

Human Arterial Proteoglycans Increase the Rate of Proteolytic Fusion of Low Density Lipoprotein Particles*

(Received for publication, March 11, 1997, and in revised form, May 27, 1997)

Markku O. Pentikäinen‡, Erno M. P. Lehtonen‡, Katariina Öörni‡, Sari Lusa\$,
Pentti Somerharju\$, Matti Jauhiainen¶, and Petri T. Kovanen‡||

From the ‡Wihuri Research Institute, 00140 Helsinki, the §Department of Medical Chemistry, Institute of Biomedicine, 00014 University of Helsinki, and the ¶Department of Biochemistry, National Public Health Institute, 00300 Helsinki, Finland

Low density lipoprotein (LDL) particles can undergo fusion in the arterial intima, where they are bound to proteoglycans. Here we studied the effect of human arterial proteoglycans on proteolytic fusion of LDL *in vitro*. For this purpose, an assay was devised based on fluorescence resonance energy transfer that allowed continuous monitoring of fusion of proteoglycan-bound LDL particles. We found that addition of human arterial proteoglycans markedly increased the rate of proteolytic fusion of LDL. The glycosaminoglycans isolated from the proteoglycans also increased the rate of fusion, demonstrating that this effect was produced by the negatively charged sulfated polysaccharides in the proteoglycans. Furthermore, heparin, chondroitin 6-sulfate, and dextran sulfate, three commercially available sulfated polysaccharides, also increased the rate of LDL fusion, with heparin and chondroitin 6-sulfate being as effective as and dextran sulfate more effective than human proteoglycans. The ability of the sulfated polysaccharides to increase the rate of proteolytic fusion of LDL depended critically on their ability to form insoluble complexes with LDL, which, in turn, resulted in an increased rate of LDL proteolysis and, in consequence, in an increased rate of LDL fusion. The results reveal a novel mechanism regulating LDL fusion and point to the potentially important role of arterial proteoglycans in the generation of LDL-derived lipid droplets in the arterial intima during atherogenesis.

Human atherosclerosis is characterized by an initial accumulation of lipid in the extracellular matrix of the arterial intima in the form of lipid droplets and vesicles (1, 2). There is substantial evidence that a fraction of the lipid droplets is derived directly from plasma low density lipoprotein (LDL)¹ particles that have entered the intima (3, 4). However, the mechanisms leading to the extracellular accumulation of lipid are difficult to study in man: human atherosclerosis takes a long time to develop, and serial samples from areas susceptible to atherosclerosis are impossible to obtain in man.

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|| To whom correspondence should be addressed: Wihuri Research Inst., Kallioliinantie 4, 00140 Helsinki, Finland. Tel.: 358-9-636494; Fax: 358-9-637476; E-mail: Petri.kovanen@wihuri.fimnet.fi.

¹ The abbreviations used are: LDL, low density lipoprotein(s); PG, proteoglycan; RET, resonance energy transfer; Pyr₁₀CE, cholesteryl 1-pyrenedecanoate; BODIPY-CE, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (cholesteryl BODIPY® FL C₁₂); CETP, cholesterol ester transfer protein; HPLC, high pressure liquid chromatography; GAG, glycosaminoglycan.

In hypercholesterolemic experimental animals, accumulation of extracellular lipid in the arterial intima is much faster. Thus, in Watanabe heritable hyperlipidemic rabbits, lipid accumulation similar to that observed microscopically in human atherosclerosis develops in months (5), and in cholesterol-fed New Zealand White rabbits, in days to weeks (5–7). The most rapid accumulation of lipid droplets and vesicles in the arterial intima so far observed was in New Zealand White rabbits that had been infused intravenously with large amounts of human LDL (8). In these animals, extracellular accumulation of aggregates of lipid droplets was found in the subendothelially located proteoglycan (PG)-rich layer of the arterial intima as little as 2 h after the infusion. This experiment provided two valuable insights into the initiation of atherosclerosis: (i) in the normal arterial intima, extracellular lipid droplets can be formed directly from plasma LDL (in contrast to being first taken up by intimal cells and then released from these cells), and (ii) formation of lipid droplets from LDL particles (particle fusion) in the extracellular matrix can be rapid. *In vitro* experiments have demonstrated that LDL particles in the fluid phase do not fuse until they have undergone modifications that labilize their structure (9, 10). In the arterial intima, however, a fraction of the LDL particles is bound to PGs (11), and so it is possible for fusion to take place between the particles of this bound fraction. Indeed, by electron microscopic techniques, proteolytic fusion of heparin PG-bound LDL has been observed on the surface of mast cell granules *in vitro* (12).

The present methods cannot monitor the kinetics of fusion between LDL particles bound to PGs. Therefore, we devised an LDL fusion assay based on measurement of fluorescent resonance energy transfer (RET), a method widely used in liposome fusion studies (13). RET occurs when an excited fluorescent probe molecule excites a different fluorescent probe molecule in its close proximity. Different fluorescent probes in the core lipids of separate LDL particles are, on average, too far apart for RET to occur, but during fusion, mixing of the core lipids of the LDL particles brings the fluorescent probes into close proximity, thus allowing RET. In our experiments, we incorporated Pyr₁₀CE and BODIPY-CE, two cholesteryl ester analogues with different fluorescent spectra, into the core lipids of two different samples of LDL particles and studied LDL fusion in a mixture of the two LDL preparations. In this system, RET can be detected by monitoring BODIPY emission upon excitation of the pyrene. This method made it possible, for the first time, to compare the rates of LDL fusion in the fluid phase and when bound to PGs. We found that fusion of LDL is faster when the particles are bound to PGs.

EXPERIMENTAL PROCEDURES

Materials—Chymostatin, α -chymotrypsin (from bovine pancreas), dextran sulfate sodium salt (M_r = 5000), Dextralip 50, and heparin

were from Sigma. Pyr₁₀CE and BODIPY-CE were from Molecular Probes, Inc. EDTA disodium salt dihydrate and the butyl-Toyopearl 650(M) column (5.0 × 15 cm) from were Merck. [1,2-³H]cholesteryl linoleate and *N*-succinimidyl [2,3-³H]propionate (³H-labeled Bolton-Hunter reagent) were from Amersham Corp. Heparin-Sepharose, Mono Q HR 5/5, and Superose HR 10/30 columns and dextran sulfate sodium salt (*M_r* = 500,000) were from Pharmacia Biotech Inc. 1-Palmitoyl-2-oleoylphosphatidylcholine was from Avanti Polar Lipids. All other lipids were from Sigma. Chondroitin 6-sulfate was from Seikagaku. Bio-Gel A-5m was from Bio-Rad. NuSieve GTG low-melting-point agarose was from FMC Corp. BioProducts. Celite 545 (acid-washed) was from Fluka. The 5-μm NH₂ column (0.3 × 25 cm) was from Spherisorb.

Preparation of LDL—Human LDL (*d* = 1.019–1.050 g/ml) was isolated from plasma of healthy volunteers by sequential ultracentrifugation (14). LDL was labeled with [³H]cholesteryl linoleate as described previously (9). Apolipoprotein B-100 of LDL was tritiated by the Bolton-Hunter procedure (15) to yield [³H]apoB-100. The amounts and concentrations of LDL are expressed in terms of protein.

Purification and Assay of Cholesterol Ester Transfer Protein (CETP)—Purification of CETP was started from plasmapheresis plasma, which was ultracentrifuged at a density of 1.21 g/ml, and the density fraction *d* > 1.21 g/ml was applied to a butyl-Toyopearl 650(M) column (5.0 × 15 cm) and run essentially as described by Ohnishi *et al.* (16). The fractions containing CETP activity were pooled and applied to a 1.5 × 8.5-cm heparin-Sepharose column equilibrated with 25 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 1 mM EDTA. During this step, the two plasma lipid transfer proteins can be separated: the phospholipid transfer protein binds to the column, and CETP elutes in the unbound fraction (17). The unbound CETP-active fraction was dialyzed against Mono Q anion-exchanger equilibration buffer (25 mM Tris-HCl and 1 mM EDTA, pH 7.4) and then applied to a Mono Q HR 5/5 column and run using a Merck-Hitachi HPLC system. The column was eluted with a linear gradient of NaCl (0–0.5 M) prepared in equilibration buffer. The CETP-active fractions eluted in the NaCl range of 80–110 mM and were stored at –20 °C until use. The activity of CETP in the different purified batches varied between 20 and 30 nmol of cholesteryl esters transferred per h/ml. Purified CETP did not contain phospholipid transfer protein, lecithin:cholesterol acyltransferase, or hepatic lipase activity, nor were these proteins detected by Western blot analysis using specific antibodies against phospholipid transfer protein, lecithin:cholesterol acyltransferase, or hepatic lipase.

Labeling of LDL with Fluorescent Cholesteryl Esters—Microemulsions were prepared essentially as described (18). For a typical preparation, 5866 nmol of cholesteryl linoleate, 845 nmol of triolein, 1411 nmol of cholesterol, 1970 nmol of 1-palmitoyl-2-oleoylphosphatidylcholine, and 652 nmol of either Pyr₁₀CE or BODIPY-CE in chloroform were combined, and the solvent was removed under a stream of nitrogen. Any residual solvent was removed by vacuum desiccation for 2 h. The lipids were dissolved in 200 μl of dry 2-propanol, heated to 60 °C, and injected in 66-μl aliquots into 1.1 ml of buffer A (150 mM NaCl, 5 mM Tris-HCl, and 1 mM EDTA, pH 7.4) at 18 °C (18). The microemulsions containing either Pyr₁₀CE or BODIPY-CE were incubated with 6 mg of LDL in the presence of 450 μl of CETP in 4.5 ml of buffer A for 20 h at 37 °C. The probe-containing lipoproteins were separated from the CETP and donor microemulsions by density gradient ultracentrifugation and size-exclusion chromatography. Briefly, the density of the samples was adjusted to 1.019 g/ml by addition of 310 μl of *d* = 1.21 g/ml KBr solution, and the samples were layered over 2 ml of *d* = 1.1 g/ml KBr solution. The samples were centrifuged at 40,000 rpm for 18 h at 4 °C in a Ti-50 rotor (Beckman Instruments), and the LDL in the middle of the tube was collected. The LDL preparations were applied to a Bio-Gel A-5m column (1 × 60 cm) and eluted with buffer A at 6 ml/h. Fractions containing native-sized LDL were pooled, analyzed, and used for the experiments.

The mass compositions of LDL, Pyr₁₀CE-LDL, and BODIPY-CE-LDL were 5.8, 5.2, and 7.2% triacylglycerol; 7.8, 5.7, and 5.3% free cholesterol; 34, 33, and 32% cholesteryl ester; 26, 29, and 29% phospholipids and 26, 27, and 26% protein, respectively. The amounts of Pyr₁₀CE and BODIPY-CE incorporated into LDL were 9.4 and 10.5 nmol/mg of apoB-100, respectively. Native LDL, Pyr₁₀CE-LDL, and BODIPY-CE-LDL eluted from a heparin affinity column at 271, 275, and 276 mM NaCl, respectively, revealing that incorporation of fluorescent probes did not influence the strength of binding between LDL and glycosaminoglycans.

Isolation, Purification, and Modification of Human Aortic Proteoglycans—PGs from the intima/media of human aortas obtained at autopsy within 24 h of accidental death were prepared exactly as described (19). The disaccharide composition of PGs was analyzed by HPLC using a 5-μm NH₂ column after treatment of PGs with chondroitinases ABC

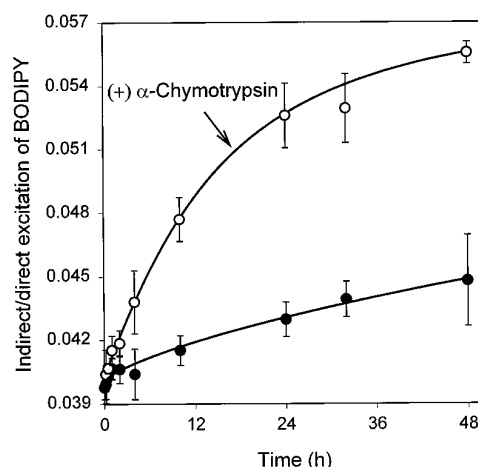


FIG. 1. Effect of proteolysis on resonance energy transfer in a mixture of Pyr₁₀CE-LDL and BODIPY-CE-LDL. Pyr₁₀CE-LDL (25 μg) and BODIPY-CE-LDL (25 μg) were incubated at 37 °C in the absence or presence of α-chymotrypsin (50 μg) in 500 μl of buffer A. At the times indicated, fluorescence was measured as described under "Experimental Procedures."

and AC (20). The PG preparation contained 56% chondroitin 6-sulfate, 25% chondroitin 4-sulfate, and 19% dermatan sulfate. The amounts of PGs are expressed in terms of their glycosaminoglycan (GAG) contents.

Modifications of LDL—For typical experiments, equal amounts of Pyr₁₀CE-LDL and BODIPY-CE-LDL were mixed and dialyzed against buffer B (6 mM KCl, 4.4 mM CaCl₂, 1.5 mM MgCl₂, and 5 mM Tris-HCl, pH 7.2). The mixture (containing 50 μg/ml Pyr₁₀CE-LDL and 50 μg/ml BODIPY-CE-LDL) was then incubated in the presence and absence of the indicated amounts of α-chymotrypsin and the indicated amounts of PGs or GAGs in buffer B supplemented with 20 μM butylated hydroxytoluene at 37 °C. Chymostatin was able to fully inhibit proteolysis and fusion of LDL, demonstrating that fusion of LDL was caused by chymotryptic activity of the α-chymotrypsin preparation used in this study.

Measurement of Fluorescent RET—Fluorescence measurements were performed at 37 °C with a Hitachi F-4000 spectrofluorophotometer equipped with a thermostated cuvette holder. Excitation and emission slit widths were set at 1.5 and 10 nm, respectively, in all experiments except those for measurement of the fractions from size-exclusion chromatography, where the slits were set at 5 and 10 nm, respectively. Excitation and emission wavelengths were set at 346 and 395 nm for direct excitation of pyrene, at 346 and 530 nm for indirect excitation of BODIPY, and at 510 and 530 nm for direct excitation of BODIPY. RET is expressed as the ratio of indirect to direct excitation of BODIPY. The base line of RET (~0.04) is caused by direct excitation of BODIPY at 346 nm.

Thin-section Electron Microscopy—Modified LDL (250 μg in 250 μl) was cast into a 2% GTG low-melting-point agarose gel. Small pieces of the gel were fixed in 3% glutaraldehyde at +4 °C for 18 h. The fixed samples were stained with the osmium/tannic acid/*para*-phenylenediamine technique as described (21) and processed for electron microscopy. Thin sections were viewed in a Jeol JEM-1200EX transmission electron microscope at the Institute of Biotechnology, Electron Microscopy, University of Helsinki (Helsinki, Finland).

Other Assays—Protein was determined by the method of Lowry *et al.* (22) using bovine serum albumin as standard. Glycosaminoglycans were assayed by the method of Bartold and Page (23) using commercial heparin as standard. Cholesterol and triglycerides were measured enzymatically (24) using commercial reagents (Boehringer Mannheim). Phospholipids were assayed with the phospholipase D/choline oxidase/peroxidase method (25) using commercial reagents (Wako Bioproducts). The activity of α-chymotrypsin was assayed spectrophotometrically using *N*-benzoyl-L-tyrosine ethyl ester as substrate exactly as described (26). 1 *N*-benzoyl-L-tyrosine ethyl ester unit of chymotryptic activity induces a change of 0.001 absorbance units/min at 256 nm.

RESULTS

We incorporated Pyr₁₀CE and BODIPY-CE into the core lipids of different samples of LDL particles and studied the effect of α-chymotrypsin on LDL fusion in a mixture of the two LDL preparations by measuring RET. As shown in Fig. 1,

incubation of Pyr₁₀CE-LDL and BODIPY-CE-LDL in the presence, but not in the absence, of α -chymotrypsin led to a rapid increase in RET. The small increase in RET in LDL incubated in the absence of α -chymotrypsin is not caused by spontaneous aggregation or fusion of the particles since at 24 h 95% of the particles eluted in the position of native LDL in size-exclusion chromatography (data not shown). Size-exclusion chromatography of LDL particles proteolyzed for 48 h (Fig. 2) showed that the large particles that eluted in the void volume of the column displayed a high RET, which gradually decreased toward the fractions containing native-sized LDL, which displayed a RET similar to that of native LDL particles (Fig. 1). Previous studies have shown that after a prolonged incubation of LDL with α -chymotrypsin, no intact apoB-100 is left (10). Moreover, experiments using size-exclusion chromatography have shown that after such proteolysis, both LDL eluting in the void volume and LDL eluting at the position of native LDL have lower protein/lipid ratios than native LDL (19). Thus, proteolysis of LDL particles alone does not lead to an increased RET, whereas fusion of the proteolyzed LDL particles does. In a preliminary experiment, we found that also vortexing LDL, a

method shown to generate both fused and aggregated LDL particles (27), increased RET. Thus, at 15 s, RET was increased from 0.58 to 0.74, and at 60 s, RET plateaued at 0.92.

After validation of the RET fusion assay, we next investigated the effect of human arterial proteoglycans on LDL. When LDL was incubated with human arterial PGs in the absence of α -chymotrypsin, a slow linear increase in RET was observed that did not differ from the increase in RET when LDL was incubated alone (Fig. 3A). When a small amount of α -chymotrypsin (5 μ g, *i.e.* one-tenth of the amount used in Figs. 1 and 2) was added, no increase in RET above the base line was observed (Fig. 3B, ●). However, when also human arterial PGs were added, a significant progressive increase in RET resulted over the 24-h incubation period. To investigate whether the increased rate of proteolytic fusion of LDL was caused by negatively charged GAGs, the effect of GAGs isolated from the arterial PG preparation used was also studied. Results similar to those with PGs were obtained (Fig. 3B), revealing that it is the GAGs in human arterial PGs that stimulate the proteolytic fusion of LDL.

Next, we studied the morphology of the modified LDL particles (Fig. 4). For this purpose, native LDL (*panel A*) was incubated with human arterial PGs (*panel B*), α -chymotrypsin (*panel C*), or both (*panel D*), and samples were taken for thin-section electron microscopy. As shown in *panel B*, arterial PGs caused extensive aggregation of LDL without affecting the size of the individual LDL particles. Proteolysis of LDL (*panel C*) resulted in the formation of some particles with increased diameters (up to 50 nm), some of which displayed extensions of membranous material. However, the sample containing LDL that had been proteolyzed in the presence of human arterial PGs (*panel D*) contained only large aggregates of lipid droplets (diameters up to 100 nm) and large amounts of membranous material both extending from fused LDL particles and as separate vesicle-like structures. This morphologic study indicated that in the presence of α -chymotrypsin, human arterial PGs can dramatically alter the structure of LDL.

To study further the effects of sulfated polysaccharides on proteolytic LDL fusion, we compared the rates of proteolytic LDL fusion in the presence of dextran sulfate, chondroitin 6-sulfate, and heparin (Fig. 5). In the absence of α -chymotrypsin (*panel A*), none of these polyanions markedly increased RET during a 7.5-h incubation. In sharp contrast, clear differences were noticed when dextran sulfate, chondroitin 6-sulfate, and heparin were incubated with LDL in the presence of α -chymotrypsin (*panel B*). The rate of proteolytic LDL fusion was increased most markedly by dextran sulfate, with the effects of heparin and chondroitin 6-sulfate being weaker. When the samples were examined by thin-section electron microscopy and the degree of fusion was estimated, fusion was most ex-

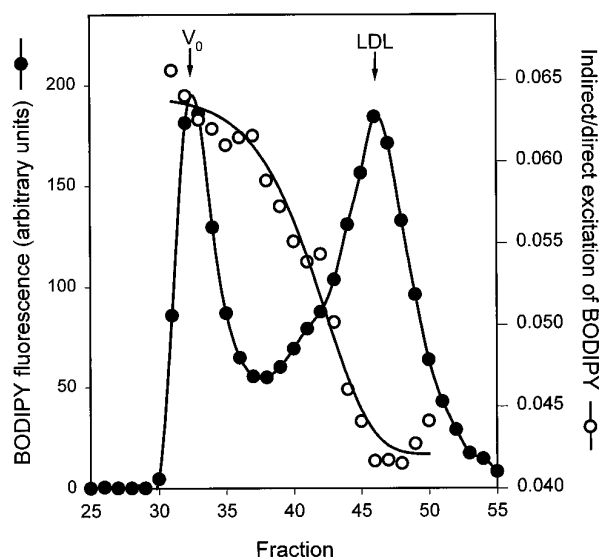
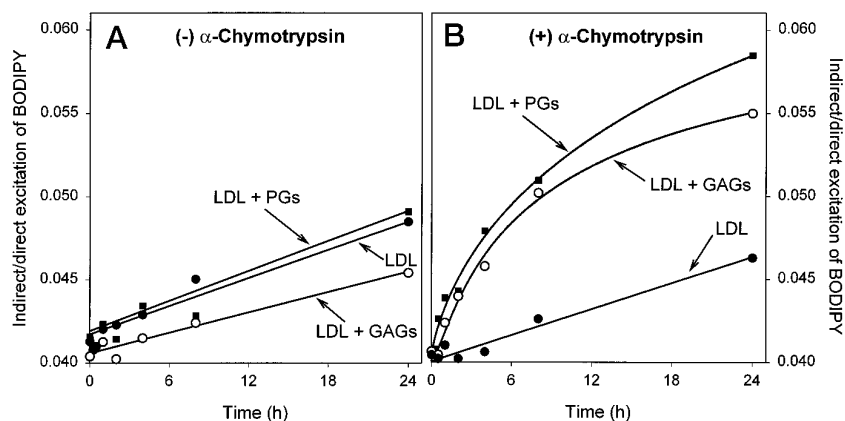


FIG. 2. Effect of particle size on resonance energy transfer in a mixture of Pyr₁₀CE-LDL and BODIPY-CE-LDL. Pyr₁₀CE-LDL (125 μ g) and BODIPY-CE-LDL (125 μ g) were incubated for 48 h at 37 °C with α -chymotrypsin (25 μ g) in 500 μ l of buffer A, and the sample was then applied to a gel filtration system consisting of two Superose 6 HR 10/30 columns connected in series and eluted with buffer A at 4 °C at a flow rate of 0.5 ml/min. 500- μ l fractions were collected, and fluorescence was measured as described under "Experimental Procedures." Elution of Pyr₁₀CE-LDL and BODIPY-CE-LDL is shown as BODIPY fluorescence.

FIG. 3. Effect of proteoglycans from human aorta and glycosaminoglycans on the proteoglycans on resonance energy transfer in a mixture of Pyr₁₀CE-LDL and BODIPY-CE-LDL in the absence (A) and presence (B) of α -chymotrypsin. Pyr₁₀CE-LDL (25 μ g) and BODIPY-CE-LDL (25 μ g) were incubated at 37 °C in 500 μ l of buffer B containing 5 μ g of human arterial proteoglycans or glycosaminoglycans derived from the proteoglycans in the absence (A) or presence (B) of 5 μ g of α -chymotrypsin. At the times indicated, fluorescence was measured as described under "Experimental Procedures."



tensive when LDL was incubated with dextran sulfate and α -chymotrypsin (data not shown).

Under the incubation conditions used, the sulfated polysaccharides formed insoluble complexes with LDL. To determine the relationship between the formation of insoluble complexes with LDL and the rate of proteolytic fusion of LDL, we incubated different amounts of dextran sulfate with LDL and measured the amount of insoluble complexes formed (sedimentation at low speed centrifugation) and the rate of proteolytic LDL fusion. As shown in Fig. 6A, as little as 5 μ g of dextran sulfate precipitated practically all of the LDL (50 μ g) present in the incubation system. When the rate of proteolytic fusion of LDL was followed (*panel B*), addition of increasing amounts of dextran sulfate (0–15 μ g) progressively increased RET in the samples, with the maximal effect being achieved with 5 μ g. Thus, the amount of dextran sulfate required to maximally increase the rate of LDL fusion was similar to that required for maximal sedimentation of LDL. In the absence of α -chymotrypsin, dextran sulfate did not cause significant changes in RET during the 3-h incubation (data not shown).

To determine whether the size of the polyanions affected the rate of proteolytic LDL fusion, we studied the rate of proteolytic LDL fusion in the presence of dextran sulfates of different molecular weights. Dextran sulfates with M_r values of 500,000 and 50,000 were equally effective in promoting proteolytic LDL

fusion, but dextran sulfate with a M_r of 5000 failed to induce fusion (data not shown). In accord with the above results, we also found that dextran sulfates with M_r values of 500,000 and 50,000 were able to form insoluble complexes with LDL, whereas dextran sulfate with a M_r of 5000 did not form such complexes (data not shown).

Previous studies have shown that the degree of LDL fusion is directly proportional to the degree of proteolytic degradation of LDL (9). Therefore, we studied whether the rate of LDL proteolysis is affected by sulfated polysaccharides. As shown in Fig. 7, the amounts of dextran sulfate that had triggered the formation of insoluble complexes with LDL and that had increased the rate of proteolytic fusion of LDL markedly increased the rate of proteolysis of LDL as well. In an additional experiment, we found that in the presence of dextran sulfate, the rate of proteolysis with 0.15 μ g of α -chymotrypsin was even higher than that with 5 μ g of α -chymotrypsin in the absence of dextran sulfate. Similarly, heparin, chondroitin 6-sulfate, and human arterial PG stimulated LDL proteolysis, although to a lesser degree (data not shown). Thus, it appears that one mechanism by which the sulfated polysaccharides increase the rate of proteolytic LDL fusion is by increasing the rate of LDL proteolysis.

Finally, we performed a set of experiments to elucidate the mechanism of the increased rate of LDL proteolysis in the presence of PGs and GAGs. We measured the activity of α -chymotrypsin (5 μ g) with a small molecular weight molecule, *N*-benzoyl-L-tyrosine ethyl ester, as substrate in the absence and presence of dextran sulfate (50 μ g) and found it to be the same (130 versus 140 units/min) under both conditions. Thus, the increased rate of proteolysis could not be explained by increased catalytic activity of α -chymotrypsin in the presence of GAGs. Next, we compared the rate and degree of proteolysis of native LDL and LDL reisolated from complexes with dextran sulfate by α -chymotrypsin and found that they were similar (11.0 versus 11.9% of trichloroacetic acid-soluble apoB-100 fragments after 1 h of proteolysis). Thus, the increased rate of proteolysis could not be explained by irreversible conformational change in apoB-100 after binding to GAGs. Moreover, we studied the binding of both LDL and α -chymotrypsin to a dextran sulfate affinity column and found that LDL bound strongly and was eluted at \sim 450 mM NaCl, whereas α -chymotrypsin bound weakly to the column, with the bound enzyme being eluted at a NaCl concentration of $<$ 50 mM. The rate of proteolytic fusion of LDL was markedly increased by dextran sulfate at 150 mM NaCl (Fig. 5), which totally prevented the binding of α -chymotrypsin to the LDL-dextran sulfate complexes. Thus, binding of α -chymotrypsin to the complexes was not required for the dextran sulfate effect. However, there was no increase in the rate of proteolysis of LDL in the presence of

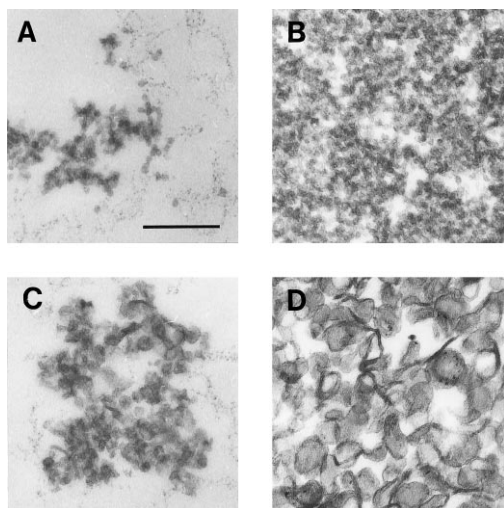
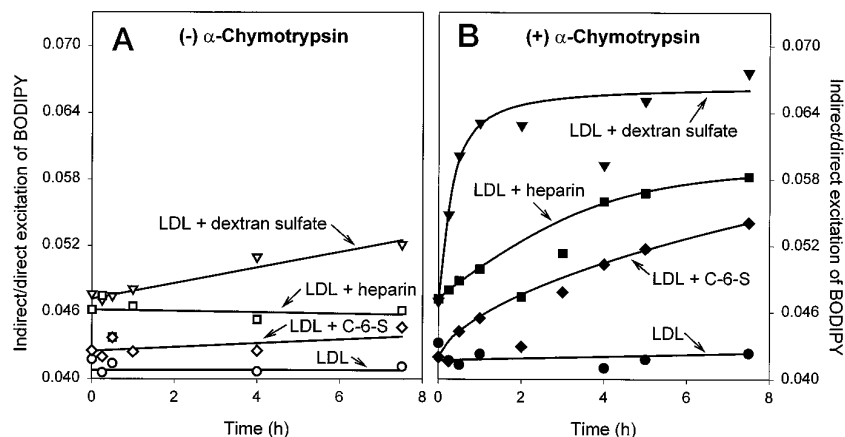


FIG. 4. Transmission electron microscopy of native and variously treated LDL particles. Native LDL (250 μ g) (A) was incubated for 6 h at 37 $^{\circ}$ C in 250 μ l of buffer B containing human arterial proteoglycans (25 μ g) (B), α -chymotrypsin (25 μ g) (C), or both human arterial proteoglycans and α -chymotrypsin (D). The samples were embedded in agarose and subjected to electron microscopy as described in detail under "Experimental Procedures." Bar = 200 nm.

FIG. 5. Effect of dextran sulfate (M_r = 500,000), heparin, and chondroitin 6-sulfate on resonance energy transfer in a mixture of Pyr₁₀CE-LDL and BODIPY-CE-LDL in the absence (A) and presence (B) of α -chymotrypsin. Pyr₁₀CE-LDL (25 μ g) and BODIPY-CE-LDL (25 μ g) were incubated at 37 $^{\circ}$ C in 500 μ l of buffer B containing 5 μ g of dextran sulfate (M_r = 500,000), heparin, or chondroitin 6-sulfate (C-6-S) in the absence (A) or presence (B) of 5 μ g of α -chymotrypsin. At the times indicated, fluorescence was measured as described under "Experimental Procedures."



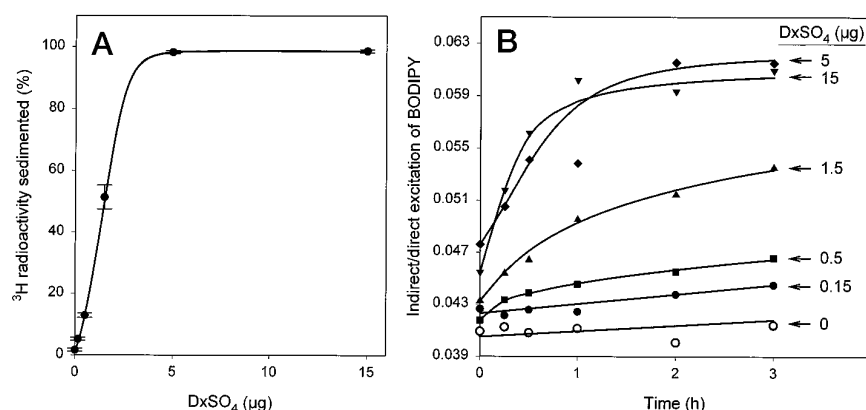


FIG. 6. Effect of dextran sulfate on sedimentation of LDL (A) and resonance energy transfer in a mixture of $\text{Pyr}_{10}\text{CE-LDL}$ and BODIPY-CE-LDL during proteolysis by α -chymotrypsin (B). A, [^3H]cholesteryl linoleate-labeled LDL (50 μg) was incubated for 30 min at 37 °C in the absence or presence of the indicated amounts of dextran sulfate (DxSO_4 ; $M_r = 500,000$) in 500 μl of buffer B. The samples were centrifuged at $10,000 \times g$ for 10 min, and [^3H]cholesteryl linoleate-labeled LDL in the supernatants and pellets were quantified by liquid scintillation counting. B, $\text{Pyr}_{10}\text{CE-LDL}$ (25 μg) and BODIPY-CE-LDL (25 μg) were incubated at 37 °C in the absence or presence of the indicated amounts of dextran sulfate ($M_r = 500,000$) in 500 μl of buffer B containing 5 μg of α -chymotrypsin. At the times indicated, fluorescence was measured as described under "Experimental Procedures."

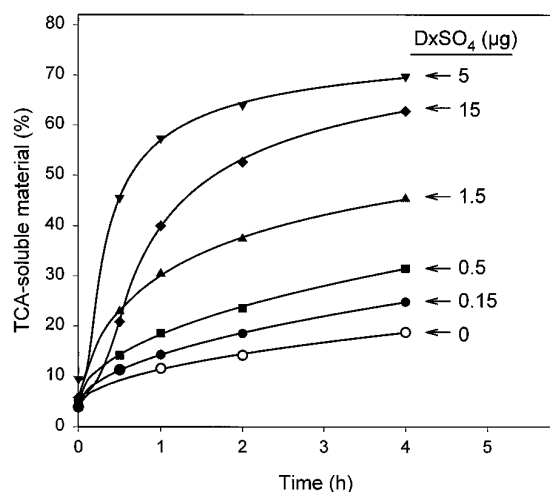


FIG. 7. Effect of dextran sulfate on the rate of proteolysis of LDL by α -chymotrypsin. ^3H -apoB-100-LDL (50 μg) was incubated at 37 °C with α -chymotrypsin (5 μg) and the indicated amounts of dextran sulfate (DxSO_4) in 500 μl of buffer B. At the times indicated, 100- μl aliquots were withdrawn for analysis of trichloroacetic acid (TCA)-soluble material as described in detail under "Experimental Procedures."

dextran sulfate when formation of insoluble complexes between LDL and dextran sulfate was prevented by the presence of 250 mM NaCl (6.9 and 6.4% of trichloroacetic acid-soluble apoB-100 fragments after 1 h of proteolysis in the presence and absence of dextran sulfate, respectively). Thus, it appears that the increased rate of LDL proteolysis in the presence of PGs or GAGs depends solely on the formation of insoluble complexes between these sulfated mucopolysaccharides and LDL.

DISCUSSION

The novel method for studying LDL fusion based on fluorescent resonance energy transfer allowed continuous monitoring of particle fusion and, most important, was not perturbed by particle aggregation. With this method, we were able to show that human arterial PGs and GAGs increase the rate of proteolytic LDL fusion.

Why is the rate of proteolytic fusion of LDL increased in the presence of PGs or GAGs? Their addition to a system containing both LDL and α -chymotrypsin was found to lead to two parallel phenomena: (i) formation of insoluble complexes (aggregation) between LDL and PGs or GAGs and (ii) an increased rate of LDL proteolysis. Aggregation of native LDL particles by

PGs or GAGs in the absence of α -chymotrypsin did not lead to particle fusion. In contrast, proteolysis of LDL, in the absence of PGs or GAGs, can result in particle fusion (9). Since PGs and GAGs increase the rate of LDL proteolysis (this study), one explanation for the increased rate of LDL fusion in the presence of PGs or GAGs is that they increase the rate of LDL proteolysis. To assess whether fusion of proteolyzed LDL is promoted by the formation of insoluble complexes between the proteolyzed particles and PGs or GAGs independently of the proteolysis-stimulating effect of PGs and GAGs, LDL was first proteolyzed by α -chymotrypsin, proteolysis was then stopped by addition of a protease inhibitor, and dextran sulfate or anti-apoB-100 antibody was added to the reaction mixture. Under these conditions, aggregation of the proteolyzed particles did not by itself trigger particle fusion (data not shown). Thus, it appears that PGs and GAGs accelerate the rate of fusion solely by increasing the rate of LDL proteolysis. However, we cannot exclude the possibility that these sulfated mucopolysaccharides contribute to the rate of particle fusion independently of their proteolysis-stimulating effect on LDL.

What is the mechanism underlying the increased rate of proteolysis of apoB-100 of LDL in the presence of PGs or GAGs? In light of our results, it appears that the increased rate of LDL proteolysis in the presence of PGs or GAGs depends solely on the formation of insoluble complexes between these sulfated mucopolysaccharides and LDL, and not on increased catalytic activity of α -chymotrypsin. The formation of LDL-GAG aggregates may thus provide microenvironments favorable for proteolytic modification of LDL. Interestingly, it has been found that the formation of soluble complexes between LDL and PGs or GAGs increases the rate of apoB-100 proteolysis by trypsin, a finding that was suggested to result from increased exposure of the amino acids lysine and arginine of apoB-100 (28).

In the arterial intima, PGs are heterogeneous, with some having a high affinity and others a low affinity for LDL (29). From our finding that dextran sulfate, the polyanion with the highest affinity for LDL, accelerated the proteolytic fusion of LDL most strongly, we infer that the same relationship between affinity for LDL and ability to accelerate LDL particle fusion will also hold in the arterial intima. GAGs with the highest binding affinity for plasma LDL are present in human arteries with high susceptibility to atherosclerosis (30), especially at branch sites (31). In addition, PGs with high affinity for LDL are produced by smooth muscle cells of synthetic phenotype (32) that are present at arterial sites susceptible to

the development of atherosclerosis (33). The above observations are consistent with the notion that there are specific atherosclerosis-prone (micro)environments in which LDL particles undergo rapid fusion. Interestingly, in the experiments in which large amounts of human LDL were infused intravenously into New Zealand White rabbits, electron microscopic analysis of the subendothelial space revealed small foci of fused LDL particles (8).

Atherosclerotic lesions of human aorta and coronary arteries contain mast cells capable of releasing granules that are complexes between heparin proteoglycans and fully active endopeptidases with either tryptic (tryptase) or chymotryptic (chymase) activity (34). Indeed, many of these such mast cells have expelled their granules into the extracellular fluid, where also LDL is present (35). The protease-containing granules then bind the apoB-100 component of LDL (36) and may proteolyze it. Therefore, it is conceivable that proteolytic modification of LDL may take place in the human arterial intima.

The arterial intima is also the site of both lipolytic (37) and oxidative (38) modifications of LDL. We recently found that these two types of LDL modification, in addition to proteolytic modification, render the particles unstable and induce their fusion *in vitro* (10), but the effect of immobilization of LDL by arterial PGs on the ability of these modifications to induce fusion of LDL is unknown. Future studies on modification of the PG-bound LDL particles should provide new insight into the actual processes leading to LDL fusion in the arterial intima during atherogenesis.

Acknowledgments—The excellent technical assistance of Päivi Hiironen is gratefully acknowledged. We also thank Ritva Keva for technical assistance in CETP purification and analysis.

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