Abstract—Foam cell formation is a key event in the onset and progression of atherosclerotic lesions. We have previously reported that internalization of aggregated low density lipoproteins (agLDLs) by vascular smooth muscle cells (VSMCs) produces cholesteryl ester (CE) accumulation in these cells. The aim of this study was to analyze whether the low density lipoprotein receptor–related protein (LRP) mediates the uptake of agLDL by VSMCs. First, immunocytochemistry and fluorescence microscopic analysis with the use of anti-LRP antibodies indicated that there was a high expression of LRP in VSMCs. Confocal microscopic analysis with the use of agLDLs labeled with fluorochrome 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine and anti-LRP antibodies showed the colocalization of agLDL and LRP. The second approach was to analyze the effect of LRP ligands on agLDL internalization; lactoferrin strongly inhibited CE accumulation from agLDLs (85.0±5.7% at 25 μg/mL) by impairing agLDL binding. Coincubation of agLDL with anti-LRP antibodies decreased in a dose-dependent manner agLDL-derived CE accumulation (from 20% at 12.5 μg/mL to 80% at 50 μg/mL). The third approach was to evaluate whether antisense LRP oligodeoxynucleotides were able to block agLDL internalization. Treatment of VSMCs with 5 μmol/L antisense LRP oligodeoxynucleotides reduced agLDL-derived CE accumulation by 84±2%. In conclusion, these results from immunologic, biochemical, and molecular interventions demonstrate that LRP mediates the binding and internalization of agLDL in human VSMCs. Because LRP is highly expressed in VSMCs and the uptake of 1 LDL aggregate amounts to the deposition of several hundreds of LDL particles, the uptake of agLDL through LRP could have a crucial role for lipid deposition in VSMCs. (Arterioscler Thromb Vasc Biol. 2000;20:1572-1579.)

Key Words: aggregated LDL ■ cholesteryl ester accumulation ■ vascular smooth muscle cells ■ LDL receptor–related protein ■ antisense oligodeoxynucleotides

One of the main events in the atherogenic process is the accumulation of lipids, mainly cholesteryl esters (CEs). Vascular smooth muscle cells (VSMCs) synthesize proteoglycans, extracellular matrix components involved in the focal deposition of cholesterol-rich particles. The uptake of matrix-retained lipoproteins by VSMCs and macrophages produces CE accumulation and leads to foam cell formation. Macrophages become foam cells through the uptake of diversely modified LDLs, whereas the aggregation of LDLs seems to be a key condition for lipid accumulation in VSMCs. Recently, we have shown that aggregation of CEs in VSMCs from in vitro–aggregated LDL (agLDL) is dependent on agLDL concentration and the degree of aggregation. AgLDLs obtained by vortexing LDL in vitro share structural characteristics with LDL aggregates present in atherosclerotic lesions.

In macrophages, phagocytosis and/or scavenger receptors mediate cholesterol accumulation from different types of modified LDL. In human lesions, scavenger receptors are present at high levels in macrophages but not in VSMCs. VSMCs express scavenger receptors only after stimulation with certain growth factors, and a direct involvement of these receptors in VSMC foam cell formation has not been demonstrated. We hypothesized that the LDL receptor–related protein (LRP) was the receptor for agLDL internalization in VSMCs. LRP is present in macrophages and VSMCs from atherosclerotic lesions and from normal vessels. LRP is a 600-kDa multifunctional endocytic receptor that belongs to the LDL receptor gene family. After synthesis, LRP is cleaved into 515-kDa (α-chain) and 85-kDa (β-chain) subunits. LRP has been shown to act as an endocytosis-mediating receptor for several ligands, including lactoferrin, thrombospondin, protease–anti-protease complexes, plasma lipoproteins such as apoE-enriched VLDL lipoprotein lipase and lipoprotein lipase-triglyceride–rich lipoprotein complexes, and Lp(a).

Therefore, the aim of the present study was to demonstrate whether LRP was responsible for the binding and internalization of agLDLs in VSMCs. We have found that both antibodies, anti-LRP and lactoferrin (a ligand of LRP), strongly inhibited agLDL binding and CE accumulation derived from agLDL internalization. In addition, VSMCs treated with antisense LRP oligodeoxynucleotides (ODNs) were unable to accumulate CE from agLDL, unlike sense...
LRP ODN–treated cells. These results seem to support our hypothesis and show that LRP mediates agLDL binding and internalization in VSMCs.

Methods

Materials

Cell culture medium and reagents were from GIBCO Laboratories. Anti–LDL receptor antibodies (clone C7, RPN 537) were purchased from Amersham. Bovine lactoferrin, α-M, and polyinosinic acid (5’) were obtained from Sigma Chemical Co. α-M (1 mg) was activated with 200 mmol/L methylamine-HCl in 50 mmol/L Tris-HCl for 8 hours, pH 8.0 at 25°C. The excess of methylamine was removed by dialysis against PBS. Activated α-M migrated with the same mobility by nonradioactive protein sequencing, confirming the conformational change. Anti–LDL receptors antibodies (clone C7, RPN 537) were purchased from Molecular Probes, Inc. Monoclonal antibodies against LRP α chain (A2 MRα-2) and LRP β chain (A2 MRβ-2) were kindly provided by Drs. S.K. Moestrup and J. Gliemann (University of Aarhus, Aarhus, Denmark). The bichiniconic acid protein assay was from Pierce.

VSMC Culture

Primary cultures of human VSMCs were obtained from nonatherosclerotic areas of the human aorta from explanted hearts obtained at transplant operations performed at the Hospital de la Santa Creu i Sant Pau, Spain. VSMCs were obtained by a modification of the explant technique as described previously. 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 in medium 199 (M199) supplemented with 5% FCS, 50 mmol/L Tris-HCl for 8 hours, pH 8.0 at 25°C. The excess of methylamine was removed by dialysis against PBS. Activated α-M migrated with the same mobility by nonradioactive protein sequencing, confirming the conformational change. Anti–LDL receptors antibodies (clone C7, RPN 537) were purchased from Molecular Probes, Inc. Monoclonal antibodies against LRP α chain (A2 MRα-2) and LRP β chain (A2 MRβ-2) were kindly provided by Drs. S.K. Moestrup and J. Gliemann (University of Aarhus, Aarhus, Denmark). The bichiniconic acid protein assay was from Pierce.

Immunocytochemistry

Cells were seeded in glass coverslips (Laboratory-Tek), grown to confluence, then fixed with methanol for 5 minutes, and blocked with PBS/1% BSA. Afterward, cell monolayers were incubated with primary antibodies diluted in PBS/1% BSA/0.1% Triton X-100 for 2 hours at room temperature. Monoclonal antibodies against smooth muscle cell α-actin (1:25 dilution, clone 1A4), von Willebrand factor (1:25 dilution, clone F8/86), α-LRP (1 μg/mL), and β-LRP (1 μg/mL) were used. Coverslips were then washed and incubated with an FITC-conjugated goat anti-mouse IgG (1:20 dilution) for 1 hour in the dark. Results were evaluated with an Olympus Vanox AHR3 microscope, and photographs were taken with Kodak Ektachrome (ASA 400) daylight films.

LDL Preparation and Dil Labeling

Human LDLs (density 1.019 to 1.063 g/mL) were obtained from pooled sera of normocholesterolemic volunteers and isolated by sequential ultracentrifugation. LDLs were dialyzed against 3 doses of 200 vol of 150 mmol/L NaCl, 1 mmol/L EDTA, and 20 mmol/L Tris-HCl, pH 7.4, overnight and once against 150 mmol/L NaCl. LDL protein concentration was determined by the bichiniconic acid method, and cholesterol concentration was determined by a commercial kit (Boehringer). The average total cholesterol content of human LDLs was ~2 mg/mg LDL protein. LDLs used in the experiments were <48 hours old. The purity of LDLs was assessed by agarose gel electrophoresis (Paragon System, Beckmann). Thiobarbituric acid reactive substances (TBARS) were measured as an indirect evaluation of lipid peroxidation. TBARS levels were <1.2 mmol malonaldehyde per milligram protein LDL. LDLs were labeled with Dil by a modification of the method described by Beisiegel et al., which involves incubating LDLs (1 mg/mL) in PBS/0.5% BSA with 100 μL of diiodoindocarbocyanine (Dil) for 24 hours or until the desired concentration was achieved. After Dil–LDL incubation, several samples were stained with anti-LRP α-chain antibodies to analyze the colocalization of Dil–LDL and LRP. Samples were analyzed by a laser-scanning confocal fluorescence microscope (Leica TCS NT). The software program used was TCSNT, version 1.3.237 (Leica).

Determination of Free and Esterified Cholesterol Content

In the experiments performed to analyze the effect of anti-LRP antibodies, arrested cells were incubated for 18 hours with native LDL or agLDL in the absence or presence of increasing concentrations (12.5, 25, and 50 μg/mL) of anti-LRP α-chain antibodies, or nonimmune IgG. In other experiments, arrested cells were incubated with increasing concentrations (25, 50, or 100 μg/mL) of lactoferrin, α-M, or polyinosinic acid. At the end of this period, cells were extensively washed, twice with PBS, twice with PBS/1% BSA, and twice with PBS/1% BSA/heparin 100 U/mL, before harvesting into 1 mL of 0.15 mol/L NaOH. Lipid extraction and thin-layer chromatography were performed as previously described. The spots corresponding to free cholesterol (FC) and esterified cholesterol were quantified by densitometry against the standard curve of cholesterol and cholesterol palmitate, respectively, with the use of a computing densitometer (Molecular Dynamics).

ODN Treatment

Phosphorothioate antisense ODNs were designed to hybridize to the initiation site on the human LRP mRNA. It was a 15-mer (5’-CGGCGGGGTCAGCG-3’) that was complementary to nucleotides 466 to 481 of LRP mRNA. The corresponding sense ODN and a random ODN (5’-TAGCTTGTAGTGAGG-3’) were used as a control. Fasta analysis (in the Genetic Computer Group package) indicated that these sequences would not hybridize to other receptor sequences (including LDL receptors) in the GenBank database. These ODNs contained the phosphorothioate modification in all positions to avoid degradation by nucleases. They were synthetized by using phosphoramidite chemistry and purified by high-performance liquid chromatography (MWG Biotech). The lyophilized product was dissolved at a stock concentration of 5 mmol/L in diethylpyrocarbonate-treated water and stored at ~20°C.

VSMCs were grown in normal medium and treated with ODNs during the quiescence period (48 hours). Antisense and sense ODNs (5, 10, and 20 μmol/L) were added to the medium at the beginning of the first 24 hours of the arresting period. Then the medium was replaced by a new medium containing the ODNs at the same concentrations and maintained for a further 24 hours. AgLDLs (100 μg/mL) were added to the medium at the beginning of the first 24 hours of the arresting period. Then the medium was replaced by a new medium containing the ODNs at the same concentrations and maintained for a further 24 hours. AgLDLs (100 μg/mL) were added to the medium at the beginning of the first 24 hours of the arresting period. Then the medium was replaced by a new medium containing the ODNs at the same concentrations and maintained for a further 24 hours. AgLDLs (100 μg/mL) were added to the medium at the beginning of the first 24 hours of the arresting period. Then the medium was replaced by a new medium containing the ODNs at the same concentrations and maintained for a further 24 hours.
mg/mL) were added to nontreated and to antisense, sense, and random ODN-treated VSMCs 12 hours before ending the second 24 hours of the arresting period. Then the cells were exhaustively washed, photographed, and harvested into 1 mL of 0.15 mol/L NaOH. The determination of FC and CE content was performed as previously described.8,40

Statistical Analysis
Data are expressed as mean±SEM. A Statview (Abacus Concepts) statistical package for the Macintosh computer system was used for all analyses. Multiple groups were compared by 1-factor ANOVA. A value of P<0.05 was considered to be statistically significant.

Results
LRP Levels in VSMCs
Immunolabeling of VSMCs with anti-LRP antibodies showed that this receptor is highly expressed in VSMCs (Figure I, which can be found online at http://atvb.ahajournals.org). All nuclei (blue) are surrounded by anti-α-subunit or anti-β-subunit staining (Figure IB and ID). Figure IA and IC shows, in high-magnification photographs, the perinuclear labeling of α and β subunits.

Colocalization of LRP and Dil-LDL
Confocal microscopy was performed to study the colocalization of agLDL and LRP during the internalization of agLDL by VSMCs. VSMCs were incubated with Dil-nLDL or Dil-agLDL for 30 minutes at 4°C. After removal of unbound LDL, the bound lipoprotein fraction was left to internalize for 4 hours at 37°C. VSMCs were then washed and incubated for 4 hours at 37°C. Then VSMCs were washed, fixed, and incubated with anti-LRP β chain. Photomicrographs show VSMCs incubated with Dil-agLDL and anti-LRP β chain (A) or Dil-nLDL and anti-LRP β chain (B). Photomicrographs are representative of 2 different experiments. Magnification ×5000.

Comparison of Lactoferrin (Ligand of LRP) and Polyinosinic Acid (Ligand of Scavenger Receptor) on CE Accumulation Derived From AgLDL
VSMCs were coincubated with agLDLs (100 μg/mL) and increasing concentrations (25, 50, and 100 μg/mL) of either lactoferrin, a ligand of LRP, or polyinosinic acid, a ligand of scavenger receptors. Polyinosinic acid only slightly reduced
CE accumulation from agLDLs. On the contrary, lactoferrin, at the lowest concentration tested (25 μg/mL), produced an inhibition of 85.0 ± 5.7% (Figure 2). Activated α2 M, at 50 μg/mL, did not have any effect on CE accumulation derived from agLDLs. Preincubation of VSMCs with lactoferrin (25 μg/mL) for 1 hour completely abolished endocytosis of agLDL (Figure II, which can be found online at http://atvb.ahajournals.org), indicating that the decrease in CE accumulation produced by lactoferrin was due to the inhibition of agLDL binding to LRP.

These results indicate that scavenger receptors are not dominant receptors for agLDL uptake in VSMCs and suggest that LRP mediates agLDL uptake.

Comparison of Effect of Anti-LRP Antibodies, Anti-LDL Receptor, and Nonimmune IgG on CE Accumulation Derived From AgLDL

To further analyze whether LRP mediates the uptake of agLDL by VSMCs, we assessed the effect of monoclonal antibodies against the α chain of LRP (A2 MRα-2)43,44 compared with the effect of monoclonal antibodies against LDL receptors (clone C7) or nonimmune IgG. Figure 3 shows that anti-LRP antibodies decreased in a dose-dependent manner the CE accumulation induced by agLDL uptake. In contrast, nonimmune IgG did not show any significant effect. The anti-LDL receptor antibody did not show any significant effect at a concentration of 12.5 or 25 μg/mL. Anti-LDL receptor antibodies decreased CE increase induced by native LDL in a dose-dependent manner (Figure III, which can be found online at http://atvb.ahajournals.org). On the contrary, nonimmune IgG did not show any significant effect.

Effect of AgLDL on Cholesterol Content of VSMCs Treated With Antisense LRP ODNs

LRP expression was blocked by antisense LRP ODNs to determine whether agLDL internalization could be suppressed in the treated cells. AgLDL-derived CE accumulation (153 ± 4.41 μg CE per milligram protein) was significantly reduced (84 ± 2% inhibition at 5 μmol/L and 91 ± 5% at 20 μmol/L) in antisense ODN–treated VSMCs (Figure 4) but not in sense ODN– or random ODN–treated cells. FC content was only slightly reduced by antisense ODN treatment. The lack of effect of sense and random ODNs on agLDL binding and CE accumulation support the specificity of antisense ODN treatment. The lack of effect of sense and random ODNs on agLDL binding and CE accumulation support the specificity of antisense ODN treatment. In addition, native LDL–derived CE increase was not diminished by antisense LRP ODN treatment (Figure 5).

Figure 6 shows microphotographs of representative cells after incubation of nontreated and antisense and sense LRP ODN–treated VSMCs with agLDL. Pictures were taken after the first wash with PBS to eliminate agLDL that was not
As shown, untreated VSMCs (Figure 6A) had many aggregates of LDL bound (arrows) on the cell surface, whereas antisense LRP ODN–treated (5 μmol/L) VSMCs did not (Figure 6B). Sense ODN–treated (Figure 6C) or random ODN–treated (photography not shown) VSMCs also have many LDL aggregates bound to the cell surface. The ODN treatment of VSMCs did not seem to induce morphological changes of VSMCs.

**Discussion**

Several lipoprotein receptors may be involved in LDL uptake in the arterial wall.20–22 Among them, the scavenger receptors (highly expressed in macrophages) and LRP (highly expressed in VSMCs) would be candidates for agLDL uptake. In the present study, high concentrations of polyinosinic acid, a ligand of the scavenger receptor, only slightly inhibited agLDL-derived CE accumulation in VSMCs. These results are in agreement with those showing that neither the uptake of LDL aggregates, obtained by vortexing13 or binding with proteoglycans,5,12 nor the uptake of agLDL isolated from injured aortas45,46 is mediated by scavenger receptors. We hypothesized that the particular structure of the binding domain of the LRP, composed of multiple copies of 3 of the 4 structural motifs found in the extracellular binding domain of the LDL receptor,23 would facilitate the interaction with agLDL, composed of hundreds of LDL particles.5,9 Indeed, lactoferrin, a nondissociable ligand for LRP, has been shown to be highly effective in preventing agLDL binding and CE accumulation in the cultured VSMCs at very low concentrations. A similar concentration of lactoferrin has been used by other authors to inhibit the uptake of chylomicron remnants.47 The high effectiveness of lactoferrin could be due to the sequestration of LRP by lactoferrin into the endosomes and the impairment of LRP recycling.25 The experiments using Dil-agLDL indicate that, as with other ligands, lactoferrin inhibits the first step of the internalization process, the binding of agLDL to the cell surface. Because in binding experiments, lactoferrin competes with lipoprotein lipase/VLDL, apoE/VLDL, and remnant chylomicrons but not with tissue plasminogen activator/plasminogen activator inhibitor type-I or αM,26,47,48 it is conceivable that agLDL could share the ligand binding site with other LRP lipoprotein ligands.

The uptake of agLDL can be also be blocked by treatment of the cells with antibodies against the LRP α chain. These antibodies strongly inhibited CE accumulation, indicating the involvement of the LRP α chain on agLDL binding. The antibody concentrations used were similar to those used by...
other authors to inhibit the uptake of other LRP ligands.26,29 It seems that structurally unrelated ligands appear to contain homologous positively charged domains that are involved in receptor binding.49–52 We do not know whether the striking ability of agLDL to induce cholesterol accumulation in VSMCs is due to the presence of various apoB-100 binding sites in each particle or to the configuration of a new LRP-recognizable epitope after LDL modification. There are controversial results concerning the capacity of LRP to interact with apoB-100; Véniant et al53 demonstrated in a mice model that there is no binding of apoB-100 to LRP. However, other authors have demonstrated the specific binding of apoB-100 to purified LRP.54

In macrophages, phagocytosis has been considered to be the main mechanism for agLDL uptake, and controversial results have been published concerning the involvement of the classic LDL receptor.12–14 In VSMCs, we have evidenced that anti-LDL receptor antibodies, which specifically inhibit intracellular CE increase induced from native LDLs, do not have any effect on CE accumulation from agLDL; therefore, the classic LDL receptor does not seem essential for agLDL uptake. These results are in agreement with our previous finding showing a different pattern for Dil-nLDL and Dil-agLDL internalization in VSMCs.8 Finally, the involvement of LRP on agLDL binding and internalization has also been demonstrated by the lack of agLDL binding and CE accumulation in antisense LRP ODN–treated VSMCs. From these results, we can conclude that a functional LRP is required for CE accumulation from agLDL in VSMCs. A direct heparan sulfate proteoglycan–mediated internalization, as postulated for apoE-triglyceride–rich lipoprotein particles,41 seems not to be the main pathway for agLDL in VSMCs. However, it is likely that the process called “ligand transfer” from heparan sulfate proteoglycans to LRP (demonstrated with other LRP ligands)55,56 can also occur with agLDL and needs to be investigated.

Our results demonstrate for the first time that in VSMCs, cells with very high levels of LRP expression, LRP mediates the binding and internalization of agLDL and that in the absence of LRP function, VSMCs are unable to accumulate cholesterol. LRP, contrary to the LDL receptor, has multiple binding sites and is not regulated by intracellular cholesterol. Therefore, LRP-mediated endocytosis can be considered as a low-specificity high-capacity mechanism that allows the uptake of large amounts of ligand. Because LRP is highly expressed in atherosclerotic plaques and because subendothelial LDL retention and aggregation are key events in atherogenesis, the uptake of agLDL through LRP could have a crucial role in VSMC-lipid deposition in atherosclerotic plaques.

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