Differential Role of Heparan Sulfate Proteoglycans on Aggregated LDL Uptake in Human Vascular Smooth Muscle Cells and Mouse Embryonic Fibroblasts

Vicenta Llorente-Cortés,* Marta Otero-Viñas,* Lina Badimon

Objective—Low density lipoprotein (LDL) receptor–related protein (LRP) binds and internalizes aggregated LDL (agLDL) in human vascular smooth muscle cells (VSMCs). To analyze the contribution of proteoglycans (PGs) to agLDL uptake in human VSMCs, in wild-type mouse embryonic fibroblasts (MEF line), and in LRP-deficient mouse embryonic fibroblasts (PEA13 line).

Methods and Results—PGs in the medium and cellular and extracellular matrix have been isolated by metabolic radiolabeling with [35S]Na2SO4 and characterized by selective digestion with heparinase I and III (4 U/mL each) and chondroitinase ABC (2 U/mL). To examine the contribution of PGs and LRPs to agLDL internalization, nonexpressing and LRP-expressing cells, treated or not with polysaccharidase, were incubated with agLDL (25, 50, and 100 μg/mL) for 18 hours. In human VSMCs, agLDL was unable to induce cholesteryl ester (CE) accumulation in antisense LRP-oligodeoxynucleotide–treated cells, and heparan sulfate (HS)-PG depletion leads to a reduction of the CE accumulation. In mouse fibroblasts, PEA13 compared with MEF showed lower, but still considerable, CE accumulation, and HS-PG depletion almost completely inhibited CE accumulation.

Conclusions—In MEF, HS-PGs can function alone as receptors that bind and internalize agLDL in the absence of LRP, but in human VSMCs, although HS-PGs facilitate agLDL binding to the cells, LRP is essential for agLDL internalization. (Arterioscler Thromb Vasc Biol. 2002;22:1905-1911.)

Key Words: heparan sulfate proteoglycans ■ human vascular smooth muscle cells ■ mouse embryonic fibroblasts ■ aggregated LDL ■ LDL-related protein

In the intima, proteoglycans (PGs) are in the pericellular space of the endothelial and smooth muscle cells and are also the major constituents of the extracellular matrix (ECM).1 Vascular smooth muscle cells (VSMCs) secrete most chondroitin sulfate (CS)-PGs associated with hyaluronan, a glycosaminoglycan (GAG). Indeed, versican (a CS-like PG) is the main PG structuring the ECM.2–4 Several studies demonstrate that CS-PGs act as sites for apoB-100 lipoprotein retention by positively charged heparin-binding domains on apoB-100 or apoE and negatively charged GAG chains of the PGs.2,4 The GAGs of versican induce alterations in the LDL particle that lead to the formation of fused and aggregated LDL (agLDL).5,6 VSMCs also synthesize heparan sulfate (HS)-PGs, which can be secreted (perlecan) or shed from the cell surface (perlecan, syndecan, or glypican).7,8 In contrast to CS-PGs, which play a major role in LDL retention and modification in arterial intima, HS-PGs may act as potential receptors for atherogenic lipoproteins9–11 or facilitate the uptake of ligands by a process called ligand transfer to lipoprotein receptors, such as the LDL receptor–related protein (LRP).12–16

We previously demonstrated that LRP binds and internalizes agLDL in human VSMCs.6,17 The aim of the present study was to analyze the contribution of PGs and LRPs on agLDL internalization by human VSMCs and fibroblasts. HS-PGs and CS-PGs from the medium and from cellular and ECM fractions have been characterized by metabolic radiolabeling and selective digestion of PGs with heparinase I and III (HSI&III)18,19 and chondroitinase ABC (ChABC),20 respectively. To examine the contribution of PGs and LRPs on agLDL internalization, human VSMCs, which do not express LRP (antisense LRP-oligodeoxynucleotide [ODN]–treated VSMCs),6,17 and LRP-deficient mouse embryo fibroblasts (PEA13 line)21 in parallel with LRP-expressing cells (either treated or not with polysaccharidase) were incubated with increasing concentrations of agLDL (25, 50, and 100 μg/mL).

We have found that in VSMCs, CS-PGs are the major component of cellular matrix and ECM. In contrast, HS-PGs are more abundant than CS-PGs in cellular and ECM fractions of wild-type mouse embryonic fibroblasts (MEF line). Although HSI&III and ChABC treatment completely degrades HS-PGs and CS-PGs, respectively, only HS-PG cleav-
age has consequences for agLDL internalization in both cell types. However, there are marked differences in the role of HS-PGs between human VSMCs and fibroblasts. In fibroblasts, HS-PGs alone can function as receptors that bind and internalize agLDL in the absence of LRP. In contrast, in human VSMCs, although HS-PGs facilitate the agLDL binding to the cells, LRP is essential for agLDL internalization.

Methods

Materials

Cell culture medium and reagents were from Gibco Laboratories. MEF (CRL-2214) and PEA13 (CRL-2216) fibroblasts were from American Type Culture Collection. Benzamidine-HCl, Triton X-100, α-amino caproic acid, guanidinium-HCl, BSA, cetylpyridinium chloride, heparinase I (heparin lyase I, EC 4.2.2.7), heparinase III (heparin lyase III, heparitinase I; EC 4.2.2.8), and ChABC (ChABC lyase, EC 4.2.2.4) were from Sigma Chemical Co. HiTrap Q ion exchange columns and [35S]Na2SO4 (100 mCi/mmol) were from Amersham Pharmacia Biotech. Bicinchoninic acid protein assays were from Pierce. Four percent to 12% Tris-glycine gels from Amersham Pharmacia Biotech. Bicinchoninic acid protein assays were from Pierce. Four percent to 12% Tris-glycine gels and Sypro Ruby protein gel staining were from Bio-Rad, and assays were from Pierce. Four percent to 12% Tris-glycine gels and Sypro Ruby protein gel staining were from Bio-Rad, and ENHANCE (NEF981G) was from NEN Life Sciences.

Cell Culture

Primary cultures of human VSMCs were obtained from human coronary arteries of explanted hearts at transplant operations performed at the Hospital de la Santa Creu i Sant Pau. VSMCs were obtained by a modification of the explant technique that we described previously.6,17 Explants were incubated at 37°C in a humidified atmosphere of 5% CO2. Outgrown cells were suspended in a solution of trypsin/EDTA and subcultured. They grew in monolayers.

LDL Preparation and Modification

Human LDLs (density 1.019 to 1.063 g/mL) were obtained from pooled sera of normocholesterolemic volunteers, isolated by sequential ultracentrifugation, and dialyzed. The model system of agLDL was generated by vortexing as previously described.6,19,24

ECM Ultrastructure

Cells grown on coverslips were treated or not with heparinase I (4 U/mL) or ChABC (2 U/mL) at 37°C for 2 hours. They were then fixed with glutaraldehyde (1.6%), washed, cryoprotected in 10% methanol, and cryofixed by projection against a copper block cooled to −196°C with the use of Cryovacublock de Reichert-Jung (Leica). The frozen samples were stored at −196°C in liquid nitrogen until subsequent use. Samples were freeze-dried and coated with platinum and carbon using a freezeetching unit (model BAF 060, BAL-TEC). A rotary shadowing of the exposed surface was made by evaporating 10 nm of carbon evaporated at a 75° angle. The replica was separated from the coverslip by immersion in 38% hydrofluoric acid, washed twice in distilled water, and digested with 5% sodium hypochlorite for 5 to 10 minutes. Finally, the replicas were washed several times in distilled water, broken into small pieces, and picked up on copper grids coated with plastic for electron microscopy. All electron micrographs were obtained by using an electron microscope (Hitachi HU-600), operating at 75 kV.

Radio-labeling and Digestion of PGs

Cells were synchronized in medium containing 0.2% FCS for 2 days. Then the medium was removed, and fresh DMEM (10% FCS) containing 20 μCi/mL [35S]Na2SO4 was added and maintained for 3 days to biosynthetically label PGs as previously described.22 CS-PGs and HS-PGs were digested by adding a mixture of HSκIII (4 U/mL each) or ChABC (2 U/mL), respectively, to the incubation media for 2 or 18 hours. Control cells without PG enzymatic digestion were processed in parallel.

Isolation of PGs

After the 3 days of labeling, the culture medium from cells treated or not with enzymes for 2 or 18 hours in the absence or presence of agLDL (100 μg/mL) were transferred to tubes. Protease inhibitors were added to a final concentration of 10 mmol/L EDTA, 10 mmol/L α-amino caproic acid, and 1 mg/mL benzamidine-HCl, and the medium was stored at −20°C until use.

Cells were washed with PBS containing 50 μg/mL heparin for 30 minutes at room temperature. The heparin-containing buffer was removed, and the cells were washed 3 times with PBS without heparin and dissolved by 2 extractions (5 mL each) of buffer containing 1% Triton X-100, 0.15 mol/L NaCl, 10 mmol/L Tris, 5 mmol/L MgCl2, 2 mmol/L EDTA, 0.255 mmol/L dithiothreitol, and 1 μmol/L AEBSF, pH 7.2. After incubation for 30 minutes under gentle shaking, the cellular extract was removed and stored at −20°C until use. The remaining matrix was washed with PBS and solubilized by 2 extractions (5 mL each) of 8 mol/L urea, 2 mmol/L EDTA, 0.5% Triton X-100, and 20 mmol/L Tris-HCl (pH 7.5) containing protease inhibitors (1 mg/mL benzamidine-HCl and 10 mmol/L α-amino-γ-caproic acid). The wells were left overnight at 4°C before the ECM extract was collected with a cell scraper.

The culture media and cellular and ECM extracts were dialyzed against binding buffer (8 mol/L urea, 2 mmol/L EDTA, 0.5% Triton X-100, and 20 mmol/L Tris-HCl, pH 7.5) for 48 hours at 4°C and were then chromatographed on a Hi Trap Q (5-mL column equilibrated with binding buffer at a flow rate of 5 mL/min. The 35S-labeled PG-containing fractions were collected after elution with a linear NaCl gradient (0.25 to 3 mol/L NaCl) and dialyzed at 4°C against water.

PG Characterization by SDS-PAGE

Equal amounts of protein from VSMCs and fibroblasts were loaded on a precasted 4% to 12% Tris-glycine gel, and SDS-PAGE was run for 2 hours at 60 V. The proteins were then fixed with methanol/acetate acid, stained with Sypro Ruby protein gel stain, and observed under UV light to control the equal protein loading. The gels were impregnated with ENHANCE before drying. The dried gels were placed for autoradiography at −80°C for 14 days before they were developed.

LDL Preparation and Modification

Human LDLs (density 1.019 to 1.063 g/mL) were obtained from pooled sera of normocholesterolemic volunteers, isolated by sequential ultracentrifugation, and dialyzed. The model system of agLDL was generated by vortexing as previously described.6,19,24

Determination of Intracellular Cholesterol Content

Arrested VSMCs or fibroblasts were untreated or treated with HSκIII (4 U/mL each) or ChABC (2 U/mL) for 2 hours before the addition of increasing concentrations of agLDL (25, 50, 100 μg/mL) to the incubation medium containing the enzymes. After 18 hours, cells were exhaustively washed and harvested into 1 mL of 0.10 mol/L NaOH. Lipid extraction and thin-layer chromatography (TLC) were performed as previously described.6,17,24 The spots corresponding to free cholesterol and cholesterol esters (CEs) were quantified by densitometry against the standard curve of cholesterol and cholesterol palmitate, respectively, by using a computing densitometer (Molecular Dynamics).

Results

Characterization of PGs Synthesized by Human VSMCs and MEF

Human VSMCs and MEF were metabolically labeled with [35S]Na2SO4 for 3 days, and PGs from the medium and cellular and ECM fractions were purified from untreated or
Synthesized [35S]GAGs were cell-associated (58% in human VSMCs and 56% in MEF). Approximately 36% and 40% of the remaining newly synthesized GAGs were in the ECM (7% in human VSMCs and MEF, respectively, and the ECM). On average, 17% versus 7.5% and 2.5% versus 0.7% of the area of the peaks were obtained from every fraction of enzymatic treatment (in the absence or presence of agLDL) in any cell type.

The autoradiographic analysis of the eluted PGs (Figure 1) revealed differences in the pattern of bands susceptible to being degraded by HSI&III and ChABC between human VSMCs and MEF. In human VSMCs, there is a defined band at the beginning of the polyacrylamide gel in the cell and ECM fractions that was degraded by HSI&III treatment and that, according to its size, could be perlecan.25,26 In MEF, the main bands degraded by HSI&III seem to be mostly syndecans.27 Additionally, there are high amounts of bands that are susceptible to being degraded by ChABC; these bands were especially abundant in the cell fraction and were different in size for human VSMCs and MEF. Protein loading was determined to be equal for enzymatically treated and untreated cells.

### Table: HS-PGs and CS-PGs Content of the Medium, Cell, and ECM Fraction From Human VSMCs and MEF

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Untreated Cells</th>
<th>HIS&amp;III-Treated Cells</th>
<th>ChABC-Treated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PG (Total)</td>
<td>HS-PGs (Remaining)</td>
<td>CS-PGs (Remaining)</td>
</tr>
<tr>
<td>Human VSMCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>16.78±2.80</td>
<td>17.23±1.30</td>
<td>0.00±0.40</td>
</tr>
<tr>
<td>Cell</td>
<td>27.48±1.65</td>
<td>25.93±2.63</td>
<td>1.56±0.97</td>
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<tr>
<td>ECM</td>
<td>3.32±0.42</td>
<td>2.52±0.22</td>
<td>0.80±0.20</td>
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<td>MEF</td>
<td>15.02±0.49</td>
<td>16.63±0.93</td>
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<tr>
<td>Medium</td>
<td>20.55±4.26</td>
<td>13.96±3.24</td>
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<td>0.60±0.05</td>
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<td>ECM</td>
<td>100</td>
<td>74.5±1.5</td>
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<table>
<thead>
<tr>
<th></th>
<th>(Remaining)</th>
<th>(Digested )</th>
<th>(Digested )</th>
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<td>Human VSMCs</td>
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<td></td>
<td></td>
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<tr>
<td>Medium</td>
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<td>Cell</td>
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<td>76±3</td>
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<tr>
<td>ECM</td>
<td>2.52±0.22</td>
<td>0.80±0.20</td>
<td>2.12±0.49</td>
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<tr>
<td>MEF</td>
<td>74.5±1.5</td>
<td>24±3.5</td>
<td>63±7</td>
</tr>
</tbody>
</table>

a, Results are expressed as the area of the peaks in cpm (×10^3) and are shown as the mean of one experiment performed in duplicate.
b, Results are expressed as a percentage of the peak areas obtained from every fraction of untreated cells.

d, Results are determined to be equal for enzymatically treated and untreated cells.
MEF (Figure 2D). Similar photographs were obtained by ChABC treatment of the cells.

Role of PGs and LRPs on agLDL Internalization in Human VSMCs and MEF

To reveal the role of HS-PGs and CS-PGs on agLDL internalization, HS-PGs and CS-PGs were selectively degraded in human VSMCs and MEF. It has been previously demonstrated that heparinase treatment did not influence LRP-binding capacity in fibroblasts. agLDL internalization experiments were performed by incubating LRP-expressing VSMCs and MEF and non–LRP-expressing cells (antisense LRP-ODN–treated VSMCs and PEA13, respectively), either enzymatically treated or not, with increasing concentrations of agLDL for 18 hours.

We have previously demonstrated that the increase in CE content observed in VSMCs reflects the cholesterol that enters the cell as LDL. An initial period of prolonged cell surface contact, facilitated by cell PGs, in which CE hydrolysis exceeds protein degradation (selective uptake), has previously been described in macrophages. A similar process cannot be excluded in CE accumulation induced by agLDL in human VSMCs and fibroblasts. As shown in Figure 3A, although agLDL induced a high intracellular cholesterol accumulation in a dose-dependent manner in human VSMCs (from 44.87 ± 1.77 μg CE per milligram protein at 25 μg/mL...
HS-PG and LRP Role in agLDL Uptake in Human VSMCs and MEF

Figure 3. Role of PGs and LRPs on CE accumulation derived by agLDL in human VSMCs and MEF. Human VSMCs/antisense LRP-ODN–treated VSMCs and MEF/PEA13 were nonenzymatically treated or incubated with HSI&III (4 U/mL) or ChABC (2 U/mL) for 2 hours before addition of agLDL. A, TLC showing the effect of increasing concentrations of agLDL (25, 50, and 100 μg/mL) on the CE accumulation induced by agLDL in VSMCs/antisense LRP-ODN–treated cells (left) and MEF/PEA13 (right). B, Line graphs showing quantification of CE in VSMCs/MEF (solid symbols) and antisense LRP-ODN–treated VSMCs/PEA13 (open symbols) either nonenzymatically treated (rhombuses) or treated with HSI&III (squares) or ChABC (triangles). Results are expressed as micrograms CE per milligram protein and are shown as a mean±SEM of 3 samples from 2 different experiments.

As previously shown, agLDL (100 μg/mL) induced a CE accumulation of 81.81±1.6 μg CE/mg protein (at 100 μg/mL), agLDL was unable to induce CE accumulation in antisense LRP-ODN–treated VSMCs, in agreement with previous results.6,17 HS-PG depletion leads to a reduction in the CE accumulation derived from agLDL at each analyzed concentration (31.68±2 versus 44.87±1.77 μg CE/mg protein at 25 μg/mL; 45.9±3 versus 46.72±2.82 μg CE/mg protein at 50 μg/mL; and 51.1±3 versus 81.81±1.6 μg CE/mg protein at 100 μg/mL). Taken together, these results indicate that LRP is essential for agLDL internalization in VSMCs and that HS-PGs facilitate the process. In mouse fibroblasts, the mechanism seems to be different, inasmuch as PEA13 had less aggregate bound, and HSI&III-treated MEF did not show any significant effect on CE accumulation derived from agLDL in any cell type.

As previously shown, agLDL (100 μg/mL) induced a CE accumulation of 81.81±1.6 μg CE/mg protein in human VSMCs and 95.51±4 μg CE/mg protein in MEF. Considering the contribution of pathways independent of LRPs and HS-PGs in human VSMCs (4±0.75 μg CE/mg protein) and MEF (7±1.5 μg CE/mg protein), LRP alone is responsible for intracellular CE accumulation in HSI&III-treated VSMCs (51.1±2.46 μg CE/mg protein) and in HSI&III-treated MEF (18±7 μg CE/mg protein), ~58% and 12% of CE accumulation in human VSMCs and MEF, respectively. In the same way, considering the role of HS-PGs alone as responsible for intracellular CE accumulation in antisense LRP-ODN–treated VSMCs (4.59±1.0 μg CE/mg protein) and PEA13 (46±9 μg CE/mg protein), HS-PGs would account for ~1% and 41% of the CE accumulation in human VSMCs and MEF, respectively.

By subtracting LRP-mediated CE accumulation and HS-PG–mediated CE accumulation from the total CE accumulation, a percentage of 26% in human VSMCs and 25% in MEF can be ascribed to an accumulation that is accomplished by a cooperative mechanism (both pathways).

Online Figure II (available at http://www.ahajournals.org) shows photomicrographs of representative untreated and HSI&III-treated human VSMCs and MEF incubated with agLDL. Pictures were taken after the first wash with PBS to eliminate agLDL that was not bound. As shown, untreated human VSMCs (online Figure IIA) or untreated MEF (Figure IIB) had many aggregates of LDL bound (arrows) on the cell surface. In contrast, HSI&III–treated VSMCs (Figure IIC) had less aggregate bound, and HSI&III–treated MEF did not show any aggregate bound (Figure IID). As observed in the photographs, HSI&III treatment did not induce changes in the morphology of any cell type.

**Discussion**

We recently demonstrated that LRP is responsible for agLDL uptake in human VSMCs. These cells have very high levels of LRP expression and are unable to accumulate cholesterol from agLDL in the absence of LRP.6,17 Because it has been proposed that PGs may play a role in the internalization of certain LRP ligands in hepatic and arterial cells,8–16 we explored the role of HS-PGs and CS-PGs on agLDL inter-

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**Table 1.** CE Accumulation in Human VSMCs and MEF

<table>
<thead>
<tr>
<th>agLDL (μg/mL)</th>
<th>No enzyme</th>
<th>HSI&amp;III</th>
<th>ChABC</th>
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<tr>
<td>25</td>
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<td>25</td>
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<td>50</td>
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**Figure 3A.** CE accumulation in human VSMCs and MEF treated with agLDL, HSI&III, and ChABC.

**Figure 3B.** Line graphs showing quantification of CE in VSMCs/MEF (solid symbols) and antisense LRP-ODN–treated VSMCs/PEA13 (open symbols) either nonenzymatically treated (rhombuses) or treated with HSI&III (squares) or ChABC (triangles). Results are expressed as micrograms CE per milligram protein and are shown as a mean±SEM of 3 samples from 2 different experiments.
nalization in human VSMCs and MEF by degrading PGs with specific polysaccharidase. Chromatographic and autoradiographic results indicate some differences in HS-PG and CS-PG composition between VSMCs and MEF, especially in the cellular and ECM fractions. The amount and molecular weight of CS-PGs were higher in VSMCs than in MEF. HS-PGs were more abundant in MEF compared with human VSMCs. Additionally, although perlecan was the main HS-PG associated with VSMCs, syndecans were most abundant in MEF. However, the differences in HS-PG contribution to agLDL internalization make extrapolation from one cell type to another not suitable for target-specific cell internalization mechanisms. The main mechanism for agLDL internalization in human VSMCs is mediated by LRPs.

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**Figure I**. Representative chromatography of PG synthesized by human VSMC and MEF. PG of the medium, cellular and ECM fractions from untreated (romboid), HSI&III-treated cells (squares) and ChABC-treated cells (triangles) were applied onto an anion-exchange column equilibrated in a buffer containing urea 8 mol/L, Tris 20 mmol/L, EDTA 2 mmol/L, 0.5% Triton X-100, pH 7.5. PG were eluted with a linear NaCl gradient at 0.5 ml/min and fractions (2.5 mL) were collected. Total counts in each fraction were determined. Y-axis scales are adjusted to the counts obtained in each fraction.

**Figure II**. Effect of HS-PG depletion on agLDL binding by human VSMC and MEF. non-enzymatically treated VSMC (A) and MEF (B) or HSI&III (4 U/mL)-treated VSMC (C) and MEF (D) were incubated with agLDL (100 µg/mL) for 18 hours. VSMC were then washed with PBS and photographed (magnification x 100). Arrows indicate LDL aggregates bound to the cell surface.
Figure I.

Human VSMC

I. MEDIUM

II. CELL

III. ECM

MEF
Figure II.