Lipoprotein–Proteoglycan Complexes From Atherosclerotic Lesions Promote Cholesteryl Ester Accumulation in Human Monocytes/Macrophages

Parakat Vijayagopal, Sathanur R. Srinivasan, Bhandaru Radhakrishnamurthy, and Gerald S. Berenson

Lipoprotein–proteoglycan complexes from human atherosclerotic lesions were studied to determine their ability to stimulate cholesteryl ester accumulation in human monocytes/macrophages. Complexes containing apolipoprotein (apo) B lipoproteins and proteoglycans were extracted from fatty streaks and fibrous plaque lesions of human aortas by extraction with 0.15 M NaCl. Fractionation of the complex with Bio-Gel A-50m yielded a single fraction from fatty streaks and two fractions from fibrous plaques. The complexes were further purified by anti–apo B affinity chromatography and analyzed for apolipoproteins, lipids, and glycosaminoglycans. Apo B was the only apolipoprotein present in the complexes. Although the complexes from fatty streaks and fibrous plaques contained varying proportions of hyaluronic acid, chondroitin 6-sulfate, and dermatan sulfate, heparin was present in only the fibrous plaque complexes. All three lipoprotein–proteoglycan complexes increased the rate of incorporation of [14C]oleate into cholesteryl [14C]oleate and stimulated cholesteryl ester accumulation in monocytes/macrophages. However, the complexes from fibrous plaques were more potent than those from fatty streaks in this regard. Cholesteryl ester synthesis that is mediated by the uptake of the complexes was dose dependent and showed apparent saturation, suggesting that cell surface binding may be required. Chloroquine, a lysosomotropic agent, inhibited cholesteryl ester synthesis that is induced by the complexes, indicating that lysosomal hydrolysis was essential. Cholesteryl ester synthesis that is mediated by the complexes was inhibited 70–79% by polyinosinic acid. Furthermore, excess unlabeled fibrous plaque complexes significantly inhibited the binding and internalization of in vitro 125I-low density lipoprotein (LDL)–proteoglycan complexes and 125I-acetylated-LDL and not 125I-LDL. These results suggest the involvement of the scavenger receptor in the uptake of the complexes. Phagocytosis played a minor role in the metabolism of these ligands because cytochalasin D inhibited cholesteryl ester synthesis, which is mediated by fibrous plaque complexes, by 7.5–25%. Cholesteryl ester synthesis increased linearly over 32 hours in macrophages incubated with the complexes, indicating an apparent lack of downregulation of binding sites. This resulted in the appearance of intracellular oil red O–positive lipid droplets. These studies show for the first time that apo B lipoprotein–proteoglycan complexes isolated from human atherosclerotic lesions can induce cholesteryl ester accumulation in monocytes/macrophages. (Arteriosclerosis and Thrombosis 1992;12:237–249)
monocytes/macrophages, which possess high-affinity receptors for the lipoprotein. However, macrophages accumulate cholesteryl ester when they are incubated with acetylated LDL (acetyl-LDL) or malondialdehyde-modified LDL. Macrophages from the mouse also take up oxidized LDL, LDL–dextran sulfate complex, LDL isolated from human atherosclerotic lesions, and β–very low density lipoprotein (β-VLDL) leading to intracellular cholesteryl ester accumulation.

Proteoglycans are ubiquitous components of all tissues, including the blood vessel wall. They consist of one or more chains of glycosaminoglycans, which are covalently linked to a protein core. Certain classes of proteoglycans selectively form complexes with plasma apo B–containing lipoproteins, particularly LDL. The isolation of such lipoprotein–proteoglycan complexes from atherosclerotic lesions has led to the hypothesis that the uptake of these complexes by macrophages could stimulate intracellular cholesteryl ester accumulation and their subsequent transformation into foam cells. Earlier studies by us and others have provided partial support to the aforementioned hypothesis. These studies show that complexes of LDL and aortic proteoglycans that are formed in vitro are taken up by macrophages, resulting in intracellular cholesteryl ester accumulation. To obtain further experimental support for the hypothesis, in the present study we have isolated lipoprotein–proteoglycan complexes from human atherosclerotic lesions and determined their ability to stimulate cholesteryl ester synthesis and accumulation in human monocytes/macrophages. The results indicate that intact in vivo lipoprotein–proteoglycan complexes induce foam cell formation in human monocytes/macrophages.

**Methods**

**Materials**

RPMI-1640 medium was purchased from GIBCO (Grand Island, N.Y.). Plastic tissue-culture dishes were obtained from Costar (Cambridge, Mass.). Bio-Gel A-50m and unipore membranes (0.8 μm) were purchased from Bio-Rad (Richmond, Calif.). We obtained cyanogen bromide-Sepharose CL-4B from Pharmacia (Piscataway, N.J.) and Histopaque, a-amino-η-caproic acid, N-ethylmaleimide, benzamide hydrochloride, phenylmethylsulfonyl fluoride, EDTA, butylated hydroxytoluene (BHT), chondroitin ABC lyase, chondroitin ACII lyase, hyaluronidase (Streptomyces hyalurolyticus), heparinase, cytochalasin D, and polyinosinic acid from Sigma (St. Louis, Mo.). 1-[1-14C]oleic acid (50 mCi/mmole) came from ICN Biomedicals (Costa Mesa, Calif.), and 1,2,6,7-3H(N)-cholesteryl oleate (82.9 Ci/mmol) from New England Nuclear (Wilmington, Del.). Anti-human apo C-I, apo C-II, and apo C-III were a kind gift from Larry Wong, Department of Physiology, Louisiana State University Medical Center. Anti-human apo A-I and apo E were available in the laboratory.

Lipoproteins and Lipoprotein-Deficient Serum

LDL (d=1.019–1.063 g/ml) was isolated from pooled human serum by sequential ultracentrifugation. EDTA (0.05%) and BHT (10 μM) were added to the blood immediately after collection. The purity of LDL was determined by agarose gel electrophoresis. LDL was acetylated as described by Fraenkel-Conrat. Human lipoprotein-deficient serum (d>1.215 g/ml) was prepared as described by Brown et al. Lipoproteins and lipoprotein-deficient serum were sterilized by membrane filtration before they were used for cell culture experiments.

LDL and acetyl-LDL were labeled with 125I and used within 2 weeks. More than 98% of the radioactivity in LDL and 96% of the radioactivity in acetyl-LDL were capable of being precipitated by 10% trichloroacetic acid.

Proteoglycan Aggregate

A chondroitin sulfate–dermatan sulfate proteoglycan aggregate from bovine aortas containing hyaluronic acid and link protein was available from earlier studies. The isolation and detailed characterization of this proteoglycan has been previously described.

Monocyte/Macrophage Culture

Monocytes were isolated from heparinized human blood by the Ficol-Hypaque gradient method. The heparinized blood was diluted with an equal volume of sterile phosphate-buffered saline (PBS), and 8-ml aliquots were layered over 6 ml Histopaque in a 15-ml sterile plastic centrifuge tube. The tube was centrifuged at 500g for 30 minutes at 20°C. The mononuclear cell preparations were washed three times with calcium- and magnesium-free PBS containing 10 mM EDTA to remove platelets. The cell preparation was suspended in RPMI-1640 containing 20% homologous serum (vol/vol), penicillin (100 units/ml), and streptomycin (100 μg/ml) to a final concentration of 2x10^6 cells/ml. Aliquots (1 or 2 ml) of the cell suspension were dispensed in 22-mm, 35-mm, or 60-mm culture dishes and incubated in a humidified 5% CO2 (5%) incubator at 37°C. After 3 hours, nonadherent cells were removed from the culture dishes by three washes with serum-free medium. The cells were then incubated in fresh medium for 7–8 days before the experiments were initiated. The culture medium was changed every 3 days. The cells were classified as macrophages by their morphology on Wright’s-stained smears, nonspecific esterase staining, and their ability to ingest latex particles.

Lipoprotein–Proteoglycan Complexes

In vitro complexes. In vitro complexes of unlabeled or 125I-labeled LDL and the proteoglycan aggregate from bovine aortas were prepared in 0.001 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4) containing 30 mM CaCl2 as previously described. The LDL–proteoglycan complex was dissolved in the appropriate medium, and aliquots were added to
macrophage cultures. Cholesterol and uronic acid contents of the complexes were determined as measures of LDL and proteoglycan, respectively.

**In vivo complexes.** In vivo lipoprotein–proteoglycan complexes were isolated from human atherosclerotic lesions by a modification of the procedure of Srini-

Vijayagopal et al.13 Lesioned human aortas were obtained at autopsy within 12 hours of death from the Pathology Service, Charity Hospital (Table 1). In some cases complexes were also isolated from lesioned aortic segments obtained at surgical resection. Grossly visible fatty streaks and fibrous plaque lesions were dissected out at 4°C, and where possible they were isolated from the same vessel. The lesions from each vessel were extracted individually. To increase yield, in some instances two to three extracts from similar lesions from different vessels were pooled before further purification. Grossly calcified, ulcerated, or thrombosed lesions were not used. The lesions were finely minced and extracted with 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4) (5 vol/g tissue) containing protease inhibitors (0.1 M e-aminocaproic acid, 0.005 M benzamidine hydrochloride, 0.005 M N-ethylmaleimide, and 0.01 M phenylmethylsulfonyl fluoride), antioxidants (0.05% EDTA, 20 μM BHT), and the antibiotic penstrep (hereafter referred to as buffer A) at 4°C for 24 hours with gentle shaking. The extracts from fatty streaks and fibrous plaques were centrifuged (1,000 g for 30 minutes at 4°C) to obtain the opalescent middle layer containing lipoprotein–proteoglycan complexes, which were then fractionated by gel-exclusion chromatography on Bio-Gel A-50m. Columns were eluted with buffer A at 4°C. The column fractions were monitored for cholesterol and uronic acid as measures of lipoprotein and proteoglycan, respectively. All peaks positive for both compounds were pooled and concentrated by ultrafiltration. They were stored at 4°C and used for further purification within 2 weeks.

**Affinity Chromatography**

Antiserum against human LDL (d = 1.03–1.05 g/ml) that contained only apo B as apolipoprotein was raised in the goat, and the anti-apo B was purified by chromatography on an LDL-Sepharose affinity column. The purified antibody (14–16 mg protein) was coupled to cyanogen bromide–Sepharose CL-4B (2 g), and a 7-ml column was prepared. Preliminary studies indicated that this affinity column could retain about 8–9 mg LDL–apo B. The lipoprotein–proteoglycan complexes (80–90 mg protein) from the Bio-Gel column were applied to the anti–apo B affinity column in buffer A. The nonadherent materials were washed off the column with buffer A until the optical absorption at 280 nm of the effluent reached baseline. The apo B–containing complex was then eluted with 0.15 M NaCl, which was adjusted to pH 11 with NH4OH. Fractions of 1.0 ml were collected and monitored for absorption at 280 nm. The individual fractions were immediately dialyzed against buffer A, and aliquots were used for analysis of cholesterol and uronic acid. Fractions positive for both compounds were pooled. The entire procedure was conducted at 4°C. On the day before an experiment, the complexes were dialyzed overnight at 4°C against the appropriate macrophage culture medium and sterilized by membrane filtration (0.45 μm).

**Characterization of Lipoprotein–Proteoglycan Complexes**

The lipoprotein–proteoglycan complexes purified by anti–apo B affinity chromatography were characterized with respect to their apoproteins, proteoglycans, and lipids. Apo B was identified by double immunodiffusion and quantified by the electroimmunoassay procedure of Laurell.25

The nature of proteoglycans in the complexes was determined by analysis of constituent glycosaminoglycans. For this, the complexes were lyophilized and delipidated by extraction with ethanol/diethyl ether (3:1, vol/vol) followed by chloroform/methanol (2:1, vol/vol). The glycosaminoglycans were B eliminated from the residual proteoglycans by treatment with alkaline borohydride;26 the relative proportions of individual glycosaminoglycans were determined by cellulose acetate electrophoresis before and after treatment with nitrous acid,28 hyaluronidase, chondroitin ACII lyase or chondroitin ABC lyase,29 or heparinase. The ratio of chondroitin 4- and 6-sulfates was determined by disaccharide analysis.30 The lipoprotein–proteoglycan complexes were also analyzed for free and esterified cholesterol,31 phospholipids,32 and total protein.33 Triglycerides were determined by the Abbott V.P. enzymatic procedure.
Dissociation of Lipoprotein–Proteoglycan Complexes

The affinity-purified lipoprotein–proteoglycan complexes were dissociated to isolate the constituent lipoproteins and proteoglycans. The complexes in buffer A were adjusted to a solvent density of 1.063 g/ml by the addition of solid potassium bromide. The solution was centrifuged at 114,000g for 20 hours at 17°C. The lipoproteins were recovered from the top of the centrifuge tube and dialyzed against buffer A at 4°C.

To obtain the proteoglycans, the complexes were dissociated in 1.5 M NaCl and the solvent density was adjusted to 1.33 g/ml by the addition of solid cesium chloride. The solution was centrifuged at 114,000g for 16 hours at 8°C. The proteoglycans were recovered from the bottom two fifths of the centrifuge tube and dialyzed against buffer A at 4°C.

Metabolic Studies

Cholesterol esterification. The effect of lipoprotein–proteoglycan complexes from atherosclerotic lesions on the incorporation of [14C]oleate into cellular cholesterol [14C]oleate was determined as previously described. Briefly, after 7–8 days in culture, human monocytes/macrophages were washed and incubated in RPMI-1640 medium containing 10% human lipoprotein–deficient serum (medium A) and 0.2 mM [14C]oleate–albunmin in the presence of various lipoprotein–proteoglycan complexes for 16 hours at 37°C. Control incubations were done without the cells, and these values were subtracted from the experimental values. For time-course studies, cells were incubated for up to 32 hours with 100 µg cholesterol/ml of each ligand. The cells were then washed and their lipids extracted and separated by thin-layer chromatography. Cholesterol ester spots were identified by the cholesteryl oleate standard and quantified in a liquid scintillation spectrometer. Results were corrected for the recovery of the internal [3H]cholesteryl oleate standard. The solvent-extracted cell layer was dissolved in 1 ml 0.2N NaOH, and an aliquot was used for protein assay.

Cholesterol ester accumulation. Human monocytes/macrophages were plated at a density of 2×10^6 cells/60-mm culture dish and incubated in medium A containing 150 µg cholesterol/ml of various lipoprotein–proteoglycan complexes. After 48 hours the cells were washed thoroughly and extracted twice with hexane/isopropanol (3:2, vol/vol). To correct for loss during the procedure, an internal standard of [3H]cholesterol (10,000 dpm) was added to each dish before extraction. The lipid extracts were combined and evaporated to dryness under nitrogen. Cholesterol and cholesteryl ester were separated by silica gel-G thin-layer chromatography and quantified by the procedure of Bowman and Wolf with the modification that the chromophore was measured spectrofluorometrically.

Lipoprotein binding and internalization. The binding and internalization of in vitro 125I-LDL–proteoglycan complexes, 125I-acetyl LDL, and 125I-LDL were assayed as described by Hurt et al with minor modifications. In brief, monocytes/macrophages were cultured for 7 days in 35-mm tissue-culture dishes. On day 8, the cells were washed and incubated in 1 ml medium A containing 10 µg cholesterol/ml of 125I-labeled ligands. For the competition studies a 40-fold excess of unlabeled competing ligands was added. After 6 hours at 37°C, the medium was removed and the cells were washed four times with PBS containing 2 mg/ml bovine serum albumin and once with PBS alone. The cells were then exposed to 1 ml of 5 mg/ml trypsin solution for 10 minutes at 37°C to release the cell surface–bound ligands. After removing the trypsin solution, the cells were washed with 1 ml each of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 ml PBS. The washings were combined with the trypsin medium, and an aliquot was counted to determine the cell surface–bound radioactivity. The cell layer was dissolved in 1 ml 0.2N NaOH, and an aliquot was counted to quantify the internalized lipoproteins. A second aliquot was assayed for protein content. Parallel incubations were done without cells, and the results were subtracted from the corresponding experimental values.

Morphological studies. For morphological studies macrophages were plated on glass slides (tissue-culture-chamber slides [Lab-Tek]) and incubated in 0.5 ml medium A in the presence or absence of lipoprotein–proteoglycan complexes (150 µg cholesterol/ml). After 48 hours at 37°C, the cells were washed, fixed, and stained with oil red O as previously described. The cells were photographed with an Olympus photomicroscope.

Other analytical methods. Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen. Total cholesterol in column effluents was assayed by a commercial enzymatic reagent kit (Autoflo cholesterol 236691, Boehringer Mannheim Diagnostics, Houston). Lipid peroxidation was determined by thiobarbituric acid assay using malondialdehyde as a standard.

Each data point represents the average of duplicate or triplicate assays, and intraexperimental variability was less than 10%. The statistical significance was analyzed by Student's t test.

Results

Isolation of Lipoprotein–Proteoglycan Complexes

Lipoprotein–proteoglycan complexes were extracted from fatty streaks and fibrous plaque lesions of human aortas. To prevent the dissociation of the complexes during isolation, a mild extraction procedure was followed. The soluble extracts contained both cholesterol and uronic acid with a high cholesterol to protein mass ratio, indicating the presence of lipoproteins and proteoglycans.
Figure 1 shows a representative elution profile of cholesterol and uronic acid in the soluble extracts from fatty streaks and fibrous plaques lesions on a Bio-Gel A-50m column. The lipoprotein–proteoglycan complexes from fatty streaks eluted as a single peak at the V₀ of the Bio-Gel column (Figure 1A). In contrast, the complexes from the fibrous plaque lesions were resolved into two peaks, one eluting at the V₀ (complex 1) and the other eluting in the inclusive volume (complex 2), with elution volume slightly lower than that of human LDL (Figure 1B). The fractions positive for both cholesterol and uronic acid were pooled for further purification. Although these fractions also contained proteins, the bulk of the extracted proteins emerged after the LDL elution volume.

**Affinity Chromatography**

To isolate complexes consisting of apo B-containing lipoproteins and proteoglycans, we subjected the complexes obtained from gel-exclusion chromatography to anti–apo B affinity chromatography. A similar approach was used earlier by Camejo et al.37 to isolate lipoprotein–proteoglycan complexes from human atherosclerotic lesions. After removing the nonadherent material, the adherent fraction was eluted from the affinity column and its composition determined. As shown in Table 2, all of the immunoreactive apo B and most of the uronic acid from the various complexes were associated with the adherent fractions. The nonadherent fractions accounted for most of the protein (90–92%) and cholesterol (81–88%) present in the Bio-Gel-purified complexes. No apo B was detected in these fractions from the various complexes; the presence of other apolipoproteins was not investigated. The anti–apo B affinity-purified complexes were subjected to further characterization and metabolic studies.

**Characterization of Lipoprotein–Proteoglycan Complexes**

Table 3 shows the yield of the various affinity-purified complexes. In terms of cholesterol, apo B, and uronic acid, the yield was significantly higher for fibrous plaque complex 1 than for the fatty streak complex (p<0.05); for fibrous plaque complex 2, the yield was significantly higher than for the fatty streak complex material in terms of cholesterol and uronic acid only (p<0.05). Double immunodiffusion of the complexes from fatty streaks and fibrous plaques isolated by anti–apo B affinity chromatography showed no cross reactivity with anti–apo E, anti–apo C-I, apo C-II, and apo C-III, or anti–apo A-I, thus

**TABLE 2. Composition of Lipoprotein–Proteoglycan Complexes by Anti–Apolipoprotein B Affinity Chromatography**

<table>
<thead>
<tr>
<th>Site/type</th>
<th>Total protein</th>
<th>Apo B</th>
<th>Cholesterol</th>
<th>Uronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty streaks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>92±12</td>
<td>1.9±0.3</td>
<td>108±16</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>85±9</td>
<td>ND</td>
<td>87±12</td>
<td>0.2±0.05</td>
</tr>
<tr>
<td>Adherent</td>
<td>5.0±1.0</td>
<td>1.7±0.2</td>
<td>8.0±2.0</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td><strong>Fibrous plaque</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex 1</td>
<td>85±10</td>
<td>1.8±0.2</td>
<td>93±10</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>78±11</td>
<td>ND</td>
<td>82±11</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>Adherent</td>
<td>4.0±0.8</td>
<td>1.6±0.15</td>
<td>6.2±1.6</td>
<td>1.6±0.05</td>
</tr>
<tr>
<td>Complex 2</td>
<td>79±11</td>
<td>2.4±0.16</td>
<td>89±12</td>
<td>1.7±0.09</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>71±7</td>
<td>ND</td>
<td>75±10</td>
<td>0.3±0.01</td>
</tr>
<tr>
<td>Adherent</td>
<td>4.5±1.6</td>
<td>2.1±0.08</td>
<td>7.3±2.5</td>
<td>1.3±0.03</td>
</tr>
</tbody>
</table>

*Extracts from three aortas were used.
Apo, apolipoprotein; ND, not detected.
various complexes was chromatographed on a col-
arin, hyaluronic acid, chondroitin 6-sulfate, and der-
chondroitin 6-surfate and hyaluronic acid and a small
complex from fibrous plaques contained hep-
amount of dermatan sulfate (5%). In contrast, the
plex from fatty streaks contained predominantly
of the three apo B-containing complexes. The com-
contained intact proteoglycans.

gh/cosaminoglycans from the protein core of proteo-
umn of Sepharose CL-6B, all of it was excluded from
buoyant density, the proteoglycans were sedimented
at a density of 1.33 g/ml. Because of their high
composition of the affinity-purified fractions from
fatty streaks and fibrous plaques. The chemical com-
positions of the three complexes were similar except
that the complex from fatty streaks contained rela-
tively less uronic acid and more triglycerides than the
complexes from fibrous plaques. Camejo et al have
reported a similar composition for affinity-purified
lipoprotein-proteoglycan complexes isolated from
human aortas by saline extraction.

The interaction of lipoproteins and proteoglycans
is mostly ionic in nature. Therefore, the complexes
were dissociated in a high-salt solution, and the
proteoglycans were recovered by ultracentrifugation
at a density of 1.35 g/ml. Because of their high
buoyant density, the proteoglycans were sedimented
and recovered free of lipoproteins at the bottom of
the centrifuge tube. When this material from the
various complexes was chromatographed on a col-
umn of Sepharose CL-6B, all of it was excluded from
the column (data not shown). After treatment with
alkaline borohydride, which involves β-elimination of
the column (data not shown). After treatment with
alkaline borohydride, which involves β-elimination of
cholesterol ester synthesis in monocytes
macrophages. There was no difference in the electrophoretic mobility
of the two lipoproteins (data not shown). Choles-
teryl ester synthesis mediated by 100 μg cholester-
ol/ml of the two lipoproteins also did not differ
(nmol±SD, cholesteryl [14C]oleate formed/16 hr/mg
protein, untreated LDL 3.8±0.6, affinity-purified
LDL 3.5±0.5). These studies showed that the elution
conditions did not functionally modify apo B.

Cholesterol ester synthesis. We tested whether the
affinity-purified lipoprotein–proteoglycan complexes
stimulated cholesterol ester synthesis in monocytes/
macrophages. As shown in Figure 2A, lipoprotein-
proteoglycan complexes from fatty streaks and fi-
brous plaques (patient 9) stimulated cholesterol ester
synthesis. This stimulation was dose dependent and
showed apparent saturation at a cholesterol concen-
tration of 100 μg/ml for the complex from fatty
streaks. When it is compared on the basis of chole-
sterol content, complex 2 from fibrous plaques was

| Table 4. Percent Composition of Lipoprotein–Proteoglycan Complexes From Atherosclerotic Lesions Isolated by Anti–Apolipoprotein B Affinity Chromatography*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Free cholesterol</th>
<th>Esterified cholesterol</th>
<th>Phospholipids</th>
<th>Triglycerides</th>
<th>Uronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty streak complex</td>
<td>27</td>
<td>5</td>
<td>36</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>Fibrous plaque complex 1</td>
<td>29</td>
<td>6</td>
<td>34</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Fibrous plaque complex 2</td>
<td>25</td>
<td>8</td>
<td>38</td>
<td>14</td>
<td>6</td>
</tr>
</tbody>
</table>

*Three separate samples from the anti-apliprotein B affinity column were pooled for analysis of composition.
TABLE 5. Glycosaminoglycan Composition of Lipoprotein-Proteoglycan Complexes

<table>
<thead>
<tr>
<th>Glycosaminoglycan (%)</th>
<th>Heparin</th>
<th>Hyaluronic acid</th>
<th>Chondroitin 6-sulfate</th>
<th>Dermatan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty streak complex</td>
<td>0</td>
<td>16</td>
<td>79</td>
<td>5</td>
</tr>
<tr>
<td>Fibrous plaque complex 1</td>
<td>16</td>
<td>25</td>
<td>46</td>
<td>13</td>
</tr>
<tr>
<td>Fibrous plaque complex 2</td>
<td>28</td>
<td>22</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

The proteoglycans isolated from affinity-purified complexes from fatty streaks and fibrous plaques were treated with alkaline borohydride, and the relative proportions of constituent glycosaminoglycans were determined as described in "Methods."

more potent than complex 1, which in turn was more potent than the complex from fatty streaks (p<0.005). At 100 μg cholesterol/ml, the esterification stimulatory activity of complexes 1 and 2 from fibrous plaque was significantly (59% and 76%, respectively) higher than that of the complex from fatty streaks (p<0.005). Similar results were obtained when these experiments were repeated with complexes isolated from three additional aortas (patients 5, 11, and 14). Lipoprotein-proteoglycan complexes isolated from pooled fatty streaks and fibrous plaque extracts also gave similar results (data not shown). Therefore, the difference in cholesterol esterification stimulatory activity between complexes from fibrous plaques and fatty streaks always persisted, although some variability existed among different aortas. The three complexes were significantly more potent than LDL in stimulating cholesteryl ester synthesis (Figure 2B). The complex from the fatty streaks and fibrous plaque of complex 1 was less effective than acetyl-LDL in its ability to induce cholesterol esterification; however, fibrous plaque complex 2 was equally effective (Figure 2B).

Figure 3 shows the time course of synthesis of cholesteryl [14C]oleate by macrophages incubated with the complexes isolated by anti-apo B affinity chromatography or LDL. Unlike macrophages incubated with LDL, cells incubated with the various complexes from atherosclerotic lesions showed a linear increase in cholesteryl ester synthesis over a 32-hour incubation period. Complex 2 from fibrous plaques produced the highest increase and the complex from fatty streaks the lowest. Chloroquine at a concentration of 15 μM completely blocked the stimulation of cholesteryl ester synthesis induced by all three complexes (data not shown).

Studies were conducted to determine the mechanism of uptake of lipoprotein–proteoglycan complexes in macrophages. We tested the ability of polyinosinic acid, an inhibitor of the scavenger receptor, to prevent cholesteryl ester synthesis mediated by the complexes. Figure 4 shows that polyinosinic acid inhibited cholesteryl ester synthesis elicited by fibrous plaque complexes 1 and 2 (patient 8). The maximum inhibition for the two complexes was 70% and 75%, respectively. In contrast, cholesteryl ester synthesis mediated by acetyl-LDL was completely inhibited by polyinosinic acid.

Competitive binding studies involving LDL, acetyl-LDL, and lipoprotein–proteoglycan complexes were performed to further determine the role of the

TABLE 6. Thiobarbituric Acid-Reactive Substances (TBARS) of Lipoprotein-Proteoglycan Complexes Isolated From Atherosclerotic Lesions

<table>
<thead>
<tr>
<th>Complex</th>
<th>Plasma LDL</th>
<th>F.S. Complex</th>
<th>F.P. Complex 1</th>
<th>F.P. Complex 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS* (nmol/mg protein)</td>
<td>1.28</td>
<td>1.28</td>
<td>1.28</td>
<td>1.31</td>
</tr>
</tbody>
</table>

*Each data point represents the average of duplicate assays. LDL, low density lipoprotein.
scavenger receptor in the uptake of the complexes from atherosclerotic lesions. Because the specific labeling of the apo B component of the fibrous plaque complex was not feasible, we used in vitro complexes of $^{125}$I-LDL and a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aortas in place of the fibrous plaque complex for competitive binding studies (Table 7). Excess unlabeled fibrous plaque complex 2 (patients 15 and 16) and an in vitro LDL–proteoglycan complex almost completely inhibited the binding and internalization of the $^{125}$I-LDL–proteoglycan complex. Acetyl-LDL produced a 58% suppression and LDL had no effect. Similarly, fibrous plaque complex 2 suppressed the binding and internalization of $^{125}$I-acetyl-LDL by 87%, and the unlabeled in vitro LDL–proteoglycan complex produced a 55% suppression; LDL had no effect. In contrast, the binding and internalization $^{125}$I-LDL were not inhibited by excess unlabeled fibrous plaque complex 2, the LDL–proteoglycan complex, or acetyl-LDL (data not shown). (As shown in Figure 4, polyinosinic acid at 50 µg/ml inhibited cholesteryl oleate synthesis mediated by fibrous plaque complex 2 from the additional aortas by 79%.) These results further indicated that the scavenger receptor was involved in the uptake of lipoprotein–proteoglycan complexes in monocytes/macrophages.

To test whether phagocytosis played any role in the uptake and subsequent stimulation of cholesteryl ester synthesis in macrophages, we tested the ability of cytochalasin D, an inhibitor of phagocytosis, to inhibit the stimulation of cholesteryl oleate synthesis. During a 16-hour incubation, cytochalasin D (10 µg/ml) decreased cholesteryl ester synthesis mediated by fibrous plaque complex 1 by 25% and fibrous plaque complex 2 by 7.5% (Table 8). However, at these concentrations the drug did not affect the acetyl-LDL–mediated cholesteryl ester synthesis in the cells.

Experiments were performed to test whether the cholesterol esterification stimulatory activity of the lipoprotein–proteoglycan complexes was associated with the intact complex and/or its constituent lipoproteins. Complex 2 from fibrous plaque was dissociated, and the lipoproteins were recovered by ultracentrifugation at a density of 1.063 g/ml. Figure 5 compares the ability of intact complex 2 from pooled fibrous plaques and its lipoprotein component to stimulate cholesteryl ester synthesis in monocytes/macrophages. The lipoprotein alone stimulated cholesteryl ester synthesis. This stimulation was almost fivefold greater than that mediated by plasma LDL (data not shown). However, at all concentrations the intact complex was twice as effective as the constituent lipoprotein in stimulating cholesteryl ester syn-
were then incubated in RPMI-1640 containing 10% lipoprotein-DMSO. After 16 hours at 37°C, the cellular content of [14C]cholesterol oleate was determined. Each value represents the average of duplicate assays. LDL, low density lipoprotein.

Fibrous plaque

<table>
<thead>
<tr>
<th>LDL-proteoglycan complex (in vitro)</th>
<th>Bound (nmol/mg cell protein)</th>
<th>Internalized (nmol/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.2±0.02</td>
<td>0.9±0.01</td>
</tr>
<tr>
<td>Acetyl-LDL</td>
<td>2.3±0.6</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>Fibrous plaque complex 2</td>
<td>0.4±0.05</td>
<td>1.3±0.3</td>
</tr>
</tbody>
</table>

Table 8. Effect of Cytochalasin D on Cholesteryl Ester Synthesis in Monocytes/Macrophages Incubated With Anti-Apo B Affinity-Purified Lipoprotein-Proteoglycan Complexes From Fibrous Plaque or Acetylated Low Density Lipoprotein

Table 7. Effect of Various Ligands on the Binding and Internalization of the [125I]-Low Density Lipoprotein-Proteoglycan Complex and [125I]-Acetylated Low Density Lipoprotein in Monocytes/Macrophages

<table>
<thead>
<tr>
<th>Unlabeled competitor ligand</th>
<th>Bound (nmol/mg cell protein)</th>
<th>Internalized (nmol/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>5.1±0.8</td>
<td>8.8±0.8</td>
</tr>
<tr>
<td>Acetyl-LDL</td>
<td>2.3±0.6</td>
<td>3.8±0.5</td>
</tr>
</tbody>
</table>

Discussion

In agreement with our earlier reports, the present study shows that human atherosclerotic lesions contain intact lipoprotein-proteoglycan complexes that can be isolated by mild extraction procedures. By gel-exclusion chromatography, the crude extract from fatty streaks was resolved into a high-molecular-weight fraction containing lipoproteins and proteoglycans. In contrast, the extracts from fibrous plaque lesions contained both high- and low-molecular-weight complexes. We previously found that the fibrous plaque extract contained only a high-molecular-weight complex. The discrepancy may result from the use of more sensitive assay procedures for cholesterol and uronic acid in the present study, which helped to identify the low-molecular-weight complex in the fibrous plaque extract.

The use of an anti-apo B affinity column offers a convenient way of isolating apo B–lipoprotein–proteoglycan complexes from atherosclerotic lesions. The functional properties of LDL with respect to uptake by macrophages were not altered when it was eluted from the affinity column by alkaline buffers. This strongly indicates that the elution procedure did not modify apo B in the complexes. The complexes purified from fatty streaks and fibrous plaques con-
cholesterol was determined as described under "Methods." Each glycan complexes isolated by anti-apolipoprotein B affinity chromatography. After 48 hours the cellular content of [14C] cholesteryl oleate was determined. Each data point is the average of triplicate assays.

![Graph](attachment:image.png)

**Figure 5.** Cholesteryl ester synthesis by monocytes/macrophages incubated with anti-apolipoprotein (apo) B affinity-purified lipoprotein-proteoglycan complex 2 from fibrous plaque or its constituent lipoproteins. Macrophages were cultured for 7 days. The cells were then incubated in RPMI-1640 containing 10% lipoprotein-deficient serum, 0.2 mM [14C]oleate-albumin, and the indicated concentrations of affinity-purified complex 2 from fibrous plaque, its constituent lipoproteins, or plasma low density lipoprotein. After 16 hours at 37 °C, the cellular content of [14C] cholesteryl oleate was determined. Each data point is the average of triplicate assays.

Tained apo B--containing lipoproteins and proteoglycans but no other apolipoproteins. This suggests that plasma LDL could be the source of apo B in the complexes. The lipid composition of the complexes (Table 4) also resembles that of LDL. However, immunoreactive apo B constitutes only 34–46% of total proteins in the various complexes. Whether the remainder of the proteins represents modified apo B proteins remains to be determined. In this context it may be pointed out that Hoff and Clevidence41 identified albumin and fibronectin in addition to apo B and apo E in large cholesteryl ester–rich particles isolated from human atherosclerotic lesions.

Although the protein and lipid composition of the complexes from fatty streaks and fibrous plaques were very similar, the fatty streak complex contained relatively less uronic acid than did the two fibrous plaque complexes. As previously noted,13 the complexes also differed in glycosaminoglycan composition of their constituent proteoglycans. Although the two complexes from fibrous plaques contained varying amounts of heparin, this glycosaminoglycan was completely absent in the fatty streak complex. Of all the glycosaminoglycans, heparin has the most potent ability to form complexes with apo B–containing lipoproteins in vitro.42 The presence of heparin in aortic tissue has been previously described.43 However, it is also likely that the fibrous plaque complexes might have acquired mast cell–derived heparin with the progression of the lesions.

Earlier studies,15–18,44 including our own, have shown that complexes of LDL and artery wall proteoglycans formed in vitro stimulate cholesteryl ester synthesis in macrophages. However, the present study shows for the first time that lipoprotein–proteoglycan complexes isolated from human atherosclerotic lesions induce intracellular cholesteryl ester accumulation in human monocytes/macrophages. Morphological and biochemical observations indicate that the increased accumulation results from the uptake of the complexes followed by increased cholesteryl ester synthesis.

Polyinosinic acid significantly inhibited cholesteryl ester synthesis mediated by the complexes from fibrous plaque. This suggests that the scavenger receptor plays a major role in the uptake of the complexes in monocytes/macrophages. This is supported by the observation that the binding and internalization of 125I-acetyl-LDL was suppressed by 87% by excess unlabeled fibrous plaque complex 2. The partial inhibition by an excess of unlabeled in vitro LDL–proteoglycan complex differs in affinity for the scavenger receptor. Because excess fibrous plaque complex 2 and LDL failed to suppress the binding and internalization of 125I-acetyl-LDL and 125I-LDL–proteoglycan complexes, respectively, it is unlikely that the apo B/E receptor plays a role in the uptake of the complexes in monocytes/macrophages. These observations differ from those of Hurt et al,18 who found that in vitro LDL–proteoglycan complexes were internalized through the apo B/E receptor in monocytes/macrophages. The discrepancy may result from the use of macrophages at different stages of maturation in the two studies. We performed our experiments with cells cultured for 7 days to ensure transformation into macrophages, and Hurt et al used cells cultured for 3 days. Apo B/E receptor and
scavenger receptor activities are known to change dramatically during the maturation of human monocytes into macrophages. 45

Because polyinosinic acid did not completely inhibit the cholesteryl oleate synthesis elicited by the aortic lipoprotein–proteoglycan complexes, we considered phagocytosis as an additional mechanism for the uptake of the complexes. This is because (the complexes are mostly soluble) some of the complexes might remain as particulate material due to their larger size and consequently be internalized by the cells through phagocytosis. Thus, cytochalasin D, an inhibitor of phagocytosis in macrophages, inhibited cholesteryl ester synthesis mediated by fibrous plaque complex 1 by 25% and complex 2 by 7.5%. These data suggest that although phagocytosis contributed to some of the uptake of the complexes, it is only a minor pathway by which these complexes are metabolized in monocytes/macrophages.

Cholesterol esterification induced by the uptake of the lipoprotein–proteoglycan complexes seems to require endocytosis and lysosomal degradation of the complexes because chloroquine, a lysosomotropic agent, completely inhibited this process.

The uptake of the complexes by macrophages resulted in the substantial intracellular accumulation of both unesterified and esterified cholesterol. A similar observation was made by Hurt and Camejo 39 of human monocytes/macrophages incubated with LDL–proteoglycan complexes formed in vitro. In this respect the human cells seem to differ from mouse peritoneal macrophages. The mouse cells do not increase their intracellular content of unesterified cholesterol when they are incubated with LDL–proteoglycan complexes formed in vitro. 15

Several studies have reported that lipid peroxidation of LDL by chemical or biologic processes leads to its increased uptake by macrophages. 7, 46, 47 However, in the current study excessive peroxidation of the lipoprotein–proteoglycan complexes that led to their increased uptake by macrophages and consequently cause enhanced synthesis of cholesteryl ester did not occur during the postmortem time interval. This is because we did not find any difference between the TBARS of complexes isolated from postmortem tissue and fresh tissue obtained at surgery. In addition, these complexes did not differ in their ability to stimulate cholesteryl ester synthesis in monocytes/macrophages. Therefore, the mild lipid peroxidation of lipoprotein–proteoglycan complexes may result from changes associated with the pathogenesis of atherosclerosis rather than the result of postmortem changes.

When compared with plasma LDL the lipoprotein component of the affinity-purified complex by itself was able to stimulate cholesterol esterification in monocytes/macrophages. This could result from the oxidative modification of the lipoproteins during the atherogenic process (Table 6). Morton et al 40 previously reported that an LDL-sized lipoprotein particle isolated from human atherosclerotic lesions induced greater stimulation of cholesteryl ester synthesis in mouse peritoneal macrophages than plasma LDL. However, the intact lipoprotein–proteoglycan complex in our study was twofold more effective than the constituent lipoprotein in stimulating cholesteryl ester synthesis. This indicates that the association of apo B-containing lipoproteins with artery wall proteoglycans makes the former more recognizable by monocytes/macrophages. Whether this increased recognition results from subtle changes in the lipoproteins induced by interaction with proteoglycans cannot be answered in the present study.

There are major differences in the esterification stimulatory activity among the different complexes from lesions. Lipoprotein–proteoglycan complexes
from fibrous plaque lesions were considerably more potent than the complex from fatty streaks in inducing cholesterol esterification in macrophages. This could result from differences in the chemical composition of the complexes. As previously mentioned the complex from fatty streaks lacks heparin, and the two complexes from fibrous plaques contain varying proportions of this glycosaminoglycan. Our recent studies indicate that the interaction of a high-affinity heparin subfraction with LDL stimulates intracellular cholesteryl ester accumulation in mouse peritoneal macrophages. It is likely that heparin-containing lipoprotein complexes are more recognizable by macrophages than are complexes that lack this glycosaminoglycan.

In summary, the present study shows that lipoprotein–proteoglycan complexes isolated from human atherosclerotic lesions are taken up by human monocytes/macrophages that induce intracellular cholesteryl ester synthesis and accumulation. Uptake of such complexes by resident macrophages could be a potential mechanism of foam cell formation in atherosclerosis.

References

Vijayagopal et al. Macrophage Uptake of Aortic Lipoprotein–Proteoglycan Complexes 249

47. Henriksen T, Mahoney EM, Steinberg D: Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. *Proc Natl Acad Sci USA* 1981;78:6499–6503

**KEY WORDS** • monocytes/macrophages • cholesterol esterification • lipoprotein–proteoglycan complexes
Lipoprotein-proteoglycan complexes from atherosclerotic lesions promote cholesteryl ester accumulation in human monocytes/macrophages.

P Vijayagopal, S R Srinivasan, B Radhakrishnamurthy and G S Berenson

doi: 10.1161/01.ATV.12.2.237

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/2/237

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/