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Human Monocyte-derived Macrophages Secrete Two Forms of Proteoglycan-Macrophage Colony-stimulating Factor That Differ in Their Ability to Bind Low Density Lipoproteins*

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This study evaluated whether human monocyte-derived macrophages synthesize specific types of proteoglycans with lipoprotein-binding capability that could contribute to lipid retention in the arterial wall. After labeling with either [^{35}S]SO_4 or [^{35}S]methionine, macrophages secreted a high molecular mass proteoglycan, with glycosaminoglycan chains of ∼18 kDa and core protein bands of ∼100 and 55 kDa. Both core protein bands were recognized by an antibody to PG-100, an antibody that recognizes the proteoglycan form of macrophage colony-stimulating factor (PG-100/PG-MCSF). The interaction between PG-100/PG-MCSF and low density lipoproteins (LDL) was examined by gel mobility shift. In this system, PG-100/PG-MCSF was resolved further into two forms. The two forms had the same core proteins but differed in their overall size and glycosaminoglycan content. The larger form contained glycosaminoglycan chains that were entirely chondroitin ABC lyase-sensitive, whereas the smaller form contained chains that were sensitive to both chondroitin ABC lyase and heparinase. Both forms bound native LDL with high affinity, but the larger form bound LDL with higher affinity than the smaller form. The glycosaminoglycan chains of PG-100/PG-MCSF, but not the core proteins, were responsible for binding to native LDL. Mildly oxidized LDL and methyl-LDL, which have an electrophoretic charge similar to that of native LDL, also bound PG-100/PG-MCSF. In contrast, extensively oxidized LDL and acetyl-LDL, which are more electronegative than native LDL, did not bind to either form of PG-100/PG-MCSF. The demonstration of two forms of human monocyte-derived macrophage PG-100/PG-MCSF which bind LDL may represent an additional role for macrophages in the extracellular trapping of lipoproteins in atherosclerosis.

The monocyte-derived macrophage is an important cell in the pathogenesis of atherosclerosis (1). In addition to its ability to phagocytose large amounts of lipid, the macrophage is capable of secreting many factors that contribute to lesion development. Included among these secreted factors are proteoglycans (2), which are a heterogeneous group of molecules that have the common structure of a core protein to which glycosaminoglycan chains are covalently attached.

The interactions of proteoglycans with other macromolecules in the arterial wall can affect numerous processes, including vascular permeability, hemostasis, thrombosis, and lipoprotein metabolism (3–8). Proteoglycan-lipoprotein interaction, leading to lipoprotein retention in the arterial intima, has been hypothesized to be the major cause of extracellular lipid accumulation within atherosclerotic lesions (6). This proteoglycan-mediated extracellular lipid accumulation has a number of important biological consequences, including increased susceptibility of lipoproteins to oxidation (9), increased lipoprotein aggregation (5), and rapid internalization of lipoprotein-proteoglycan complexes by both macrophages (10) and smooth muscle cells (11). All of these events potentially can contribute to foam cell formation and development of atherosclerotic lesions.

To date, much of the understanding of arterial proteoglycan structure and function has been derived from studies of arterial smooth muscle cell proteoglycans. However, less is known about the proteoglycans secreted by monocyte-derived macrophages, which also could contribute to proteoglycan-mediated lipid retention and foam cell formation in the arterial wall. For example, macrophages from other sources, such as cholesterol-enriched pigeon peritoneal macrophages and P388D1 macrophage-like cells, synthesize proteoglycans that can bind low density lipoproteins (LDL)[1] (12, 13). In addition, the differentiated THP-1 macrophage cell line synthesizes cell surface proteoglycans that bind lipoprotein lipase (15), which has been shown to enhance the binding of LDL to extracellular matrix proteoglycans of the arterial wall (16). Although these studies have not identified specific types of proteoglycans, they have suggested that the deposition of these proteoglycans in the extracellular matrix by macrophages may play an important role during atherogenesis.

The overall goal of this study was to determine whether or not human monocyte-derived macrophages (HMDM) synthesize specific types of proteoglycans with lipoprotein binding capability that could contribute to lipid retention in the arterial wall. We demonstrate that HMDM synthesize and secrete two major proteoglycan forms that differ in size, glycosaminoglycan composition, and affinity for native LDL (N-LDL). Both forms bind LDL with high affinity. The larger form contains only chondroitin sulfate/dermatan sulfate and binds to N-LDL with higher affinity than the smaller form, which contains chondroitin sulfate/dermatan sulfate and heparan sulfate. Further, the predominant component of these secreted proteoglycans is identified as the proteoglycan form of the growth factor macrophage colony-stimulating factor (PG-MCSF). The finding that HMDM secrete a novel proteoglycan with lipoprotein-binding

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[1] The abbreviations used are: LDL, low density lipoprotein; HMDM, human monocyte-derived macrophages; N-LDL, native LDL; PG-MCSF, proteoglycan form of macrophage colony-stimulating factor; PAGE, polyacrylamide gel electrophoresis.

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capability suggests an additional role for the macrophage in the pathogenesis of atherosclerosis.

EXPERIMENTAL PROCEDURES

Cell Culture

Monocyte-derived Macrophages—Human monocytes were isolated by density gradient ultracentrifugation by the method of Böyum (17), as described previously (18), and maintained in RPMI 1640 medium supplemented with 20% autologous serum containing 2 mM glutamine (2×10^6 cells/35-mm dish). Cells were fed twice weekly for 8–10 days by which time they were differentiated into macrophages (19). Monocyte-derived macrophages were metabolically labeled with either 50 μCi/ml $^{35}SO_4$- or ^{35}S-labeled methionine (ICN, Costa Mesa, CA) in fresh medium for 48 h. Labeled medium was harvested, and protease inhibitors (0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, and 0.1 mM phenylmethylsulfonyl fluoride) were added (20). The radiolabeled cell layer was harvested with 8 M urea buffer, 0.25 M NaCl, 0.5% Triton X-100, containing protease inhibitors (20).

Smooth Muscle Cells—Human and monkey vascular smooth muscle cells were isolated from the thoracic aorta by the explant method and maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum, as described previously (20). Smooth muscle cells were metabolically labeled with 50 μCi/ml $^{35}SO_4$ in fresh medium for 48 h. Labeled medium was harvested with protease inhibitors.

Proteoglycan Isolation and Characterization

Isolation—Medium from metabolically labeled macrophage cultures or smooth muscle cells was applied to DEAE-Sephacel minicolumns equilibrated in 8 M urea buffer, 0.25 M NaCl, 0.5% Triton X-100 and were eluted with 8 M urea buffer containing either 3 M NaCl, 0.5% Triton X-100, or a gradient of 0.25–1 M NaCl (20). This ion exchange chromatography step served to remove free radiolabel and to concentrate proteoglycan samples.

Isolation of Proteoglycans from Agarose—$^{35}SO_4$-Labeled proteoglycans were resolved further by agarose gel electrophoresis and characterized. $^{35}SO_4$-Proteoglycans were applied to a single wide lane of an agarose gel and electrophoresed for 7 h at 60 V and 4 °C. A portion of the gel was sliced into 0.5-cm segments for scintillation counting and location of the proteoglycan species. The proteoglycan species that were resolved by this method were cut out of the agarose, which then was melted and diluted 10-fold with 8 M urea buffer containing 0.5% Triton X-100 and 0.25 M NaCl. The diluted agarose was reconcentrated on DEAE-Sephacel minicolumns, and proteoglycans were eluted with 8 M urea buffer containing 0.5% Triton X-100 and 3 M NaCl (21).

Analysis of Molecular Size—$^{35}SO_4$-Proteoglycan and [^{35}S]methionine-labeled core protein molecular sizes were characterized by SDS-PAGE under reducing conditions according to the procedure of Laemmli (22). Chondroitin and dermatan sulfate glycosaminoglycan chains were removed by digestion with 0.03 unit/ml chondroitin ABC lyase (ICN, Costa Mesa, CA) in 0.3 M Tris-HCl, pH 8.0, 0.6 mg/ml bovine serum albumin, and 18 mM sodium acetate with protease inhibitors for 3 h at 37 °C (20). Heparan sulfate glycosaminoglycan chains were removed by digestion with 20 units/ml heparinase I (Sigma) in 0.1 M Tris-HCl, pH 7.0, 10 mM calcium acetate, and 18 mM sodium acetate with protease inhibitors for 3 h at 37 °C followed by the addition of 20 units/ml heparinase II (Sigma) and incubation for overnight at 41 °C (20). The ^{35}S-labeled intact proteoglycans and core proteins (after enzyme digestion) were visualized by fluorography of dried gels treated previously with Enlightning™ (NEN Life Science Products) and exposed to Kodak XAR-2 film at −70 °C.

Analysis of Hydrodynamic Size—The hydrodynamic size of $^{35}SO_4$-proteoglycans was analyzed on Sepharose CL-4B (0.7 × 73 cm) and CL-6B (0.7 × 63 cm) molecular sieve columns equilibrated in 8 M urea buffer with 0.5% Triton X-100 (20). Fractions of 1 ml were collected, and an aliquot of each fraction was assayed for radioactivity by liquid scintillation counting. The elution position of free $^{35}SO_4$ was used as a marker for the total volume (V_t).

Glycosaminoglycan Isolation and Analysis—Glycosaminoglycan chains were released from proteoglycans by reductive β-elimination with 1 M sodium borohydride in 50 mM NaOH for 4 h at 45 °C (20). The reaction was terminated by neutralizing the sample with glacial acetic acid. Glycosaminoglycan chains then were applied to a Sepharose CL-6B column (0.7 × 63 cm) in 0.2 M Tris, pH 7.0, with 0.2 M NaCl for analysis of chain length by size exclusion chromatography (23). The elution position of free $^{35}SO_4$ was used a marker for the V_t).

Immunoblot Analysis—Core proteins of chondroitin ABC lyase- or heparinase I- and II-digested samples were separated on SDS-PAGE 4–12% gradient gels and transferred to nitrocellulose membranes at 20 V for 70 min in 40 mM Tris, 50 mM glycine with 20% methanol and 0.0375% SDS in a semidry electrophoretic transfer apparatus (Bio-Rad). Membranes were blocked with 2% calf serum in 50 mM Tris-buffered saline, pH 7.4, with 0.05% Tween 20 (TBST) and then incubated overnight at 4 °C with rabbit antiserum prepared against recombinant human versican (1:1,000 in blocking buffer) (kindly provided by Dr. R. LeBaron, San Antonio TX) (24), a peptide sequence near the NH_2 terminus of human biglycan (1:1,000) (kindly provided by Dr. L. Fisher, NIH) (25), a peptide from human decorin (1:1,000) (also provided by Dr. L. Fisher) (25), bovine glomeruli heparan sulfate proteoglycan (1:100) (Chemicon, El Segundo CA), or human osteosarcoma PG-100/PG-MCSF (1:1,000) (kindly provided by Dr. H. Kresse, University of Munster, Munster, Germany) (26). Nitrocellulose membranes were washed with 0.1% calf serum in TBST before incubation with alkaline phosphatase-conjugated goat anti-rabbit antiserum (1:2,000 dilution in TBST with 0.1% bovine serum albumin) (Boehringer Mannheim) for 1 h at room temperature. After washing, the membranes were developed by enhanced chemiluminescence (Pierce) and visualized by fluorography on Kodak XAR-2 film.

Lipoprotein Preparation

Isolation—LDLs were isolated from plasma of normal human volunteers as described previously (27). In brief, LDL (d = 1.019–1.063 g/ml) was separated from normal human plasma by preparative ultracentrifugation in a Beckman VTi 50 vertical rotor (28) and purified by sequential density gradient ultracentrifugation (27).

Modification—N-LDL was oxidized in the presence of copper by incubation of LDL (300 μg/ml) in the presence of 5 μM copper sulfate for 4 h at room temperature (mildly oxidized LDL) or 18 h at 37 °C in air (extensively oxidized LDL) (29). LDL was acetylated as described (30). In brief, LDL (1–3 mg/ml) in 0.15 M NaCl, with 0.01% EDTA, was added to an equal volume of a saturated sodium acetate solution with continuous stirring on ice, after which four 1.5-μl aliquots of acetic anhydride were added every 15 min (acetyl-LDL). Reductive methylation of LDL was performed as described (31). 1 mg of sodium borohydride and 5 μl of 7.4% aqueous formaldehyde were added to 6–10 mg of LDL. Additional 5-μl aliquots of formaldehyde were added every 6 min for 30 min (methyl-LDL). The reaction was stopped by dialysis against 0.15 M NaCl, with 0.01% EDTA. The extent of modification was assessed by measuring thiobarbituric acid-reactive substances (32), trinitrobenzenesulfonic acid reactivity, and electrophoretic mobility (30).

Analysis of Lipoprotein-Proteoglycan Binding by Gel Mobility Shift Assay

Binding of LDL to proteoglycans, glycosaminoglycans, or core protein was measured using a modification (33) of a gel mobility shift assay developed for assessing the binding of proteins to DNA (34). 10 μl of $^{35}SO_4$-proteoglycans, $^{35}SO_4$-glycosaminoglycans, or [^{35}S]methionine-core proteins (in 10 mM Hepes, pH 7.2, with 140 mM NaCl, 2 mM $MgCl_2$, and 5 mM $CaCl_2$), were mixed with 10 μl of increasing concentrations (0–1 mg/ml) of N-LDL or modified LDL in the same buffer and incubated for 1 h at 37 °C. 3 μl of glycerol:bromphenol blue (1:1 v/v) was added to the samples, and 20 μl was loaded into wells of Nu-Sieve (FMC Bioproducts, Rockland, ME) agarose gels (0.7% in 10 mM Hepes, pH 7.4, with 2 mM $CaCl_2$ and 4 mM $MgCl_2$). Samples were electrophoresed for 3 h at 60 V 4 °C with buffer recirculation. The gels were fixed with 0.1% cetyl pyridinium chloride in 70% EtOH for 2 h, air dried, and analyzed by autoradiography. In this system, free $^{35}SO_4$-proteoglycans migrate toward the front of the gel, whereas $^{35}SO_4$-proteoglycans complexed to lipoproteins remain near the origin of the gel. Autoradiograms were scanned with a Hewlett-Packard Scan Jet IIcx using ImageQuant software. Binding constants were determined from quantification of free $^{35}SO_4$-proteoglycans migrating at the front of the gel with SAAM II software using Michaelis-Menten equations (SAAM Institute, Seattle).

RESULTS

Ion Exchange Chromatography of $^{35}SO_4$-Labeled Material Synthesized by HMDM—The distribution of newly synthesized proteoglycans into the medium and cell layer of macrophage cultures was determined from the yields of $^{35}SO_4$-labeled medium in the two compartments after ion exchange chromatography. The majority (~75%) of the newly synthesized proteoglycans appeared in the medium after a 24-h label, whereas ~25% was associated with the cell layer.

HMDM $^{35}SO_4$-labeled medium was applied to DEAE-Sepha-

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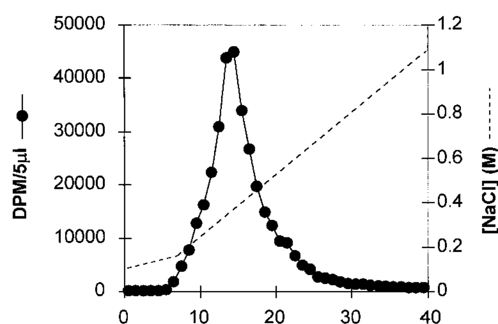

FIG. 1. **Ion exchange chromatography of $^{35}SO_4$-labeled material synthesized by HMDM.** HMDM were metabolically labeled with $^{35}SO_4$ for 48 h. Medium (●) was applied to analytical DEAE-Sephacel columns in 8 M urea buffer containing 0.5% Triton X-100 with 0.25 M NaCl and eluted with a gradient of 0.25–1 M NaCl in 8 M urea buffer containing 0.5% Triton X-100 (*dashed line*).

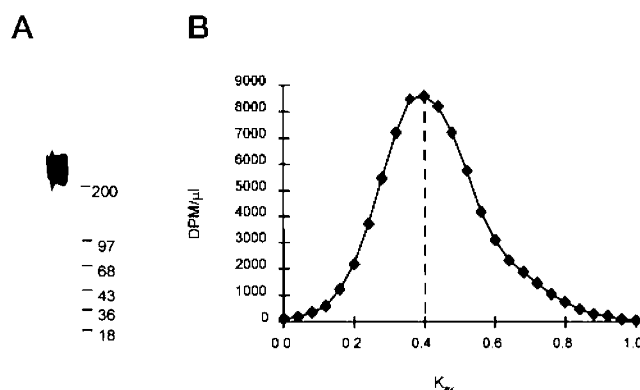

FIG. 2. **HMDM synthesize high molecular mass proteoglycans.** *Panel A*, proteoglycans secreted into the medium were purified by ion exchange chromatography and characterized by SDS-PAGE (4–12% gradient with a 3.5% stacking gel). *Panel B*, secreted proteoglycans were characterized by size exclusion chromatography on Sepharose CL-4B under dissociative conditions (8 M urea buffer with 0.5% Triton X-100). The *dashed line* indicates the mean hydrodynamic size (K_{av}) of the intact proteoglycan.

cel and eluted with a 0.25–1 M NaCl gradient. Secreted $^{35}SO_4$-labeled material eluted as a single peak at 0.5 M NaCl (Fig. 1), which is approximately the same concentration of NaCl required to elute the proteoglycans secreted by arterial smooth muscle cells in culture (20). The elution profile of $^{35}SO_4$-labeled material associated with the cell layer was similar to that of the secreted material (data not shown). Because the majority of the newly synthesized proteoglycans was secreted into the medium and because the same ionic strength was required to isolate proteoglycans by ion exchange chromatography from both the medium and cell layer, only the medium compartment was characterized further.

SDS-PAGE and Molecular Sieve Chromatography of Monocyte-derived Macrophage Proteoglycans—To characterize the hydrodynamic size and types of proteoglycans synthesized by monocyte-derived macrophages, $^{35}SO_4$-labeled medium was subjected to SDS-PAGE and size exclusion chromatography. On SDS-PAGE under reducing conditions, $^{35}SO_4$-labeled material secreted into the medium migrated as a single broad band, with an apparent molecular mass between 200 and 350 kDa (Fig. 2*A*).

To assess whether these proteoglycans could be resolved further by hydrodynamic size, ^{35}S-labeled material was chromatographed on Sepharose CL-4B molecular sieve columns under dissociative conditions (21). The $^{35}SO_4$-labeled material was not resolved further but eluted within the inclusion volume of the column as a single peak at K_{av} = 0.40 (Fig. 2*B*). It should be noted that because of the nonglobular chromatographic nature of intact proteoglycans, this is not a method for determination of the molecular mass of proteoglycan species.

To determine whether all of the $^{35}SO_4$-labeled material was proteoglycans and to determine the size of the glycosaminoglycan chains associated with the proteoglycan, the chains were released from the core proteins by reductive alkaline β-elimination and analyzed for hydrodynamic size by molecular sieve chromatography. Associative conditions were used to preserve the structure of the glycosaminoglycan chains for further characterization. On Sepharose CL-6B, the intact proteoglycan eluted within the inclusion volume of the column with a peak at K_{av} = 0.18. After reductive alkaline β-elimination, the elution profile shifted to a K_{av} = 0.40, indicating that the liberated glycosaminoglycan chains had an apparent molecular mass of ~18 kDa (23) (Fig. 3*A*). Fractions corresponding to free glycosaminoglycan chains were pooled and reconcentrated by DEAE-ion exchange chromatography for further characterization.

Size exclusion chromatography and digestion of glycosaminoglycan chains with specific enzymes aided in determination of the general types of proteoglycans present. Glycosaminoglycan chains purified by Sepharose CL-6B (Fig. 3*A*) were treated with chondroitin ABC lyase or heparinase treatment, and the degradation products were analyzed by Sepharose CL-4B size exclusion chromatography (Fig. 3*B*). When the total free glycosaminoglycan chains were treated with chondroitin ABC lyase, ~80% of the $^{35}SO_4$ radioactivity eluted at the column V_t on Sepharose CL-4B, whereas ~20% of the radioactivity was not degraded. Treatment of free glycosaminoglycan chains with heparinases I and II resulted in ~10% of the $^{35}SO_4$ radioactivity eluting at V_t, whereas the remaining ~90% was not degraded and eluted with the same mean K_{av} = 0.40 as the undigested chains. Thus, macrophages secrete proteoglycans of high apparent molecular mass which contain primarily chondroitin sulfate/dermatan sulfate glycosaminoglycans and a minor amount of heparan sulfate glycosaminoglycans.

Identification of Macrophage Proteoglycan Core Proteins—[^{35}S]Methionine-proteoglycans were treated with chondroitin ABC lyase and heparinase for analysis SDS-PAGE. With chondroitin ABC lyase treatment, macrophage proteoglycans were found to contain two core protein bands of ~100 and ~55 kDa (Fig. 4*A*, *lane 2*). No core protein bands were visualized after treatment of the proteoglycans with heparinase I and II (Fig. 4*A*, *lane 3*).

To identify the macrophage core protein bands, a series of Western immunoblots was performed using a panel of antibodies against known proteoglycans. Core proteins for the major proteoglycans secreted by smooth muscle cells, versican, biglycan, and decorin, were not detected in macrophage proteoglycan preparations (Fig. 4*B*). However, an antibody against PG-100/PG-MCSF recognized both the 100-kDa and 55-kDa core protein bands (Fig. 4*C*, *lane 1*). In some, but not all, experiments, this antibody also recognized another band at ~65 kDa (Fig. 4*C*, *lane 1*). This band also was seen in some experiments in the enzyme control conditions (data not shown). In contrast, medium from radiolabeled cultures of non-human primate arterial smooth muscle cells did not contain immunodetectable PG-100/PG-MCSF (Fig. 4*C*, *lane 2*).

Analysis of Lipoprotein Binding Properties of Macrophage Proteoglycans—The lipoprotein-binding capacity of the proteoglycans secreted by HMDM was evaluated using an agarose gel mobility shift assay. In this system, free $^{35}SO_4$-proteoglycans migrate toward the front of the gel, whereas $^{35}SO_4$-proteoglycans complexed to lipoproteins remain near the origin of the gel. In the absence of any lipoprotein, the $^{35}SO_4$-proteoglycans were resolved into two bands by this system (Fig. 5*A*). In the presence of increasing concentrations of lipoprotein, both pro-

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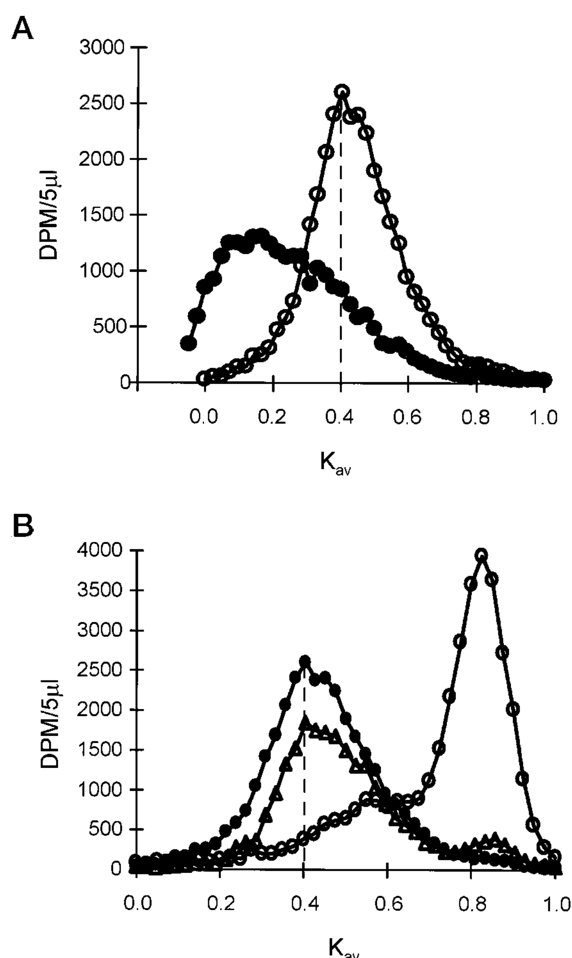

FIG. 3. **Proteoglycans secreted by monocyte-derived macrophages contain primarily chondroitin sulfate/dermatan sulfate, with a minor amount of heparan sulfate disaccharides.** *Panel A*, secreted proteoglycans were characterized by size exclusion chromatography on Sepharose CL-6B under associative conditions (0.2 M Tris, pH 7.0, with 0.2 M NaCl) before (●) and after (○) alkaline borohydride treatment. The *dashed line* indicates the mean hydrodynamic size of the free glycosaminoglycan chains. *Panel B*, glycosaminoglycan chains were characterized by size exclusion chromatography on Sepharose CL-4B under associative conditions (0.2 M Tris, pH 7.0, with 0.2 M NaCl) before (●) or after chondroitin ABC lyase (○) or heparinase (△) treatment. The *dashed line* indicates the mean hydrodynamic size of the undigested free glycosaminoglycan chains.

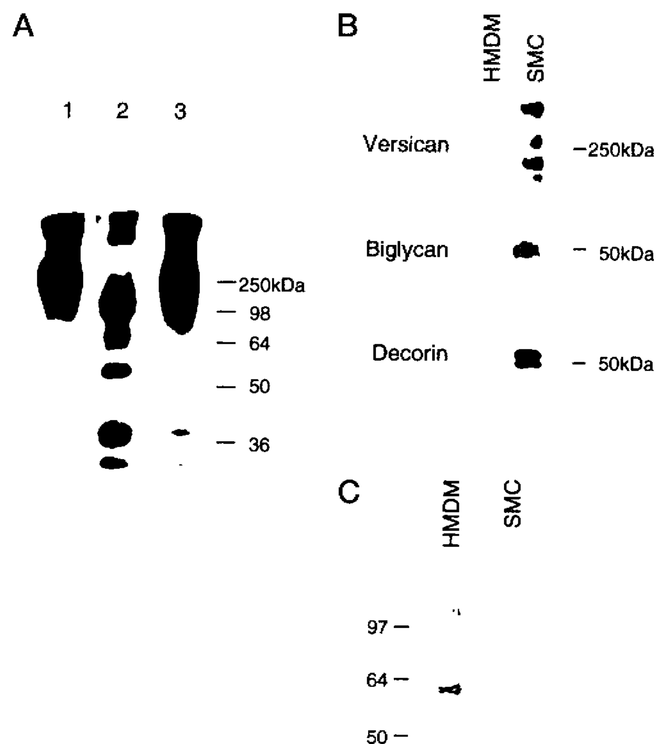

FIG. 4. **Monocyte-derived macrophages synthesize PG-100/PG-MCSF.** *Panel A*, the molecular sizes of [^{35}S]methionine-labeled core protein-secreted proteoglycans were characterized by 4–12% SDS-PAGE after treatment of the intact proteoglycan (*lane 1*) with chondroitin ABC lyase (*lane 2*) or heparinase (*lane 3*). *Panel B*, core protein bands of $^{35}SO_4$-labeled macrophage (HMDM) or smooth muscle cell (*SMC*) proteoglycans were prepared by chondroitin ABC lyase treatment, run on 4–12% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to versican, biglycan, and decorin. *Panel C*, core protein bands of $^{35}SO_4$-labeled macrophage or smooth muscle cell proteoglycans were prepared as described for *panel B* and immunoblotted with antibodies to PG-100/PG-MCSF.

teoglycan bands bound to N-LDL (Fig. 5*A*) with high affinity. However, the slower migrating upper band (Band 1) exhibited higher affinity binding (K_a = 1.5 × 10^{-7} M; n = 3) to N-LDL than did the faster migrating lower band (Band 2) (K_a = 7.7 × 10^{-7} M; n = 3).

The binding of macrophage proteoglycans to several forms of modified LDL also was examined. $^{35}SO_4$-Labeled proteoglycan binding to mildly oxidized LDL (Fig. 5*B*) and methyl-LDL (Fig. 5*C*), two forms of modified LDL with a charge similar to that of N-LDL (data not shown), was similar to the binding to N-LDL (Fig. 5*A*). In contrast, neither proteoglycan band bound to acetyl-LDL (Fig. 5*D*) or to extensively oxidized LDL (Fig. 5*E*), two forms of modified LDL with decreased electronegativity (data not shown). Thus, the overall negative charge of LDL is important in its binding to macrophage proteoglycans.

The contribution of the glycosaminoglycan chains and the core protein to the interaction of the macrophage proteoglycans with N-LDL also was determined. Free glycosaminoglycan chains were prepared from $^{35}SO_4$-labeled material by reductive alkaline β-elimination and size exclusion chromatography as shown in Fig. 3*B*. In the agarose gel mobility shift assay the free glycosaminoglycan chains migrated as a single band with higher electrophoretic mobility (Fig. 5*F*) than either of the two intact proteoglycan bands (Fig. 5*A*). In the presence of increasing concentrations of lipoprotein, the free glycosaminoglycan chains also bound to N-LDL (Fig. 5*F*). In addition, [^{35}S]methionine-core protein was prepared by chondroitin ABC lyase digestion of [^{35}S]methionine-labeled material. This resulted in some aggregation, as seen at the origin of the gel (Fig. 5*G*). However, free core protein migrated as a single band of slower electrophoretic mobility than either of the two intact proteoglycan bands. The intensity of this band did not diminish with increasing concentrations of N-LDL, indicating that the free core protein did not bind N-LDL (Fig. 5*G*). Thus, the glycosaminoglycan chains, but not the core protein, of the macrophage proteoglycans contribute to the interaction with N-LDL.

SDS-PAGE and Molecular Sieve Chromatography of Macrophage Proteoglycan Isolated from Agarose Gel—The two macrophage proteoglycan bands that were resolved by electrophoresis in agarose were isolated and characterized further. $^{35}SO_4$-Proteoglycans were applied to an agarose gel in the absence of lipoproteins and electrophoresed for 7 h to achieve further resolution of the bands. The two bands then were cut out of the gel and reconcentrated on DEAE-Sephacel for characterization.

Both bands consisted of intact proteoglycan, as determined by SDS-PAGE and molecular sieve chromatography. On SDS-PAGE, Band 1 migrated as a broad band with an apparent molecular mass between 150 and 350 kDa, and Band 2 mi-

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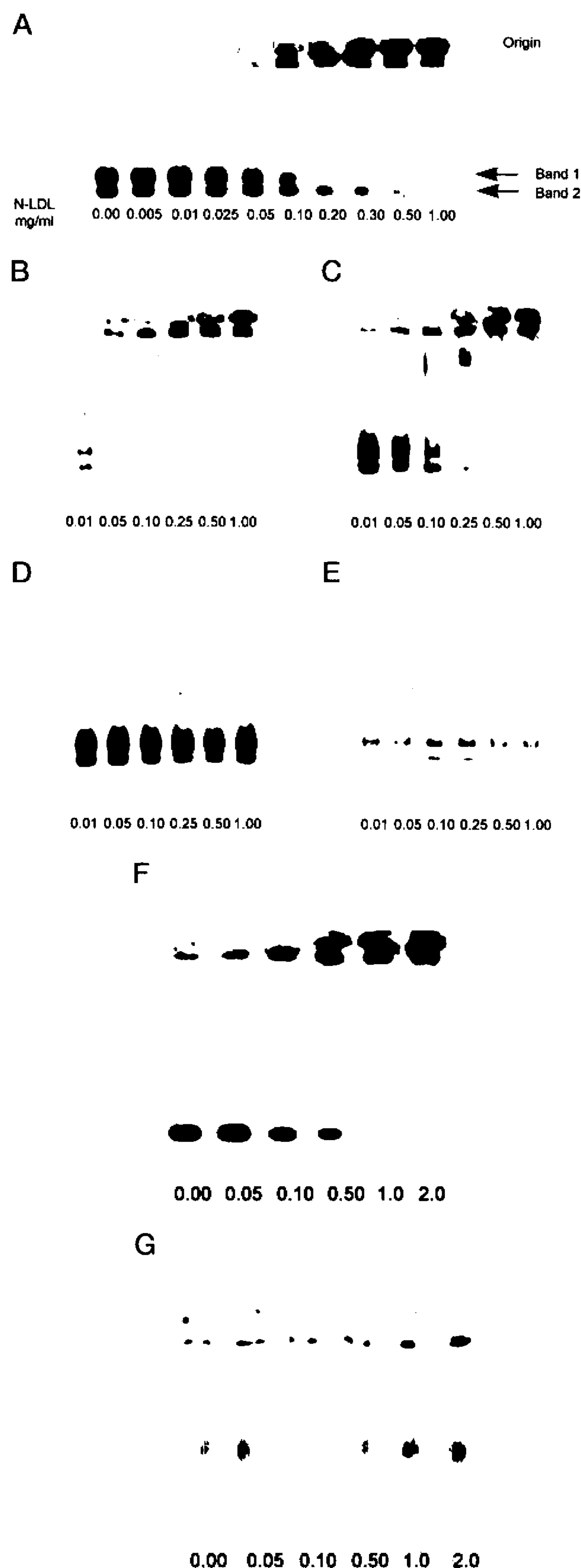

FIG. 5. **Monocyte-derived macrophage proteoglycans bind N-LDL.** Intact proteoglycan, glycosaminoglycan chains, or core protein were evaluated for their lipoprotein-binding capacity by electrophoresis in an agarose gel mobility shift assay (3 h in 0.7% agarose). *Panel A*, $^{35}SO_4$-proteoglycans (5,000 dpm/*lane*) were incubated for 1 h with 0–1 mg/ml N-LDL before electrophoresis. *Panel B*, $^{35}SO_4$-proteoglycans (5,000 dpm/*lane*) were incubated for 1 h with 0–1 mg/ml mildly oxidized LDL bfore electrophoresis. *Panel C*, $^{35}SO_4$-proteoglycans (5,000 dpm/*lane*) were incubated for 1 h with 0–1 mg/ml methyl-LDL before electrophoresis. *Panel D*, $^{35}SO_4$-proteoglycans (5,000 dpm/*lane*) were incubated for 1 h with 0–1 mg/ml acetyl-LDL before electrophoresis. *Panel E*, $^{35}SO_4$-proteoglycans (5,000 dpm/*lane*) were incubated for 1 h with 0–1 mg/ml extensively oxidized LDL before electrophoresis. *Panel F*, $^{35}SO_4$-glycosaminoglycan chains of $^{35}SO_4$-proteoglycans, prepared by alkaline borohydride treatment and repurified by size exclusion and ion exchange chromatography, were incubated for 1 h with 0–2 mg/ml N-LDL before electrophoresis. *Panel G*, [^{35}S]methionine-core protein of [^{35}S]methionine-labeled proteoglycans, prepared by chondroitin ABC lyase digestion, were incubated for 1 h with 0–2 mg/ml N-LDL before electrophoresis.

grated as a broad band with an apparent mass between 150 and 300 kDa (Fig. 6*A*).

On Sepharose CL-6B, Band 1 eluted with a peak at K_{av} = 0.16 (Fig. 6*B*), and Band 2 eluted with a peak at K_{av} = 0.18 (Fig. 6*C*). After reductive alkaline β-elimination, the elution profile of both bands shifted, and the liberated glycosaminoglycan chains of both bands eluted with the same mean K_{av} = 0.45 on Sepharose CL-6B (Fig. 6, *B* and *C*). Fractions corresponding to the free chains of Bands 1 and 2 were pooled and reconcentrated by DEAE-ion exchange chromatography for further characterization with chondroitin ABC lyase and heparinase treatment.

The degradation products after treatment with these enzymes were analyzed by Sepharose CL-4B size exclusion chromatography. When free glycosaminoglycan chains of Band 1 were treated with chondroitin ABC lyase, all of the $^{35}SO_4$ radioactivity eluted with a mean K_{av} = 1.0 at the column V_t (Fig. 7*A*). After free glycosaminoglycan chains of Band 2 were treated with chondroitin ABC lyase, 87% of the $^{35}SO_4$ radioactivity eluted with a mean K_{av} = 1.0 at the column V_t, but 13% was not degraded. Correspondingly, ~15% of Band 2 was heparinase-sensitive (Fig. 7*B*). Thus, the proteoglycan species in Band 1 was slightly larger than that in Band 2, whereas the glycosaminoglycan chains of both bands were of the same size. Band 1 consisted entirely of chondroitin sulfate/dermatan sulfate glycosaminoglycans, and Band 2 consisted of primarily chondroitin sulfate/dermatan sulfate glycosaminoglycans with a minor component of heparan sulfate disaccharides.

By Western immunoblotting, the antibody against PG-100/PG-MCSF recognized core protein bands of 100, 65, and 55 kDa in both Bands 1 and 2 (data not shown).

Analysis of the Role of Heparan Sulfate Glycosaminoglycans in the Binding of Macrophage Proteoglycans to LDL—The role of heparan sulfate glycosaminoglycans in the differential binding of the two forms of PG-100/PG-MCSF to LDL was assessed using the gel mobility shift assay. Macrophage proteoglycans were treated with buffer alone or heparinase I and II before incubation of LDL and analysis by gel mobility shift. Treatment with buffer alone conditions had no effect on the ability of either Band 1 or Band 2 to bind LDL (Fig. 8*A*) compared with the standard assay conditions (Fig. 5*A*). Treatment with heparinase had no effect on the ability of Band 1, which does not contain heparan sulfate, to bind LDL ($K_a = 1.5 \times 10^{-7}$ M). By densitometric scanning, 75.2 ± 0.6% of the radiolabel in heparinase-treated Band 1 was bound by 1 mg/ml LDL compared with 80.2% ± 4.2% of undigested Band 1. However, the ability of Band 2, the heparan sulfate-containing species, to bind LDL was reduced markedly after digestion with heparinase (K_a = 1.9×10^{-6} M) (Fig. 8*B*). Only 25.4 ± 0.4% of the radiolabel in heparinase-treated Band 2 was bound by 1 mg/ml LDL compared with 51.9 ± 10.8% of undigested Band 2. Thus, the difference in LDL binding affinities of macrophage proteoglycan Bands 1 and 2 is the result of the difference in the glycosaminoglycan content of the two bands.

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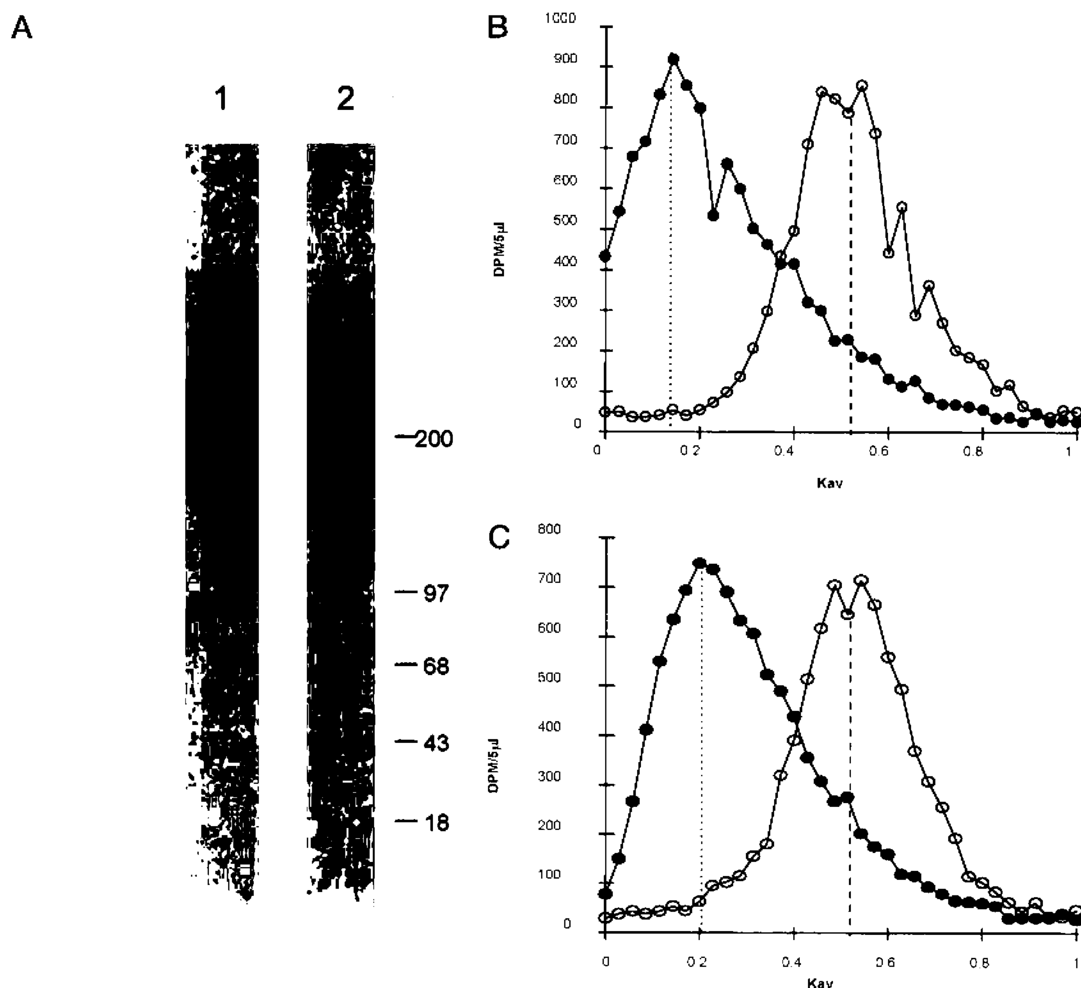

FIG. 6. **Macrophages secrete two species of proteoglycan that differ in size.** The two macrophage proteoglycan bands that were resolved by agarose electrophoresis were isolated and characterized by SDS-PAGE and molecular sieve chromatography. *Panel A*, SDS-PAGE (4–12% gradient with a 3.5% stacking gel) of Band 1 (*lane 1*) and Band 2 (*lane 2*). Size exclusion chromatography of Band 1 (*panel B*) and Band 2 (*panel C*) on Sepharose CL-6B under associative conditions (0.2 M Tris, pH 7.0, with 0.2 M NaCl) before (●) and after (○) alkaline borohydride treatment is shown. The *broken lines* in *panels B* and *C* indicate the mean hydrodynamic size (K_{av}) of the intact proteoglycan (*dotted lines*) and free glycosaminoglycan chains (*dashed lines*).

DISCUSSION

The monocyte-derived macrophage plays a critical role in the pathogenesis of atherosclerosis. In addition to its phagocytic function, it secretes many products (1). Several of these may be involved in atherogenesis, including proteoglycans. In the present study, HMDM in culture were shown to synthesize and secrete two forms of PG-100/PG-MCSF which differed slightly in size and glycosaminoglycan content. The larger form contained solely chondroitin sulfate/dermatan sulfate glycosaminoglycans. The smaller form contained chondroitin sulfate/dermatan sulfate and heparan sulfate glycosaminoglycans. The two proteoglycan forms exhibited differential binding for N-LDL, which could be attributed to the difference in their glycosaminoglycan content. Core proteins (~100 and 55 kDa) for PG-100/PG-MCSF were associated with both proteoglycan forms and accounted for the majority of the macrophage-secreted proteoglycan. The relationship between the two core protein bands is yet to be established. They may represent individual monomers of the PG-MCSF heterodimer (35), or they may arise by proteolysis.

PG-100 first was identified as a major secretory product of osteosarcoma cells and fibroblasts. It is a member of the small interstitial proteoglycan family, with a core protein of ~100 kDa, hence its name (36). It subsequently was found to be identical to PG-MCSF on the basis of its amino acid sequence (26). MCSF is a survival, growth, and differentiation factor for mononuclear phagocytic cells (35), and it is secreted by a variety of cell types, including monocytes activated with phorbol esters (37), indomethacin (38), and interleukin-4 (39) but not by resting monocytes. The predominant form of MCSF secreted by mouse L cells is a proteoglycan (40). MCSF is secreted as both a glycoprotein and a proteoglycan by Chinese hamster ovary cells transfected with human MCSF cDNA (41). The present study demonstrates that MCSF also is secreted as a proteoglycan by well differentiated, but not activated, HMDM.

MCSF is potentially an important molecule in the pathogenesis of atherosclerosis. In addition to its role as a growth factor for mononuclear phagocytic cells, MCSF has a role in cholesterol metabolism *in vivo* and *in vitro*. It also inhibits lesion formation in the Watanabe heritable hyperlipidemic rabbit (42). *In vitro*, MCSF enhances the uptake and degradation of N-LDL, acetylated LDL, and oxidized LDL by macrophages (43, 44). It also stimulates scavenger receptor expression (45), cholesterol esterification (43), and cholesterol efflux by macrophages (44). *In vivo*, MCSF lowers plasma cholesterol in normal (46) and Watanabe heritable hyperlipidemic rabbits (44) and in non-human primates (46).

Although the proteoglycan form of MCSF shares the growth factor role of the non-proteoglycan form of MCSF (47), it is not yet known if PG-MCSF also has a role in cholesterol metabo-

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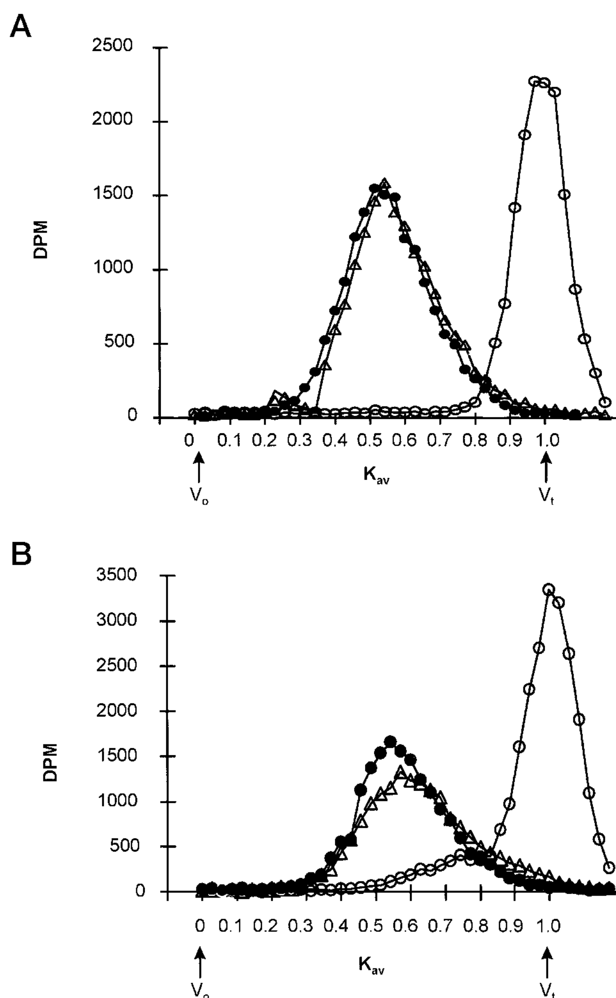

FIG. 7. **The two forms of macrophage proteoglycans differ in disaccharide content.** Glycosaminoglycan chains of the two macrophage proteoglycan bands that were resolved by agarose electrophoresis were characterized by size exclusion chromatography on Sepharose CL-6B under associative conditions (0.2 M Tris, pH 7.0, with 0.2 M NaCl) before (●) or after chondroitin ABC lyase (○) or heparinase (△) treatment.

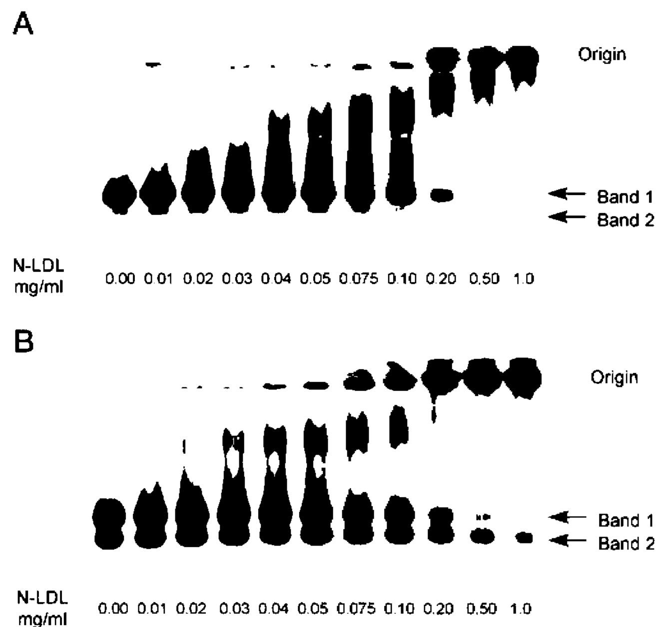

FIG. 8. **The difference in disaccharide content accounts for the differential LDL binding of the two proteoglycan species.** Macrophage $^{35}SO_4$-proteoglycans were treated without (*panel A*) or with (*panel B*) heparinase I (3 h at 37 °C) and II (overnight at 41 °C) before incubation for 1 h with 0–1 mg/ml N-LDL and electrophoresis in the gel mobility shift assay.

lism. However, PG-MCSF has unique properties that are not shared by the non-proteoglycan form of MCSF. PG-MCSF has been shown to bind collagen type V (47), which is a minor extracellular matrix component in normal tissue (48) but is increased in atherosclerotic vessels (49, 50). PG-MCSF also has been shown previously to bind LDL (51). In the present study, we have characterized this interaction further. With the use of an agarose gel mobility shift assay, binding constants for the interaction could be determined. The high affinity binding (10^{-7} M) of PG-100/PG-MCSF for N-LDL is entirely attributable to the free chondroitin sulfate glycosaminoglycan chains, not the core protein, and is consistent with the putative role of ionic charge in lipoprotein-proteoglycan interactions (5, 6). The role of ionic charge in these interactions is supported further by the demonstration that acetylation and extensive copper oxidation, both modifications that increase the net negative charge of LDL, result in a loss of binding to the negatively charged residues on the proteoglycan. Macrophage PG-100/PG-MCSF could be resolved into two proteoglycan species by agarose gel electrophoresis. The two forms were found to have the same core proteins and similar length glycosaminoglycan chains but to differ slightly in intact proteoglycan size and glycosaminoglycan composition. The presence of heparan sulfate on the smaller proteoglycan form contributed significantly to its affinity for LDL because digestion with heparinase significantly reduced its ability to bind LDL. The lower affinity of the heparan sulfate-containing proteoglycan form for LDL compared with that of the non-heparan sulfate-containing form was consistent with other studies that have reported that heparan sulfate binds LDL less well than chondroitin sulfate and dermatan sulfate glycosaminoglycans (52). The difference in binding of the two forms after heparinase treatment was of interest. If the glycosaminoglycans remaining on the smaller form after heparinase treatment were identical to those of the larger form, the heparinase-treated smaller form would have been expected to exhibit LDL binding similar to that of the larger non-heparan sulfate-containing form. However, binding to LDL of the smaller form was reduced markedly by treatment with heparinase. The remaining glycosaminoglycans on the smaller proteoglycan were entirely chondroitin ABC lyase-sensitive. This enzyme cleaves chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate, suggesting that these two proteoglycan species have further differences in their composition which might contribute to their different binding affinities for N-LDL.

In contrast to what is known about proteoglycans secreted by smooth muscle cells, much less is known about those secreted by human monocytes and monocyte-derived macrophages. *In vitro*, human blood monocytes have been reported to synthesize primarily chondroitin sulfate proteoglycans (53). The glycosaminoglycan chains of these proteoglycans are regulated by cell density (54), platelet products such as platelet-derived growth factor and platelet factor 4 (55), activators such as phorbol myristate acetate and lipopolysaccharide (56), and differentiation of monocytes into macrophages (57). Cell type specificity is also an important factor in the regulation of both glycosaminoglycan chains and core proteins of proteoglycans. The core protein of the major proteoglycan synthesized by both monocytes and macrophages of the U-937 neoplastic cell line has been identified as serglycin (58). Although HMDM have been shown to synthesize RNA for serglycin, the core protein for this proteoglycan was not demonstrated in these cells (58).

In data not shown here, a very minor band of approximately 19 kDa could be detected on 4–20% SDS-PAGE after chondroitin ABC lyase treatment of [^{35}S]methionine-proteoglycans from the HMDM. This molecular mass corresponds to that of the serglycin core protein (59). However, because this band represented less than 1% of the [^{35}S]methionine-labeled material, it was not characterized further.

The role of the monocyte-derived macrophage as a source of proteoglycans in the thickened intima of atherosclerotic blood vessels is yet to be established. However, the ability of the macrophage secretory product, PG-MCSF, to bind a variety of molecules known to be present in atherosclerotic blood vessels suggests a potentially important function for this proteoglycan. Along with other well characterized proteoglycan molecules, including those secreted by smooth muscle cells, macrophage-derived proteoglycans may participate in LDL binding and trapping within the arterial wall. Such trapping of LDL may result in prolonged retention of LDL and increased susceptibility to oxidative modification. The absence of binding of proteoglycans to extensively oxidized LDL suggests that after oxidation, the LDL may be "liberated" from the proteoglycan molecule. This liberated oxidized LDL then may be available for binding to other matrix molecules (14). Additionally, the glycosaminoglycan chains of PG-MCSF may serve to anchor MCSF in the vascular extracellular matrix where it can participate in the survival and differentiation of mononuclear phagocytic cells. Thus, macrophage-derived proteoglycans in the vascular wall may be important molecules in atherogenesis.

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