A Secreted Soluble Form of LR11, Specifically Expressed in Intimal Smooth Muscle Cells, Accelerates Formation of Lipid-Laden Macrophages

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Objective—Macrophages play a key role in lipid-rich unstable plaque formation and interact with intimal smooth muscle cells (SMCs) in early and progressive stages of atherosclerosis. LR11 (also called sorLA), a member of low-density lipoprotein receptor family, is highly and specifically expressed in intimal SMCs, and causes urokinase-type plasminogen activator receptor-mediated degradation of extracellular matrices. Here we investigated whether the secreted soluble form of LR11 (solLR11) enhances adhesion, migration, and lipid accumulation in macrophages using animal models and cultured systems.

Methods and Results—Immunohistochemistry showed solLR11 expression in thickened intima of balloon-denuded rat artery. Macrophage infiltration into the cuff-injured artery was markedly reduced in LR11-deficient mice. In vitro functional assays using THP-1-derived macrophages showed that solLR11 (1 μg/mL) significantly increased acetylated low-density lipoprotein uptake by THP-1 cells and cell surface levels of scavenger receptor SR-A 1.7- and 2.8-fold, respectively. SolLR11 dose-dependently increased the migration activity of THP-1 macrophages and adhesion to extracellular matrices 2.0- and 2.1-fold, respectively, at 1 μg/mL. These effects of solLR11 were almost completely inhibited by a neutralizing anti-urokinase–type plasminogen activator receptor antibody.

Conclusion—SolLR11, secreted from intimal SMCs, regulates adhesion, migration, and lipid accumulation in macrophages through activation of urokinase-type plasminogen activator receptor. The formation of lipid-laden macrophages in atherosclerotic plaques possibly is regulated by SolLR11 of intimal SMCs. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: atherosclerosis ■ foam cells ■ macrophages ■ scavenger receptors ■ smooth muscle cells

The early recruitment of monocytes to the arterial neointima, their subsequent differentiation to macrophages, and lipid accumulation are key events in the pathogenesis of atherosclerosis.1-2 Coincidentally, smooth muscle cells (SMCs) migrate and accumulate in the developing neointimal lesion, where intimal SMCs secrete extracellular matrices, such as elastin, collagen and proteoglycans, inflammatory cytokines, and several proteases.3-4 Recent functional studies using genetically modified animals or cells have revealed that certain receptors belonging to the family of low-density lipoprotein (LDL) receptor relatives (LRs) are important regulators of migration, proliferation, and secretory functions of SMCs.5-10 We have demonstrated that LR11 is abundantly and specifically expressed in intimal SMCs during intimal thickening in a variety of experimental models of atherogenesis, and that its expression is elevated in early stages of neointimal formation.11-13 LR11 enhances the migration of SMCs by increasing cell-surface urokinase-type plasminogen activator (uPA) receptor (uPAR) levels. LR11 is secreted in soluble form from isolated cultured SMCs, especially in the logarithmic growth phase, and tumor necrosis factor-α converting enzyme is responsible for the shedding of the large ectodomain of LR11.14-15 This secreted soluble form of LR11 has biological activity toward SMC migration, different from that of the membrane-bound form.11,16 This finding strongly suggested a solLR11-mediated interaction of intimal SMC and other players, particularly macrophages, in the intima. However, the role of intimal SMCs in the process of lipid accumulation in macrophages has not been well characterized.

The uPAR on monocytes/macrophages is implicated in the pathological infiltration of monocytes into the intima and in the process of foam cell formation.17-18 Cell-surface expression of uPAR is significantly elevated in monocytes of subjects with acute myocardial infarction and contributes to enhanced cell adhesion in vitro.17 In apoE deficient mice, overex-
pression of human uPAR in macrophages enhances cell adhesion to the aortic wall, and targeted overexpression of uPA, a ligand of uPAR, in macrophages accelerates atherosclerosis with increased foam cell formation.

Thus, soLR11 might be expected to modify the macrophage foam cell formation through the activation of uPAR-mediated extracellular matrix degradation. Here we demonstrate the presence of soLR11 in hyperplastic intima, and show that soLR11 deficiency drastically reduces the infiltration of lipid-laden macrophages into the intima of LR11 mice on a high-fat diet using a cuff-injury model. Cell culture experiments showed that recombinant soLR11 increases the migration and adhesion of macrophages to extracellular matrix and SMCs through enhanced expression of adhesion molecules, as well as lipid accumulation through scavenger receptors. These results support a novel function of intimal SMCs in the regulation of macrophage-foam cell formation in the process of atherosclerosis.

Materials and Methods

Antibodies and Cells

Preparation and properties of the monoclonal and polyclonal antibodies against human and mouse LR11, 5–4–30–19–2 and pm11, respectively, were described previously. Monoclonal antibodies against SR-A (KT022) was obtained from Wako (Tokyo, Japan). Polyclonal or monoclonal antibodies against uPAR (AF807), VLA-4 (BBA37) and PSGL-1 (MAB996) and recombinant PDGF-BB (520-BB) were from R&D systems (Minneapolis, Minn). Monoclonal (BBA37) and PSGL-1 (MAB996) and recombinant PDGF-BB (520-BB) were from R&D systems (Minneapolis, Minn). Monoclonal antibody against Mac-3 was from BD Pharmingen (San Diego, CA). Monoclonal antibodies against uPAR (AF807), VLA-4 (BBA37) and PSGL-1 (MAB996) and recombinant PDGF-BB (520-BB) were from R&D systems (Minneapolis, Minn). Monoclonal antibody against Mac-3 was from BD Pharmingen (San Diego, Calif). Primary cultures of SMCs were prepared from the isolated medial layer of rat aorta as described. COS7 cells were from ATCC (CRL-1651; Manassas, Va). THP-1 cells were obtained from ATCC (TIB-202) and maintained in RPMI 1640 containing 10% fetal bovine serum. THP-1 macrophages were differentiated to macrophages by treatment with 200 nM of phorbol 12-myristate 13-acetate (PMA; Promega, Madison, Wis) for 24 hours at 37°C in the presence or absence of purified soLR11 at 1 µg/mL (unless indicated otherwise) and/or of the indicated antibodies.

Animal Experiments

All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Ethics Board. Male Wistar rats (Charles River Laboratories, Japan), weighing 400 to 450 grams, were anesthetized, and the left common carotid artery was denuded by balloononing as described. The left carotid arteries were isolated at 7 or 14 days after injury and used for histochemical and Western blotting. Germline-transmitted chimeras obtained were crossbred with C57BL6/J females, and resulting heterozygous offspring were born in Mendelian ratios. All mice born were maintained under standard animal house conditions with a 12-hour light/dark cycle and were fed ad libitum with regular chow diet.

Immunohistochemistry and Western Blot

Serial paraffin-embedded sections (5 µm) were used for immuno- and immunohistostaining as described. Briefly, sections were pretreated with 3% H2O2 to inactivate endogenous peroxidase. Slides were then stained with anti-LR11 (pm11, 1:50) or anti-Mac3 (1:25) for 1 hour at 25°C in the presence of 0.1% bovine serum albumin. Vectastain ABC-AP kit (Vector Laboratories) was used with biotin-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Wako) according to the manufacturer’s instructions. Slides were counterstained with hematoxylin-eosin and elastica von Gieson. Western blot analysis was performed as described previously using anti-LR11 (pm11, 1:500), anti-MLR11 (pm11, 1:250), anti-SR-A (1:250) and anti-uPAR (1:250).

Construction, Expression, and Purification of soLR11

Materials and Methods for this study are fully described in the online data supplement section (please see http://atvb.ahajournals.org). Briefly, we first constructed an expression plasmid for the soluble form of LR11 lacking 104 C-terminal amino acids containing the transmembrane region. COS7 cells were transfected with the expression construct and soLR11 was purified using Ni2+-chelating chromatography. The biological activity of purified soLR11 was confirmed by a SMC migration assay.

Adhesion and Migration

Cell adhesion was determined in 96-well plates as described. Wells were coated with 5 µg/mL collagen or fibronectin for 2 hours at 37°C. THP-1 macrophages were fluorescently labeled by loading with Cellcei-AM dye for 1 hour at 5 × 105 cells/mL in RPM1 containing 1% fetal bovine serum. Cellcei-loaded cells were then added to the extracellular matrix coated plates at 2.5 × 104 cells/well, and incubated for 30 minutes at 37°C. Nonadherent cells were removed by gently washing with phosphate-buffered saline, and adherent cells were analyzed by measuring fluorescence using a fluorescence microplate reader, SPECTRAMax GEMINI XS (Molecular Devices, Menlo Park, Calif). Cell migration was measured in a 96-well micro-Boyden chamber with collagen type I-coated filters as described. The lower chamber contained RPMI 1640 with 5 ng/mL PDGF-BB, and THP-1 macrophages were added to the upper chamber and incubated for 4 hours at 37°C. Migrated cells were quantitated using a fluorescence microplate reader.

Acetyl-LDL Uptake

THP-1 macrophages were seeded on 96-well culture plates and incubated with the indicated concentrations of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (DiL-AcLDL) for 4 hours at 37°C. Then, unincorporated DiL-AcLDL was removed by washing with phosphate-buffered saline. DiL-AcLDL uptake was measured using a fluorescence microplate reader.

Statistics

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

Results

LR11, Expressed in Intimal SMCs, Is Secreted as a Soluble Form in the Intima of Balloon-Denuded Artery

A soluble form of LR11 is secreted from cultured SMCs and induces the migration activity of SMCs together with the membrane-anchored form. To investigate the pathophysio-
logical relevance of solLR11 in the process of neointimal formation, the expression of soluble and membrane-anchored LR11 proteins were analyzed in the rat balloon injury model. Immunohistochemistry and Western blot showed that LR11 is highly and specifically expressed in intimal SMCs, and that its expression is higher at day 7 after injury than at day 14 (Figure 1A). This is in agreement with the finding that LR11 is specifically expressed in the proliferating phase of SMCs in culture. Using the samples of thickened intima obtained at day 14, secreted solLR11 with reduced molecular size compared with that of membrane-bound LR11, was detected in intimal homogenates, as expected from the results in cultured SMCs (Figure 1B).

Macrophage Infiltration and Lipid Accumulation in Intima of Cuff-Injured Artery Is Inhibited in LR11 Knockout Mice

Blocking LR11’s function by neutralizing antibody significantly reduced neointimal thickening in cuff-injured femoral artery in mice. We have recently established LR11 knockout mice, in which the coronary arterial structure appears histopathologically normal (Jiang et al, submitted). To clarify the role of solLR11 in neointimal formation, we applied cuff injury in femoral artery in the LR11−/− mice on a high-fat diet. Infiltration of Mac3-positive macrophages and lipid accumulation in macrophages were detected at 7 days after cuff placement, and elastin-rich neointimal thickening was observed at day 28 in wild-type mice on a high-fat diet (Figure 2). The intimal thickness at day 28 after cuff injury was significantly reduced in the LR11−/− mice compared with the mice on normal chow diet (Jiang et al, submitted). Surprisingly, infiltration of Mac3-positive and lipid-laden macrophages was significantly decreased in the SMC-rich early neointima. These data suggest that LR11 is involved in lipid accumulation and macrophage infiltration into the intima at an early stage of injury-induced neointimal formation.

Expression, Purification, and Biological Activity of Recombinant SolLR11

To investigate the mechanism of decrease in intimal lipid-laden macrophages after cuff injury, we analyzed the effect of solLR11 on macrophages using the established cell line, THP-1. Recombinant solLR11 was expressed using a COS7 expression system and purified by single step Ni2+-chelating chromatography (supplemental Figure I, available online at http://atvb.ahajournals.org). The addition of purified recombinant solLR11 at 1 to 100 μg/mL strongly increased the PDGF-induced migration activity of SMCs when compared with SMCs transfected with vector alone or vector containing full-length LR11 (supplemental Figure I). The enhancement of SMC’s migrating activities by LR11s were completely blocked by anti-LR11 antibody.
SoLR11 Increases Scavenger Receptor Expression and Lipid Accumulation in THP-1 Macrophages

Because LR11KO mice showed reduced lipid-containing macrophages (Figure 2), we next investigated the effect of soLR11 on the regulation of scavenger receptor expression and lipid accumulation of THP-1 macrophages. THP-1 macrophages were cultured for 24 hours in the presence or absence of PMA and/or soLR11 at 1 μg/mL, followed by Western blot of plasma membrane preparations probed with anti-SR-A and anti-uPAR antibodies. Although soLR11 did not induce SR-A protein expression in the absence of PMA, it increased SR-A expression 2.8-fold in its presence (Figure 3A). The cell-surface level of uPAR was increased by soLR11, likely because of the soLR11-mediated stabilization of uPAR. To test whether soLR11 affects lipid accumulation in macrophages, we evaluated Dil-AcLDL uptake in THP-1 macrophages (Figure 3B). In the undifferentiated THP-1 cells, there was no significant Dil-AcLDL uptake, and soLR11 did not affect Dil-AcLDL uptake (data not shown). However, in THP-1 macrophages, soLR11 at 1 to 100 μg/mL significantly increased Dil-AcLDL uptake (Figure 3C). Addition of neutralizing anti-LR11 or anti-uPAR antibodies almost totally inhibited the increase in Dil-AcLDL uptake by the cells (Figure 3D). These data indicate that soLR11 stimulates lipid uptake via SR-A, and that the accelerated lipid accumulation in macrophages may be attributable to the LR11-mediated upregulation of uPAR levels.

Recombinant SoLR11 Increases Adhesion and Migration of THP-1-Derived Macrophages

We next investigated the effect of soLR11 on the adhesion of THP-1-derived macrophages (THP-1 macrophages) in vitro using the recombinant protein. THP-1 cells were differentiated to macrophages by the treatment with 200 nM PMA for 24 hours, and then the cells were labeled with fluorescent dye Calcein-AM for quantitative analysis by the in vitro adhesion assay. SoLR11 at 1 μg/mL significantly increased the adhesion of THP-1 macrophages to collagen and fibronectin (Figure 4A) 1.8- and 2.1-fold, respectively. The neutralizing anti-LR11 antibody completely blocked soLR11-induced increase in adhesion. Next, we tested the effect of soLR11 on the adhesion of macrophages to SMCs, because of the drastic decrease in macrophage recruitment in intima of cuff-injured artery in LR11−/− mice, principally caused by proliferating SMCs. Pretreatment of THP-1 macrophages with 1 μg/mL soLR11 increased cell adhesion to cultured SMCs 1.6-fold (Figure 4B). The addition of neutralizing antibodies against VLA-4 and PSGL-1 completely inhibited the increased adhesion by soLR11, as observed with anti-LR11 or anti-uPAR antibodies. Thus, we analyzed the effect of soLR11 on the expression of adhesion molecules. SoLR11 enhanced the expression of cell-surface VLA-4 in the presence and absence of PMA (Figure 4C).

We next tested the effect of soLR11 on the migratory functions of THP-1 macrophages by using the Boyden chamber method. SoLR11 itself did not affect migration of THP-1 macrophages in vitro (data not shown). When cells were preincubated with 1 μg/mL soLR11 for 12 hours, PDGF-BB-induced migration of THP-1 macrophage was 2.0-fold greater than in the absence of soLR11 (Figure 4D). The stimulatory effect of soLR11 was decreased by addition of neutralizing anti-LR11 or anti-uPAR antibodies. These data indicate that soLR11 induces adhesion and migration.
activities of macrophages through uPAR-mediated pathways, possibly through increasing the levels of cell-surface adhesion molecules.

**Discussion**

In this study, we have shown that LR11 is secreted in a soluble form from intimal SMCs in a balloon injury model, and that LR11-deficient mice show drastically decreased lipid-accumulating macrophages in early intimal formation after cuff injury in mice on a high-fat diet. Functional analysis of recombinant solLR11 demonstrated that solLR11 can regulate the functions of THP-1 macrophages toward foam cell formation, such as lipid incorporation, adhesion, and migration. The inducing effect on foam cell formation of solLR11 was almost abolished by functional neutralization of solLR11 or of its target protein, uPAR. Based on these results, we propose a new role of intimal SMCs in the regulation of monocyte/macrophage functions involving the secretion of soluble LR11.

Although LR11 was originally identified as a type I transmembrane protein, significant amounts of LR11 are shed from cultured SMCs, IMR32 and BON cells, and hydra as a soluble form of the large extracellular domain cleaved off by metalloprotease. In CHO cells, it was demonstrated that tumor necrosis factor-α convertase is responsible for the proteolytic cleavage of LR11. However, the physiological function of solLR11 is still poorly understood because of the lack of availability of recombinant protein. We have reported that solLR11, secreted from cultured cells as well as the membrane-bound form, enhance SMC migration, and that the expression of solLR11 largely depends on the differentiation stage of SMCs. The medial contractile type does not express solLR11, whereas the intimal synthetic type does, consistent with the expression of embryonic myosin isoform SMemb. These data suggest that LR11-expressing cells likely perform diverse functions via secretion of soluble LR11 and/or expression of membrane-bound LR11, respectively.

We detected solLR11 protein by Western blot of thickened intima obtained 14 days after balloon injury (when neointimal formation is almost accomplished). Although the level of solLR11 expression was lower than that of the membrane-
bound form, solLR11’s expression at an earlier stage is likely higher than that at late stages, because solLR11 was specifically expressed in rapidly proliferating SMCs in culture.11 The macrophage infiltration into the intima and lipid accumulation was greatly decreased in LR11 knockout mice compared with those in wild-type mice (Figure 2). Because the expression of LR11 was barely detectable in monocytes/macrophages, we hypothesize that the soluble form of LR11 from intimal SMCs affects macrophage functions that facilitate progression of atherosclerosis, especially in early neo-intimal formation. With the preparation of recombinant solLR11, we were able to obtain experimental support for our above hypothesis concerning the role of solLR11 in macrophage function.

Macrophages express a variety of scavenger receptors which are involved in uptake of modified LDL and atherogenesis.2,26,27 SR-A is highly expressed almost exclusively in differentiated macrophages, and is implicated in increased foam cell formation in atherogenesis.28,29 We showed that solLR11 enhanced SR-A expression and Di-AcLDL accumulation in THP-1 macrophages in vitro, suggesting a possible role of solLR11 in the formation of lipid-rich plaques. Furthermore, solLR11 significantly enhanced monocyte adhesion not only to extracellular matrices but also to the cultured SMCs in vitro. Increased adhesion and infiltration of circulating monocytes is believed to be the key event in early stage of atherogenesis. Furthermore, the direct association between monocytes and SMCs is implicated in the prolonged retention of monocytes in atherosclerosis, and increases matrix metalloproteinase-1 production, possibly leading to the formation of unstable plaque.30 Monocyte adhesion to SMCs is mediated, eg, by vascular cell adhesion molecule-1, and immunohistochemical analysis showed the abundant expression of vascular cell adhesion molecule-1 in SMCs in human atherosclerotic lesions.31 PDGF-BB and angiotensin II are implicated in the enhanced binding of monocytes to cultured SMCs. Thus, solLR11 is probably involved in monocyte accumulation at activated areas in plaques at which SMCs actively migrate and proliferate, and prolongs on-site retention of macrophages.

SolLR11 increased cell-surface uPAR levels in THP-1 monocytes/macrophages. Moreover, solLR11-enhanced lipid uptake, adhesion, and migration of THP-1 macrophages were almost completely blocked by neutralizing anti-uPAR antibodies as well as anti-LR11 antibodies. The increased expression of uPAR on monocytes/macrophages is implicated in the adhesion, differentiation, and increased metalloproteinase expression in the cells. Moreover, uPAR expression is increased in circulating monocytes in patients with acute myocardial infarction compared with that in patients with chronic stable angina.17 LR11 upregulates cell surface uPAR levels in SMCs by inhibition of its catabolism, which is mediated by LRP1,
another member of the LDLR family. LR11 is also abundantly expressed in monocytes/macrophages; hence, it is likely that LR11 regulates macrophage differentiation and lipid accumulation in plaques by increasing uPAR levels in monocytes/macrophages.

In summary, SMCs and macrophages coexist in plaques throughout the progressive stage of atherogenesis. SolLR11, which is secreted from activated SMCs in the intima, likely is a coregulator of scavenger receptor expression, lipid accumulation, adhesion, and migration of monocytes/macrophages at an early stage of neointimal formation. The uPAR-mediated effects were observed at the same concentration range (0.1 to 10 μg/mL) of recombinant solLR11 in cultured macrophages as that required for the migration of SMCs (Figure 1D). Although the pathophysiological concentrations of solLR11 in intima is difficult to determine, the increase in levels of intimal solLR11 in injured arteries, and the loss of infiltrated macrophages in LR11-KO mice strongly suggest that intimal SMCs locally secrete sufficient amounts of solLR11. Nevertheless, the elucidation of the significance of interactions of SMCs and macrophages involving solLR11 requires further analyses using various models for atherosclerosis. Clearly, the regulation of solLR11 function in the arterial wall is a promising target not only for such studies but also for therapeutic amelioration of atherosclerosis with unstable plaque.

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Disclosures

None.

References

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Supplementary Method

For preparation of recombinant solLR11, we first constructed expression plasmid by amplifying rabbit LR11 cDNA by PCR using following primers: LR11U1 (5’-AAAAGAATTCCGCCACCATGGCGACACGGAGCAGCAGGAGGGAGT-3’) and LR11L6300 (5’-AAAATCTAGACTCCCCGCAGATCTGGCTGCCAAAGG-3’) designed to remove C-terminal 104 amino acids containing transmembrane region. An amplified product was subcloned into pcDNAmycHis (Invitrogen Corp., Carlsbad, CA) to add C-terminal myc and His6 tags confirmed by sequencing. SolLR11 cDNA including tags was then subcloned into pEFcDNA for expression study. COS7 cells were transfected with the expression construct using the Lipofectamine 2000 (Invitrogen Corp.) according to the manufacture’s instruction. Cells were harvested at 24 hours post transfection with the lysis buffer: 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5% glycerol, 12 mM imidazole, complete-EDTA free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Then the cells were homogenized with a polytron homogenizer, followed by a Potter homogenizer, and the homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was re-centrifuged at 100,000 x g for 1 hour. The resulting supernatant was applied to a HisTrap HP column (GE Healthcare, Piscataway, NJ), then the column was washed with 10X volume of buffer followed by elution with imidazole at 0-500 mM linear gradient. The elution fractions were collected and dialyzed against PBS and stored at -80°C until use.
Supplementary Result

We prepared solLR11 expression construct in which the region from membrane-spanning domain to C-terminus is replaced with short c-myc and His6 tags for purification and detection, and subcloned into mammalian expression vector (Supplemental Figure I-A). We could not express recombinant solLR11 by using baculovirus or yeast expression systems which are usually used for large scale recombinant protein expression. So far, transient expression in COS7 cells showed highest levels of solLR11 protein expression than that of other stably expressed clones among tested in mammalian expression systems. The solLR11 protein expression was increased by 2 ~ 4-fold by the addition of PMA into the transfection medium during 24 hours transfection. Although we have reported that solLR11 was secreted from cultured SMCs into culture medium, the recombinant solLR11 was not secreted into the culture medium from COS7 cells transfected solLR11 expression construct unlike native solLR11 (Supplemental Figure I-B). Inclusion bodies were not observed, and a majority of the solLR11 protein was able to be recovered in soluble cytosolic fraction by mechanical homogenization of cells. Crude cell extract was prepared and applied to Ni\(^{2+}\) chelating chromatography and solLR11 was purified by imidazole linear gradient elution (Supplemental Figure 1C).
Supplementary Figure I

A

Cytoplasmic domain  
TM  
TACE

C

Extracellular domain

LR11

SolLR11

Vps10/sortin receptor like domain
YWTD containing repeat
Ligand binding repeat
Fibronectin type III domain

Transmembrane domain
Cytoplasmic domain
Endocytosis signal
c-myc tag
His tag

B

1 2 3 4

220 kDa
Anti-LR11
Anti-c-myc

C

LR11
CBB
**Supplementary Figure I.** Expression and purification of recombinant solLR11. **A,** Schematic structure of solLR11. C-terminal 103 amino acids containing transmembrane domain and TACE cleavage site were removed and c-myc tag and His6 tags were attached at the C-terminus. **B,** COS7 cells were transfected with full-length LR11 or solLR11 expression construct, and whole cell lysate and culture medium extract were subjected to Western blot. Lane1, 2: COS7 whole cell lysate, lane 3, 4: COS7 culture medium extracts, lane 1, 3: solLR11/COS7, lane 2, 4: full length LR11/COS7. **C,** Cytosolic fraction of COS7 transfectant cells (500 cm² X 10 culture dishes) were applied to HisTrap HP column and eluted with imidazole linear gradient. Each fraction was subjected to SDS-PAGE followed by CBB staining and Western blot with anti-LR11 antibody. **D,** Dose dependent effect of solLR11 on the migration of cultured SMCs. SMCs were preincubated with indicated concentrations of solLR11. **E,** Enhanced
migration activity of SMCs by solLR11. SMCs were preincubated with 1 μg/mL solLR11 or transfected with full-length LR11 construct. Cell migration was measured with micro-Boyden chamber as described in Materials and Methods. Data are expressed as mean ± SD, n= 4 ~ 6. (*P<0.05, **P<0.01)