Effect of Arterial Proteoglycans and Glycosaminoglycans on Low Density Lipoprotein Oxidation and Its Uptake by Human Macrophages and Arterial Smooth Muscle Cells

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The reversible interaction of low density lipoprotein (LDL) with arterial chondroitin sulfate proteoglycans (CSPGs) or glycosaminoglycans (GAGs) selects LDL particles with a high affinity for sulfated GAGs and also induces modifications in apolipoprotein B (apo B) and the lipid organization of the lipoprotein. In the present work we studied the effect that the reversible interaction with sulfated polysaccharides has on the susceptibility of LDL to in vitro oxidation. For this purpose soluble, nonaggregated CSPG- or GAG-treated LDL was subjected to oxidation in the presence of 5 μM CuSO₄ for as long as 48 hours. The rate of formation of thiobarbituric acid-reactive substances, the decrease in isoelectric point, the increase in relative electrophoretic mobility of LDL, the higher degradation rate by human macrophages, and the lower degradation rate by human arterial smooth muscle cells showed that LDLs exposed to CSPGs and GAGs were significantly more susceptible to oxidation than native LDL. Results from competition experiments indicate that C6S-treated LDL after 4 hours of oxidation is taken up via the acetylated LDL receptor in human macrophages. Coincubation of lipoproteins with human macrophages or human arterial smooth muscle cells for 24 hours also indicated that C6S-treated LDL was more susceptible to cell-induced modifications than native LDL. The occurrence in vivo of similar processes may contribute to focal retention, increased rate oxidation of LDL in the arterial intima, and foam cell formation during atherogenesis. (Arteriosclerosis and Thrombosis 1992;12:569–583)

KEY WORDS • atherogenesis • human macrophages • proteoglycans • chondroitin 6-sulfate • low density lipoprotein oxidation rate

Atherosclerotic lesions are characterized by the focal accumulation of apolipoprotein B (apo B)-containing lipoproteins in the extracellular matrix of the arterial intima and the presence of macrophage-derived foam cells.1–3 Several lines of evidence indicate that the proteoglycans (PGs) of the extracellular matrix contribute to the deposition of apo B lipoprotein in arterial lesions.4–11 The interaction of low density lipoprotein (LDL) with human arterial chondroitin sulfate proteoglycans (CSPGs) or glycosaminoglycans (GAGs) induces changes in the apo B lipoprotein and lipid organization that can be observed by low-angle x-ray analysis,12 differential scanning calorimetry,13 and proteolytic pattern analysis.14 In addition, the reversible interaction of LDL with arterial CSPGs has been shown to increase LDL's in vitro uptake by human macrophages15 and human arterial smooth muscle cells (ASMCs).16 These effects are probably caused by both the structural modifications on LDL induced by the formation of reversible complexes with the intimal PGs and the selection of LDL particles with high affinity for CSPG.17

Biological modifications of LDL have been proposed to play an important role in the development of atherosclerosis. There is evidence that lipid peroxidation is a likely in vivo process responsible for LDL modification.18–20 However, oxidative modification is unlikely to occur in the circulation, but it may take place in the vascular wall, which may be devoid of efficient antioxidant mechanisms.19 Although the in vivo mechanism involved in oxidation of LDL is poorly understood, its occurrence in the extracellular environment of the arterial wall may contribute to the atherogenicity of LDL.21 This could cause 1) migration of monocytes/macrophages, which accumulate at the sites of lesion progression in response to chemoattractants and growth factors produced during peroxidation22–24; 2) unregulated uptake of aggregated LDL by macrophages22; and
LDL was labeled before the reversible interaction with than 5% of the total radioactivity was lipid extractable. Precipitated with 15% (wt/vol) trichloroacetic acid. Less cellular uptake, iodination was performed with 125I-

iodination procedures was between 50 and 180 cpm/ng tyramine cellobiose. The specific activity for both labeling of the lipoproteins with 125I was performed with 125I-

Materials

Sodium [125I]iodide (14.4 mCi/µg iodine) was purchased from Amersham International (Amersham, England). Plastic culture dishes and tubes were purchased from Nunclon (Delta, Denmark). Ficoll-Hypaque and PD-10 Sephadex columns were purchased from Pharmacia (Uppsala, Sweden). Millex-GV (non-protein binding filters for lipoprotein and medium filtering) were purchased from Millipore S.A. (Molsheim, France). All cell-culture supplies were obtained from Flow Laboratories (Irvine, Scotland). Salts, buffer substances, and solvents used in this work were of analytical grade and were purchased from Merck (Darmstadt, FRG).

Lipoproteins

Differential centrifugation in potassium bromide solutions containing 1 mg/ml Na2EDTA was used for the preparation of LDL within the density range 1.019-1.063 g/ml. The lipoproteins were maintained in potassium bromide with 1 mg/ml Na2EDTA and 0.2 mM phenylmethylsulfonyl fluoride at 2-4°C. Just before use, the lipoproteins were equilibrated with the appropriate solution using PD-10 gel exclusion columns (Pharmacia). In the present experiments lipoproteins from four different donors were used.

For the studies of cellular degradation, conventional labeling of the lipoproteins with [125I]tyramine was performed with the iodine monochloride method. For the studies of cellular uptake, iodination was performed with [125I]-tyramine cellobiose. The specific activity for both iodination procedures was between 50 and 180 cpm/ng protein. More than 99% of the radioactivity could be precipitated with 15% (wt/vol) trichloroacetic acid. Less than 5% of the total radioactivity was lipid extractable. LDL was labeled before the reversible interaction with CSPGs or GAGs. Acetylation of labeled LDL (Ac-LDL) was performed with acetic anhydride.

Methods

Culture of Human Macrophages and Arterial Smooth Muscle Cells

Human monocyte-derived macrophages (HMDMs) were obtained from buffy coats by the Ficoll-Hypaque procedure. In the present experiments cells from 10 donors were used. Mononuclear cells were washed five times with calcium- and magnesium-free phosphate-buffered saline (PBS), pH 7.2, containing 10 mM Na2EDTA to remove the platelets. Mononuclear cells were resuspended at 2-5x10^6 cells/ml in serum-free RPMI-1640 medium supplemented with 24 mM NaHCO3, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids. One milliliter of cell suspension was seeded in 16-mm plastic wells, and the monocytes were allowed to adhere overnight. Mononuclear cell preparations consist of 70% lymphocytes (nonadhering cells) and 30% monocytes. Non-adherent cells were eliminated the next day by six washes with PBS, and adherent monocytes (10^6) were cultured with 1 ml RPMI-1640 medium supplemented with 10% (vol/vol) fetal calf serum, 10% (vol/vol) human serum, 24 mM NaHCO3, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids (medium A). The yield was practically 100%. The rationale for initially plating the cells in serum-free medium overnight followed by serum supplementation was to avoid the possible activation of monocytes/macrophages by lymphocytes because of the presence of alloantigens in pooled human sera and fetal calf serum. The experiments were performed with HMDMs that were cultured for 8-12 days after plating. Twenty-four hours before the addition of labeled lipoproteins, the cells were washed three times with 1 ml PBS, pH 7.2, containing 2 mg/ml bovine serum albumin (PBS-BSA), and three times with 1 ml PBS, pH 7.2. The cells were then incubated in 1 ml culture medium containing 5 mg protein/ml apo B-free lipoprotein-deficient human plasma (medium B) to induce LDL receptor expression. All the incubations were performed at 37°C in a 5% CO2 humid atmosphere.

Primary cultures of human ASMCs from the inner media of human uterine arteries were established using a previously described explantation technique. The experiments were carried out using cells between passages 4-8. Bulk preparation of the cells for the experiments was made in Waymouth's MB medium supplemented with human serum and fetal calf serum at concentrations of 10% (vol/vol) each, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids. Cells were harvested by trypsinization and passed at 2x10^4 cells/ml per well (16-mm diameter) in 24-well plates. The cells were allowed to proliferate for 7 days (90% confluence) and were preincubated with lipoprotein-deficient Waymouth's MB medium 24 hours before adding the lipoproteins.
Treatment of Low Density Lipoprotein With Human Arterial Chondroitin Sulfate Proteoglycans, Chondroitin 6-Sulfate, and Heparin

Human arterial CSPGs were extracted from human aortic intima−media segments obtained from victims of traffic accidents within 24 hours postmortem. The isolation and characterization of the CSPG aggregates have been described in detail.34,39 The composition of the arterial CSPGs used in these experiments was 60–65% C6S, 10–20% chondroitin 4-sulfate, and 10–20% dermatan sulfate. C6S (shark cartilage) GAGs were of special grade and were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Hep (grade 1) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

The effect of arterial CSPGs and GAGs on LDLs was studied under two conditions: 1) after reversible complexes of LDL with CSPGs and GAGs were formed at low ionic strength, collected by centrifugation, washed, and solubilized with physiological ionic solutions and 2) after binding of LDL at low ionic strength to GAGs that were immobilized on agarose columns and later eluted by raising the ionic strength. With the first treatment, LDLs modified by arterial PGs (CSPG-LDL), C6S-LDL, or Hep-LDL were prepared by adding four sequential 10-μl aliquots of arterial CSPG (1 mg/ml GAGs, as calculated from the hexuronate content; x3.3) or GAG (1 mg GAG/ml) solution to 1 ml LDL solution (2 mg apo B protein/ml). In a typical reaction <0.02 mg CSPG or GAG per milligram of apo B protein was used.17 The lipoprotein, CSPG, C6S, and Hep were previously equilibrated in 5 mM HEPES buffer containing 20 mM NaCl, 4 mM CaCl2, 2 mM MgCl2, pH 7.2 (buffer A) and kept on ice. After 30 minutes approximately 60% of the LDL was precipitated by CSPG, C6S, or Hep, and the complexes were collected by centrifugation at 10,000g at 4°C for 10 minutes. The supernatant containing the nonprecipitated LDL was removed, and the pellets were washed with buffer A and redissolved without mechanical stirring in 5 mM HEPES buffer containing 150 mM NaCl, 4 mM CaCl2, 2 mM MgCl2, pH 7.2 (buffer B), or in RPMI-1640 or Waymouth's MB cell culture medium for HMDMs and human ASMCs, respectively. Under these conditions the CSPG-LDL, C6S-LDL, and Hep-LDL were recovered as nonaggregated solutions with no signs of protein or lipid hydrolytic degradation and with a molecular size similar to that of native LDL (N-LDL).15,40 The solutions of modified LDL contained <10 μg CSPG or C6S per milligram of LDL protein.

LDL was also complexed to Hep and C6S that were covalently bound to Sepharose in 5-cm-long, 1-cm-diameter columns filled with 2 ml gel. Hep-Sepharose gel was purchased from Pharmacia. C6S was attached to N-3-hydroxysuccinimide-activated Sepharose (Affi-gel, Bio-Rad, Richmond, Calif.). A similar Sepharose column without the immobilized Hep or C6S was used as a blank control. After washing and equilibration of the columns in buffer A (see above), 1-ml aliquots of LDL (1 mg apo B protein/ml), also equilibrated in buffer A, were loaded at the same time in parallel in the Hep or C6S-Sepharose columns and in the Sepharose control column. By stopping the flow, the LDLs were allowed to remain in the columns for 2 hours at 20°C and were then eluted with one column volume of buffer B (see above) made with 200 mM NaCl. Under these conditions the eluted LDLs from the experimental and blank columns were collected in a solvent of very similar composition and without the presence of GAGs. No differences in the levels of thiobarbituric acid–reactive substances (TBARS) and 232-nm absorption have been observed in the LDL before and after elution from both columns,41 thus suggesting that no detectable oxidation of the LDL occurred in the columns.

In Vitro Oxidation of Native Low Density Lipoprotein, Acetylated Low Density Lipoprotein, and Low Density Lipoprotein Treated With Arterial Chondroitin Sulfate Proteoglycans and Glicosaminoglycans

Labeled and unlabeled N-LDL, Ac-LDL, CSPG-LDL, C6S-LDL, Hep-LDL, Hep-Sepharose-LDL, C6S-agarose-LDL, and Sepharose-N-LDL were adjusted to 250 μg protein/ml with buffer B and incubated at 37°C with 5 μM CuSO4 in acid-washed borosilicate glass tubes with Teflon-lined, loosely tightened screw caps for up to 48 hours. At different time periods as indicated in the tables and figures, 0.5-ml aliquots were withdrawn and diluted to a concentration of 25 μM by addition of a 1 mM ethanolic solution of butylated hydroxytoluene (BHT) to arrest the oxidative process until they were analyzed. Control samples were incubated at 37°C with 5 μM CuSO4 in the presence of 20 μM BHT that was added to the samples before addition of the copper.

Cell Modification of Chondroitin 6-Sulfate-Low Density Lipoprotein and Native Low Density Lipoprotein

The susceptibility of C6S-LDL compared with that of N-LDL to cell-induced oxidation was studied by coincubating 125I-tyramine-cellobiose–labeled lipoproteins (50 or 200 μg protein/ml) for 24 hours with HMDMs and human ASMCs. Because activated HMDMs and polymorphonuclear leukocytes, prominent at the sites of inflammation, are known to produce reactive species of oxygen,28 we also wanted to study the susceptibility of the lipoproteins to oxidation by human macrophages activated with interferon gamma (IFN-γ). For this purpose HMDMs cultured for 8 days were treated with 100 units/ml human recombinant IFN-γ (Boehringer, Mannheim, FRG) 24 hours before and during the coincubation with the lipoproteins. HMDMs, IFN-γ-treated HMDMs, and ASMCs were cultured in 16-mm culture dishes in 250 μl serum-free RPMI-1640 medium containing 200 μg protein/ml N-LDL or C6S-LDL 1) with 5 μM CuSO4, 2) without 5 μM CuSO4, or 3) with 5 μM CuSO4 plus 20 μM BHT to arrest the copper-catalyzed oxidation. Incubations were done at 37°C, 5% CO2, for 24 hours. Control dishes without cells were incubated in parallel under identical conditions. The medium containing the lipoproteins was removed after 24 hours of coincubation with or without cells. Oxidation was arrested by adding BHT to a final concentration of 20 μM to those samples that did not contain BHT during the coincubation. Na2EDTA, at a final concentration of 10 μM, was added to all the samples. Aliquots were removed for measurement of TBARS, agarose electrophoresis, isoelectric point analysis, and 10% polyacrylamide gel electrophoresis. The remaining
sample was equilibrated in culture medium using PD-10 columns (Pharmacia), and aliquots were removed to measure radioactivity and protein content. The samples were then incubated with human ASMCs cultured in 16-mm culture dishes in 0.5 ml lipoprotein-deficient Waymouth's MB medium containing 10 μg protein/ml of each type of preincubated lipoprotein. The cellular uptake was determined as described below.

**Lipoprotein Uptake and Degradation by Human Macrophages and Human Arterial Smooth Muscle Cells**

N-LDL, CSPG-LDL, C6S-LDL, Hep-LDL, Hep-Sepharose-LDL, and Sepharose-N-LDL labeled with 50–70 cpn/ng protein were oxidized as described above for different periods of time as indicated in the figures. HMDMs cultured for 8–12 days and postconfluent human ASMCs were incubated with lipoprotein-deficient RPMI-1640 and Waymouth's MB cell medium (medium B), respectively, for 24 hours before addition of the different labeled lipoproteins. N-LDL, CSPG-LDL, and C6S-LDL that had been oxidized for different periods and equilibrated in cell-culture medium were added at the concentrations indicated in the figures to cells cultured in 24-well plastic dishes. After 4 hours' incubation, the extent of lysosomal degradation was determined by measuring the content of chloroform-extracted, acid-soluble [125I]-monooiodotyrosine in the medium. Cell surface-bound lipoproteins were released by trypsinization of the cells. The release of surface-bound lipoprotein by Hep (LDL receptor) or polyniosinic acid (scavenger receptor) would have been a more classical and easier method to use instead of trypsinization. However, the structural modifications induced in the LDL particle by the reversible interaction with sulfated polysaccharides will most probably affect the Hep or polyniosinic acid binding properties of LDL. The amount of N-LDL, CSPG-LDL, or C6S-LDL that could be released from the cell surface, before or after oxidation, would depend on the capacity of each type of lipoprotein to bind to either Hep or polyniosinic acid but would not affect lipoprotein that is actually bound to the cell surface. For this reason we considered it more appropriate to measure the amount of lipoprotein bound to the cell surface by releasing it with trypsin treatment, as previously reported by us and others. Adherent cells were washed once with PBS containing 2 mg BSA/ml and twice with PBS alone. The cells were treated with 5 mg/ml trypsin in 1 ml calcium- and magnesium-free Earle's medium for 10 minutes at 37°C. The medium with trypsin was transferred to a tube, and the trypsinized macrophages were washed with 0.5 ml Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum and 0.5 ml PBS. The trypsin medium and the two washes were mixed, and the radioactivity corresponding to the cell surface-bound lipoprotein was counted. Treatment with trypsin does not produce detachment of the macrophages. Adherent macrophages were dissolved by the addition of 0.4 ml 0.2N NaOH (2×) and transferred to a tube. The radioactivity corresponding to the amount of lipoprotein internalized by the cells was counted. In the case of human ASMCs, the treatment with trypsin detached the cells from the plastic dish surface. The trypsinized cells plus two washes of the wells were centrifuged for 5 minutes at 3,000 rpm. The supernatant was transferred to another tube and the pellet washed once with PBS. The two supernatants were pooled and counted to evaluate the radioactivity corresponding to the ASMC-surface-bound lipoproteins. The cell pellets were dissolved in 1 ml 0.2N NaOH. Aliquots from the cell extraction were used to measure protein content.

Competition experiments were performed as described above, except for the addition of a constant amount of 4- or 24-hour-oxidized [125I]-labeled LDL or C6S-LDL (5 μg protein/ml) in the absence or presence of as much as 20× unlabeled lipoprotein competitors as indicated in the figure legend.

When [125I]-tyramine cellobiose-labeled lipoproteins were used to measure lipoprotein cell uptake, the cell medium was removed and the cells were washed once with PBS containing 2 mg/ml BSA and twice with PBS alone. The amount of intracellular lipoprotein was determined after treatment with trypsin as described above.

In all cell assays, parallel dishes without cells were included as controls for nonspecific adsorption or degradation of the label. Radioactivity and protein values in these dishes were subtracted from those of the dishes with cells. The experiments were performed in duplicate or quadruplicate and repeated at least once.

**Analytical Procedures**

Protein was determined by the Bradford procedure using bovine gamma globulin as the standard (Bio/Rad protein assay, Bio-Rad). [125I] radioactivity was measured with an LKB 1282 Compgamma Universal Gamma Counter (LKB, Bromma, Sweden). Analytical isoelectric focusing of LDL and C6S-LDL before and after oxidation was carried out on 0.5-mm-thick 1% agarose gels containing 10% (wt/vol) sorbitol, 1% Tween-20, and 5% (vol/vol) of a mixture of Pharmalyte 4–6.5 and 5–8.4. Estimation of TBARS was carried out essentially as described. Agarose electrophoresis was performed on a 0.5% gel. Single 10–20-μl applications of lipoproteins were applied to each lane of the gel. The electrophoresis was run with barbital buffer, pH 8.5, at 170 V for 45 minutes. The gels were lipid stained with a solution of fat red 7B (0.86%) and oil red O (0.015%) in 60% ethanol. The lipoprotein mobility was expressed as a ratio of the sample mobility to that of the unoxidized control LDL.

**Results**

**Effect of Arterial Chondroitin Sulfate Proteoglycans on Low Density Lipoprotein Oxidation Rate**

LDLs that interacted with human arterial CSPGs at low ionic strength (buffer A) and that were dissociated by raising the salt concentration to physiological levels (buffer B) were oxidized at a faster rate than N-LDL when exposed to copper. This was shown by the rate of appearance of TBARS and the changes in the surface charge of the lipoproteins (Figure 1). Agarose isoelectric focusing of the lipoproteins showed that CSPG-LDL changed its isoelectric point from 5.20 to 4.80 after 4 hours of copper-catalyzed oxidation whereas it took 48 hours of oxidation to change the isoelectric point of...
intracellular lipoprotein (dashed line and open symbols) was determined after treatment of the macrophages with trypsin. Macrophages were incubated with lipoprotein-deficient medium containing the indicated concentrations of each \(^{125}\)I-labeled lipoprotein. After 4 hours, the extent of lipoprotein degradation was arrested with 25 \(\mu\)M butylated hydraxyltoluene (BHT). Human monocyte-derived macrophages of chondroitin sulfate proteoglycans (CSPG)–LDL after different periods of copper-catalyzed oxidation. \(^{125}\)I-labeled lipoproteins (250 \(\mu\)g/ml) were incubated at 37°C with 5 \(\mu\)M CuSO\(_4\) without butylated hydraxyltoluene (BHT) or in the presence of BHT as a control (lane C). Note that native LDL and CSPG-LDL were incubated for different periods of time. Copper-catalyzed oxidation was arrested with 25 \(\mu\)M BHT. Standard proteins indicated at left are human carbonic anhydrase (\(pI\) 6.55), bovine carbonic anhydrase (\(pI\) 5.85), \(\beta\)-lactoglobulin \(A\) (\(pI\) 5.20), and soybean trypsin inhibitor (\(pI\) 4.55). At the bottom of the gels, the thiobarbituric acid-reactive substances (TBARS) values (nanomoles of malondialdehyde per milligram of protein) and the isoelectric point (\(pI\)) of the lipoproteins are indicated.

N-LDL from 5.20 to 5.00. Therefore, CSPG-LDL was a more negatively charged particle at physiological pH than was N-LDL after a comparable oxidation period. This effect was inhibited by 20 \(\mu\)M BHT added to the samples before addition of copper.

These differences in physical properties between CSPG-LDL and N-LDL after oxidation were reflected in their uptake and degradation by human macrophages. Figure 2 shows a saturable degradation curve for CSPG-LDL after 4 hours of oxidation, indicating the presence of specific recognition sites for oxidized CSPG-LDL in human macrophages. CSPG-LDL after 4 hours of oxidation was degraded to a significantly higher extent than was N-LDL after 48 hours of oxidation at protein concentrations from 5 to 50 \(\mu\)g/ml. Similar results were obtained for the amounts of lipoproteins that remained internalized in the macrophages after trypsin release of cell surface-bound lipoproteins (data not shown).

**Effect of Glycosaminoglycans on Low Density Lipoprotein Oxidation Rate**

The interaction of LDL with human arterial PGs has been shown to occur by the specific interaction between the negatively charged CS GAG moiety of the PGs and the positively charged regions in the apo B lipoprotein. To show that the increase in the LDL oxidation rate induced by human arterial PGs was due to this specific interaction with GAGs and not to an effect of possible contaminant proteins present in PG preparations, we decided to perform the experiments with commercially available sulfated GAGs.

LDLs insulubilized with C6S and with Hep at low ionic strength and dissociated by raising the salt concentration to physiological levels were oxidized at a faster rate than was N-LDL after exposure to copper. This was shown by the rate of appearance of TBARS and the changes in the relative electrophoretic mobility (Table 1). This effect was inhibited by 20 \(\mu\)M BHT added to the samples before addition of copper (Table 1). Similar to the results obtained by LDL interaction with human arterial CSPG, C6S-LDL and Hep-LDL were also more negatively charged particles at physiological pH than was N-LDL after comparable periods of copper-catalyzed oxidation.

In general there was a linear relation between the appearance of TBARS and the relative electrophoretic mobility, except for Ac-LDL, oxidized Ac-LDL, and the GAG-treated LDLs that were extremely oxidized. This nonlinearity may have been caused by the bimodal shape of the TBARS and diene formation curves that increase linearly at early oxidation stages but decrease at late stages. On the other hand, the relative electrophoretic mobilities increased continuously because of oxidative modifications of the lysine and probably the arginine and histidine residues that occurred under the conditions used. This phenomenon was especially noticeable in those lipoproteins that were more susceptible to oxidation, such as Hep-LDL, C6S-LDL, and CSPG-LDL. For example, at the 24-hour oxidation point in experiment 1 (Table 1), the TBARS yield for Hep-LDL and N-LDL appear similar (12 nmol malondialdehyde/mg protein) although the relative electrophoretic mobility for Hep-LDL was higher (3.3) than that for N-LDL (2.3). Another source of variation in the TBARS yield among LDL preparations from different donors is their dissimilar content of endogenous antioxidants and unsaturated fatty acid composition. Large amounts of GAGs or CSPGs that remain associated...
with LDL may increase its electrophoretic mobility. However, after dissolving the pellet, no measurable sulfated polysaccharide remained associated with the LDL.14 In experiments 1 and 2, similar amounts of GAGs were used to precipitate the lipoproteins. Because GAGs by themselves do not contribute TBARS, we attribute the observed differences to a dissimilar endogenous susceptibility of the two LDL preparations used.

**Degradation of Chondroitin 6-Sulfate—Low Density Lipoprotein and Low Density Lipoprotein by Human Arterial Smooth Muscle Cells and Human Macrophages After Similar Periods of Copper-Catalyzed Oxidation**

HMDMs in culture are known to express a receptor for N-LDL and scavenger receptor(s) for chemically modified LDL.51-52 Unstimulated human smooth muscle cells, contrary to macrophages, have been reported to express the N-LDL receptor but not the scavenger receptor(s). 53 We then decided to evaluate the effects of varying degrees of oxidation on the ability of LDL and C6S-LDL to interact with the N-LDL receptor or the scavenger receptor(s) by measuring the lipoprotein uptake, binding, and degradation by cultured human ASMCs and HMDMs. The same lipoprotein preparations were incubated in parallel with human ASMCs and HMDMs. Figure 3 shows the results obtained with human ASMCs. As expected, the degradation of N-LDL by human ASMCs after 4 or 24 hours’ oxidation decreased compared with unoxidized N-LDL. However, the degradation of C6S-LDL after only 4 hours of oxidation decreased significantly more than the degradation of N-LDL oxidized for as long as 24 hours. These results indicate a higher degree of oxidation in C6S-LDL than in N-LDL after similar periods of copper-catalyzed oxidation. Contrary to what we expected, higher amounts of C6S-LDL oxidized for 4 or 24 hours than N-LDL also oxidized for similar periods of time were found to be bound and associated with the ASMCs. Although the amounts of oxidized C6S-LDL found associated with human ASMCs were lower than the amounts measured in HMDMs (Figure 4), they increased progressively with the extent of oxidation. Similar results were observed with arterial CSPG-LDL and Hep-LDL (data not shown).

Figure 4 shows the results obtained after the incubation with HMDMs. Degradation, cell-surface binding, and uptake were much higher for C6S-pretreated LDL than for N-LDL exposed to copper-catalyzed oxidation for comparable periods (Figure 4). C6S-LDL oxidized for 4 hours was degraded five times more than N-LDL oxidized for 24 hours. Similar results were obtained with Hep-LDL (data not shown).

These increases and decreases in lipoprotein degradation observed with HMDMs and ASMCs, respectively, reflect the higher extent of oxidative modifications of C6S-LDL and Hep-LDL when compared with N-LDL oxidized for similar periods of time.

Table 2 shows the extent of copper-catalyzed oxidation for LDL that was bound to Hep and the LDL in the control column, as measured by TBARS and relative electrophoretic mobility. Also in this case, the interaction with the sulfated polysaccharides accelerated the oxidative alterations when compared with LDL that was exposed to a similar column with Sepharose only (control column). Similar results were obtained with LDL passed through a C6S-agarose column (data not shown).

Human macrophages degraded much more LDL eluted from the Hep column (Hep-Sepharose) than LDL eluted from the control column (Sepharose-LDL) after comparable periods of copper-catalyzed oxidation.

### Table 1. Thiorbarbituric Acid–Reactive Substances Contents and Relative Electrophoretic Mobilities of Lipoproteins Used in Cell Experiments

<table>
<thead>
<tr>
<th>Lipoprotein (250 µg protein/ml)</th>
<th>TBARS (nmol MDA/mg protein)</th>
<th>Relative electrophoretic mobility (Exp 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-LDL+BHT</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Native LDL+BHT</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Native LDL, 4-hr oxidation</td>
<td>1.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Native LDL, 8-hr oxidation</td>
<td>2.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Native LDL, 24-hr oxidation</td>
<td>12.0</td>
<td>2.3</td>
</tr>
<tr>
<td>C6S-LDL+BHT</td>
<td>0.0</td>
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</tr>
<tr>
<td>C6S-LDL, 4-hr oxidation</td>
<td>31.0</td>
<td>2.3</td>
</tr>
<tr>
<td>C6S-LDL, 8-hr oxidation</td>
<td>38.0</td>
<td>2.5</td>
</tr>
<tr>
<td>C6S-LDL, 24-hr oxidation</td>
<td>36.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Heparin-LDL+BHT</td>
<td>1.9</td>
<td>1.0</td>
</tr>
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<td>Heparin-LDL, 4-hr oxidation</td>
<td>8.8</td>
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</tr>
<tr>
<td>Heparin-LDL, 24-hr oxidation</td>
<td>12.0</td>
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<tr>
<td>Ac-LDL+BHT</td>
<td>0.24</td>
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<td>Ac-LDL, 6-hr oxidation</td>
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<td>3.5</td>
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<tr>
<td>Ac-LDL, 24-hr oxidation</td>
<td>46.0</td>
<td>3.5</td>
</tr>
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</table>

Lipoproteins (250 µg protein/ml) were incubated at 37°C for the indicated periods of time in the presence of 5 µM CuSO₄. Oxidation was arrested by refrigeration and addition of 25 µM butylated hydroxytoluene (BHT). Thiorbarbituric acid–reactive substances (TBARS) values are averages of duplicates determinations. Relative electrophoretic mobility is the ratio of the mobility of the lipoprotein to that of the native low density lipoprotein (LDL) incubated with CuSO₄ in the presence of BHT. Exp, experiment; MDA, malondialdehyde; C6S, chondroitin 6-sulfate; Ac, acetylated.
Figure 3. Bar graphs of degradation (panel A), cell-surface binding (panel B), and internalization (panel C) of \(^{125}\text{I}\)-labeled low density lipoprotein (LDL) and chondroitin 6-sulfate-LDL (C6S-LDL) by arterial human smooth muscle cells after different periods of copper-catalyzed oxidation. \(^{125}\text{I}\)-labeled LDL and C6S-LDL (250 \(\mu\)g protein/ml) were incubated with 5 \(\mu\)M CuSO\(_4\) at 37°C for 0, 4, or 24 hours. Copper-catalyzed oxidation was arrested with 25 \(\mu\)M butylated hydroxytoluene. Thiobarbituric acid–reactive substances contents and relative electrophoretic mobilities of these lipoproteins are shown in Table 1. Human arterial smooth muscle cells were incubated in Waymouth's MB lipoprotein-deficient medium containing 5 \(\mu\)g protein/ml (○) or 10 \(\mu\)g protein/ml (■) of each labeled lipoprotein. After 4 hours' incubation, the extent of lipoprotein degradation (A), the amount of labeled lipoprotein bound to the cell surface (B), and the amount internalized by the macrophages (C) were determined as described in “Methods.” Values represent the mean of four determinations and the positive value of the standard deviation.

Figure 5 shows that LDL eluted from Hep-Sepharose and oxidized for only 6 hours was degraded to the same extent as LDL eluted from a control Sepharose column and oxidized for 24 hours. In contrast, in ASMCs only 2 hours of copper-catalyzed oxidation were needed with LDL eluted from Hep-Sepharose and 24 hours were needed with LDL from control Sepharose to almost completely abolish the degradation. These results probably reflect the higher extent of oxidative modification of the LDL eluted from Hep-Sepharose when compared with LDL eluted from the Sepharose column and then oxidized for similar periods.

In these experiments although LDL was degraded as expected, no increase in cell-associated LDL was found when compared with LDL obtained from a control column. This result is different from that obtained with GAG-treated LDLs obtained with the pelleting procedure (Figure 3). This dissimilarity may have been caused by a more extensive structural modification introduced into the LDL during the pelleting and redissolving procedures. Another possibility, supported by experiments from our laboratory,\(^{17}\) is that the pelleting procedure selects LDL particles with a higher affinity for the PG and the LDL.
receptor. A third possibility is that the small amount of GAGs or CSPGs added to the cells with the pelleted and redissolved LDL preparation could increase the binding to the cells. However, we have shown that addition of CSPGs to the cells with the LDL, if anything, decreased the binding. 17

**Nature of Recognition Sites for Oxidized Chondroitin 6-Sulfate–Low Density Lipoprotein on Human Macrophages**

Competition experiments were performed to analyze which binding sites recognize oxidized C6S-LDL in human macrophages. Figure 6 shows the results from the experiments in which macrophages were incubated with 5 μg protein/ml 125I–C6S-LDL oxidized for 4 or 24 hours in the absence or presence of 100 μg protein/ml unlabeled potential lipoprotein competitors (N-LDL, C6S-LDL, or Ac-LDL after 0, 4, 8, and 24 hours of copper-catalyzed oxidation). The TBARS contents and the relative electrophoretic mobilities of the labeled and unlabeled lipoproteins used in these experiments are shown in Table 1. Unlabeled oxidized N-LDL and C6S-LDL were ineffective competitors that failed to block the uptake and degradation of

---

**FIGURE 4.** Bar graphs of degradation (panel A), cell-surface binding (panel B), and internalization (panel C) of 125I-labeled low density lipoprotein (LDL) and chondroitin 6-sulfate–LDL (C6S-LDL) by human monocyte–derived macrophages after different periods of copper-catalyzed oxidation. 125I-labeled LDL and C6S-LDL (250 μg protein/ml) were incubated with 5 μM CuSO4 at 37°C for 0, 4, or 24 hours. Copper-catalyzed oxidation was arrested with 25 μM butylated hydroxytoluene. Thiobarbituric acid–reactive substances contents and relative electrophoretic mobilities of the lipoproteins are shown in Table 1. Human macrophages were incubated in RPMI-1640 lipoprotein-deficient medium containing 5 μg protein/ml (□) or 10 μg protein/ml (●) of each type of labeled lipoprotein. After 4 hours' incubation, the extent of lipoprotein degradation (A), the amount of labeled lipoprotein bound to the cell surface (B), and the amount internalized (C) were determined as described in “Methods.” Values represent the mean of four determinations and the positive value of the standard deviation.
TABLE 2. Thiobarbituric Acid–Reactive Substances Contents and Relative Electrophoretic Mobilities of Low Density Lipoprotein After Elution

<table>
<thead>
<tr>
<th>Lipoprotein (250 μg protein/ml)</th>
<th>TBARS (nmol MDA/mg protein)</th>
<th>Relative electrophoretic mobility (exp 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin-Sepharose + BHT</td>
<td>0.45</td>
<td>1.5</td>
</tr>
<tr>
<td>Heparin-Sepharose, 2-hr oxidation</td>
<td>32.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Heparin-Sepharose, 6-hr oxidation</td>
<td>35.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Heparin-Sepharose, 24-hr oxidation</td>
<td>34.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Sepharose CL-4B + BHT</td>
<td>0.45</td>
<td>1.5</td>
</tr>
<tr>
<td>Sepharose CL-4B, 2-hr oxidation</td>
<td>0.70</td>
<td>1.6</td>
</tr>
<tr>
<td>Sepharose CL-4B, 6-hr oxidation</td>
<td>1.21</td>
<td>1.8</td>
</tr>
<tr>
<td>Sepharose CL-4B, 24-hr oxidation</td>
<td>5.04</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Lipoproteins (250 μg protein/ml) were incubated at 37°C for the indicated periods of time in the presence of 5 μM CuSO₄. Oxidation was arrested by refrigeration and addition of 25 μM butylated hydroxytoluene (BHT). Thiobarbituric acid–reactive substances (TBARS) values are averages of duplicate determinations. Relative electrophoretic mobility is the ratio of the mobility of the lipoprotein to that of native low density lipoprotein (LDL) incubated with CuSO₄ in the presence of BHT after elution of the LDL from a heparin-Sepharose CL-4B or a Sepharose CL-4B (control) column. Effect of binding to heparin on LDL susceptibility for copper-catalyzed oxidation is shown. MDA, malondialdehyde; Exp, experiment.

FIGURE 5. Bar graphs of human monocyte-derived macrophage (upper panel) and human arterial smooth muscle cell (lower panel) degradation of low density lipoprotein (LDL) eluted from Sepharose or heparin-Sepharose columns and oxidized for different periods of time. LDL was complexed to heparin that was covalently bound to Sepharose as described in “Methods.” ¹²⁵I-labeled LDLs eluted from the heparin column and from a Sepharose control column (250 μg protein/ml) were incubated with 5 μM CuSO₄ at 37°C for 0, 2, 6, or 24 hours. Copper-catalyzed oxidation was arrested with 25 μM butylated hydroxytoluene. Thiobarbituric acid–reactive substances contents and relative electrophoretic mobilities of the lipoproteins used are shown in Table 2. Human macrophages were incubated with RPMI-1640 lipoprotein-deficient medium containing 5 μg protein/ml (●) or 10 μg protein/ml (●) of each labeled lipoprotein. After 4 hours' incubation, the extent of lipoprotein degradation was determined. Values represent the mean of four determinations and the positive value of the standard deviation.
N-LDL oxidized for 24 hours was ineffective in inhibiting the uptake and degradation of \( 125^{\text{I}} \)-C6S-LDL oxidized for 4 hours. This result suggested either the presence of a specific receptor for oxidized C6S-LDL or differences in the affinity for a common receptor. To evaluate these possibilities, HMDMs were incubated with 5 \( \mu \)g/ml \( 125^{\text{I}} \)-C6S-LDL after 4 hours' oxidation or with \( 125^{\text{I}} \)-LDL after 24 hours' oxidation in the absence or presence of increasing concentrations of unlabeled potential lipoprotein competitors (C6S-LDL oxidized for 4 hours, LDL oxidized for 24 hours, and Ac-LDL). Figure 7 shows the results obtained with these experiments. LDL oxidized for 24 hours failed to inhibit the degradation of \( 125^{\text{I}} \)-C6S-LDL oxidized for 4 hours. However, unlabeled C6S-LDL oxidized for 4 hours could efficiently inhibit the degradation of \( 125^{\text{I}} \)-LDL oxidized for 24 hours. Ac-LDL inhibited the degradation of both \( 125^{\text{I}} \)-C6S-LDL oxidized for 4 hours and \( 125^{\text{I}} \)-LDL oxidized for 24 hours. These results suggest that oxidized LDL and oxidized C6S-LDL share the same uptake mechanisms as Ac-LDL. However, as suggested above, the affinity of these lipoproteins for a common receptor on the human macrophage appears to be related to the extent of the oxidative modification induced in the lipoproteins. The observation that C6S-LDL after 24 hours' oxidation was an effective competitor against C6S-LDL oxidized for only 4 hours, but not vice versa, supports this idea.

Results from these competition experiments also suggest that for both oxidized \( 125^{\text{I}} \)-LDL and oxidized \( 125^{\text{I}} \)-C6S-LDL, >90% of both uptake and degradation measured at 5 \( \mu \)g protein/ml was specifically due to a saturable high-affinity process. Thus, the similar unlabeled ligand competed for more than 90% of the total.

Modification of Native Low Density Lipoprotein and Chondroitin 6-Sulfate–Low Density Lipoprotein by Coincubation With Human Arterial Smooth Muscle Cells and Human Macrophages

Experiments with copper-catalyzed oxidation in vitro showed that C6S-LDL was more susceptible to oxidation than was N-LDL. The next question to answer was whether C6S-LDL was also more susceptible to in vitro cell-induced modifications than was N-LDL. Because ASMCs express receptors for N-LDL but not for other types of modified LDL, we evaluated possible cell-induced modification(s) on the lipoproteins by the ability of C6S-LDL and N-LDL that were preincubated with cells to interact with the N-LDL receptor in human ASMCs in a subsequent incubation period. TBARS and isoelectric points of the lipoproteins were also used as parameters to evaluate the cell-induced modifications on the lipoproteins. \( 125^{\text{I}} \)-tyramine cellobiose–labeled C6S-LDL and N-LDL (200 \( \mu \)g protein/ml) were coincubated for 24 hours with HMDMs, IFN-\( \gamma \)-treated HMDMs, ASMCs, and without cells. The coincubation was done in the absence or presence of 5 \( \mu \)M CuSO\(_4\), or with 5 \( \mu \)M CuSO\(_4\) plus 20 \( \mu \)M BHT. Table 3 shows the uptake by human ASMCs of C6S-LDL and the LDL before and after coincubation. Table 4 shows the isoelectric point values and TBARS contents of the lipoproteins after the coincubation period. The following results indicate that C6S-LDL was more susceptible to cell-induced modifications than was N-LDL: 1) a significant decrease in the lipoprotein uptake by human
FIGURE 7. Plots showing a comparison of the ability of unlabeled lipoproteins to inhibit human monocyte-derived macrophage degradation of $^{125}$I-chondroitin 6-sulfate–low density lipoprotein (C6S-LDL) after 4 hours' oxidation (upper panel) and $^{125}$I-LDL after 24 hours' oxidation (lower panel). Human monocyte-derived macrophages were cultured for 10 days and then incubated with lipoprotein-deficient medium containing 5 µg protein/ml $^{125}$I-C6S-LDL oxidized for 4 hours (upper panel) or $^{125}$I-LDL oxidized for 24 hours and in the absence or presence of increasing concentrations of the following unlabeled lipoproteins as competitors: LDL oxidized for 24 hours (□), acetylated (Ac) LDL (○), and C6S-LDL oxidized for 4 hours (▲, ▽). After 4 hours' incubation, the extent of lipoprotein degradation was measured as indicated in “Methods.” Values are expressed as percentages. The 100% value for the uptake of $^{125}$I-C6S-LDL oxidized for 4 hours was 147±10 ng/mg cell protein/hr and for $^{125}$I-LDL oxidized for 24 hours, 17±4 ng/mg cell protein/hr. Values represent the mean of four determinations.

TABLE 3. Human Smooth Muscle Cell Uptake of $^{125}$I-Tyramine Cellobiose–Labeled Low Density Lipoprotein and Chondroitin 6-Sulfate–Low Density Lipoprotein Preincubated for 24 Hours With or Without Cells

<table>
<thead>
<tr>
<th>Lipoprotein (10 µg/ml)</th>
<th>Without cells</th>
<th>ASMCs (1×10⁶)</th>
<th>HMDMs (1×10⁶)</th>
<th>HMDMs+IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>216±12</td>
<td>132±43</td>
<td>279±15</td>
<td>240±4</td>
</tr>
<tr>
<td>LDL+Cu²⁺</td>
<td>150±12</td>
<td>111±16</td>
<td>132±15</td>
<td>144±8</td>
</tr>
<tr>
<td>LDL+BHT+Cu²⁺</td>
<td>171±21</td>
<td>123±12</td>
<td>165±18</td>
<td>135±16</td>
</tr>
<tr>
<td>C6S-LDL uptake before preincubation</td>
<td>307±27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6S-LDL</td>
<td>150±12</td>
<td>165±23</td>
<td>234±24</td>
<td>216±45</td>
</tr>
<tr>
<td>C6S-LDL+Cu²⁺</td>
<td>180±18</td>
<td>39±9</td>
<td>30±2</td>
<td>44±5</td>
</tr>
<tr>
<td>C6S-LDL+BHT+Cu²⁺</td>
<td>222±21</td>
<td>51±4</td>
<td>132±15</td>
<td>120±24</td>
</tr>
</tbody>
</table>

Low density lipoprotein (LDL) and chondroitin 6-sulfate (C6S)–LDL (200 µg protein/ml) equilibrated in serum-free RPMI-1640 medium were incubated in 24-millwell dishes (0.5 ml/well) with human arterial smooth muscle cells (ASMCs), human monocyte-derived macrophages (HMDMs), HMDMs treated with interferon gamma (IFN-γ) (100 units/ml), or without cells (control). CuSO₄ (5 µM) and butylated hydroxytoluene (BHT) (20 µM) were added at the times indicated. After 24 hours of incubation at 37°C and 5% CO₂, media containing the lipoproteins were removed and transferred to tubes containing 5 µM Na₂EDTA. BHT was added to those lipoproteins that were not preincubated with BHT. Preincubated lipoproteins were equilibrated in RPMI-1640 medium containing lipoprotein-depleted plasma and incubated with human ASMCs (10 µg protein/ml). After 4 hours of incubation, ASMCs were treated with trypsin and the intracellular contents of $^{125}$I-tyramine cellobiose–lipoproteins were measured. Values represent mean±SD of four determinations expressed as nanograms per milligram of cell protein per hour.
It is possible that some differences in the oxidation rate between C6S-LDL and N-LDL could have been caused by changes in the concentration of endogenous or exogenous antioxidants and chelators introduced during the pelleting and solubilization during the preparation of C6S-LDL. To distinguish this possibility from the effects associated with structural alterations induced by association with sulfated polysaccharides, the LDL was modified with Hep and C6S immobilized on agarose columns. With this design, the only difference in the solutions of LDL eluted from the columns should be those introduced by the noncovalent association with the immobilized Hep or C6S. The resulting observation was that LDL after such interaction was more susceptible to copper-catalyzed oxidation than the LDL exposed to a control column with agarose only. This result does not support the possibility that changes in the concentrations of antioxidants or chelators introduced during precipitation were the cause of the increased susceptibility to oxidation. The observed increase in LDL susceptibility to oxidation after Hep interaction should be considered when using Hep affinity chromatography as a method for the purification and/or subfractionation of LDLs.

We have previously reported that unoxidized N-LDL and CSGP-LDL have similar isoelectric point values. In the present work, CSGP-LDL and GAG-LDL were more negatively charged particles at physiological pH than was N-LDL after comparable copper-catalyzed oxidation periods. One may suspect that this increase in the negative charge of CSGP-LDL or GAG-LDL during copper-catalyzed oxidation could be due to Ca\(^{2+}\)/Mg\(^{2+}\)-mediated associations between the LDL particle and some residual CSPGs or GAGs, even at high ionic strength. However, no differences in the isoelectric point value or electrophoretic mobility were detected between the control samples, N-LDL, and CSGP-LDL or GAG-LDL that were also incubated with the same concentration of copper but in the presence of the antioxidant BHT. Therefore, after oxidation, the acquisition of a more negative charge by CSGP-LDL or

### Table 4

<table>
<thead>
<tr>
<th>Lipoprotein (200 µg/ml)</th>
<th>Without cells</th>
<th>ASMCs ((1 \times 10^5))</th>
<th>HMDMs ((1 \times 10^6))</th>
<th>HMDMs+IFN-γ ((1 \times 10^4))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pl</td>
<td>TBARS</td>
<td>pl</td>
<td>TBARS</td>
</tr>
<tr>
<td>LDL</td>
<td>5.12</td>
<td>1.6</td>
<td>4.92</td>
<td>0.4</td>
</tr>
<tr>
<td>LDL+Cu(^{2+})</td>
<td>5.12</td>
<td>1.6</td>
<td>4.92</td>
<td>0.4</td>
</tr>
<tr>
<td>LDL+BHT+Cu(^{2+})</td>
<td>5.22</td>
<td>0.7</td>
<td>5.02</td>
<td>0.2</td>
</tr>
<tr>
<td>C6S-LDL</td>
<td>5.30</td>
<td>0.7</td>
<td>5.02</td>
<td>0.2</td>
</tr>
<tr>
<td>C6S-LDL+Cu(^{2+})</td>
<td>5.12</td>
<td>1.6</td>
<td>5.02</td>
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<td>C6S-LDL+BHT+Cu(^{2+})</td>
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<td>0.4</td>
<td>5.02</td>
<td>0.2</td>
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</tbody>
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Low density lipoprotein (LDL) and chondroitin 6-sulfate (C6S)–LDL (200 µg protein/ml) equilibrated in serum-free RPMI-1640 medium were incubated with human arterial smooth muscle cells (ASMCs), human monocyte-derived macrophages (HMDMs), HMDMs treated with interferon gamma (IFN-γ) (100 units/ml), or without cells as the control. CuSO₄ (5 µM) and butylated hydroxytoluene (BHT) (20 µM) were added at the times indicated. After 24 hours of incubation at 37°C and 5% CO₂, media containing the lipoproteins were removed and transferred to tubes containing 5 µM Na₂EDTA. BHT was added to those lipoproteins that did not have BHT during the preincubation. Aliquots were taken for isoelectric point (pl) and thiobarbituric acid-reactive substances (TBARS) determinations.

TBARS values are the means of duplicate determinations and are expressed as nanomoles of malonaldehyde equivalents per milligram of protein.

We have previously reported that unoxidized N-LDL and CSGP-LDL have similar isoelectric point values, suggesting that the modifications induced on the lipoproteins by the cells, especially on N-LDL, were due to oxidative processes as previously reported by another group. However, BHT was not so efficient in blocking the human ASMC-induced alterations in C6S-LDL. Superoxide release during coincubation with lipoproteins was significantly lower in human ASMC cultures (7±1 µmol/µg cell protein/hr) compared with HMDMs (24±2 µmol/µg cell protein/hr [n=3]). These results suggest that during coincubation with ASMCs, processes other than oxidation may be involved in the alteration induced on the lipoproteins. N-LDL and C6S-LDL were equally susceptible to cell-induced modifications when the lipoprotein concentration was decreased to 50 µg protein/ml during the coincubation with the cells (data not shown).

### Discussion

We have previously reported that the reversible interaction of LDL with arterial CSPGs increases its in vitro uptake by HMDMs and human ASMCs through the N-LDL receptor. There is experimental evidence for the two possible reasons that could explain this effect: 1) structural modifications induced in the LDL particle by the formation of reversible complexes with the sulfated GAGs and 2) the selection of LDL particles with different structural properties and capacities to interact with CSPGs and cells.

In the present study we have shown that this specific, reversible interaction of LDL with CSPGs or sulfated GAGs increased the susceptibility of LDL to copper-catalyzed oxidation. The enhanced rate of formation of TBARS associated with the lipoprotein, the increased anodic electrophoretic mobility (decrease in isoelectric point value), the higher uptake and degradation by the scavenger receptor in human macrophages, and the decreased recognition by the N-LDL receptor on human ASMCs clearly indicated that CSGP-LDL and GAG-LDL were significantly more susceptible to oxidation than N-LDL.
GAG-LDL must be due to the copper-catalyzed oxidative modification of the lipoprotein, e.g., derivation of lysine residues by lipid peroxides.49

Although we do not know which specific alteration may be responsible for the increased susceptibility to oxidation, the results from low-angle x-ray diffraction12 and differential scanning calorimetry13 indicate that the interaction with CSPGs provokes a loss of organization in the lipid core of the LDL. In addition, the extent of exposure of trypsin-sensitive peptide bonds of apo B-100 is increased.48 Such structural alterations could increase the affinity for copper and make the LDL particle more permeable to neutral and charged oxygen-centered free radicals, thereby increasing its susceptibility to oxidation.41

Regarding the selection of LDL particles, we have previously reported that CSPGs can be used to select subfractions of LDL with different structural properties and capacities to interact with human macrophages. The LDL subclasses with smaller size, higher density, and lower ratio of surface polar lipid to core nonpolar component are the particles with a higher affinity for PG.37 Similar LDL particles have been reported to have an enhanced susceptibility to in vitro oxidation.55 Therefore, we cannot discard the possibility that the increased susceptibility to oxidation after interaction of LDL with CSPG or GAGs could be due to a combination of structural modifications introduced in the LDL particles and selection of LDL subclasses that are indeed more susceptible to oxidation.

C6S-LDL was more susceptible to cell-induced modifications than N-LDL in the presence of copper. The level of lipoprotein oxidation induced by HMDMs was not enhanced further by addition of human recombinant IFN-γ. Similar results have been reported by another group.56 Human ASMCs were more efficient in modifying lipoproteins than were HMDMs, judging by their effect on cell uptake, TBARS content, and isoelectric point value. This result may be considered in light of a previous study, in which HMDMs were reported not to oxidize LDL despite their ability to produce large amounts of reactive oxygen intermediates.57 However, larger amounts of human ASMCs than HMDMs were used during the coculture in our experiments. Therefore, we could not draw firm conclusions about the ability of the different cells to modify the lipoproteins.

Modification of LDL by oxidation involves derivatization of lysine residues of apo B-100 by the lipid products generated during lipid oxidation.40 This reduction in the total positive charge of the oxidized lipoprotein has been shown to permit its recognition by the Ac-LDL receptor in macrophages.58 We have previously reported that the interaction of LDL with arterial CSPG or C6S induces an increase in the surface exposure of lysine- and arginine-rich apo B-100 regions.11,12,59 Therefore, CSPG and C6S may also potentiate the modification of lysine residues during LDL oxidation, which would allow further uptake of oxidized C6S-LDL through the scavenger receptor in human macrophages.

Competition experiments indicated that after 4 hours of oxidation, C6S-LDL was degraded by the same mechanism as Ac-LDL in human macrophages. Although Ac-LDL inhibited by 80% the macrophage degradation of 125I-C6S-LDL oxidized for 4 hours, Ac-LDL oxidized for as long as 24 hours was the most effective competitor, inhibiting essentially all the binding and degradation of 125I-C6S-LDL oxidized for 4 hours. This suggests the existence of recognition epitopes on C6S-LDL oxidized for 4 hours that are not present on Ac-LDL but that can be produced by oxidizing Ac-LDL. Further oxidation of C6S-LDL for as long as 24 hours generated a modified form that was still degraded by the macrophages but that was not competed for by any type of competitor used, except itself. Probably after long periods of oxidation what remained from the lipoprotein is internalized by human macrophages through mechanisms that do not involve any specific receptor.

A specific receptor for oxidized LDL has been proposed in studies of mouse peritoneal macrophages and liver Kupffer cells in rats.59-61 However, our competition experiments indicated that C6S-LDL oxidized for 4 hours and LDL oxidized for 24 hours were internalized by the Ac-LDL receptor in human macrophages. One problem with the interpretation and comparison of competition experiments with oxidized lipoproteins is the different levels of oxidation between lipoproteins. The ideal situation would be to compare lipoproteins with identical levels of oxidation. However, this is difficult to achieve, especially when different types of modified lipoproteins and different donors for LDL preparations are used.

Human ASMCs did not degrade oxidized C6S-LDL; however, contrary to what we expected, more oxidized C6S-LDL than oxidized LDL was found to be associated with the cells. One possible explanation for these results is that the interaction with GAGs increases the lipoprotein affinity for the cell membrane. Another possibility could be the presence of a low number of scavenger receptors, as reported in rabbit arterial SMCs.62 The reversible interaction with arterial PGs has been reported to stimulate lipoprotein uptake by human ASMCs.63 The increase in lipoprotein uptake was partly mediated by the receptor for N-LDL and could be competed for by an excess of unlabeled N-LDL. Why the previous exposure to PGs would have such an effect is unclear at present, but it may be related to the changes induced in the structure of LDL and/or the selection of LDL particles to a higher affinity for the LDL receptor, as reported in human macrophages.12-14,17 Further experiments need to be done to clarify the results obtained with human ASMCs. If a low number of scavenger receptors are present in human ASMCs, then it will be interesting to investigate whether upregulation by phorbol esters62 enhances the binding, accumulation, and degradation of oxidized CSPG- or GAG-treated LDL by these cells. Oxidation of C6S-LDL may allow the particles to be internalized but not degraded by ASMCs. These types of observations could be related to the findings of Rosenfeld and collaborators,64,65 who observed smooth muscle foam cells in the LDL receptor-deficient rabbit. In addition, the same group has identified lipid-protein adducts, probably originated from LDL, in the extracellular compartment and that associated with SMCs in arterial lesions of the Watanabe hyperlipemic rabbit.86

The focal accumulation of apo B lipoproteins by specific retention in the extracellular compartment appears to precede lesion development.67 Intimal CSPGs
may increase the LDL susceptibility to oxidation by inducing structural modifications and by increasing the residence time in the arterial wall, thus allowing further hydrolytic and oxidative modifications of LDL to take place. In addition, as suggested by Haberland and collaborators,20 in vitro experiments point to a possible mechanism by which oxidation could reduce the number of positive charges on the LDL surface, thereby reducing the association with intimal PGs and making the modified particles available to cells.66 The occurrence in vivo of similar processes may contribute to the retention and rapid oxidation of LDL in the arterial intima and to foam cell formation during atherogenesis.

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