Enhanced Expression of the LDL Receptor Family Member LR11 Increases Migration of Smooth Muscle Cells In Vitro

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Background—LR11, a member of the LDL receptor family, is highly expressed in vascular smooth muscle cells (SMCs) of the hyperplastic intima but not media. To further clarify the involvement of LR11 in the process of atherosclerosis, we have characterized the migration and invasion activities of LR11-overexpressing SMCs.

Methods and Results—LR11 cDNA was transfected into the rat SMC line A7r5. Compared with mock cells (C-1), in the presence of platelet-derived growth factor-BB, the transfected cells (R-1 and R-2) showed 3.5- to 4.0-fold higher expression of LR11 protein, 1.7- to 1.8-fold increased migration, and 2.0- to 2.2-fold elevated invasion activities, respectively. The increases were essentially abolished by the addition of receptor-associated protein, anti-LR11 antibodies, or apolipoprotein E. Immunological analyses showed that urokinase-type plasminogen activator receptor (uPAR) levels were increased in LR11-overexpressing cells. Anti–urokinase-type plasminogen activator (uPA) and anti-uPAR antibodies reduced the migration and invasion activities of R-1 and R-2 cells to baseline levels. Receptor-associated protein, anti-LR11 antibodies, and apolipoprotein E decreased uPAR expression in the LR11-overexpressing cells by ≈50%. Cellular catabolism of uPAR was significantly decreased in R-1 and R-2 cells compared with control. Cultured SMCs isolated from intima of atherosclerotic rabbit aortas showed increased expression levels of LR11 and uPAR and enhanced migration and invasion compared with SMCs from medial layers.

Conclusions—Overexpression of LR11 induces enhanced migration and invasion activities of intimal SMCs in vitro, probably through its regulation of the uPA/uPAR system. (Circulation. 2002;105:1830-1836.)

Key Words: atherosclerosis ■ lipoproteins ■ receptors ■ plasminogen

Historical studies have revealed that receptors belonging to the LDL receptor family are markedly induced during formation of atherosclerotic lesions (reviewed in References 1 and 2).1,2 For instance, the very low-density lipoprotein receptor (VLDLR/LR8) is highly expressed in smooth muscle cells (SMCs), macrophages, and endothelial cells in rabbit atherosclerotic lesions. The expression of LDL receptor-related protein (LRP) is also induced in atheromata. We and others have discovered and molecularly characterized a novel, unusually complex and highly conserved member of the LDL receptor gene family, LR11.3–6 The predominant domain of this type I membrane protein consists of a cluster of 11 LDL receptor ligand binding repeats,3 hence its designation. Our recent studies with two experimental models of atherogenesis have demonstrated marked induction of LR11 during intimal thickening.7 Immunohistochemical and in situ hybridization analyses showed that LR11 is predominantly localized to SMCs in the intima. These results suggested a functional significance of LR11 in the (patho)physiology of SMCs in atheromata.

We studied the function of LR11 in migration of SMCs by using LR11-overexpressing SMCs generated by transfection of rabbit LR11 cDNA into a rat cell line. As evaluated by two distinct methods for cellular migration in vitro, LR11 caused increased migration activities of SMCs. We also assessed the effects of LR11 overexpression on the plasminogen activator (PA)-mediated migration system, which has been invoked in the processes of arterial remodeling, neuronal migration, and cancer invasion.8–10

Methods

Cells

A7r5, a rat embryonic aortic SMC line, was obtained from the American Type Cell Culture. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 40 mg/L gentamicin, and 2 mmol/L glutamine, 4.5 g/L glucose. The recombinant expression plasmid pBKCMVLR11 was constructed by cloning a 7-kilobase fragment carrying the entire protein coding sequence of the rabbit LR11 cDNA into the vector pBKCMV. The transfection of A7r5 cells was carried out with pBKCMVLR11 or pBKCMV (control)
and a proprietary nonliposomal lipid (Effectan, Qiagen). Three clones, R-1, R-2, and C-1 (control), were used for the experiments. Primary cultures of rabbit medial and intimal SMCs (Takasugi, Tokyo, Japan) were prepared from the medial and intimal layers of atherosclerotic aortas from atherogenic diet-fed rabbits as described and used at passages 3 and 4.

**Blot Analyses and Immunocytochemistry**

Cell membranes were prepared and solubilized as described. For analysis of phosphorylated extracellular signal-regulated kinase (ERK), cells were starved for 48 hours in 0.3% FBS–DMEM followed by the addition of platelet-derived growth factor (PDGF)-BB (R&D Systems) at 10 ng/mL for 10 minutes. For the analysis of cell-surface urokinase-type plasminogen activator receptor (uPAR), cells were treated with 0.5 U/mL of phosphatidylinositol-specific phospholipase C (Sigma-Aldrich) for 2 hours and the media recovered. For inhibition assays, cells were starved for 24 hours, and the medium was changed to 10% FBS–DMEM containing receptor-associated protein (RAP) (10 μg/mL), anti-LR11 antibody (1:2 dilution), or apolipoprotein E (apoE; 50 μg/mL, Cosmo Bio) for 24 hours. Equal amounts of membrane protein or phosphatidylinositol-specific phospholipase C (PF-PLC)–conditioned medium were subjected to SDS-PAGE under reducing (Western blot) and nonreducing (ligand blot) conditions. Electrophoretic transfer of the proteins to polyvinylidene fluoride membranes (NEF Life Science) was performed for 2 hours on ice. For Western blotting, anti-LR11 antibody (1:2 dilution) or polyclonal antibodies against rat uPAR (American Diagnostica), PDGF-β receptor (Upstate Biotechnology), or phosphorylated ERK 1/2 (New England Biolabs), were used. Development was performed with the electrochemiluminescence detection reagents. The signals were quantified by densitometric scanning with National Institutes of Health Image software. Recombinant human RAP was produced as glutathione S-transferase (GST)-fusion protein in _Escherichia coli_ strain DH5α, and ligand blotting with 125I-labeled RAP-GST was performed as reported. Northern blot analysis was described previously. The membranes were probed with a 360-bp polymerase chain reaction–amplified fragment corresponding to nucleotides 301 to 660 of rat uPAR (AF07789) or human β-actin cDNA (R&D Systems, Abingdon). Filters were exposed to Fuji imaging plates for the Bioimaging analyzer (Fuji Bas 2000). Immunocytochemical studies were performed with anti-LR11 antibody (1:2 dilution) and rabbit anti-rat uPAR antibody (1:10 dilution) as described.

**Migration and Invasion**

Migration of cells was measured essentially as previously described in a 96-well micro-Boyden chamber (its surface was coated with a type I collagen). Invasion assays were performed with Transwell (Corning) 24-well plates coated with a collagen gel. For inhibitory assays, cells were preincubated with RAP (10 μg/mL), anti-LR11 antibody (1:1 dilution), apoE (25 to 50 μg/mL), aprotinin (100 KIU/mL, Sigma-Aldrich), anti–urokinase-type plasminogen activator (uPA) antibody (10 μg/mL, NeoMarkers), or anti-uPAR antibody (10 μg/mL) for 30 minutes at 37°C, and then aliquots of the cell suspension were added to the upper or inner chamber. The lower or outer chamber contained 1% FBS–DMEM with or without 10 ng/mL PDGF-BB. Inhibitors were added to both chambers and were present throughout the experiment. After a 4-hour incubation at 37°C, the cells on the upper surfaces were washed, fixed, and stained by Diff-Quik (International Reagents). The number of cells that migrated to the lower surface of the filters was determined microscopically by counting. The data are presented as “fold-increase” of the migrated or invaded cell numbers in the absence of PDGF-BB in the lower or outer chambers.

**Binding and Metabolic Labeling Assays**

To determine cell binding of PDGF-BB, monolayers of cells were used for the assay as described. Cells were incubated for 4 hours at 4°C with 1 to 40 ng/mL of 125I-labeled human recombinant PDGF-BB (Amersham–Pharmacia Biotech) in the presence or absence of 10 μg/mL of unlabeled PDGF-BB. Cell-associated radioactivity was determined by γ-counting after cell lysis. Metabolic labeling of uPAR was performed as described. Cells were cultured in methionine-free DMEM for 12 hours, followed by the incubation in the same medium with [35S]-methionine (10 μCi/mL) for 24 hours. The cells were chased for 1 hour with DMEM, washed, and incubated in fresh DMEM with 0.5 mmol/L uPA–plasminogen activator inhibitor (PAI)-1 complex for 6 hours. uPA–PAI-1 complexes were formed by reacting 0.25 mmol/L 2-chain uPA (American Diagnostica) with an equimolar concentration of PAI-1 (Calbiochem) for 10 minutes at 37°C.

**Statistics**

The results are shown as mean±SD for each index, respectively. Comparisons of data were performed by means of the Student’s _t_ test; a value of _P_<0.05 was considered significant.

**Results**

**Enhanced Migration and Invasion of LR11-Overexpressing SMCs**

Figure 1A shows the expression of LR11 protein in the stably transformed cells, R-1 and R-2. Levels of LR11 in R-1 and R-2 cells were 3.5- and 4.0-fold higher than in the control (C-1) cells; note that the monoclonal antibody cross-reacts with endogenous rat LR11. R-1 and R-2 cells bound 125I-labeled RAP, shown to bind to native LR11 in brain more efficiently than C-1 cells (Figure 1B). All cells showed the same level of a RAP-binding protein of ~500 kDa, probably presenting LRAP. In the presence of FBS and PDGF-BB, the migration activities of transformed cells were significantly greater than those of mock cells (Figure 1C). The increase in migration activities after addition of PDGF-BB was dose dependent. As shown in Figure 1D, at every time point, PDGF-BB–directed invasion through a collagen barrier was also greater for R-1 and R-2 cells than for C-1 cells. These data, obtained with two different methods for measuring cell migration and invasion, suggest that LR11 is involved in enhanced migration of SMCs in vitro.

**Effects of RAP, Anti-LR11 Antibody, and ApoE on Migration of SMCs**

To test whether enhanced migration is related to LR11 function, we studied the effects of anti-LR11 antibody, RAP, and apoE on SMC migration. Anti-LR11 antibody and RAP inhibited the migration of C-1 control cells by ~20% (Figure 2A, panel a). Both reagents inhibited the number of migrated R-1 and R-2 cells by 40% and 47%, respectively (panel b), that is, twice as much as the parent cells. RAP and anti-LR11 antibody also inhibited the invasion of C-1 cells by ~25% and of R-1 and R-2 cells by ~50% after 24 hours (Figure 2B). ApoE, shown to bind LR11 with high affinity in vitro, inhibited PDGF-induced migration of C-1 cells in a dose-dependent manner by a maximum of ~30% at 50 μg/mL. In contrast, migration of R-1 and R-2 cells was inhibited by 22%, 35%, and 62% by 5, 25, and 50 μg/mL apoE, respectively (Figure 2C, panel b); again, apoE abolished the enhanced migration activities of LR11-overexpressing cells to the levels of mock cells. Taken together, increased migration activities observed in the LR11-transfected cells most likely are caused by overexpression of LR11 protein.
Involvement of the uPA-uPAR System in LR11-Enhanced Migration

The mechanisms of PDGF-BB–directed SMC migration include binding, subsequent signaling by mitogen-activated protein kinase, and extracellular proteolytic processes, for example, through the uPA-uPAR system. There were no differences in PDGF-BB binding to R-1, R-2, and C-1 cells or the levels of immunoreactive PDGF-β receptor among these cells (Figure 3A). Also, there were no detectable differences in the levels of phosphorylated ERK 1/2 (Figure 3B) or in the secretion of matrix metalloproteinase-3, matrix metalloproteinase-9, and plasminogen (data not shown) among the three cell lines. Immunoblotting revealed that PI-PLC–releasable uPAR expression in R-1 and R-2 cells was 4.4-fold greater than control, with total uPAR expression 1.7-fold higher (Figure 4A). Furthermore, immunocytochemical staining showed that uPAR antigen was detected in R-1 cells but much less in C-1 cells (Figure 4B). Thus, our analyses revealed that LR11 overexpression is accompanied by an increase in uPAR, suggesting an involvement of the uPA-uPAR system in LR11-enhanced migration.

We therefore performed blocking experiments with aprotinin (a plasmin inhibitor), anti-uPA, and anti-uPAR antibodies. As shown in Figure 5A, anti-uPA and anti-uPAR antibodies inhibited the migration of C-1 cells by only 15% and 35%, respectively (panel a), but of R-1 and R-2 cells by 54% and 58% (panel b). Figure 5B shows that both antibodies inhibited the invasion of R-1 and R-2 cells by ~60%, but that of C-1 cells only by one half as much. Aprotinin significantly inhibited invasion by 26% in R-1 and R-2 cells and by 19% in C-1 cells, respectively, but did not significantly inhibit migration.

Effects of RAP, Anti-LR11 Antibodies, and ApoE on uPAR Expression

Because RAP, anti-LR11 antibodies, and apoE inhibit the migration activities of LR11-overexpressing SMCs to control levels (Figure 2), we investigated the effects of these proteins on uPAR expression in SMCs. There was no significant inhibition by RAP, anti-LR11 antibodies, and apoE on uPAR expression in C-1 cells (Figure 6A); on the other hand, RAP, anti-LR11 antibodies, and apoE decreased uPAR expression by 52%, 58%, and 42%, respectively, in LR11-overexpressing cells (Figure 6B). The correlation of inhibi-
tory effects on both uPAR and migration of proteins interacting with LR11 strongly suggest that uPAR plays a significant role in increased migration and invasion of LR11-overexpressing SMCs.

**uPAR Transcripts and Degradation of Protein**

To elucidate the mechanism for uPAR induction in LR11-overexpressing cells, we analyzed transcript levels and uPAR protein stability. uPAR mRNA (1.5 kb) levels in R-1 and R-2 cells and in C-1 cells were identical (Figure 7A). Catabolism of uPAR protein was studied after incubation of the prelabeled cells with uPA–PAI-1 complex, followed by the immunoprecipitation of the $^{35}$S-methionine-labeled uPAR (Figure 7B). In C-1 cells, within 6 hours, 43% less $^{35}$S-uPAR was precipitated under identical conditions. The levels in R-1 and R-2 cells decreased only by 9% and 2%, respectively, significantly less than in control cells. Thus, LR11-overexpression in SMCs is associated with decreased catabolism of uPAR.

**Expression of LR11 and uPAR Proteins in Intimal SMCs With Enhanced Migration Activity**

Finally, we attempted to correlate migration and invasion activities with LR11 levels in cultured SMCs prepared from intimal and medial layers of rabbit atherosclerotic aortas. As shown in Figure 8A, the average migration activity of intimal SMCs was significantly higher than that of medial SMCs, as previously observed. Invasion activity of intimal SMCs was 1.6-fold higher than in medial SMCs (Figure 8B). The level of LR11 protein was clearly higher in intimal SMCs (I-1, I-2, and I-3) than in medial SMCs (M-1, M-2, and M-3) (Figure 8C). The same was observed for uPAR levels. These findings were in agreement with the results described above using LR11-overexpressing A7r5 cells.

**Discussion**

We showed increased PDGF-BB–induced migration and invasion caused by overexpression of LR11 in SMCs in vitro, because the increase is diminished by anti-LR11 antibodies and by RAP to baseline levels. RAP blocks the binding of PDGF-BB to LR11, and decreases migration and invasion.
ligands to LR11, VLDLR, LRP, and megalin 2,12 and has been suggested to inhibit the migration and invasion of SMCs by interfering with the internalization of the uPA–PAI-1 complex. 17–20 Thus, our results strongly suggest that in LR11-overexpressing SMCs, the LR11-mediated activation of the uPA-uPAR system is implicated in enhanced migration activities.

Of particular relevance is the high level of LR11 expression in SMCs in intimal layers of atheromatous lesions, whereas in medial layers, the receptor was not detectable.7 We found that both LR11 and uPAR are elevated in intimal SMCs compared with the cells from medial layers (Figure 8), again suggesting that LR11 and

the uPA-uPAR system cooperate in mediating SMC migration in atheromatous PDGF-BB, a potent chemoattractant and mitogen, modulates the migration of SMCs from the media to the intima, where it induces SMC proliferation.21 However, because we did not find significant effects of LR11 overexpression on PDGF-BB cell-surface binding nor on the expression of PDGF-β receptor and phosphorylated ERK (Figure 3), it is unlikely that LR11 acts through the mitogen-activated protein kinase signal pathway to enhance migration and invasion of SMCs.

The uPAR system plays a crucial role in several complex biological processes.9,22 Receptor binding of uPA provides cells with a localized proteolytic potential by stimulating conversion of plasminogen to active plasmin, required for proteolytic degradation of certain basement membrane components.9 uPAR has been identified as a vitronectin receptor,23 which could be important in evaluating the migration and invasion experiments reported

Figure 5. Effects of aprotinin and antibodies against uPA and uPAR on migration (A) and invasion (B) of C-1 cells (a) and R-1 and R-2 cells (b). Cells were preincubated in the absence or presence of 100 KIU/mL aprotinin, 10 µg/mL anti-uPA antibody, or 10 µg/mL anti-uPAR antibody. n=12 in all cases. *P<0.05, **P<0.01.

Figure 6. Effects of RAP, anti-LR11 antibody, and apoE on uPAR expression of C-1 cells (A) and R-1 and R-2 cells (B). Cells were incubated for 24 hours in medium with 10 µg/mL RAP, anti-LR11 antibody (1:2 dilution), or 50 µg/mL apoE. PI-PLC-treated medium was analyzed by immunoblotting with anti-uPAR antibody. The photograph shown is representative of 4 independent experiments (R-1). n=12 in all cases. *P<0.01.

Figure 7. Transcript levels (A) and catabolism (B) of uPAR protein in C-1 (C), R-1 (R1), and R-2 (R2) cells. A, Poly (A)+ RNA (10 µg/lane) was subjected to Northern blot analysis with probes specific for uPAR or β-actin. Data shown are representative of 3 independent experiments. In B, cells were pulse-chase–labeled with 35S-methionine, and uPAR was immunoprecipitated. Signal intensity after 6-hour chase (6) was calculated as percentage of control, the intensity after the pulse (0). n=3. *P<0.01.
Here, members of the LDL receptor family function in endocytosis and subsequent degradation of many ligands, including uPAR-bound uPA–PAI-1 complexes. Anti-LRP antibodies and RAP inhibit migration and invasion of SMCs by inhibiting the internalization of uPA–PAI–uPAR complex. In the present study, LR11-overexpressing cells showed upregulation of uPAR expression and increased activities of migration and invasion, and anti-uPA and anti-uPAR antibodies inhibited these increases. On the other hand, aprotinin significantly inhibited invasion but not migration (Figure 5). RAP not only binds to LR11 with high affinity but also inhibits migration and invasion of LR11-overexpressing cells. Furthermore, RAP and anti-LR11 antibody interfered with increased uPAR expression in LR11-overexpressing cells (Figure 6), and the catabolism of uPAR was delayed in these cells (Figure 7). We suggest that LR11 inhibits the internalization of uPA–PAI–uPAR through other LDL receptor family members; consistent with this notion, anti-LR11 antibodies reduce this inhibitory effect.

ApoE inhibits PDGF-directed migration of SMCs, extending previous findings with cultured primary cells. Acknowledgments These studies were supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology to Dr Saito and Dr Bujo and from the Austrian Science Foundation to Dr Schneider (FWF P-13940 and F-0608).

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