LRP1B Attenuates the Migration of Smooth Muscle Cells by Reducing Membrane Localization of Urokinase and PDGF Receptors

Kousei Tanaga, Hideaki Bujo, Yanjuan Zhu, Tatsuro Kanaki, Satoshi Hirayama, Kazuo Takahashi, Masahiro Inoue, Keiji Mikami, Wolfgang J. Schneider, Yasushi Saito

Objective—Studies on the involvement of low-density lipoprotein receptor relatives (LRs) in atherosclerosis have recently gained new focus because of the specific expression of certain of these receptors in the thickened intima. Here, we show that LRP1B, a member of LRs, modulates the migration of smooth muscle cells (SMCs) by increasing the degradation of membrane receptors, urokinase-type plasminogen activator receptor (uPAR), and platelet-derived growth factor receptor (PDGFR) β.

Methods and Results—Immunohistochemistry showed that LRP1B expression in human coronary arteries is localized to the intimal SMCs near the plaque surface as well as to medial SMCs. LRP1B expression levels in cultured SMCs increase at the late phase of proliferation. Cell surface and internalization assays, in combination with coimmunoprecipitation experiments, showed that LRP1B binds and internalizes uPAR. Metabolic labeling analysis demonstrated that anti-LRP1B IgY decreased the catabolism of uPAR. In addition, the anti-LRP1B antibody raised PDGFRβ protein and PDGFR-mediated phosphorylation levels of ERK1/2. Finally, the anti-LRP1B IgY enhanced the migration and invasion of SMCs in the presence of PDGF-BB.

Conclusions—LRP1B modulates the catabolism of uPAR and PDGFR, affecting the migration of SMCs. This functional characterization of LRP1B opens novel avenues for elucidating the (patho)physiological significance of SMC migration in atheromatous plaques. (Arterioscler Thromb Vasc Biol. 2004;24:1422-1428.)

Key Words: smooth muscle cells ■ LRP1B ■ PDGF receptor ■ uPA receptor ■ migration

Migration of vascular smooth muscle cells (SMCs) from the media to the intima is a key step in the development of atherosclerosis.1,2 After migration, SMCs proliferate in the intima and secrete matrices comprising elastic fiber proteins, collagen, proteoglycans, proteases such as matrix metalloproteinases, and plasminogen and its modulators, to form atheromatous plaques. The plasminogen activation system, as well as other proteinases, is thought to be important for the degradation of basement membrane and of components of extracellular matrices, which facilitates cell migration.3-5 Recent studies showed that induction of the degradation system is accompanied by high invasion ability in malignant cells because of increased migration activity.6,7 The formation of plasmin from plasminogen is catalyzed by urokinase-type plasminogen activator (uPA); the uPA activity is localized to and enhanced by the uPA receptor (uPAR) on the cell surface. The activated system on the cell surface is inhibited by binding to PA inhibitor-1 (PAI-1), followed by the internalization of these complexes by a receptor belonging to the family of low-density lipoprotein (LDL) receptor relatives (LRs), ie, LDL receptor-related protein (LRP1).8 Accordingly, loss of LRP1 expression or function is associated with an increase in the cell-surface uPAR levels.9 In certain cell types, increased uPAR levels are associated with increased cellular motility10

Recently, we have discovered that an unusually complex and highly conserved LR, LR11, enhances the migration of SMCs via elevated levels of uPAR, thereby increasing the activation of the uPA system.11 LR11 leads to uPAR immobilization in the plasma membrane because both the membrane-spanning and the secreted soluble forms of LR11 bind to and colocalize with uPAR on the cell surface.12 LR11 is highly expressed in the plaque area of apo E knockout mice, particularly in the intimal SMCs at the border between intima and media. These results suggested that SMC migration mediated by the interaction among LRs, eg, LR11 and LRP1, constitutes an important factor in the process of atherosclerosis.
We have now revealed that a recently described LR, termed LR11B, which has been cloned as a tumor suppressor gene, is highly expressed in SMCs. In cultured SMCs, LR11B gene expression increases in the late proliferating phase, later than the expression of LR11. Neutralization of LR11B function delays the catabolism and thus enhances the localization of uPAR in the cell membrane, concomitant with enhanced migration and invasion of SMCs. Furthermore, inhibition of LR11B increases the expression level of platelet-derived growth factor receptor (PDGFR) in the cell membrane, concomitant with enhanced migration and invasion of SMCs. This novel modification of uPAR and PDGFR expression by LR11B possibly contributes to the regulation of migration and proliferation of SMCs from the media into the intima during atherogenesis.

**Methods**

**Antibodies and Cells**

A polyclonal antibody against human LR11B, anti-LR11B IgY, was produced by immunizing chickens with the synthetic peptide ALNIDKTKGVL1 (residues 4152 to 4163 of human LR11B) and purified as described previously. A monoclonal antibody against human LR11, 5 to 4-30 to 19-2, was described previously. Mouse monoclonal antibodies against human very LDL (VLDL) receptor (VLDLR, sc-18824), LDL receptor (LDLR, sc-18823), and LRP1 (sc-19616) were obtained from Santa Cruz Biotechnology. Goat and rabbit polyclonal antibodies against human uPAR (AF807) and PDGFRβ (sc-432) were from R&D Systems and Santa Cruz Biotechnology, respectively. Polyclonal antibodies against total and phosphorylated ERK were from New England Biolabs. Recombinant human uPAR and PDGF-BB were from R&D Systems (Minneapolis, Minn), RAP and apoE were from Molecular Innovations (Southfield, Mich) and Cosmo Bio (Tokyo, Japan), respectively. Monoclonal antibodies against human nonmuscle myosin heavy chain (SMemb) and SM1 were from Yamasa (Chiba, Japan). Primary cultures of human SMCs were from Cambrex Bio Science (Baltimore, Md). Three different lines of SMCs were used with similar results. For cell proliferation assays, 1 x 10⁴ cells seeded in T-25 flasks were incubated for 48 hours in DMEM in the absence of fetal bovine serum (FBS). Cells were counted at 3, 6, and 9 days after using 10% FBS. Cell counts were performed on triplicate wells using a Coulter counter (Coulter Electronics).

**Cloning Strategy and Northern Blot Analysis**

For cloning of LRs expressed in SMCs, we first constructed a ag10 cDNA library using mRNA isolated from human cultured confluent SMCs, and then screened the cDNA library with JH-1, a cDNA clone for human LRP1. Preparation of poly(A)-RNA and Northern blot analysis were performed as described previously. For preparation of hybridization probes, reverse-transcription polymerase chain reaction was performed with sense and antisense primers as follows: 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' and 5'-TATGTGGTGAACCT-3' for LR11B; 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' and 5'-TATGTGGTGGAACCT-3' for LR11; and 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' and 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' for LR11B. For cloning of LRs expressed in SMCs, we first constructed a ag10 cDNA library using mRNA isolated from human cultured confluent SMCs, and then screened the cDNA library with JH-1, a cDNA clone for human LRP1. Preparation of poly(A)-RNA and Northern blot analysis were performed as described previously. For preparation of hybridization probes, reverse-transcription polymerase chain reaction was performed with sense and antisense primers as follows: 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' and 5'-TATGTGGTGAACCT-3' for LR11B; 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' and 5'-TATGTGGTGGAACCT-3' for LR11; and 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' and 5'-GGAGAAGTCGAAATGCTGTGCCAGTT-3' for LR11B.

**Immunoblotting and Immunoprecipitation**

Immunoblot analysis was performed as described previously using anti-LR11B IgY (10 µg/mL), anti-LDLR antibody (1:100), anti-VLDLR antibody (1:200), anti-LRP1 antibody (1:200), anti-uPAR antibody (1:20), anti-PDGFRβ (1:200), anti-ERK antibody (1:500), or anti-phosphorylated ERK antibody (1:500). For analysis of the effects of incubation of RAP or anti-LR11B antibody on the expression of uPAR and PDGFRβ, cells were incubated with RAP (5 µg/mL) or anti-LR11B IgY at indicated concentrations at 37°C for 4 hours before the membrane preparation. For analysis of the effects of incubation of anti-LR11B antibody on the expression of total and phosphorylated ERK, cells were incubated with anti-LR11B IgY (1:100) in the presence or absence of PDGF-BB at 5 µg/mL at 37°C for 6 hours. For immunoprecipitation, 100 µg of membrane protein was mixed with 1 µg recombinant human uPAR at 4°C for 3 hours in the presence or absence of apo E (1, 5, or 50 µg), RAP (5 µg), or secreted soluble LR11. The secreted soluble LR11 was collected from KT38 cells and concentrated 20-fold using Centricon-100 (Millipore), as described. Then, 3 µg of anti-human uPAR goat IgG was added and rotated overnight at 4°C. The LR11B-uPAR antibody complex was precipitated by protein A Sepharose. The proteins were released into 25 µL SDS sample buffer by heating to 95°C for 10 minutes.

**Immunohistochemistry**

Autopsy specimens of human coronary arteries were obtained from 68-year-old (Figure 1A through 1F and 1K through 1N) and 73-year-old (Figure 1G through 1J) men who had died of acute coronary artery disease. Paraffin-embedded sections (10 µm) of specimens were stained with antibodies (blue) against SM1 (I) and SMemb (J), respectively. Magnifications: (A to C, E, F, K to N), ×40; (D, G, H), ×80; (I, J, O to R), ×160.

**Figure 1. Immunohistochemical analysis of LR11B in human coronary arteries.** Paraffin-embedded sections (10 µm) of specimens of atherosclerotic coronary artery (A to J), sections of thinned region with coronary stent (K to N), and sections produced by atherectomy (O to R) were subjected to immunohistochemistry. Sections were stained with an antibody against LR11B (A to D, G, K, and O), LR11 (H, L, and P), α-actin (E, M, and Q), SMemb (N and R), or macrophage (F) after hematoxylin staining. Sections stained with LR11B (brown) were double-stained with antibodies (blue) against SM1 (I) and SMemb (J), respectively. Data are representative of 5 sectioned specimens, respectively. Magnifications: (A to C, E, F, K to N), ×40; (D, G, H), ×80; (I, J, O to R), ×160.
myocardial infarction. Atherectomy specimens were from a target region considered responsible for unstable angina pectoris of a 62-year-old man (Figure 1O through 1R) who gave his informed consent for this study. These specimens were obtained in accordance with guidelines of the institution. Serial paraffin-embedded sections (10 μm) of specimens were used for immunohistostaining as described. Slides were stained in the presence of 0.1% bovine serum albumin with anti-LRP1B IgY (1:2), 5 to 4+30 to 19:2 (1:50), anti-smooth muscle α-actin antibody, anti-SMemb antibody, anti-SM1 antibody, or RAM11 at 23°C for 1 hour. Vectastain ABC-AP kit and BCIP/NBT alkaline phosphatase substrate kit IV (Vector Laboratories) were used with biotin-conjugated anti-mouse IgG (H+L) goat IgG (Wako) according to the manufacturer’s instructions for the double-immunostaining using 2 different antibodies.

Migration and Invasion

Cell migration and invasion were measured as described in a 96-well micro-Boyden chamber (its surface was coated with type I collagen) and Transwell (Corning Incorporated) 24-well plates coated with collagen gel, respectively. The lower or outer chamber contained 1% FBS-DMEM with or without 5 ng/ml PDGF-BB. The cells were incubated for 4 hours or for the indicated times at 37°C in the presence or absence of anti-LRP1B IgY at 1:100 dilution.

Binding, Internalization, and Metabolic Labeling Assays

Human recombinant uPAR and RAP were iodinated using chloramine T (specific activities, 6000 to 8000 cpm/ng) as described. For the cell surface assay, the cell monolayers were prechilled on ice for 1 hour, washed with binding buffer twice, followed by incubation with [125I]-labeled ligands in the presence or absence of anti-LRP1B IgY (1:10) or 5 μg/mL RAP. The cells were incubated for 1 hour at 4°C (binding) or for 30 minutes at 37°C (internalization). To measure internalized ligand, the cells were washed twice with binding buffer and then incubated with 50 mmol/L glycine, 150 mmol/L NaCl (pH 3.0) at 4°C for 15 minutes to dissociate cell surface-bound ligands after incubation for 30 minutes at 37°C. The cells were dissolved by adding 0.1 N NaOH for 1 hour, and the extract was counted. Metabolic labeling of uPAR was performed as described. Cells were cultured in methionine-free DMEM for 12 hours, followed by incubation in the same medium containing [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 mM uPA-PAI-1 complex for 6 hours in the presence or absence of anti-LRP1B IgY at 1:10 dilution or 5 μg/mL RAP. The membrane extracts were prepared from incubated cells and immunoprecipitated using anti-LRP1B IgY for electrophoresis as described.

Statistics

The results are shown as mean±SD for each index. Comparison of data were performed using the Student t test; a value of P<0.05 was considered significant.

Results

Expression of the Human LRP1B Gene in SMCs

Intensive sequence analysis of identified clones from our human SMC cDNA library using the fragment encoding the binding domain of LR11 as a screening probe at nonstringent conditions showed that LRP1B (4 of 16 clones) was an abundant LR, as were LRP1 (3 of 16), LR11 (3 of 16), and LDLR (1 of 16 clones); 5 of the 16 clones were not identifiable. To identify and characterize the human LRP1B protein, we performed immunoblot analysis with a polyclonal chicken antibody raised against an oligopeptide derived from the LRP1B sequence. The antibody, anti-LRP1B IgY, reacted with ~105-kDa protein (corresponding to the small LRP1B subunit generated by furin processing) in extracts of brain, muscle, and heart (not shown, see Figure 2A).

We analyzed LRP1B expression in atherosclerotic lesions of human coronary arteries by immunohistochemistry (Figure 1). The receptor was abundantly expressed in the medial layer of coronary arteries and, to a lesser extent, in thickened intima (Figure 1A through 1D). The protein was predominantly localized in SMCs, and some medial SMCs showed high levels of immunoreactivity. As shown in Figure 2G and 2H, the immunostaining using neighboring slices shows the LRP1B expression was not almost overlapped with LR11, which predominates in intimal SMC. Double-staining with different isoforms of myosin heavy chain, SMemb and SM1, indicated that LRP1B in the thickened intima is localized in cells expressing SM1 but not SMemb (Figure 11
and 1J). Furthermore, sections from severe thickened regions with a coronary stent strut showed LRP1B expression in intimal SMCs (Figure 1K). The cells displaying LRP1B did not overlap with those expressing LR11 (Figure 1L). The SMCs staining for SMemb overlapped with those containing LR11, but not with LRP1B-positive cells (Figure 1N). Finally, we analyzed the immunoreactive protein using sections of specimens produced by atherectomy (Figure 1O through 1R). Again, the cells expressing LRP1B were not those that expressed LR11 or SMemb. Thus, LRP1B is expressed in SMCs of the medial layer and in thickened intimal regions.

Regulation of LRP1B Protein Expression in Proliferating SMCs

The expression of LRP1B in SMCs of the thickened intima of human coronaries suggests that LRP1B expression is induced at certain stages of proliferation and/or migration. We therefore studied the protein’s expression in cultured SMCs from human arteries. The antibody, anti-LRP1B IgY, reacted with a 105-kDa protein, which is clearly different from other LRs, such as LDLR and VLDLR, in SMCs (Figure 2A). We then analyzed LRP1B levels by immunoblotting in the course of proliferation in SMCs (Figure 2B). At the beginning of proliferation, the signal intensity was rather low; subsequently, the intensity increased and reached to 2.4-fold of control at 6 days after serum addition (note that LR11 transcript increased to 3.4-fold at 3 days, and then decreased to 2.6-fold of control at 6 days after serum addition, as previously reported). In contrast, the LRP1 transcript level did not significantly change in the proliferative phase. The intensity finally decreased to 1.6-fold of control at 9 days after serum addition, when the cell density reached confluence. These results indicate that LRP1B protein levels are regulated during the course of proliferation in SMCs. Furthermore, we performed Northern blotting to analyze the changes of mRNA levels in detail (Figure 2C). The LRP1B transcript was increased 4.5-fold at 6 days after serum addition. These results show that LRP1B expression is increased in the late phase of proliferation of SMCs, and that the regulation of its expression in these cells is different from that of other LRs.

Molecular Interaction of LRP1B and uPAR

We have recently reported that uPAR expression is important for the migration of SMCs and that uPAR levels are regulated by LR11. Based on the structural similarity of LR11 and LRP1B, we therefore analyzed the binding of uPAR to and its internalization via LRP1B, which is expressed in SMCs. As shown in Figure 3A, SMCs at 6 days after serum addition bind and endocytose 125I-uPAR in dose-dependent and saturable fashion. Specific binding of 125I-uPAR was reduced to 49% and 17%, and internalization to 62% and 26%, in the presence of anti-LRP1B IgY and RAP, respectively (Figure 3B). These results indicate that LRP1B in SMCs is indeed able to bind and internalize uPAR. Furthermore, they suggest that LRP1B can contribute 50% to the internalization of uPAR, as indicated by comparing the levels of inhibition achieved by using the specific anti-LRP1B antibody and RAP, which inhibits other LRs in addition to LRP1B.

To determine whether LRP1B binds directly to uPAR in the cell membrane, we performed coimmunoprecipitation experiments. When membrane extracts from SMCs were incubated with recombinant uPAR and the indicated concentrations of labeled 125I-uPAR (specific activity, 380 cpmpg) for 3 hours at 4°C (left) or 37°C (right). Data are the average of triplicate determinations and represent the difference between activities in the presence or absence of 1 mmol/L of unlabelled uPAR. B, Specific binding (left) and internalization (right) of uPAR at a concentration of 150 nM 125I-uPAR was determined in the absence or presence of anti-LRP1B IgY at 1:10 dilution or 5 µg/mL RAP, respectively; n=4 in all cases; P<0.05, **P<0.01. C, Membrane extracts of SMCs (100 µg) were incubated with 1 µg/mL uPAR in the presence or absence of LR11, apoE, or RAP, immunoprecipitated with anti-uPAR antibody, and subjected to Western blot analysis with anti-LRP1B and apoE. Relative amounts of the signals were determined by densitometric scanning; n=3 in all cases; *P<0.05, **P<0.01 versus control.
was inhibited by the addition of apoE or LR11 in dose-dependent fashion. Furthermore, RAP efficiently inhibited the complex formation, also. Thus, LR1P1 clearly has the capacity to bind and form complexes with uPAR.

**Inhibition of LR1P1-Mediated Catabolism of uPAR Increases Its Cell Surface Expression**

The specific binding, internalization, and complex formation with uPAR suggest that LR1P1 may regulate the level of uPAR on the cell surface. To evaluate this possibility, we studied the effect of LR1P1 on the catabolism of uPAR in SMCs. The catabolism of uPAR protein was studied after incubation of biosynthetically 35S-methionine prelabeled cells with uPA-PAI-I complex, followed by immunoprecipitation of the 35S-labeled uPAR (Figure 4A). During a 6-hour incubation, the amount of immunoprecipitatable 35S-uPAR decreased by 37%. However, in the presence of anti-LR1P1 IgY and RAP, the levels decreased only by 16% and 8%, respectively, i.e., significantly less than in the absence of these inhibitors of receptor function. Figure 4B shows the immunoblot analysis of uPAR in the membrane fraction of SMCs after the incubation with anti-LR1P1 IgY or RAP for 24 hours. The expression levels of uPAR increased 2.0- and 1.6-fold compared with control with antibody and RAP, respectively. Thus, approximately one-half of the increase in uPAR protein in the membrane fraction observed in Figure 4B is caused by the inhibition of LR1P1.

**LR1P1 Regulates the Expression and Signaling Activity of PDGFRβ**

The mechanism of PDGF-BB–directed SMC migration includes PDGFRβ-mediated signal transduction through mitogen-activated protein kinase. LR1P1 has recently been shown to form a complex with the PDGFRβ and to control the receptor’s activation.20 This finding prompted us to investigate the role of LR1P1 in the activation of PDGFRβ in SMCs. As shown in Figure 5A, membrane extract from SMCs previously incubated with increasing concentrations of anti-LR1P1 IgY showed a dose-dependent increase in PDGFRβ. The cells incubated with the antibody also showed a significant increase in the levels of phosphorylated ERK1/2 compared with cells kept in the absence of antibody (Figure 5B). Thus, blocking the function of LR1P1 induces PDGFRβ numbers and activity, thereby likely contributing to the increased SMC migration, together with the activation of uPA/uPAR system.

**Functional Inhibition of LR1P1 Induces Migration of SMCs**

Finally, we studied whether LR1P1-mediated uPAR modulation and PDGFRβ modulation is accompanied by the regulation of migration activity of SMCs. In the presence of 1% FBS, PDGF-BB–dose-dependently increased the migration activity of SMCs (Figure 6A). Presence of anti-LR1P1 IgY further increased the migration of SMCs at each concentration of PDGF-BB significantly. Also, as shown in Figure 6B, PDGF-BB–stimulated invasion through a collagen barrier of cells in the presence of anti-LR1P1 antibody was greater than in its absence. These data, obtained with 2 different methods for measuring parameters of cell mobility, indicate that the expression of functional LR1P1 on the cell surface is involved in attenuating the migration of SMCs in vitro.

**Discussion**

We have identified and characterized the product of a gene encoding a member of the LDL receptor family expressed in...
catabolism is one of the determinants for migration of SMCs from the media into the intima in the process of atherosclerosis. Analysis of the binding of uPAR to LR1B showed that 50% of the surface depletion of uPAR was mediated by LR1B at the late exponential growth phase of cultured SMCs. Metabolic analysis showed that uPAR catabolism was clearly delayed when LR11 function was blocked by RAP or a specific anti-LRP1B antibody. The complex formation of LR1B with uPAR was inhibited in the presence of LR11 and other known ligands, such as apoE and RAP, respectively. The inhibitory action of LR11 suggests that LR11 competes with LR1B and LR1 for uPAR binding; therefore, interaction of these LR with each other and with uPAR likely regulates the catabolism of uPAR during proliferation of SMCs.

Finally, inhibition of LR1B appeared to induce the expression of PDGFβ on the cell surface, resulting in the enhanced phosphorylation of ERK in the presence of PDGF. LR1B is suggested to form a complex with the PDGFβ and to control the receptor’s activation, as is LR1, in addition to uPAR. Thus, in summary, the proliferation-dependent expression of LR1B may play a role in modification of the migration activity of SMCs through the modification of PDGF and uPA signals. Studies to analyze proliferation-responsive elements in the regulatory region of the LR1B gene and detailed expression patterns using various cells are now underway.

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References


Figure 6. Migration and invasion activities of SMC. Cells were preincubated with anti-LRP1B IgY at 1:100 dilution for 4 hours. Migration (A) and invasion (B) activities were measured in the presence or absence of PDGF-BB at the indicated concentrations; n=12; *P<0.05, **P<0.01.

proliferating SMCs. This protein, LR1B, shows striking similarities to the well-known LR1.13,14 Recently, we have demonstrated that another LR, LR11, is markedly induced during intimal thickening and causes increased migration activities of SMCs.11,12,18 In addition, the expression of LR1B, the closest LR1B relative known, is induced in atheroma.21,22 Embryonic fibroblasts genetically deficient in LR1B show accelerated migration.9 These observations on LRs in cellular proliferation and migration, as well as the identification of LR1B as a late-stage tumor suppressor gene by Liu et al,13 suggest that the physiological function of LR1B might also be related to the modulation of cellular migration.

In specimens of coronary arteries, LR1B is clearly observed in intimal SMCs as well as medial SMCs; the LR1B-positive cells are different from those expressing LR11, which has been shown to predominate in SMCs12,18,19 and to control the receptor’s localization of LR1B suggests that it performs its task in various cells are now underway.


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