthenium red was omitted from the fixative (Fig 9C) or if the cultures were treated with chondroitinase ABC prior to ruthenium red staining (Fig 9D). Both scanning and transmission electron microscopy demonstrated the presence of early hematopoietic cells throughout this flocculent material (Figs 9E and F). The ruthenium red-positive layer extended a distance of at least 25 μm above the cells and consisted of aggregates of dense granules frequently joined by thin 5-nm diameter filaments (Figs 10A and B). The thin

![Image](https://www.bloodjournal.org/)

Fig 10. (A) Transmission electron microscopy of cross sections of these cultures prepared in the presence of ruthenium red reveals an extensive layer of granular material lying above a multilayer of adherent cells (S). Original magnification ×9,600; current magnification ×6,720. Bar = 1.0 μm. (B) Higher magnification of this substance reveals clumps of dark-staining granules occasionally connected to one another via thin 5-nm threads (arrows). The surface of the adherent cells (S) also stains intensely with ruthenium red. Original magnification ×60,000; current magnification ×42,000. Bar = 0.25 μm.
filaments frequently associated with the surface of flattened cells in the multilayer (Fig 10B). Prominent ruthenium red staining of the surface of the adherent cells as well as the hematopoietic cells was a consistent feature of these cultures (Figs 10 and 11). Frequently, areas of contact between early hematopoietic cells and adherent cells were stained with ruthenium red (Fig 11). Ruthenium red–positive granules were also distributed among dark-staining fibrils between adjacent cells of the multilayer (Fig 12). These dark-staining fibrils resisted elimination by chondroitinase ABC treatment whereas the granules were totally removed by this enzyme treatment. The fibrils resembled fibrils shown to contain fibronectin34 which are enriched in long-term bone marrow cultures35 and may contain heparan sulfate proteoglycan, which is known to bind to fibronectin.36

The ability of chondroitinase ABC treatment to remove the majority of the proteoglycans present in the cell layer was confirmed when prelabeled cultures were incubated with the enzyme. Chondroitinase ABC treatment removed 70% of the 35S-sulfate–labeled macromolecules present in the cell layer whereas buffer treatment alone removed 10%. The enzyme treatment did not remove adherent cells, and the conditions used to demonstrate the loss of ruthenium red–positive granules paralleled those previously described.

DISCUSSION

The major sulfated proteoglycans synthesized by human long-term bone marrow cultures can be separated into at least three families based on their relative charge, hydrodynamic size, and/or glycosaminoglycan composition. A large chondroitin sulfate proteoglycan (CSPG) containing glycosaminoglycan chains of ~38,000 mol wt predominates in both the medium and cell layer compartments of these cultures. A similar large CSPG has been observed in a number of other culture systems including arterial smooth muscle,22,23 glial cells,27,38 skin, and lung fibroblasts.39,40 These findings indicate that this class of proteoglycan can be synthesized by a variety of cell types. The other major proteoglycan present in these cultures is hydrodynamically smaller and contains glycosaminoglycan chains of nearly equal size to those of the larger CSPG, but unlike the CSPG, the glycosaminoglycan chains contain a small percentage of iduronic acid residues (~10%) as part of the total uronic acid present in the molecule. It is uncertain at this point whether the iduronate in MIIa and CIla might originate from incomplete separation from MIIb and CIlb, which are rich in iduronate. However, similar low iduronate acid–containing proteoglycans have been described in a variety of tissues41,42 and cultures of various types of cells.5,43 Interestingly, a papain-resistant, sulfate-labeled dermanan sulfate peak that contains a higher percentage of iduronate (~40%) is found in both the cell layer and medium compartments. The finding that this peak does not shift upon chromatography in Sepharose CL-6B following papain treatment indicates that the peak contains either free glycosaminoglycan chains or single chains attached to a short peptide.27,45 Similar papain-resistant peaks have been observed in cell layer extracts of a variety of cells in culture,22,27,45 and these peaks have been interpreted to represent intracellular degradation products of proteoglycans present within lysosomes.45 However, a similar molecule appears to gain access to the medium compartment in bone marrow cultures. It is uncertain at this point whether this media-derived, papain-resistant material represents degraded proteoglycan released from the cells or extracellular degradation products. Studies are currently being conducted to investigate the source of this low–molecular weight material.

Although the majority of 35S-sulfate–labeled material present in both the medium and the cell layer compartment in human bone marrow cultures contains chondroitin and/or dermanan sulfate, a small amount of 35S-sulfate activity (~5%) is present in nitrous acid–sensitive material, indicating the presence of heparan sulfate. This material was present in both medium and cell layer fractions and could be effectively separated from the chondroitin- and dermanan sulfate–containing molecules by ion-exchange chromatography. The finding that the heparan sulfate–containing material elutes before the chondroitin-dermanan sulfate–contain-

Fig 11. Occasionally, hematopoietic cells (HP) can be seen to contact adherent stromal cells (S) via knobby extensions of their cytoplasm in regions enriched in ruthenium red staining. Dark staining filamentous material (arrow) is also prominent between adherent cells in the multilayer. Original magnification ×42,000; current magnification ×24,780. Bar = 0.25 μm.
Fig 12. (A) This electron micrograph was taken from a longitudinal section through the multilayer of adherent cells (S) fixed and processed in the presence of ruthenium red. Numerous dark-staining fibrils and a large number of ruthenium red-positive granules (arrows) are present throughout the intercellular space. A high magnification (B) of the granules reveals that many of them are aggregated and connected via thin 5-nm threads. (A), original magnification ×4,320; current magnification ×4,190. Bar = 2.5 μm. (B), original magnification ×27,000; current magnification ×26,196. Bar = 0.25 μm.

ing material on the DEAE ion-exchange column indicates that the heparan sulfate present in these cultures is less charged than the chondroitin–dermatan sulfate.

The structural and compositional characteristics of the proteoglycans synthesized in the human bone marrow cultures resembles those proteoglycans synthesized by arterial smooth muscle cells in vitro. These similarities include similar ratios of HSPG to CSPG/DSPG, identical behavior on ion-exchange and molecular-sizing columns, similar distribution in a dissociative CsCl density gradient, and similar glycosaminoglycan composition and chain size. In addition, like proteoglycans from arterial smooth muscle cells, some of the proteoglycans present exist in aggregate form (data not shown). Although long-term bone marrow cultures contain a mixture of cell types including endothelial cells, fibroblasts, macrophages, adipocytes, and hematopoietic stem cells, recent studies indicate that approximately 50% of the adherent cells in the human long-term bone marrow cultures react
with monoclonal antibodies specific for smooth muscle cells. At present, it is not clear whether all proteoglycans synthesized and secreted in the bone marrow cultures originate from smooth muscle–like cells since other cell types present such as endothelial cells, fibroblasts, monocytes, and granulocytes are all capable of synthesizing and secreting proteoglycans. However, recent studies using mouse marrow cultures indicate that the adherent cells and not the nonadherent hemopoietic cells were the major source of glycosaminoglycans and that the predominant glycosaminoglycan was heparan sulfate.

The presence of a matlike arrangement of ruthenium red positive granules and filaments above the monolayer of adherent cells is unique to this culture system, since similar structures have not been observed within cultures of other cell types. The susceptibility of this material to chondroitinase ABC and the granular texture suggests that this layer is enriched in proteoglycan. The finding that hemopoietic stem cells are enmeshed in the proteoglycan-rich layer indicates close association of stem cells with a proteoglycan-rich matrix. Whether this matrix is critical in promoting stem cell interaction with the adherent cells and subsequent hemopoiesis remains to be determined. It will be of interest to examine the nature of proteoglycans present in marrow cultures which exhibit suppressed or defective hemopoiesis such as cultures derived from SI/Sld mice, or patients with leukemia or aplastic anemia.

In conclusion, proteoglycans have been implicated in morphogenetic events for some time, but the exact nature of their influence has remained enigmatic. A key question that still remains is whether these molecules function merely to stabilize the differentiated state and create the proper microenvironment for morphogenetic events to proceed or act as inductive elements directly affecting those cellular events necessary for differentiation. The development of an in vitro system which supports differentiation (ie, hemopoiesis) and which possesses macromolecules confined to the microenvironment not only offers the opportunity to address this question but also may provide insight into the mechanisms by which specific proteoglycans influence differentiation.

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