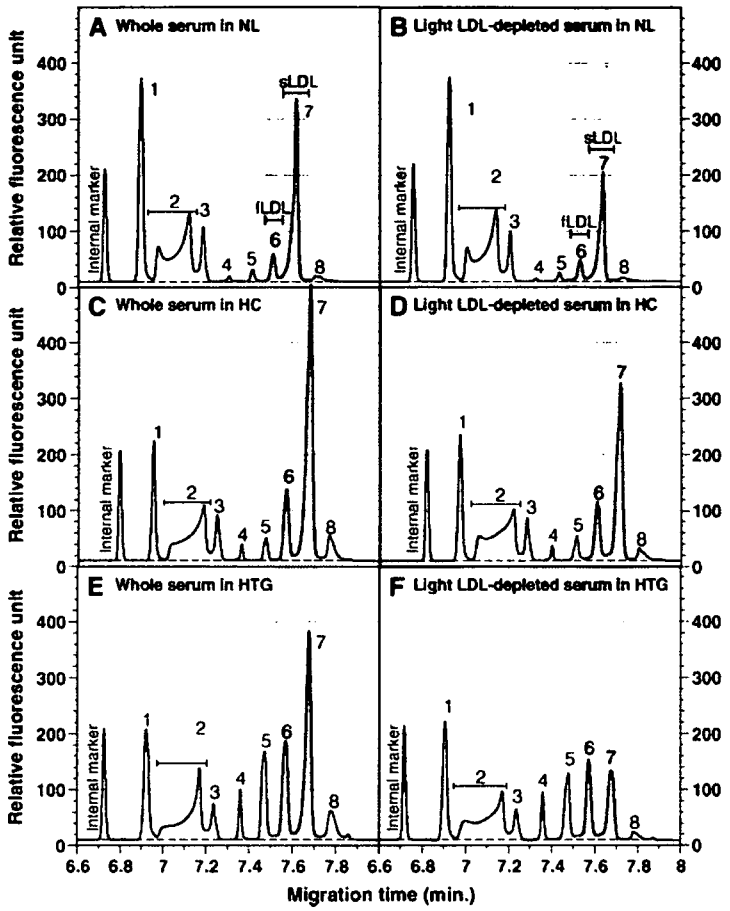

Fig. 5. Typical lipoprotein profiles as determined by cITP in whole serum (A, C, E) and serum-depleted of large, light LDLs (d = 1.019–1.044 g/ml) by heparin-Mg^{2+} precipitation (30) (B, D, F) from a normolipidemic (NL) subject (A, B), a hypercholesterolemic (HC) subject (C, D), and a hypertriglyceridemic (HTG) subject (E, F). Peaks 1–3, HDL subfractions; peak 4; chylomicron/remnant fraction; peak 5, VLDL/IDL; peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

dense LDL subclass were higher than those in the large, light LDL subclass (indicated by daggers) in all three groups (Fig. 6A, right panel), indicating that cITP fLDL is distributed mainly in the small, dense LDL subclass. However, cITP fLDL levels in both the dense and light LDL subclasses (Fig. 6A, right panel) were significantly higher (indicated by asterisks) in the HC and HTG groups than in the NL group. Similar results were obtained when cITP fLDL was expressed as a proportion of total cITP LDL (Fig. 6B). These results indicate that cITP fLDL, expressed as either a peak area relative to that of an internal marker or as a proportion of total LDL, in HC and HTG subjects was increased in both light and dense LDL subfractions, but more of the cITP fLDL was distributed in small, dense LDL subclasses.

Because the storage of samples is inevitable in most clinical studies, we evaluated the effects of short-term (**Fig. 7A**), intermediate-term (Fig. 7B), long-term (**Fig. 8A**), and very-long-term (Fig. 8B) storage of serum samples on cITP lipoprotein analysis. Fig. 7Aa shows the cITP lipoprotein profile in fresh serum from a volunteer subject (female, 62 years old). Small aliquots of fresh serum from the subject were protected with N_2 gas, snap-frozen with liquid nitrogen, and stored at −80°C for 1 day. Figure 7Ac shows the cITP lipoprotein profile in the frozen serum sample. As shown, cITP lipoprotein profiles in fresh serum (Fig. 7Aa) and the briefly stored serum (Fig. 7Ac) were similar, indicating that the storage of a serum sample under the indicated conditions for a very short time does not significantly affect cITP lipoprotein analysis. Both fresh serum and briefly stored serum were then depleted of large, light LDLs by heparin-Mg^{2+} precipitation and subjected to cITP analysis. As shown in Fig. 7Ab, d, cITP lipoprotein profiles in fresh serum and stored serum that were depleted of large, light LDLs were also similar, indicating that the storage of serum samples under the indicated conditions for a very short time does not significantly affect cITP lipoprotein subfractions in different LDL density subclasses.

The impact of intermediate-term storage of serum was

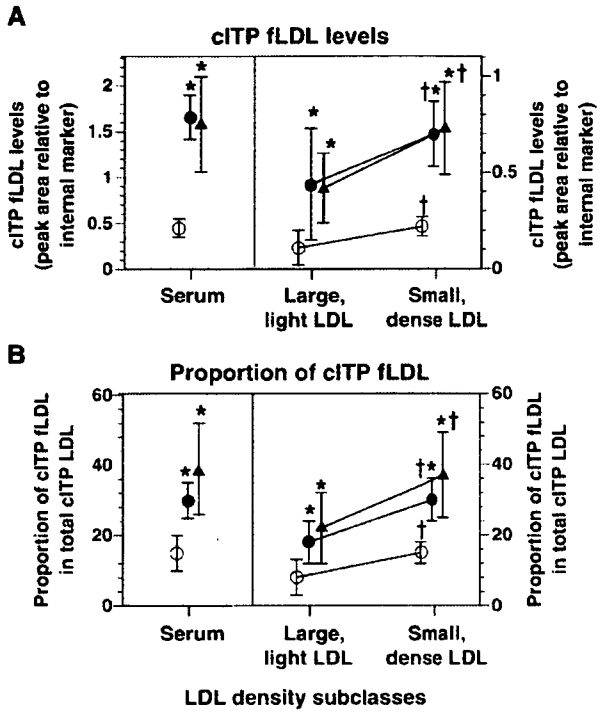

Fig. 6. Distributions of levels of cITP fLDL expressed as the peak area relative to that of an internal marker (A) and the proportion of cITP fLDL in total cITP LDL (B) in whole serum (left panel) and in light and dense LDL subclasses separated by heparin-Mg^{2+} (30) (right panel) in NL (open circles), HC (closed circles), and HTG (closed triangles) subjects. * $P < 0.05$ versus NL subjects, assessed by ANOVA and Scheffe's multiple comparison test; † $P < 0.05$, dense LDLs versus light LDLs, assessed by ANOVA.

assessed using serum samples from another volunteer (male, 36 years old), which had been used as a quality control for routine cITP analysis (Fig. 7B). Fig. 7Ba–d shows cITP lipoprotein profiles in serum of the subject that was not stored (0 Mo.) and stored at −80°C under protection with N_2 gas for 1, 2, and 3 months, respectively. As shown, cITP HDL (peaks 1–3), VLDL/IDL (peak 5), and LDL subfractions (peaks 6–8) were similar in serum samples that were not stored (Fig. 7Ba) and stored for 1 (Fig. 7Bb), 2 (Fig. 7Bc), and 3 (Fig. 7Bd) months. This result indicates that the storage of serum samples under the indicated conditions does not significantly affect these cITP lipoprotein subfractions. However, the cITP chylomicron/remnant subfraction (peak 4) seemed to be reduced in stored serum samples compared with that in serum that was not stored. This cITP subfraction was not included in the data analysis in the present study.

The impact of long-term and very-long-term storage of serum was assessed using serum samples from a 12-year-old boy (Fig. 8A) and a 12 year old girl (Fig. 8B). Fig. 8Aa, b shows cITP lipoprotein profiles in serum from the boy that was not stored and stored at −80°C for 9 months (not protected by N_2 gas), respectively. As shown, the distribution patterns of cITP HDL (peaks 1–3), VLDL/IDL (peak 5), and LDL subfractions (peaks 6–8) were not markedly different in serum samples that were not stored (Fig. 8Aa) and stored for 9 months (Fig. 8Ab). Fig. 8Ba, b shows cITP lipoprotein profiles in serum from the girl that was not stored and stored at −80°C for 18 months (not protected by N_2 gas), respectively. As shown, VLDL/IDL (peak 5) and fLDL subfractions (peak 6) were apparently different in serum samples that were not stored (Fig. 8Ba) and stored for 18 months (Fig. 8Bb). cITP HDL (peaks 1–3) and sLDL subfractions (peak 7) were less affected. These results indicate that 9 months should be the maximum duration of storage of serum samples at −80°C for cITP analysis, if not protected by N_2 gas. Very-long-term storage of serum samples is not recommended for cITP analysis.

DISCUSSION

The LDL(−) subfraction in plasma includes various forms of modified LDL, and the proportion of LDL(−) to major LDL has been used as a marker for LDL modification (1, 4, 7, 11, 12). Both type 1 and type 2 diabetes have been shown to have increased glycated LDL and a greater proportion of the LDL(−) fraction (3, 4). However, glycemic control reduced LDL(−) in type 1 diabetes but not in type 2 diabetes (3–5). This encouraged us to examine the possibility of an association between insulin resistance and LDL(−).

In the present study, we used the cITP method, originally developed by Böttcher et al. (14) and Schmitz, Mollers, and Richter (15), to separate and quantify the LDL(−) subfraction (cITP fLDL). It is not clear whether or not the proportion of cITP fLDL that is measured directly in plasma is equivalent to that in LDL separated by ultracentrifugation. Therefore, we compared cITP LDL subfractions measured directly in plasma and those measured in ultracentrifugally separated LDL. We found that the proportion of cITP fLDL in ultracentrifugally separated LDL was much lower than that in plasma (Fig. 2). Therefore, separation of LDL by ultracentrifugation may reduce the accuracy of the measurement of the proportion of LDL(−) because LDL(−) is not distributed uniformly in fractions of LDL with different densities.

We examined whether or not the two different methods for monitoring LDL subfractions used in the cITP and chromatography methods are related. We found that levels of cITP fLDL and sLDL, prestained by the lipophilic dye NBD-ceramide, are proportional to the protein content of LDL between 0.1 and 0.8 mg/ml LDL protein. This finding indicates that cITP is an accurate and sensitive method for quantifying charge-based LDL subfractions. It has the advantage that LDL subfractions can be measured directly in plasma. We also examined changes in cITP LDL subfractions during the in vitro oxidation of LDL to prove that fLDL is related to the electronegativity of LDL. We found that cITP sLDL was converted to fLDL during the oxidation of LDL and that the progressive increase in fLDL at the initial stage of oxidation paralleled

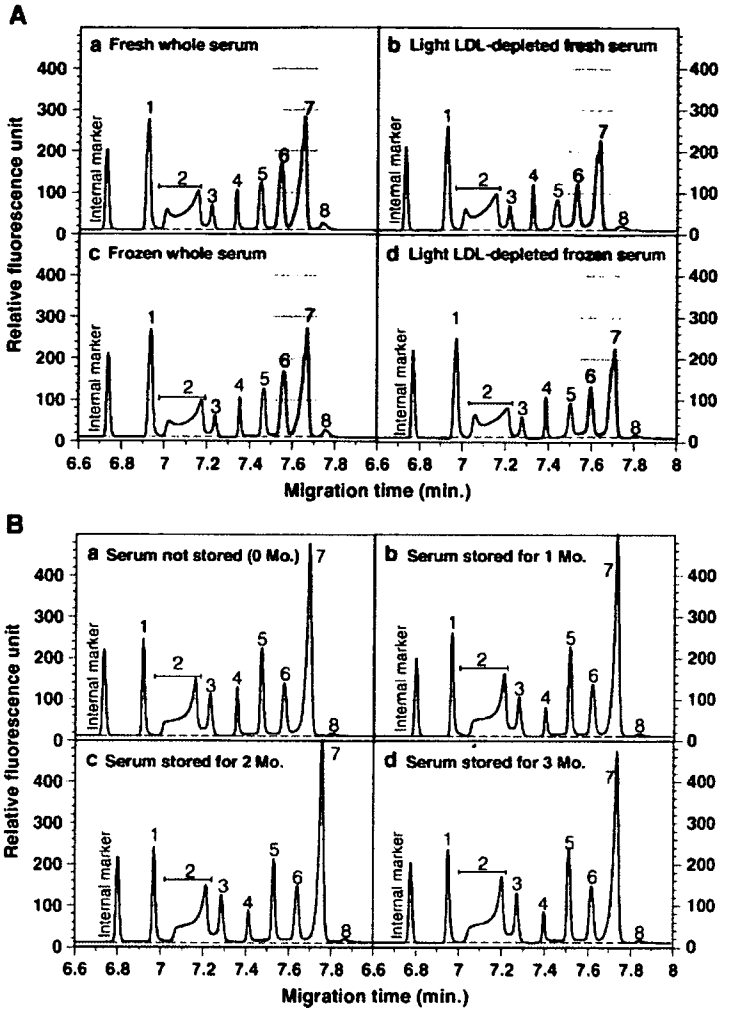

Fig. 7. A: Effects of snap-freezing serum with liquid nitrogen and short-term storage of serum at −80°C on cITP lipoprotein subfractions in serum (a, c) and serum depleted of large, light LDLs by heparin-Mg^{2+} precipitation (b, d). a: Fresh whole serum; b: fresh serum depleted of large, light LDLs; c: frozen, preserved (for 1 day) whole serum; d: frozen serum that was depleted of large, light LDLs after thawing. B: Effects of intermediate-term storage of serum at −80°C on cITP lipoprotein subfractions. a: Fresh serum without being preserved (0 Mo.); b, c, d: serum that had been stored at −80°C under N_2 gas for 1, 2, and 3 months, respectively. Peaks 1–3, HDL subfractions; peak 4; chylomicron/remnant fraction; peak 5, VLDL/IDL; peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

that in the negative charge of LDL, as indicated by the electrophoretic mobility of LDL on agarose gels. These findings indicate that cITP fLDL is related to the electronegativity of LDL and represents an electronegative subfraction of LDL.

We found that cITP fLDL is strongly and positively correlated with TG levels. This finding supports those of other authors that LDL(−) separated by anion-exchange fast protein liquid chromatography has a higher TG content than LDL(+) (7–9). Therefore, an increased TG level in plasma contributes to the qualitative modification of LDL. However, our finding disagrees with that of Cazzolato, Avogaro, and Bittolo-Bon (32), who separated LDL(−) using anion-exchange high-pressure liquid chromatography and found no significant correlation between the percentage concentration of LDL(−) in total native LDL and TG levels. Considering that the TG level was not correlated with the major LDL subfraction (cITP sLDL; data not shown) in the present study, this discrepancy suggests that the proportion of LDL(−) determined by anion-exchange chromatography may not be sensitive enough to detect changes in LDL(−) related to TG levels.

Our finding that cITP fLDL is negatively correlated with the size of LDL agrees with the finding of Sánchez-Quesada et al. (10) that most of the LDL(−) in NL subjects was contained in dense LDL subclasses. They also reported that half of the LDL(−) from patients with hypertriglyceridemia was contained in dense LDL subclasses,

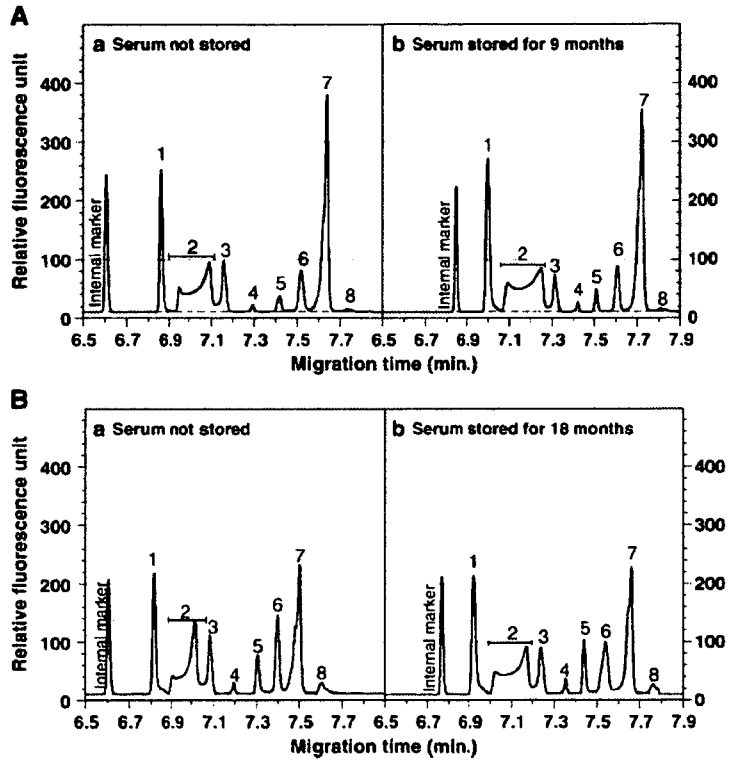

Fig. 8. A: Effects of long-term storage of serum at −80°C (not protected by N_2 gas) on cITP lipoprotein subfractions in a 12-year-old boy. a: Fresh serum without being preserved; b: serum that had been stored at −80°C for 9 months. B: Effects of very-long-term storage of serum at −80°C (not protected by N_2 gas) on cITP lipoprotein subfractions in a 12-year-old girl. a: Fresh serum without being preserved; b: serum that had been stored at −80°C for 18 months. Peaks 1–3, HDL subfractions; peak 4; chylomicron/remnant fraction; peak 5, VLDL/IDL; peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

whereas most of the LDL(−) from patients with familial hypercholesterolemia was contained in light LDL subclasses (10). Because LDL(−) from both NL and familial hypercholesterolemia subjects has been shown to have proinflammatory activity on human endothelial cells (7, 11), it could be important to establish the relationship between LDL subfractions as measured by density and charge. Therefore, we examined the distribution of the cITP fLDL subfraction in light and dense LDL subclasses in NL, HC, and HTG subjects. Hirano, Saegusa, and Yoshino (30) recently reported a novel and simple method for the quantification of small, dense LDLs in serum depleted of large, light LDLs (d > 1.044 g/ml) by heparin-Mg^{2+} precipitation. We used their method to separate large, light LDL subclasses from small, dense LDL subclasses. Our findings that HC and HTG subjects had increased levels and a higher proportion of cITP fLDL agree with those of Sánchez-Quesada et al. (10), who reported that the proportion of LDL(−) determined by ion-exchange chromatography was increased in both patients with familial hypercholesterolemia and those with HTG. Our finding that cITP fLDL was distributed more in small, dense LDL subclasses in NL, HC, and HTG subjects is consistent with that of Sánchez-Quesada et al. (10) in NL subjects. Our finding that HC and HTG subjects had increased cITP fLDL in large, light LDL subclasses supports the suggestion of Sánchez-Quesada et al. (10) that hyperlipidemia could promote the formation of light LDL(−). We found that both the proportion and the levels of cITP fLDL were also increased in small, dense LDL subclasses in HC and HTG subjects. This finding disagrees with the finding of Sánchez-Quesada et al. (10), who reported that NL subjects had a higher percentage of LDL(−) in dense LDL fractions (LDL4–6 by gradient centrifugation) than patients with familial hypercholesterolemia and HTG. This discrepancy may be attributable to the different methods used to separate LDL density subclasses and quantify charge-based LDL subfractions. However, our findings and those of Sánchez-Quesada et al. (10) consistently indicate that the quantification of the minimally modified negatively charged LDL subfraction in LDL density subclasses could be clinically important. Further investigations are needed to clarify whether or not the combination of charge-based LDL subfractions and LDL density subclasses, which can be routinely measured with the cITP method and the precipitation method, respectively, could be a novel and potentially useful marker for atherosclerosis.

This is the first study to show that HOMA-IR is associated with increased cITP fLDL levels. The origin of the association between cITP fLDL and insulin resistance is not clear. However, our finding that the relation between HOMA-IR and cITP fLDL depended on TG levels indicates that an increased TG level is a contributing factor to cITP fLDL associated with insulin resistance. Because increased TG levels are preceding factors in the development of insulin resistance in type 2 diabetic patients (33, 34), our finding suggests that an increased TG level may be an important contributor to the risk of CHD in type 2 diabetes.

We found that the relation between insulin resistance and cITP fLDL levels was independent of LDL size. This novel finding suggests that insulin resistance may contribute to the risk of CHD independent of small, dense LDLs, considering that LDL with an increased negative charge is atherogenic (1). However, because the present study did not directly compare cITP fLDL with LDL(−) isolated by anion-exchange HPLC, further investigation will be needed to determine whether or not an association between insulin resistance and cITP fLDL may explain the failure of glycemic control to reduce the proportion of LDL(−) in type 2 diabetes (4). It would also be interesting to determine whether or not antidiabetic drugs such as thiazolidinediones, which are high-affinity peroxisomal proliferator-activated receptor γ (PPARγ) ligands, may reduce cITP fLDL or LDL(−) in type 2 diabetes. However, it is possible that these drugs may at least limit the inflammatory response to LDL(−), because pioglitazone, a synthetic PPARγ activator, has been shown to inhibit the expression of vascular cell adhesion molecule-1 on human umbilical vein endothelial cells after interleukin-1β stimulation (2).

In conclusion, cITP lipoprotein analysis is an accurate and sensitive method for quantifying the charge-based LDL subfractions in human plasma, and insulin resistance is associated with an increased cITP fLDL fraction independent of LDL size. Further studies are needed to clarify whether or not cITP fLDL is useful for controlling the risk factors of CHD in type 2 diabetes.

The authors thank Ms. Yuri Saito for excellent technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan (Grants 12670712, 15790403, and 16590806), by a research grant from the Clinical Research Foundation (2003), by research grants from the Ministry of Health and Welfare, by a grant from the Uehara Memorial Foundation (2002), and by Research Grants 996006 and 026001 from the Central Research Institute of Fukuoka University.

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

Kenji Ohwaki, Hideaki Bujo, Meizi Jiang, Hiroyuki Yamazaki, Wolfgang J. Schneider, Yasushi Saito

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;27:1050-1056.**)

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$^{-/-}$ mice, overex-

Original received November 28, 2006; final version accepted February 12, 2007.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/ATVBAHA.106.137091

pression of human uPAR in macrophages enhances cell adhesion to the aortic wall,[18] and targeted overexpression of uPA, a ligand of uPAR, in macrophages accelerates atherosclerosis with increased foam cell formation.[19]

Thus, solLR11 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 solLR11 in hyperplastic intima, and show that solLR11 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 solLR11 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.[11] Monoclonal antibodies against SR-A (KT022) was obtained from Wako (Tokyo, Japan). Polyclonal or monoclonal antibodies against uPAR (AF807), VLA-4 (BBA37) and P-selectin glycoprotein ligand (PSGL)-1 (MAB996) and recombinant platelet-derived growth factor (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 aortas as described.[20] 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 cells were differentiated to macrophages (THP-1 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 solLR11 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, Chiba, Japan), weighing 400 to 450 grams, were anesthetized, and the left common carotid artery was denuded by ballooning as described.[21] The left carotid arteries were isolated at 7 or 14 days after injury and used for histochemical staining, immunohistochemistry and Western blot. Female $LR11^{-/-}$ and $LR11^{+/+}$ mice, aged ≈40 weeks fed a high-fat diet (Research Diets, Inc; 60 kcal% fat supplied from lard and soybean oil, 20 kcal% carbohydrate from sucrose and maltodextrin, and 20 kcal% protein from casein) from 3 days before surgery, were anesthetized, and the left femoral artery was sheathed with a polyethylene cuff made of PE90 tubing as described,[11] then maintained on high-fat diet. The left femoral arteries were isolated at 7 days after cuff placement and used for histochemical staining and immunohistochemistry.

Generation of Knockout Mouse

$LR11^{-/-}$ mice were generated as described (Jiang et al, submitted). Briefly, an LR11 targeting vector was constructed with short (3.3 kb) and long (4.4 kb) arms of homology and a Neo cassette (3.9 kb) to target the first exon of mouse LR11. Cultured embryonic stem cells were transfected with the LR11 targeting vector, homologous recombinant clones were selected with G418, and confirmed by Southern blotting. Germline-transmitted chimeras obtained were crossbred with C57BL6/J females, and resulting heterozygous offspring were interbred. Wild-type, heterozygous, and homozygous mutant mice 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 immunohistostaining as described.[12] Briefly, sections were pretreated with 3% H_2O_2 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 van Gieson. Western blot analysis was performed as described previously[22] using anti-LR11 (pm11, 1:500), anti-VLA-4 (1:250), anti-SR-A (1:250) and anti-uPAR (1:250).

Construction, Expression, and Purification of SolLR11

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 solLR11 was purified using Ni^{2+}-chelating chromatography. The biological activity of purified solLR11 was confirmed by a SMC migration assay.[13]

Adhesion and Migration

Cell adhesion was determined in 96-well plates as described.[23] 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 Calcein-AM dye for 1 hour at 5×10^6 cells/mL in RPMI containing 1% fetal bovine serum. Calcein-loaded cells were then added to the extracellular matrix coated plates at 2.5×10^5 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.[13] 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 (DiI-AcLDL) for 4 hours at 37°C. Then, unincorporated DiI-AcLDL was removed by washing with phosphate-buffered saline. DiI-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

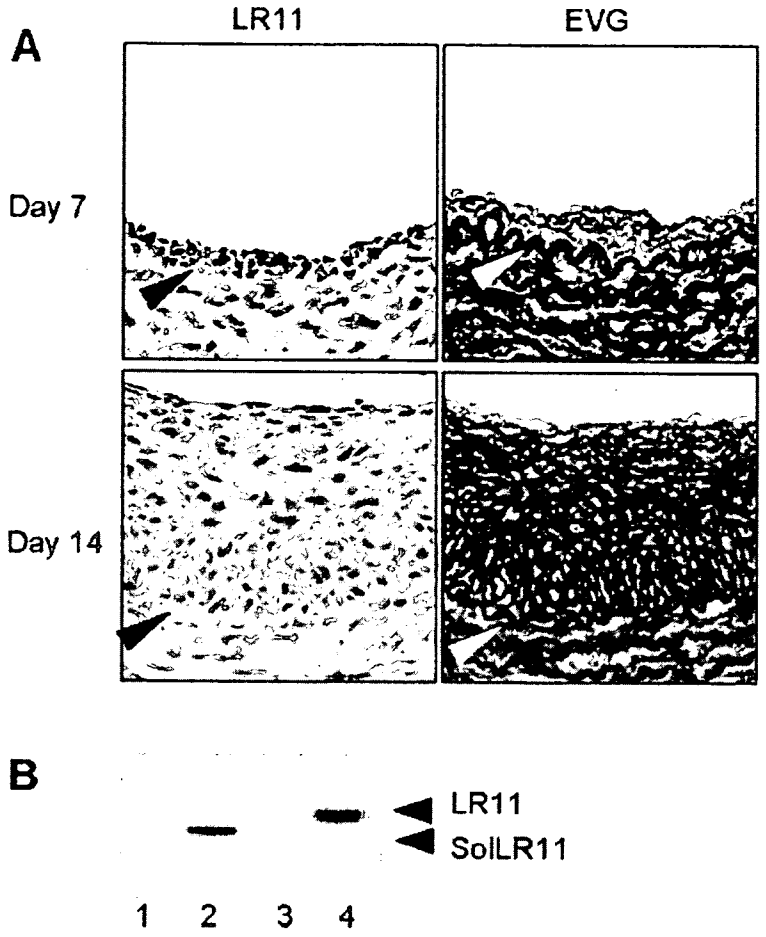

Figure 1. SolLR11 expression in intimal SMCs in balloon-denuded rat artery. A, Sections of balloon-denuded carotid artery were subjected to histological analysis using elastica van Gieson staining (EVG), and to immunohistochemistry with anti-LR11 antibody (pm11) at day 7 (top) and day 14 (bottom) after injury. Arrowheads indicate the internal elastic layers. B, Intima from day 14 balloon-denuded carotid artery was homogenized and analyzed by Western blotting with anti-LR11 antibody (pm11). Ln 1: mock/COS7; lane 2: solLR11/COS7; lane 3: medial layer extract; lane 4: intimal layer extract. Arrowheads indicate the full-length and truncated soluble LR11, respectively.

membrane-anchored form.[11] To investigate the pathophysiological 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.[11] Using the samples of thickend 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.[11] 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 hig- 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 Ni^{2+}-chelating chromatography (supplemental Figure I, available online at http://atvb.ahajournals.org). The addition of purified recombinant solLR11 at 1, 10, and 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.

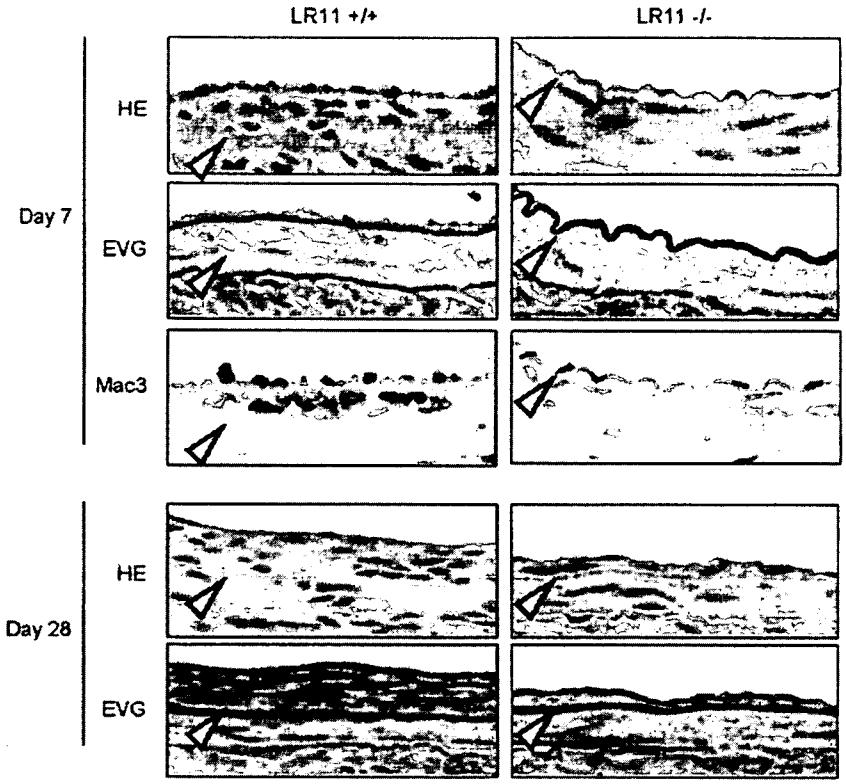

Figure 2. Reduced macrophage infiltration into intima of cuff-injured artery in LR11$^{-/-}$ mice. Sections of femoral artery obtained at day 7 and day 28 after cuff injury in LR11$^{+/+}$ and LR11$^{-/-}$ mice on a high-fat diet were subjected to histological analysis using hematoxylin & eosin (HE) and EVG staining, and to immunohistostaining with anti-Mac3 antibody. Arrowheads indicate the internal elastic layers.

SolLR11 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 solLR11 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 solLR11 at 1 μg/mL, followed by Western blot of plasma membrane preparations probed with anti-SR-A and anti-uPAR antibodies. Although solLR11 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 solLR11, likely because of the solLR11-mediated stabilization of uPAR.[11] To test whether solLR11 affects lipid accumulation in macrophages, we evaluated DiI-AcLDL uptake in THP-1 macrophages (Figure 3B). In the undifferentiated THP-1 cells, there was no significant DiI-AcLDL uptake, and solLR11 did not affect DiI-AcLDL uptake (data not shown). However, in THP-1 macrophages, solLR11 at 1 to 100 μg/mL significantly increased DiI-AcLDL uptake (Figure 3C). Addition of neutralizing anti-LR11 or anti-uPAR antibodies almost totally inhibited the increase in DiI-AcLDL uptake by the cells (Figure 3D). These data indicate that solLR11 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 SolLR11 Increases Adhesion and Migration of THP-1–Derived Macrophages

We next investigated the effect of solLR11 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. SolLR11 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 solLR11-induced increase in adhesion. Next, we tested the effect of solLR11 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 solLR11 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 solLR11, as observed with anti-LR11 or anti-uPAR antibodies. Thus, we analyzed the effect of solLR11 on the expression of adhesion molecules. SolLR11 enhanced the expression of cell-surface VLA-4 in the presence and absence of PMA (Figure 4C).

We next tested the effect of solLR11 on the migratory functions of THP-1 macrophages by using the Boyden chamber method. SolLR11 itself did not affect migration of THP-1 macrophages in vitro (data not shown). When cells were preincubated with 1 μg/mL solLR11 for 12 hours, PDGF-BB-induced migration of THP-1 macrophage was 2.0-fold greater than in the absence of solLR11 (Figure 4D). The stimulatory effect of solLR11 was decreased by addition of neutralizing anti-LR11 or anti-uPAR antibodies. These data indicate that solLR11 induces adhesion and migration

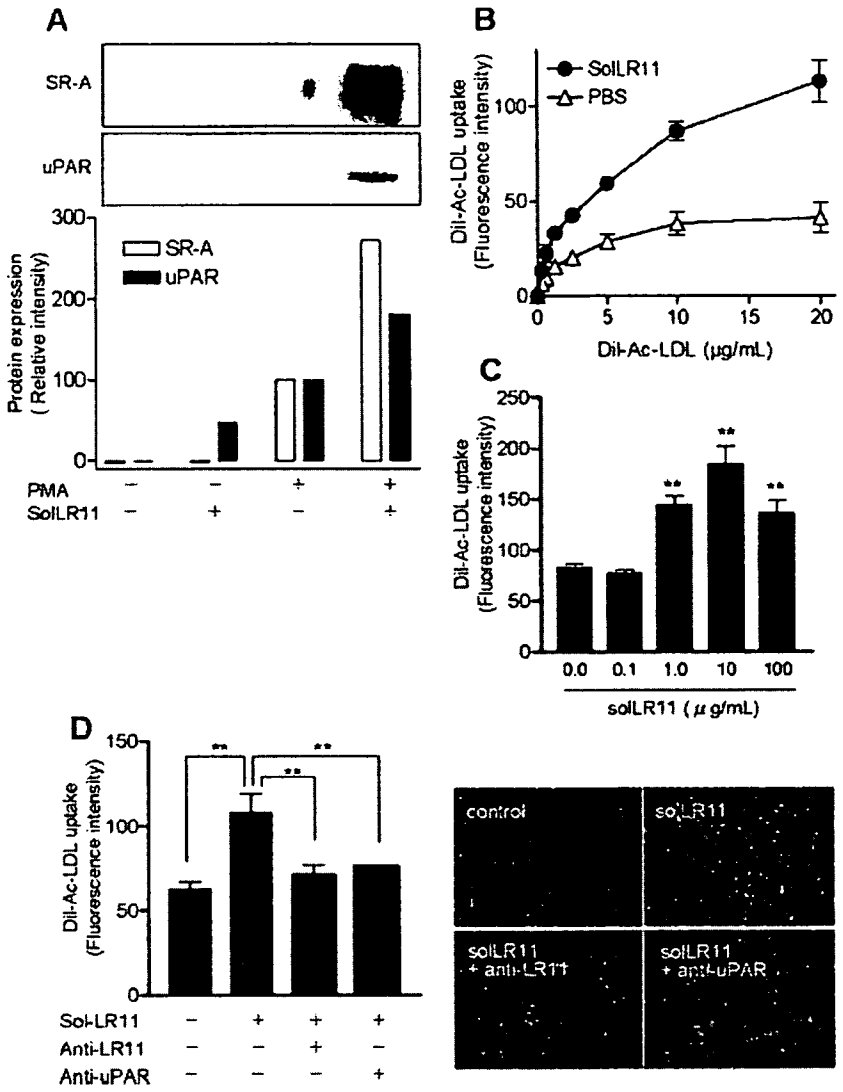

Figure 3. SolLR11 enhances cell-surface expression of SR-A and uPAR, and the uptake of modified LDL by THP-1 macrophages. A, Membranes of THP-1 monocytes or macrophages, prepared from cells obtained under the conditions indicated in the bottom panel, were subjected to Western blotting with anti-SR-A or anti-uPAR antibodies. B, THP-1 macrophages were preincubated with 1 μg/mL solLR11 (black circles) or phosphate-buffered saline (white triangles). Cells were washed and then incubated with the indicated concentrations of Dil-AcLDL in the presence or absence of excess amount of Ac-LDL. C, Dose-dependent effect of solLR11 on Dil-AcLDL uptake by THP-1 macrophages. D, The effects of anti-uPAR and anti-LR11 (5-4-30-19-2, 1:2 dilution) antibodies on solLR11-induced uptake of Dil-AcLDL (10 μg/mL) by THP-1 macrophages. DiAcLDL uptake was visualized by fluorescence microscopy and quantitative measurement was obtained using a fluorescence microplate reader. Data are expressed as mean±SD, n=6 (*$P<0.05$, **$P<0.01$).

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.[11,15,24] In CHO cells, it was demonstrated that tumor necrosis factor-α convertase is responsible for the proteolytic cleavage of LR11.[14,25] 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,[11] 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.[16] 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-

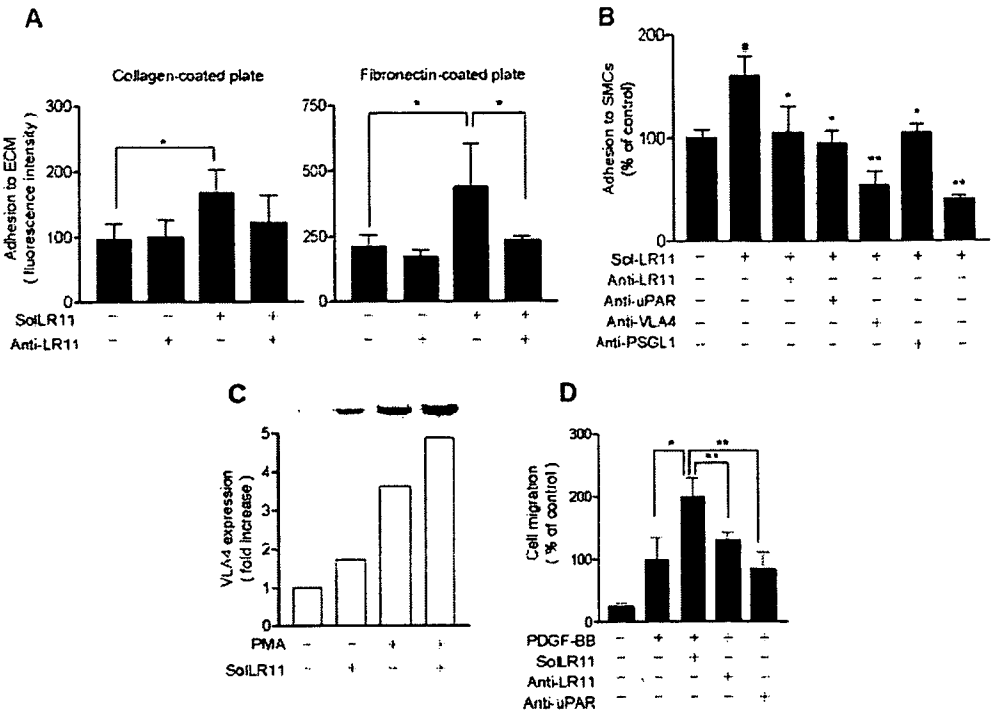

Figure 4. SolLR11 enhances adhesion and migration of THP-1 macrophages. A, THP-1 macrophages were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-uPAR antibodies. The cells were washed and then incubated on collagen- or fibronectin-coated plates. B, THP-1 macrophages were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-uPAR, anti-LR11 (5-4-30-19-2, 1:2 dilution), anti-VLA-4 or anti-PSGL-1 antibody. The cells were washed and then incubated in the cultured SMCs. C, THP-1 monocytes were treated with 200 nM PMA in the presence or absence of 1 μg/mL solLR11. Membranes were subjected to Western blotting with anti-VLA-4 antibody. D, Cells were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-LR11 (5-4-30-19-2, 1:2 dilution) and anti-uPAR antibodies. The cells were washed, and then the PDGF-induced cell migration was measured using a micro-Boyden chamber. Data are expressed as mean±SD, n=4 to 6 (*$P<0.05$, **$P<0.01$).

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 neointimal 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 DiI-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 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.[11] LRP1 is also abundantly expressed in monocytes/macrophages;[8] 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.

Sources of Funding

These studies were supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to Y.S. and H.B., and from the Austrian Science Foundation to W.J.S. (F-0608).

Disclosures

None.

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Low-dose GH supplementation reduces the TLR2 and TNF-α expressions in visceral fat

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Received 3 January 2008
Available online 15 January 2008

Abstract

The increased population of TLR2/TNF-α co-expressing adipocytes is associated with the development of insulin resistance. We have herein shown the significance of low-dose growth hormone (GH) supplementation for the regulation of TLR2 and TNF-α expressions in visceral fat using different kinds of mouse models fed with a high-fat diet. Low-dose GH supplementation reduced the increased population of TLR2/TNF-α co-expressing adipocytes in high-fat fed mice. The neutralization of IGF-1 abolished the effect of GH supplementation on the TLR2 expression using GH-overexpressing mice. IGF-1, but not GH, inhibited the FFA-induced TLR2 and TNF-α expression in 3T3-L1 cells. Finally, low-dose GH supplementation reduced the TLR2 expression without an obvious change in the visceral fat volume in ob/ob mice. These results indicate that low-dose GH supplementation possibly inhibits the high-fat induced change of the adipocytes to TLR2/TNF-α co-expressing cells through the action of IGF-1.

Keywords: Growth hormone; Toll-like receptor 2; Adipocyte; Visceral fat; Insulin-like growth factor-1

A dysfunction of adipocytes leads to an accumulation of metabolic abnormalities, such as dyslipidemia, hypertension, and glucose intolerance [1]. This functional abnormality is characterized by a disturbance in the cytokine expressions of adipocytes, causing the development of insulin resistance, a pathogenesis of the metabolic syndrome [2]. However, the regulation of cytokine secretion from adipocytes accumulated in visceral regions has not yet been fully elucidated.

We have shown that cultured adipocytes implanted in mesenteric, but not in subcutaneous, regions induce tumor necrosis factor (TNF)-α secretion in mice [3]. The TNF-α expression of visceral adipocytes is accompanied with toll-like receptor (TLR) 2 expression, and the population of TLR2/TNF-α co-expressing adipocytes is drastically induced in mice fed a high-fat diet [4]. These observations suggest that the identification of the regulator(s) for the occurrence of TLR2/TNF-α co-expressing adipocytes may provide a target for the amelioration of insulin resistance in the metabolic syndrome.

Insulin resistance accompanied with visceral fat accumulation is not only observed in the metabolic syndrome, but also in several hormonal disturbances. One such hormonal disturbance is growth hormone (GH) deficiency, which is frequently accompanied by reduced insulin sensitivity and accumulated visceral fat. Recent studies have shown the low-dose supplementation of GH to have a beneficial effect on the treatment of insulin resistance accompanied by aging and/or abdominal obesity, as well as GH deficiency itself. [5–7]. These studies have provided a novel

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doi:10.1016/j.bbrc.2008.01.035

therapeutic possibility for GH in the regulation of insulin sensitivity. On the other hand, the beneficial effect of GH raises a complicated issue to be solved, specifically the development of acromegaly-associated glucose intolerance classically observed in association with GH excess. The obvious difference in the opposite effects of GH on the regulation of insulin sensitivity seems to be largely a function of the plasma GH concentration; low dose GH supplementation may be of benefit for GH deficiency and abdominal obesity, whereas excessive GH production results in pathological acromegaly. The aim of this study is to clarify the effect of low-dose GH supplementation on insulin resistance, particularly through the regulation of TLR2/TNF-α co-expressing adipocytes using cultured adipocytes and animal models of visceral fat accumulation.

Materials and methods

Mice and blood samples. Mice were obtained from Charles River Japan. All work was carried out according to the guidelines of the Animal Care Committees of Chiba University. The levels of plasma human growth hormone (Roche), mouse insulin-like growth factor (IGF)-I (R&D Systems), and mouse insulin (Morinaga) were measured using ELISA kits. Insulin tolerance test was performed by intra-peritoneal injection of human insulin (Sigma–Aldrich) 0.5 or 2.0 U/kg body weight according to the mice models [8].

Cell culture. 3T3-L1 cells were from the American Type Culture Collection. The differentiation of preadipocytes to mature adipocytes was as described [8]. Cells were treated with DMEM supplemented with 10^{-8} M hGH (Novo Nordisk Pharma) or 10^{-8} M human IGF-1 (Jena Bioscience), with 2% free fatty acids (FFA)-free BSA (Sigma–Aldrich) overnight, and then with a fatty acid mixture composed of 500 μM myristic acid and 500 μM palmitic acid, with 10^{-8} M hGH or 10^{-8} M hIGF-1 in the presence of 2% FFA-free BSA at 37 °C for 8 h.

Implantation of 3T3-L1 cells overexpressing hGH into BALB/c nude mice. Human GH cDNA full clone was obtained by polymerase chain reaction (PCR) using human brain-derived cDNA pool using oligonucleotide primers specific for parts of human GH sequence (5′-GACGGC GATCGCCATGGGCTACAGGCTCCCGGAC-3′ and 5′-ATGCGT TTAAACGAAGCCACAGCTGCCCTCCAC-3′). The cDNA fragment subcloned into pcDNA3.1/Hygro(−), were transfected into 3T3-L1 cells using GeneJammer Transfection Reagent (Stratagene). The cells stably expressing hGH and mock transfected cells by the transfection of pcDNA3.1/Hygro(−) without hGH cDNA were cloned as described [9]. The hGH production in the conditioned medium of 3T3-L1 cells overexpressing hGH was 8.8 ± 0.9 ng/10^6 cells/24 h, whereas it was not detectable in conditioned medium of the mock cells. 3T3-L1 cells overexpressing hGH, or the mock cells were suspended at 4×10^6 cells/250 μl in Matrigel (BD Bioscience) and injected subcutaneously in the back of male BALB/c nude mice (6-week old) as described [10]. The mice were fed with high fat diet from a week after the implantation. Insulin tolerance test were performed by using human insulin (Sigma–Aldrich) 0.5 U/kg, i.p. after fasting for more than 16 h.

Isolation of single adipocytes and flow cytometry. Mesenteric fat tissues or 3T3-L1 adipocytes were collected and digested at 37 °C for 60 min with 1 mg/ml type I collagenase (Nitta Gelatin). The digested tissue was centrifuged at 400 rpm for 4 min. The floating adipocyte fraction was prepared for flow cytometry analysis. Isolated adipocytes (1×10^6 cells) were analyzed with FACS Calibur flow cytometer (BD Bioscience) as described [4].

Anti-IGF-1 antibody treatment in mice. Goat polyclonal antibody against mouse IGF-1 (R&D Systems) or normal goat IgG (R&D Systems) was injected i.p. (0.1 μg/g body weight) into male BALB/c nude mice (6-week old) at weekly intervals starting on the day of the implantation of the established 3T3-L1 cells overexpressing hGH or the mock cells. The mice were started to be fed with high fat diet from a week after the implantation. At 4 weeks after the cell implantation, insulin tolerance test was performed by using human insulin 0.5 U/kg, i.p.

RT-PCR. Quantitative RT-PCR amplifications were performed using TaqMan Gene Expression Master Mix (Applied Biosystems) as described [8]. For TLR2 and TNF-α mRNA quantification, Real-time RT-PCR amplification were performed using TLR2 primers (Mm00442346_m1, Applied Biosystems) and TNF-α primers (MA031450, Sigma Genosys). The quantification of given gene, expressed as relative mRNA level compared with a control, was calculated after normalization to 18s rRNA. All PCRs were performed in an ABI PRISM 7000 sequence system (PE Applied Biosystems.) [11].

Fat volume measurement by computed tomography (CT). From 12 weeks of age, male ob/ob mice were administered either hGH (0.5 mg/kg body weight/day) or equivalent volume of saline via mini-osmotic pumps for 4 weeks. There was no significant difference in body weight between the mice administered hGH and the mice administered PBS. The plasma hGH level was 881 ± 643 pg/ml in the mice administered hGH. After fasting for overnight, abdominal CT was performed using GE Healthcare eXplore Locus MicroCT Scanner (GE Healthcare). Visceral and subcutaneous fat volume was calculated using GE Healthcare eXplore Lucus Microview Software (ver 2.2) (GE Healthcare).

Statistical analysis. The results are shown as means ± SD for each index. Statistical significance was determined by means of the Student's *t*-test or Dunnett's multiple range test followed by ANOVA among several groups. Statistical analyses were conducted by using SPSS software (version 13.0J; SPSS Inc.). All P values quoted are two-tailed. A *P*-value of <0.05 was considered statistically significant.

Results

Low-dose GH supplementation reduces the number of TLR2/TNF-α co-expressing adipocytes in visceral fat

We have previously shown that high fat intake induces an increased number of TLR2/TNF-α-coexpressing adipocytes in mesenteric fat in mice [4]. In order to clarify the effect of low-dose GH supplementation on the increase in the population of TLR2/TNF-α co-expressing cells in the adipocytes of mesenteric fat, we performed a flow cytometry analysis of single adipocytes prepared from the mesenteric fat of high-fat fed mice after hGH administration for 2 weeks. There was no significant difference in body weight between the mice administered hGH (GH group) and the mice administered PBS (control group). The plasma hGH concentration in the GH group was 160 ± 86 pg/ml, which is similar to the GH concentrations in previous studies using low dose GH supplementation [12] (Fig. 1A). The plasma IGF-1 concentrations were higher in the GH group in comparison to those in the control group (Fig. 1B). The blood glucose levels 30 min after insulin loading were decreased in the GH group in comparison to the control group (Fig. 1C). The TLR2 mRNA expression levels in mesenteric fat were significantly decreased in the GH group in comparison to those in the control group, suggesting the inhibitory effect of low-dose GH supplementation on TLR2 expression in visceral adipocytes (Fig. 1D). A flow cytometry analysis of single adipocytes prepared from mesenteric fat showed that the high-fat-induced increase in the population of TLR2/TNF-α co-expressing adipocytes was

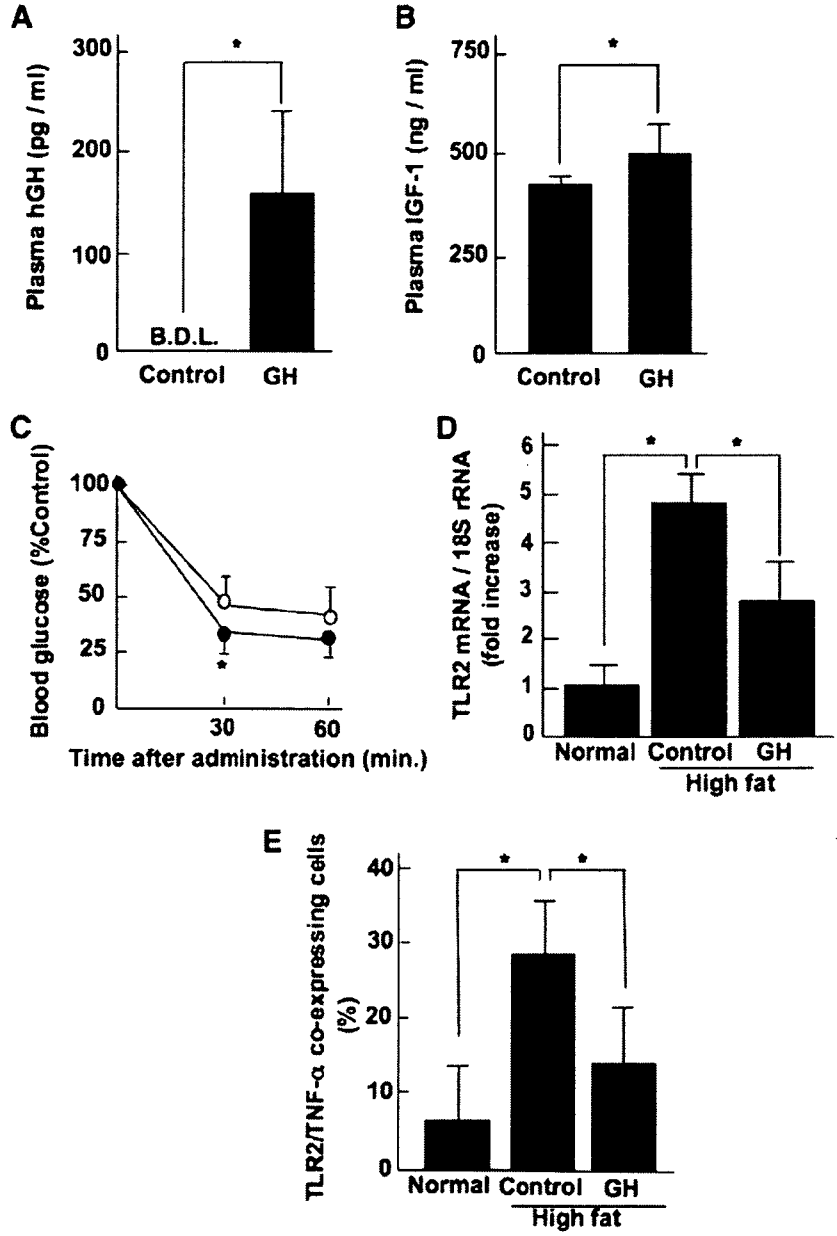

Fig. 1. The effects of low-dose hGH supplementation on reduced insulin sensitivity in high-fat fed mice. (A) The plasma hGH concentration in mice supplemented with hGH (GH) or PBS (control). Male C57BL/6J mice, which have been fed with a high-fat diet (60% fat) for 6 months, were supplemented with hGH (0.05 mg/kg/day) or PBS for 2 weeks. B.D.L., below the detection limit (less than 4 pg/ml). $n = 8$. $^*P < 0.05$ compared to the value of control. (B) The plasma IGF-1 levels in the mice supplemented with hGH (GH) or PBS (control). $n = 8$. $^*P < 0.05$ compared to the value of control. (C) Insulin tolerance test in the mice supplemented with hGH (●) or PBS alone (○). The blood glucose levels were monitored at 0, 30, and 60 min after injection of human insulin. $n = 8$. $^*P < 0.05$ compared to the value of the control. (D) The TLR2 mRNA expression in mesenteric fat tissues of the mice supplemented with hGH (GH) or PBS (control). Normal, mice fed with a normal diet. High fat, mice fed with a high-fat diet. $n = 8$. $^*P < 0.05$ compared to the value of the control. (E) Flow cytometric analyses of TLR2/TNF-α co-expressing adipocytes in the fat tissues of mice supplemented with hGH (GH) or PBS (control). Single adipocytes were prepared from mesenteric fat, and analyzed by FACS Calibur. The averaged populations of TLR2/TNF-α co-expressing adipocytes in the total cells (50,000 cells) were expressed ($n = 8$).

significantly and largely inhibited in the GH group in comparison to that in the control group (Fig. 1E). These results strongly suggest that low-dose GH supplementation reduces the increase in the population of TLR2/TNF-α co-expressing adipocytes in mesenteric fat, as well as reducing insulin resistance, in mice fed a high-fat diet.

Neutralization of IGF-1 abolishes the effect of low-dose GH supplementation on the decrease in the number of TLR2/TNF-α-coexpressing adipocytes

We next analyzed the effect of neutralization of IGF-1, an effector of GH actions for the regulation of insulin sensitiv-

ity, on the decrease in population of TLR2/TNF-α co-expressing adipocytes in visceral fat by low-dose GH supplementation. For this purpose, we established the hGH-expressing mice using cell transplantation methods as described [9]. The hGH-overexpressing 3T3-L1 preadipocytes were subcutaneously implanted into BALB/c nude mice (GH mice). The plasma GH concentrations increased 4 weeks after the implantation of hGH-overexpressing 3T3-L1 preadipocytes were significantly higher in the GH mice than those in the mock-implanted mice (mock mice) (322 ± 165 pg/ml vs 136 ± 128 pg/ml, $P < 0.05$). The plasma IGF-1 concentrations were significantly higher in the GH mice than in the mock mice (323 ± 71 ng/ml vs 267 ± 19 ng/ml, $P < 0.05$) (Fig. 2A). The blood glucose levels 30 min after insulin loading were significantly decreased in the GH mice in comparison to those in the mock mice (Fig. 2B). In accordance with the decreased insulin sensitivity, the plasma triglyceride levels were significantly lower in the GH mice in comparison to those in the mock mice (Fig. 2C). The pretreatment of mice with anti-IGF-1 antibody cancelled the ameliorating effect of GH on the insulin resistance (Fig. 2B). The TLR2 mRNA expression levels of visceral fat were significantly lower in the GH mice than those in the mock mice, and anti-IGF-1 antibody treatment significantly increased the TLR2 mRNA expression levels to those expressed in the mock mice (Fig. 2D). Therefore, circulating IGF-1 is important for the effect of the low-dose GH supplementation on the high-fat-induced insulin resistance in mice.

IGF-1, not GH, inhibits FFA-induced TLR2 and TNF-α gene expressions in 3T3-L1 adipocytes

Two different mouse models fed with a high-fat diet showed that low-dose GH supplementation suppresses the population of TLR2/TNF-α co-expressing adipocytes in visceral fat, and possibly the amelioration by GH supplementation is mediated by the effects of increased plasma IGF-1.

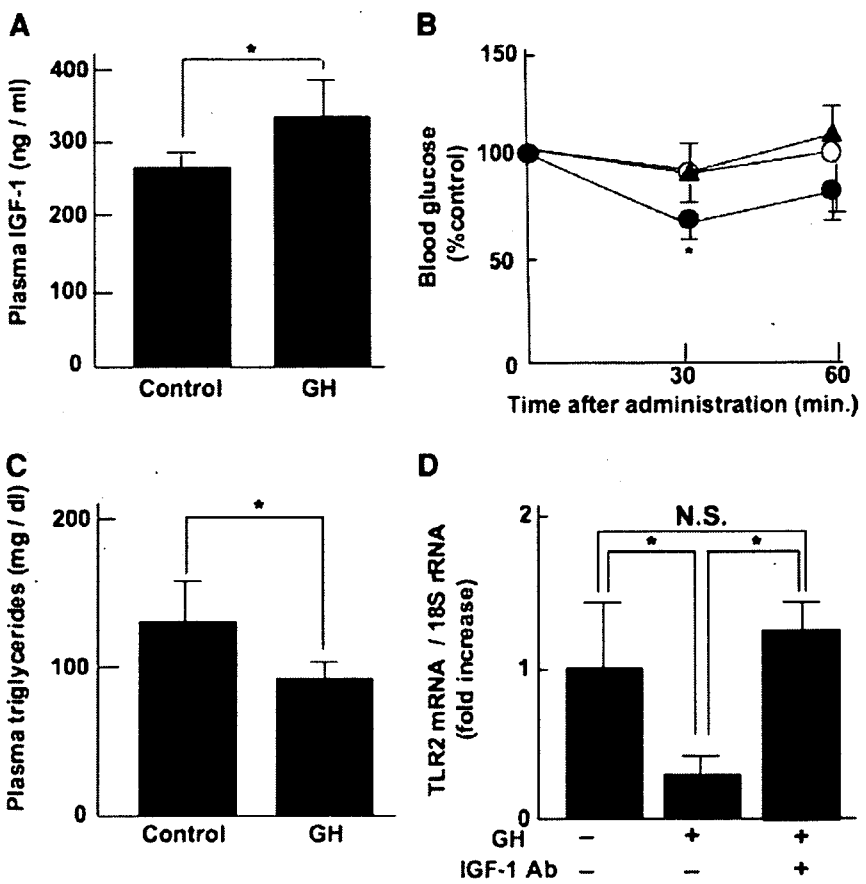

Fig. 2. Effects of IGF-1 neutralization on the actions of low-dose GH supplementation using cell transplantation models. Male BALB/c nude mouse was subcutaneously injected with 10^6 cells of hGH-overexpressing 3T3-L1 preadipocytes. A high-fat diet was started at a week after cell implantation, and continued for 3 weeks. (A) The plasma IGF-1 levels in the mice implanted with hGH-overexpressing cells (GH) or mock cells (Control). $n = 6$. $^*P < 0.05$ in comparison to the value of the control. (B) Insulin tolerance test in the mice implanted with hGH-overexpressing cells or with the mock cells (○). The mice implanted with hGH-overexpressing cells were injected with normal goat (●) or anti-mouse IGF-1 antibody (▲). Blood glucose levels were monitored at 0, 30, and 60 min after intraperitoneal insulin injection. $n = 6$. $^*P < 0.05$ compared to the value of the control. (C) The plasma triglyceride levels in the mice implanted with hGH-overexpressing cells (GH) or mock cells (control). $n = 6$. $^*P < 0.05$ compared to the value of the control. (D) TLR2 mRNA expression in mesenteric fat of the mice implanted with hGH-overexpressing cells or PBS alone. Anti-mouse IGF-1 antibody or normal goat IgG was injected after transplantation of hGH-overexpressing cells. $n = 6$. $^*P < 0.05$ compared to the value of the control. N.S., not significant.

Therefore, in order to know the role of IGF-1 in the regulation of TLR2/TNF-α co-expressing adipocytes, we analyzed the effects of IGF-1 on the TLR2 and TNF-α mRNA expressions in 3T3-L1 adipocytes (Fig. 3A). The TNF-α mRNA level was increased by the stimulation of a mixture of myristic and palmitic acids [4]. The incubation of 3T3-L1 cells with hGH did not inhibit the increased expression of TNF-α by FFAs. In contrast, IGF-1 completely inhibited the FFA-induced increase in TNF-α mRNA expression. Furthermore, IGF-1 almost inhibited all of the FFA-induced TLR2 mRNA expression in 3T3-L1 adipocytes. A flow cytometry analysis of single adipocytes prepared from 3T3-L1 adipocytes showed that the FFA-induced increase in the population of TLR2/TNF-α co-expressing adipocytes was largely inhibited by the incubation with IGF-1 (Fig. 3B). These results are in consistent with the observations made using in vivo models (see Figs. 1 and 2), thereby suggesting that IGF-1, not GH, reduces the number of TLR2/TNF-α co-expressing adipocytes in visceral fat.

Low-dose GH supplementation reduces the TLR2 mRNA expression of visceral fat before an obvious change of fat volume in obese mice

We finally examined the effect of low-dose GH supplementation on TLR2 mRNA expression in visceral fat in obese mice in order to know the relationship of TLR2 expression and fat volume in visceral fat. The plasma IGF-1 concentration significantly increased in ob/ob mice supplemented with low-dose GH (GH-ob) in comparison to ob/ob mice in the absence of supplementation (control-ob) (Fig. 4A). Measurements of the fat volume using a CT scan showed no significant difference in either the visceral or the subcutaneous fat volume between the GH-ob and the control-ob mice (Fig. 4B). In contrast, the TLR2 mRNA expression levels of visceral fat tissue were significantly decreased in the GH-ob mice in comparison to those in the control-ob mice (Fig. 4C). These results indicate that low-dose GH supplementation caused the decrease in TNF-α expression in the visceral fat before the obvious change in the visceral fat volume in the obese mice.

Discussion

An abnormal expression of cytokines in adipocytes, particularly in the visceral regions, causes the onset of metabolic syndrome through the development of insulin resistance [2]. We have shown that TNF-α expression is induced in adipocytes accumulated in the visceral, and not in the subcutaneous, regions, using a cell transplantation model [3]. The TNF-α expression in the visceral fat is closely associated with the increased population of TLR2/TNF-α co-expressing adipocytes in response to a high-fat intake [4]. The identification of the TLR2/TNF-

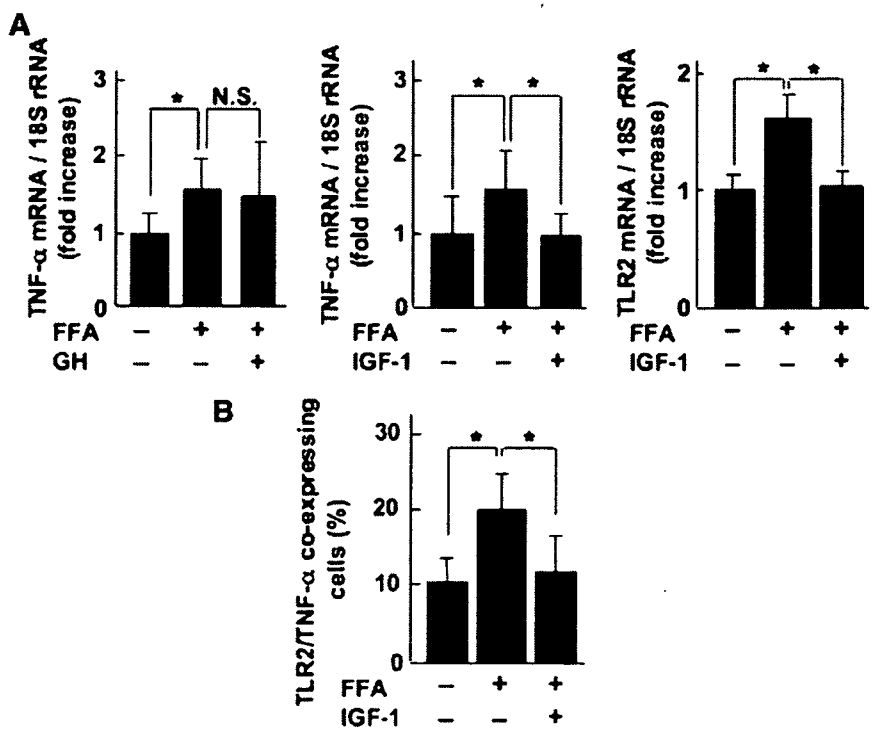

Fig. 3. Effects of GH or IGF-1 on FFA-induced TLR2 and TNF-expressions in 3T3-L1 adipocytes. (A) Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of hGH or IGF-1 for 8 h. Quantitative RT-PCR was used to measure the expression level of TNF-α gene or TLR2 gene $n = 6$. $^*P < 0.05$. (B) Flow cytometric analyses of TLR2/TNF-α co-expressing adipocytes in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of IGF-1 for 8 h, and analyzed by FACS Calibur. The averaged populations of TLR2/TNF-α co-expressing adipocytes in the total cells (20,000 cells) were expressed ($n = 3$). $^*P < 0.05$.

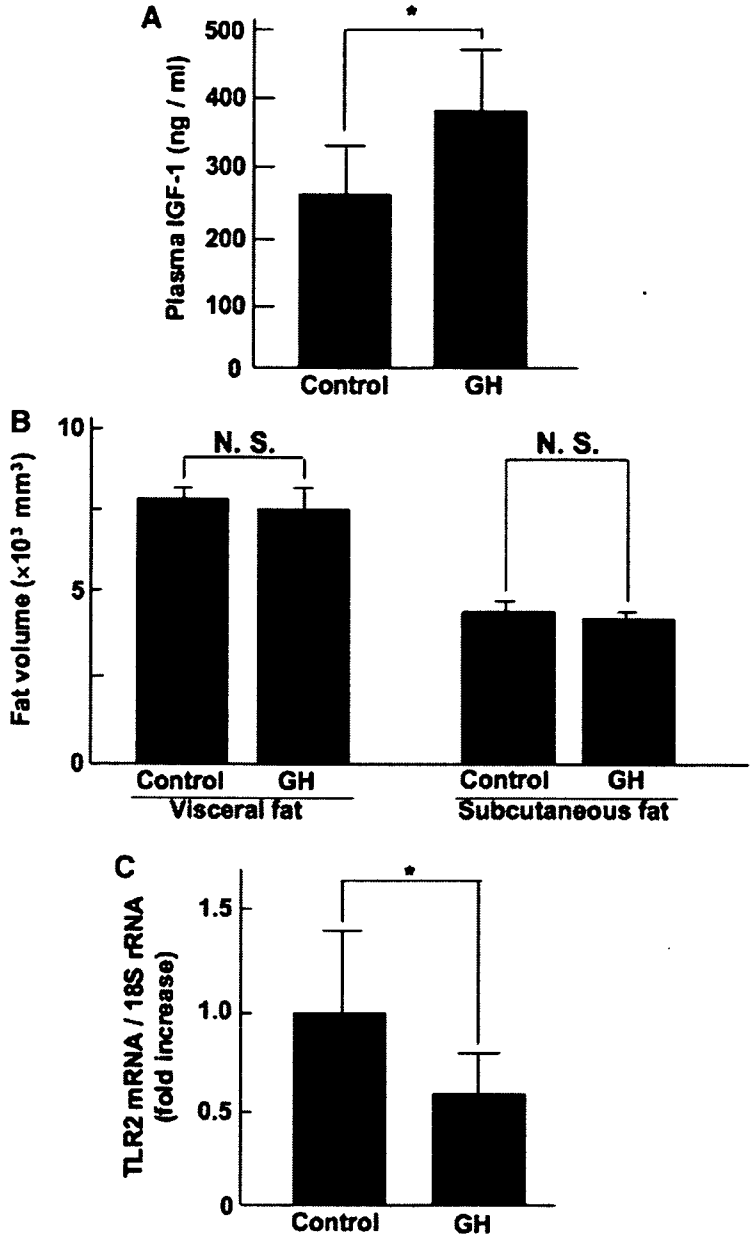

Fig. 4. Effects of low-dose hGH supplementation on the fat volume and TLR2 mRNA expression of visceral fat. Male ob/ob mice were supplemented with hGH (0.5 mg/kg/day) (GH) or PBS (Control) for 4 weeks. (A) The plasma mouse IGF-1 concentration was measured. $n = 7$. $^*P < 0.05$. (B) Visceral or subcutaneous fat volumes at 4 weeks after administration of hGH or PBS alone was measured by using CT. $n = 7$. $^*P < 0.05$. (C) The TLR2 mRNA expression levels in mesenteric fat tissue were measured by RT-PCR. $n = 7$. $^*P < 0.05$.

α co-expressing adipocytes as a regulator of TNF-α expression in the visceral fat suggested that the regulation of the occurrence of pathogenic adipocytes in the visceral fat is important for the improvement of TNF-α-mediated insulin resistance.

Low-dose GH supplementation research has recently focused on the regulation of insulin resistance accompanied by visceral obesity. Yuen et al. found that low-dose GH therapy (0.1 mg/day) improved insulin sensitivity in GH-deficient adults and also notably in subjects with the metabolic syndrome [7]. Johansson showed that GH treatment of obese men reduces the abdominal fat mass, and improved the accompanied metabolic abnormalities [6]. These clinical studies indicate that low-dose GH supplementation is potentially beneficial for metabolic abnormalities accompanied by visceral obesity, in contrast to the glucose intolerance due to the GH overproduction in acromegaly. In this context, there are relevant studies regarding the heterogenous effect of GH on metabolic abnormalities using animal models [13,14]. Based on this background, we performed this study in order to clarify the mechanism for the effect of low-dose GH supplementation on insulin resis-

tance, particularly through the regulation of the population of TLR2/TNF-α co-expressing adipocytes, which has been shown to be related to high-fat-induced insulin resistance [4]. A flow cytometry analysis clearly showed that continuous low-dose GH supplementation reduced the population of TLR2/TNF-α co-expressing adipocytes in visceral regions, and improved insulin resistance. These results using high-fat fed mice are inconsistent with the above clinical observations in obese subjects [6]. We then studied the mechanism of low-dose GH supplementation-mediated inhibition of high-fat induced TLR2 and TNF-α expressions in visceral fat using another model. The GH continuously supplemented from the subcutaneously implanted cells reduced the high-fat induced insulin resistance, and the effect was abolished by the neutralization of IGF-1, a mediator of GH action [15]. The cancellation of GH-mediated action was also observed in the inhibition of TLR2 expression in the visceral fat. Thus, our study showed that IGF-1 was a key molecule in the low-dose GH supplementation for the regulation of TLR2 and TNF-α expressions in visceral fat. The results obtained from cultured adipocytes supported the role of IGF-1 in the effect of low-dose GH supplementation.

The effect of IGF-1 on apoptosis and adipogenesis have been shown in primary cultured adipocytes [16,17]. Our results suggested that TLR2 is one of the genes regulated by IGF-1 in 3T3-L1 cells. The induction of TLR2 expression in high-fat intake could be protected by low-dose GH supplementation through the effect of IGF-1 on visceral adipocytes. The study using ob/ob mice suggested that the effect of IGF-1 on the suppression of TLR2 expression is not necessarily linked to the changes in visceral fat volume. The identification of IGF-1 as a regulator of TLR2 mRNA expression in adipocytes may contribute to the elucidation of the heterogenous functions of GH in various metabolic states. Recent clinical trials suggested that the effects of low-dose GH supplementation are mediated by its ability to increase IGF-1 without the induction of lipolysis [18]. The studies of IGF-1-mediated function on visceral adipocytes may be important for the further therapeutic application of low-dose GH (or IGF-1) supplementation in patients with metabolic syndrome and insulin resistance.

Acknowledgments

We thank for Drs. K. Aragane and J. Kusunoki (Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd) for their assistance of fat volume measurement. These studies were supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to N.R., Y.S., and H.B.

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