Biosynthesis of Chondroitin Sulfate Proteoglycan by P388D1, Macrophage-like Cell Line

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Macrophages are in large part responsible for the subendothelial deposition of lipid within the artery wall during the early stages of atherogenesis. Proteoglycans secreted by these cells may play a role in this pathological process either by trapping lipoproteins in the extracellular matrix or by enhancing the formation of lipid-laden foam cells. The synthesis and secretion of proteoglycan was studied in the P388D1 macrophage-like cell line cultured in the presence of [35S]sulfate. The radiolabeled proteoglycan had a Kd of 0.69 on Sepharose CL-2B corresponding to an Mw of 2.8 x 10^5. It consisted of approximately 13 chondroitin sulfate chains of Mw 20,000 attached to a core protein with an Mw of 18,000. The chondroitin sulfate chains contained both N-acetylgalactosamine 6-sulfate and N-acetylgalactosamine 4-sulfate residues. No disulfated N-acetylgalactosamine residues were present. The P388D1 proteoglycan bound specifically to immobilized human low density lipoprotein. These results suggest that, in the focal regions of the arterial wall in which macrophages are found during the development of fatty streaks, proteoglycans secreted by these cells may affect the transport and cellular metabolism of plasma-derived lipids. (Arteriosclerosis 8:535-543, September/October 1988)

Proteoglycans are hybrid molecules, which consist of a protein core to which is covalently attached at least one glycosaminoglycan chain. In chondroitin sulfate proteoglycans, the chains are attached to the protein core through a four sugar oligosaccharide (the chondroitin sulfate-protein core linkage tetrasaccharide). Proteoglycans are particularly abundant in connective tissue in which they clearly play a major structural role. However, non-connecctive tissue cells also synthesize both cell-associated and secreted proteoglycans, which have been implicated in a variety of functions including cell-cell interaction, cell substrate attachment, and selective molecular transport (for a review see reference 1). Macrophages in culture synthesize proteoglycans, which are found intracellularly, pericellularly, and in the medium, but their functions in these cells are not well understood. Some evidence suggests that cell-associated macrophage proteoglycans may be involved in attachment to surfaces. For example, guinea pig peritoneal macrophages synthesize a specific type of cell-surface heparan sulfate only when cultured in monolayer and not when grown in suspension. The increase of heparan sulfate on the surface of attached macrophages is consistent with the view that it plays a role in the adhesion of cells to substrata, as has been suggested for fibroblasts. Levitt and Ho* demonstrated that mitogens stimulated human peripheral blood monocytes to secrete increased amounts of a chondroitin 4-sulfate proteoglycan and suggested that it might be involved in cellular immune responses. In support of this idea, other workers have found that sulfated polysaccharides on the surface of macrophages bind to specific receptors on lymphocytes and thereby may promote interaction between these two cell types. Furthermore, receptors for glycosaminoglycans were found on the cell surface of activated, but not resident, macrophages. Secreted proteoglycans may serve as a marker of monocyte-to-macrophage differentiation. During the first day in culture, human peripheral blood monocytes secreted chondroitin 4-sulfate. After 5 days of culture on plastic, the cells developed macrophage-like characteristics and began to secrete "oversulfated" chondroitin sulfate in which about 20% of the N-acetylgalactosamine residues were sulfated in both the 4- and 6-positions. When cultured on fibronectin-coated plates, the monocytes neither developed into macrophages nor secreted oversulfated chondroitin sulfate. Furthermore, fully differentiated macrophages from human peritoneal fluid secrete oversulfated chondroitin sulfate proteoglycan immediately upon being put into culture.

Macrophages have been implicated in pathological processes that may depend on the presence of proteoglycans. One of the early events seen during the development of an atherosclerotic lesion is the invasion of the subendothelial space by blood monocytes, which then develop into macrophages. These macrophages take up and metabolize lipoprotein; in the process they retain, in their cytoplasm, cholesterol esters in the form of droplets and become foam cells. Many of these foam cells are seen in the center of the early lesion, and it is thought that their presence contributes to the accumulation of lipid in the developing fatty streak. The process whereby macrophages in vivo become foam...
cells is not well understood. In vitro, macrophages do not accumulate lipid when cultured in the presence of low density lipoprotein (LDL) unless it first has been chemically modified to increase its net negative charge.10 It is perhaps relevant to the development of atherosclerotic lesions that isolated aorta proteoglycans interact with LDL in vitro to form both soluble and insoluble complexes.11 In addition, cultured macrophages actively take up proteoglycan-LDL complexes and become foam cells morphologically similar to those in fatty streaks.12-15

The above observations suggest that proteoglycans synthesized by macrophages and adjacent cells may be involved in macrophage maturation and may have a role in cellular attachment, interaction, and metabolism. Furthermore, extracellular macrophage proteoglycans may complex with LDL in the subendothelial space and thereby enhance the formation of foam cells and the accumulation of extracellular lipid. The present article describes the molecular properties and the lipoprotein binding characteristics of the proteoglycan synthesized by the mouse-derived P388D1 macrophage-like cell line.

Methods

Materials

Guanidine hydrochloride, phenylmethylsulfonyl fluoride, benzamidine hydrochloride, N-ethylmaleimide, papain, heparin (Type II), and N-morpholinopropanesulfonic acid (MOPS) were obtained from Sigma Chemical Company. Chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate were purchased from Behring Diagnostics. Sepharose CL-4B, Sepharose CL-2B, Sephadex G-50, DEAE-Sephacel, and Sepharose-CNBr were purchased from Pharmacia, Incorporated, and electrophoresis-grade urea was from ICN Immunobiologics. Bovine nasal cartilage proteoglycan was prepared as described previously.16 LDL was isolated from human plasma by the single vertical spin method.17 35S-sulfuric acid (carrier-free) was purchased from New England Nuclear.

Analytical Methods

Protein was determined by the Lowry method.18 Proteoglycans and glycosaminoglycans were measured either as uronic acid by the carbazole method or as glycosaminoglycan with dimethylmethylene blue.20 Radioactivity was determined in a Packard Model 3330 liquid scintillation spectrometer with 0.4 ml of aqueous sample dissolved in 5 ml of ACS aqueous counting scintillant (Amersham).

Gradient gel electrophoresis was done in the presence of sodium dodecylsulfate using the discontinuous buffer system of Laemmli.21 The gels were fixed and then stained with Coomassie blue to visualize the standards. To localize 35S-labeled bands, the gels were briefly washed in distilled water, soaked in 1 M sodium salicylate for 2 hours, were dried and then were placed in contact with x-ray film for 1 week.

Cells

The P388D1 macrophage-like cell line was obtained from the American Type Culture Collection (ATCC TIB 63) and was maintained at 37°C in a 5% CO2 atmosphere. The culture medium consisted of HL-1 medium containing 2% heat-inactivated fetal bovine serum and 50 units of penicillin plus 50 μg of streptomycin sulfate per ml (Medium A). These cells accumulated cholesterol esters when incubated in the presence of acetylated LDL,22 they stained for nonspecific esterase23 and reacted with the MAC-1 anti-macrophage antibody24 as expected for a macrophage-like cell line. For passaging, the cells were scraped from the bottom of the dishes and collected by centrifugation.

Radiolabeling

After washing the cells with HL-1 medium, the macrophages were resuspended in Medium A at a concentration of 2 x 10⁸ cells per ml and incubated under the standard conditions in the presence of 50 μCi of 35S-sulfuric acid per milliliter for 24 or 48 hours.

Collection of Labeled Material from Medium and Cells

The spent Medium A was removed, each culture dish was rinsed with 0.5 volumes of HL-1 medium, and the rinse was pooled with the spent medium. The pool was centrifuged, and solid guanidine hydrochloride (0.57 g/ml of medium) was added. Protease inhibitors were added to give the indicated final concentrations: 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 5 mM benzamidine hydrochloride. The cell layer was dissolved by first adding 0.5 volumes of 4 M guanidine hydrochloride containing 1 g/100 ml Triton X-100 and the protease inhibitors and then incubating for 30 minutes. The 35S-labeled macromolecules were separated from free 35S-sulfate by chromatography in Sephadex G-50 columns (16 ml total volume) eluted with 50 mM Tris buffer (pH 7.5) containing 6 M urea and 0.15 M NaCl. Radioactive fractions in the void volume were pooled and saved.

Ion-exchange Chromatography

The Sephadex G-50 void volume pools were applied to a 2.5 ml column of DEAE-Sephacel. The column was washed sequentially with 15 ml of 50 mM Tris (pH 7.5), containing 0.15 M NaCl and 6 M urea and then with 15 ml of 50 mM sodium acetate (pH 4.0) containing 0.15 M NaCl and 6 M urea. It was eluted with a 0.15 M to 1.5 M linear gradient of NaCl in 50 mM acetate (pH 4.0) containing 6 M urea (total volume of gradient = 60 ml). Fractions of 3 ml were collected and assayed for radioactivity. Those containing radiolabeled proteoglycan, which eluted at about 0.6 M NaCl, were pooled. The column was washed and eluted with pH 4 buffers since only highly acidic molecules such as proteoglycans will bind to DEAE-Sephacel at this pH.

To effect concentration of the material from the DEAE column, it was diluted three-fold in 10 mM Tris (pH 7.5) and adsorbed to a 0.2 ml column of DEAE-Sephacel. The proteoglycan was eluted with 800 μl of 50 mM Tris (pH 7.5), containing 1 M NaCl.
Alkaline Borohydride Elimination of Glycosaminoglycan Chains and Sepharose CL-4B Chromatography

The glycosaminoglycan chains were removed from the protein core by $\beta$-elimination with 0.05 M NaOH in the presence of 1 M NaBH$_4$, (alkaline borohydride) as described by Carlson. To the radiolabeled sample (containing 2000 to 8000 cpm) were added 250 $\mu$g (dry weight) of bovine nasal cartilage proteoglycan as carrier. One volume of 0.1 M NaOH containing 2 M NaBH$_4$ was added, and the samples were incubated at 45°C for 48 hours. Glacial acetic acid was added to neutralize the borohydride, and the samples were subsequently applied to a Sepharose CL-4B column (0.5 cm x 120 cm) and eluted with 0.01 M MOPS buffer (pH 7.0) containing 0.14 M Na$_2$SO$_4$. Fractions of about 0.5 ml were collected at a flow rate of 1 ml per hour and the radioactivity of each fraction was measured. Elution of the carrier was monitored by either the carbazole assay or the dimethylmethylen blue assay. The presence of the carrier increased the recovery of the radioactive proteoglycan and allowed the effectiveness of the reaction to be monitored.

Chondroitinase Digestion

Aliquots containing 2000 to 8000 cpm of the labeled proteoglycan samples and 50 to 250 $\mu$g (dry weight) of bovine nasal cartilage proteoglycan carrier in 50 mM Tris-HCl (pH 7.3) were treated with 0.1 units (nominal) of chondroitinase ABC or AC for 20 hours at 25°C. Samples were applied to a 16 ml Sephadex G-50 column to fractionate the undigested material (void volume) from the disaccharides (near the total volume). Elution of the disaccharides was monitored using the carbazole assay for uronic acid. Complete recovery of the uronic acid in the total volume of the column indicated that the digestion reached completion. The fractions that eluted in the total volume were pooled, and the 4-sulfated and the 6-sulfated disaccharides were separated by high performance liquid chromatography (HPLC) with the method of Hjerpe et al. Separation was done on a Lichrosorb NH$_2$ column (E. Merck, Darmstadt, West Germany) with isocratic elution with 75 mM acetate buffer (pH 4.0). Elution of the carrier disaccharides was monitored at 230 nm, and the peaks were collected and assayed for radioactivity.

Papain Digestion

Aliquots containing 2000 to 8000 cpm of the labeled proteoglycan samples and 250 $\mu$g (dry weight) of bovine nasal cartilage proteoglycan carrier in 50 mM sodium acetate, 5 mM EDTA, 20 mM cysteine (pH 5.8) were incubated with 10 units (nominal) of papain at 45°C for 20 hours. Digested samples were subjected to chromatography on Sepharose CL-4B as described for the alkaline borohydride-treated samples.

Nitrous Acid Treatment

Degradation of N-sulfated glycosaminoglycans with nitrous acid, at pH 2.5, was done by the method of Shively and Conrad. The products were separated by gel chromatography on Sephadex G-50.

Affinity Chromatography on Immobilized Low Density Lipoprotein

An affinity column was prepared by reacting human LDL with CNBr-activated Sepharose using the manufacturer's instructions, with the exception that all of the buffers contained EDTA to minimize oxidation of the lipoprotein. One gram of CNBr-activated Sepharose (dry weight) was reacted with 3 mg (on a protein basis) of LDL in 4 ml of 0.1 M NaHCO$_3$ buffer (pH 9.0) containing 0.5 M NaCl and 0.01% EDTA, for 2 hours at 25°C. Excess reactive groups on the Sepharose were blocked by reaction with 1 M ethanolamine (pH 8.0) containing 0.01% EDTA. Essentially all of the protein was covalently bound to the Sepharose as determined by protein analysis on the reaction mixture before and after the reaction. Control material was prepared by the same procedure but with the lipoprotein omitted.

Affinity chromatography on the immobilized LDL was performed by the following procedure. The radiolabeled P388D$_1$ proteoglycan was diluted into interaction buffer consisting of 30 mM CaCl$_2$, 50 mM NaCl, and 0.1% bovine serum albumin in 10 mM Tris, pH 7 (total ionic strength of the interaction buffer was 150 mM). One milliliter of this mixture was allowed to flow into a 1-ml column of the immobilized LDL and incubated at room temperature for 90 minutes. After the incubation, the column was washed with 4 ml of the above buffer and then was eluted with 5 ml of 10 mM Tris buffer (pH 7), containing 1 M NaCl. Fractions of 1 ml were collected, and aliquots were assayed for radioactivity. Potential inhibitors of the binding were added to the interaction buffer along with the radiolabeled P388D$_1$ proteoglycan.

Results

When the P388D$_1$ macrophage-like cells were incubated for up to two days in the presence of $^{35}$S-sulfate, a time-dependent incorporation of radioactivity into secreted macromolecular material (void volume of the G-50 column) was observed. Approximately 11 000 cpm and 19 000 cpm/10$^6$ cells (initial seeding density) were obtained after 24 and 48 hours of incubation, respectively. The cell culture medium (Medium A, see Methods) contained approximately 1 mM sulfate (from the serum and from magnesium sulfate and streptomycin sesquisulfate in the HL-1 medium), resulting in a final specific radioactivity of about 5 $\mu$Ci/µmol of sulfate or about 10$^4$ dpm/500 ng of chondroitin sulfate chains. The macromolecular material extracted from the cell layer contained less than 10% of the total incorporated radioactivity and was not characterized. The results described below were obtained with radiolabeled material isolated from the medium after a 24-hour labeling period.

The radiolabeled material from the void volume pool of the Sephadex G-50 column was further purified on DEAE-Sephacel by using a gradient elution scheme (Figure 1). The radioactivity that eluted in the 0.2 M NaCl wash was shown to be associated with macromolecular material, as
judged by its elution in the excluded volume of a Sephadex G-50 column. It was not degraded by chondroitinase ABC or nitrous acid and most likely consisted of sulfated glycoprotein. It was not characterized further. The labeled material that eluted from the DEAE when the concentration gradient reached about 0.6 M in NaCl was used for further characterization as described below. Elution from DEAE at this high ionic strength is characteristic of proteoglycans due to their high net negative charge.28

The radiolabeled material that was eluted from the DEAE-Sephacel was subjected to gel filtration chromatography on Sepharose CL-4B. The native preparation eluted with a Kd of 0.33 (Figure 2A). When the gel filtration was done in the presence of either 4 M guanidine hydrochloride or 6 M urea, the relative elution position on Sepharose CL-4B did not change, indicating that the proteoglycan was not aggregated with itself or other medium components (data not shown). However, other aggregating components could have been removed from the proteoglycan during the ion-exchange chromatography in the presence of 6 M urea.

The DEAE-purified material was reacted with dilute alkali or digested with papain. It is known that either of these treatments releases single glycosaminoglycan chains from cartilage proteoglycan.29 After treating the preparation with alkali, the radioactivity emerged in a more included position with a Kd of 0.63 (Figure 2A). The shift to an elution position corresponding to a smaller molecular size suggested that the original material consisted of a proteoglycan from which glycosaminoglycan chains were released by β-elimination with alkali. The glycosaminoglycan chains released from the bovine nasal cartilage proteoglycan carrier also eluted from the Sepharose CL-4B with a Kd of 0.63 (arrow in Figure 2A). Therefore, the radiolabeled chains are similar in size to those from the cartilage proteoglycan, which have an average molecular weight of about 20 000.30 As indicated by the relative sizes of the radioactive peaks in Figure 2A, the recovery of the free glycosaminoglycan chains from the gel column was essentially quantitative, while that of the native proteoglycan was about 50%. The recovery of the proteoglycan was not improved by the addition of 6 M urea or 4 M guanidine hydrochloride to the elution buffer (data not shown).

After exhaustive digestion with papain, the 35S-labeled proteoglycan eluted from the Sepharose CL-4B column in a bimodal fashion (Figure 2B). The first peak had a Kd of 0.44 and most likely consisted of a papain-resistant portion of the protein core, while the following shoulder had a Kd of 0.61 and probably contained small peptides with single glycosaminoglycan chains attached. As expected, the cartilage proteoglycan carrier in the same papain digestion mixture was degraded to short peptides containing single glycosaminoglycan chains as indicated by the fact that the papain digestion products eluted at the same position as those from the alkali-treated carrier (Figures 2A and 2B). Thus, the incomplete degradation of the...
P388D, proteoglycan protein core was not the result of insufficiently rigorous digestion conditions. Most likely, the papain resistance of the 0.44 Kd material was due to the presence of closely spaced carbohydrate chains which protected a portion of the protein core from enzymatic attack and/or a domain composed of a particularly protease-resistant amino acid sequence.

Chondroitinase ABC degrades chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate chains to disaccharides but does not degrade hepanan sulfate or keratan sulfate, while chondroitinase AC will digest chondroitin 4-sulfate and chondroitin 6-sulfate only. Heparan sulfate contains N-sulfated glucosamine residues. At low pH, the sulfate groups are removed, leaving glucosamine residues, which are susceptible to deamination and cleavage in the presence of nitrous acid. Thus, the two chondroitinase enzymes and nitrous acid can be used in combination to estimate the relative content of the various glycosaminoglycans in a proteoglycan preparation. After digestion of the DEAE-purified proteoglycan with either chondroitinase ABC or chondroitinase AC, more than 95% and 92%, respectively, of the radiolabel eluted in the total volume of a Sephadex G-50 column. No degradation of the glycosaminoglycan chains by nitrous acid could be detected. Thus, the 35S-labeled glycosaminoglycan chains consisted of at least 92% chondroitin 4- or 6-sulfate and about 3% dermatan sulfate.

The disaccharides, which are released by chondroitinase ABC digestion of the proteoglycan chains, contain N-acetylgalactosamine residues which may be either unsulfated or sulfated in the 6-position only, the 4-position only, or in both positions. The HPLC method of Hjerpe et al. fractionates the chondroitinase-generated chondroitin sulfate disaccharides on the basis of sulfation position. Analysis by this procedure indicated that the N-acetylgalactosamine residues in the chondroitin sulfate chains were 82% 6-sulfated and 18% 4-sulfated. Although the elution time for the 4,6-disulfated N-acetylgalactosamine-containing disaccharide was not known, the fact that more than 95% of the label applied to the HPLC column could be recovered in the two monosulfate fractions indicated that little, if any, disulfated disaccharide was present in the digestion mixture. Since the elution from the HPLC column was monitored by assaying for 35S-sulfate radioactivity, any unsulfated disaccharide would not have been detected.

Figure 3. Polyacrylamide gel electrophoresis of the 35S-labeled proteoglycan before and after chondroitinase ABC digestion. An aliquot of the 35S-labeled material from the DEAE-Sephacel (6500 cpm) in 90 μl of 50 mM Tris (pH 7.4) containing 50 mM sodium acetate was digested with 0.05 units of chondroitinase ABC for 3 hours at 37°C. The entire mixture was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described in the text. Lane A. Undigested 35S-labeled proteoglycan (700 cpm). Lane B. Chondroitinase ABC-digested proteoglycan (6500 cpm in the total digest). The top of the running gel is marked R. A stacking gel is above the running gel.

The molecular weight of the protein core remaining after degradation of the chondroitin sulfate chains with chondroitinase ABC was estimated by electrophoresis on a polyacrylamide slab gel in the presence of sodium dodecyl sulfate. After chondroitinase ABC digestion, one or two chondroitin sulfate repeat disaccharides remain attached to the chondroitin sulfate-protein core linkage tetrasaccharides. Therefore, about 5% of the total sulfate label remained associated with the protein core of the proteoglycan after exhaustive treatment with this enzyme (data not shown), and the protein core could be detected by autoradiography of the dried gel. As shown in Figure 3, the chondroitinase digestion product migrated as a single band with an apparent size of 36 000 daltons. The size as determined by gel electrophoresis is only approximate since the residual chondroitin sulfate chains may cause the protein core to migrate anomalously. A portion of the undigested proteoglycan was retarded by the stacking gel, with the remainder barely entering the running gel (3% to 20% gradient gel). No other bands were detected when the x-ray film was exposed to the dried gel for up to 60 days.

The molecular weight of the intact proteoglycan was estimated by gel permeation chromatography on Sephacore CL-2B in the presence of 4 M guanidine hydrochloride. As shown in Figure 4, the radiolabel eluted from the column with a Kd of 0.69. According to the relationship developed by Ohno et al., this distribution coefficient corresponds to a weight average Mw of 275 000.

To determine whether or not the P388D, macrophage proteoglycan interacted with lipoprotein, the DEAE-purified preparation was incubated on a column of immobilized LDL under conditions which were shown to be optimum for binding. To minimize nonspecific binding, the interaction was done in the presence of 0.1% bovine serum albumin. More than 90% of the total applied label bound to the column of immobilized LDL (Table 1). Only 5% of the P388D, proteoglycan bound to the control
that remained after chondroitinase ABC digestion had an  

proteoglycan protein core. These chains contained both 6-sulfated and 4-sulfated \( \text{N-acetylgalactosamine} \) residues. It is not known whether the two \( \text{N-acetylglactosamine} \) sulfate isomers reside on the same chain as is true for the bovine nasal cartilage proteoglycan\(^{34} \) or whether they are on separate chondroitin sulfate chains. The proteoglycan protein core that remained after chondroitinase ABC digestion had an M, of about 36 000 as determined by polyacrylamide gel electrophoresis. Chondroitinase ABC digestion of proteoglycans degrades most of the repeat disaccharide portion of the chondroitin sulfate chains but leaves, attached to the protein core, hexa- and octasaccharides.\(^{32} \) Thirteen such hexa- and octasaccharides would account for a combined mass of 15 000 to 21 000 daltons, and the size of the P388D, proteoglycan protein core, minus the residual oligosaccharides, would be approximately 18 000 daltons. The protein core contained a domain that was resistant to cleavage by papain. Thus, the P388D, proteoglycan does not resemble the cartilage large chondroitin sulfate proteoglycan, which consists of about 100 chondroitin sulfate chains attached to a 250 000 dalton protein core.\(^1 \) However, it is similar to small chondroitin sulfate proteoglycans synthesized in culture by a variety of noncartilage cells.

Levitt and Ho\(^4 \) found that cultures of human peripheral blood mononuclear cells (containing B and T cells and monocytes) synthesized chondroitin 4-sulfate proteoglycan and heparan sulfate proteoglycan, which were found both associated with the cells and in the medium. In the presence of cell mitogens such as concanavalin A, these cells produced mainly a secreted chondroitin 4-sulfate proteoglycan with an M, of about 130 000 (Kav of 0.23 on Sepharose 6B). It was estimated that four chondroitin sulfate chains of M,=25 000 were attached to a 35 000 dalton protein core. Somewhat similar results were obtained by Kolset and Kjellén\(^{35} \) who showed that the essentially pure populations of human peripheral blood monocytes secreted a proteoglycan (Kav of 0.23 on Sepharose CL-6B) that contained chondroitin 4-sulfate chains of M,=14 000 to 17 000. The size of the chondroitinase-generated core was judged to be about 11 000 daltons. After 5 days in culture, these cells became macrophage-like and secreted a proteoglycan with slightly larger chondroitin sulfate chains (M,=22 000) containing disulfated \( \text{N-acetylglactosamine} \) residues. Further results from the same laboratory demonstrated that human peritoneal macrophages secreted a proteoglycan containing the slightly larger and more highly sulfated chains similar to those found on the human monocyte-derived macrophage proteoglycan.\(^9 \) On the other hand, the U-937 macrophage-like cell line, which was derived from a human lymphoma, secreted a proteoglycan that contained very large chondroitin 4-sulfate chains of M,=60 000.\(^{36} \) The protein cores of the proteoglycans secreted by the U-937 cell line and by the human monocytes and the monocyte-derived macrophages were susceptible to total degradation by papain.\(^{36} \)

The P388D, proteoglycan is similar also to that isolated from a murine parietal yolk sac (PYS-2) cell line. The PYS-2 proteoglycan had a molecular weight of 200 000 to 300 000, contained 10 to 15 glycosaminoglycan chains of M,=14 000 to 16 000, and contained a papain-resistant domain.\(^{37} \) After chondroitinase digestion, the PYS-2 proteoglycan yielded two polypeptides, the largest of which had a molecular weight of 34 000. Similarly, a rat yolk sac tumor cell line (L2 cells) secreted a chondroitin sulfate proteoglycan consisting of a protein core of molecular weight 10 200, as determined by cDNA analysis, with
about 14 chondroitin 4-sulfate chains attached.38 However, the L2 chondroitin sulfate chains were significantly larger ($M_r = 40,000$) than those of either the PYS-2 or the P388D proteoglycan, and the protein core did not contain papain-resistant domains.

A chondroitin 4-sulfate proteoglycan with an $M_r$ of about 250,000 was isolated from intracellular granules of mouse interleukin 3-dependent, bone marrow-derived mast cells.39 As in the human monocyte/macrophage proteoglycan, the chondroitin 4-sulfate chains, which were of $M_r = 12,000$, contained 4,6-disulfated N-acetylgalactosamine residues. The chondroitinase ABC-generated protein core had an $M_r$ of about 10,000 which was not further reduced in size by protease digestion.

It is difficult to accurately measure the molecular weight of proteoglycans because of their polydispersity and their high polysaccharide content. The glycosaminoglycans are also highly polydisperse. Furthermore, the molecular weights of the proteoglycan protein cores are usually determined on the chondroitinase-digested proteoglycans, which may contain up to 50% residual carbohydrate. If these experimental difficulties are taken into account, it is apparent that the P388D, proteoglycan does resemble those synthesized by the human monocytes and macrophages, the rodent yolk sac cells, and the mast cells. However, as opposed to the P388D, proteoglycan, none of the above proteoglycans contained any N-acetylgalactosamine mono-6-sulfate residues and only the PYS-2 and the mouse mast cell proteoglycans contained protease-resistant protein cores.

Mouse peritoneal macrophages in primary culture synthesize both cell-associated and secreted proteoglycan(s) containing heparan sulfate, dermatan sulfate, and chondroitin 4-sulfate chains.40 The proteoglycans were not purified and no information was obtained on the properties of either the individual glycosaminoglycans or the protein cores. In contrast to the mouse peritoneal cells, the P388D, cell line apparently secretes a single proteoglycan which contains chondroitin 4- and 6-sulfate chains.

One of the earliest microscopic events seen upon induction of atherosclerosis in experimental animals is the recruitment and attachment of blood-borne monocytes to focal regions of the aortic endothelium. After attachment, the cells migrate into the subendothelial space and develop into macrophages.41 The fatty streak, which is the first gross feature observed in the arterial wall after initiation of the experimental atherosclerotic diet, consists largely of foam cells and foam cell-derived extracellular cholesterol. A major portion of the foam cells have been identified by immunohistochemical methods as being cholesterol ester-filled macrophage/monocytes.42 In culture, the uptake of LDL by macrophages is under strict negative feedback control and the formation of foam cells is not observed. The manner in which foam cells are formed in vivo is not understood and is an active area of investigation in a number of laboratories. Recent results suggest that one possible mechanism for the intracellular accumulation of lipid involves the uptake of LDL complexed with proteoglycans.

Proteoglycans isolated from aorta form soluble and insoluble complexes with low density lipoprotein.11 Either desulfation of the chondroitin sulfate or specific modification of the lysine or arginine residues in LDL abolishes the interaction, suggesting that the sulfate groups on the chondroitin sulfate chains interact with the basic amino acid groups on the apolipoprotein B. Free glycosaminoglycan chains bind to LDL much more weakly than do the proteoglycans from which the chains are derived.11,43 Apparently, in the native proteoglycan, the sulfate groups are held in optimum configuration and density for maximal interaction with LDL. Recently, it has been shown that human monocyte-derived macrophages and peritoneal macrophages in culture take up proteoglycan-LDL complexes, accumulate intracellular cholesterol esters, and subsequently become foam cells.12-15 Thus, proteoglycans may play a role in the accumulation of lipid during the development of atherosclerotic lesions by enhancing foam cell formation. In addition, secreted proteoglycans may form insoluble complexes with lipoproteins and thereby trap lipids in the extracellular matrix of the arterial wall.

As discussed above, the P388D, cell line secreted a proteoglycan which contained both 4- and 6-sulfated chondroitin sulfate chains, while the mouse peritoneal macrophages and the human monocyte/macrophages secreted only the 4-sulfated isomer of this glycosaminoglycan. Results reported in the literature suggest that the sulfation pattern of macrophage chondroitin sulfate proteoglycans might be controlled by the cellular environment. For example, when guinea pig peritoneal macrophages were maintained in suspension, relatively high levels of chondroitin 6-sulfate, but no 4-sulfated chains,
were found in the cell surface compartment. On the other hand, when the cells were grown in monolayer, the opposite was true. The synthesis of the 6-sulfate by the P388D1 cell line may be related to the fact that these cells attach weakly to cell culture dishes. In contrast, the peripheral blood monocyte/macrophages and the peritoneal macrophages, cells which secrete the 4-sulfated chains, are isolated and purified by taking advantage of their ability to adhere tenaciously to culture dishes. The type of chondroitin sulfate chains synthesized by macrophages in in vivo environments is not known. However, it is possible that when macrophages are in contact with extracellular matrix components, the cells synthesize proteoglycans and/or glycosaminoglycan chains different from those produced in culture.

The finding that the P388D1 proteoglycan bound to the LDL affinity column was not unexpected in view of results obtained by others which have indicated that chondroitin 6-sulfate-containing proteoglycans and glycosaminoglycans interact strongly with LDL. Investigations with chondroitin sulfate isolated from human aortic wall showed that the 6-sulfated, as compared to the 4-sulfated chains, bound more strongly to immobilized LDL. In the present study, chondroitin 6-sulfate was more effective than chondroitin 4-sulfate in blocking the binding of radiolabeled P388D1 proteoglycan to immobilized LDL (Table 1). Furthermore, chondroitin 6-sulfate is a major component of proteoglycan-lipoprotein complexes isolated from saline-extracted atherosclerotic lesions. It is possible that when macrophages are in contact with smooth muscle cells, those produced in culture cannot be ruled out at this time. Most likely macrophages synthesize a minor portion of the extracellular proteoglycan found in the artery wall as a whole. However, in the restricted region of the subendothelial space to which these cells are recruited during fatty streak induction and development, macrophage proteoglycans could be present in significant amounts and play an important role in the deposition of lipids both by enhancing foam cell formation and by forming insoluble complexes with lipoproteins.

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