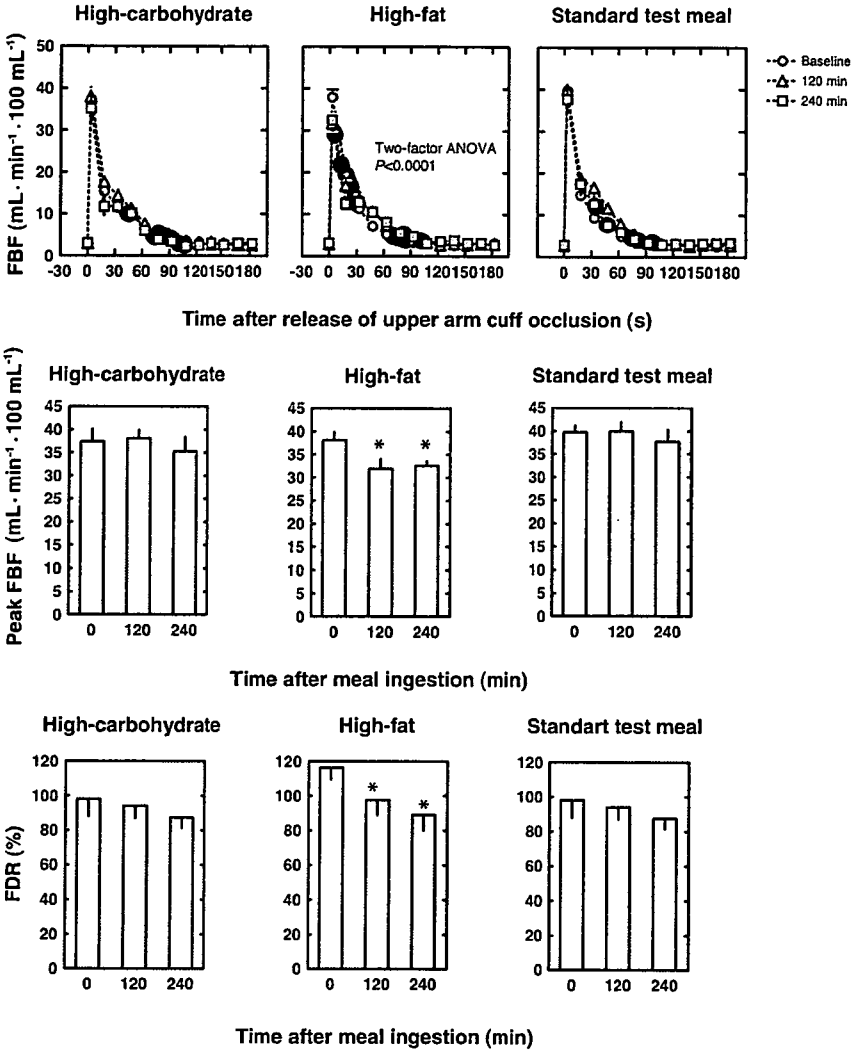

FIGURE 3. Effects of dietary composition (high-carbohydrate, high-fat, or standard test meal) on forearm blood flow (FBF) and total reactive hyperemic flow (flow debt repayment; FDR) in 12 healthy subjects (6 men and 6 women). A: Measurements were taken at baseline and during reactive hyperemia 120 and 240 min after the meals. B: Peak FBF was measured during reactive hyperemia before (0 min) and 120 and 240 min after the meals. C: Total reactive hyperemic flow (FDR) was measured during reactive hyperemia. FDR (%) = (reactive hyperemic flow/blood flow debt) × 100. Data are presented as means ± SEMs. One-factor ANOVA with Tukey's post hoc test was used to compare intragroup means. *Significantly different from 0 min, $P < 0.01$.

glucose, insulin, and serum lipid concentrations were different, and postprandial forearm endothelial function was impaired after high-fat diet but not after the high-carbohydrate and standard test meals.

Postprandial hyperlipidemia (4) and postprandial hyperglycemia independently produce endothelial dysfunction and both are now largely recognized as potential underlying mechanisms of macrovascular events in subjects with normal or impaired glucose tolerance (1, 2). Postprandial state is a complex dynamic phase. Hyperlipidemia, hyperglycemia, or changes in the bloodstream of various humoral factors are simultaneously present in the postprandial phase. Thus, the specific molecule mostly responsible for endothelial dysfunction has not been determined. We therefore examined the effects of diet composition on postprandial biochemical variables and endothelial function. The study was done on 3 separate mornings, and the order in which the 3 types of meals were ingested was randomized in a crossover design to minimize the other confounding factors.

Plasma glucose and insulin concentrations increased at 60 min and returned to baseline at 240 min after the high-carbohydrate and standard test meals but did not change after the high-fat meal. Serum FFA and triacylglycerol concentrations increased only at 120 and 240 min after the high-fat meal. Plasma adiponectin concentrations did not change during the 240 min after either test meal. Some (13, 14), but not all (15), studies reported that adiponectin concentrations changed postprandially. Esposito et al

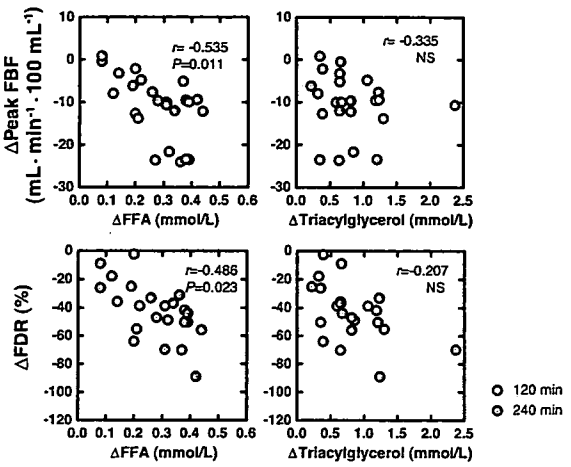

FIGURE 4. Changes from baseline in peak forearm blood flow (Δpeak FBF) and flow debt repayment (ΔFDR) and changes in serum free fatty acids (ΔFFA) and triacylglycerols 120 and 240 min after high-fat meal loading in 12 healthy subjects. After adjustment by ANCOVA with the use of time point after loading as covariance, Pearson's correlation coefficients (r) and P values were calculated.

(13) reported that adiponectin concentrations decreased from baseline after the high-fat meal, but not after the high-carbohydrate meal. Although the reason for the discrepancy between their study and ours is unknown, the difference in the percentage of energy as fat of the diets could be one reason (60% compared with 32.9%).

We reported that the high-carbohydrate and standard test meals did not produce endothelial dysfunction in healthy subjects with normal glucose tolerance (16, 17). However, it has been reported that the high-fat meal could impair endothelial function, even in healthy subjects (4–7). Our results confirm the previous findings that endothelial function is impaired after ingestion of a high-fat meal but not after ingestion of a high-carbohydrate or standard test meal.

As shown in Figure 4, decreases in peak FBF and FDR after a high-fat meal were well correlated with increases in serum FFA concentrations, but not with the other biochemical variables, including triacylglycerol, insulin, glucose, total cholesterol, HDL cholesterol, or adiponectin. Because adiponectin concentrations did not change after the meals, adiponectin oscillation is unrelated to postprandial changes in acute vascular reactivity. Our previous study showed that plasma adiponectin concentrations were well correlated with baseline (fasting) endothelial function in nondiabetic subjects (11). We postulated that there are 2 possible mechanisms by which hypoadiponectinemia decreases endothelial function: *1*) hypoadiponectinemia can cause endothelial dysfunction by decreasing insulin sensitivity, and *2*) hypoadiponectinemia may be directly linked to early atherosclerotic vascular damage and subsequent endothelial dysfunction.

In the present study, we showed that the FFA concentration, rather than the plasma adiponectin concentration, is mainly associated with postprandial endothelial dysfunction in healthy subjects. Taken together, hypoadiponectinemia could be linked to chronic vascular injury but not to acute postprandial vascular injury. It has been shown that the FBF response to the intraarterial infusion of acetylcholine was impaired acutely after systemic infusion of lipid and heparin in healthy subjects (9, 10). Previous findings support the notion that acute hyperlipacidemia directly impairs endothelial function, which was observed in this study.

The mechanism for such FFA-related endothelial dysfunction could not be drawn from this study. One can assume that hypertriglyceridemia, a cumulative effect of endothelial activation, or both may be associated with increased serum concentrations of inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (13).

Another possible explanation is the production of reactive oxygen species (ROS) by FFAs. Ceriello (7) reported that increased production of ROS, determined by nitrotyrosine, was closely associated with endothelial dysfunction after consumption of a high-fat meal. We confirmed that FFA-induced endothelial dysfunction could be protected by co-infusion of antioxidant, vitamin C, in healthy humans (10). Experimentally, we observed that FFA directly inhibited vascular response to acetylcholine in stripped aorta isolated form normal animal models (19). Also, we found that FFA directly enhanced the production of vascular ROS via up-regulation of NADPH oxidase subunit mRNA, and the inhibition of ROS production by *N*-acetylcysteine (a general antioxidant), diphenyleneiodonium, and apocynin (NADPH oxidase inhibitors) can prevent FFA-induced endothelial dysfunction (19).

In summary, we investigated the effects of diet composition on postprandial plasma concentrations of adiponectin and endothelial function in healthy subjects. Postprandial endothelial function was impaired only after ingestion of the high-fat diet and not after consumption of the high-carbohydrate and standard test meals. Because such endothelial dysfunction after a high-fat meal was closely correlated with FFA concentrations, the postprandial state could be hazardous, mostly through acute hyperlipacidemia in healthy subjects.

None of the authors had a conflict of interest.

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別紙3

研究報告書

厚生労働科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
分担研究報告書

血圧に関する診断のエビデンスに関する研究

分担研究者　伊藤貞嘉　東北大学大学院医学系研究科内科病態学講座教授

研究要旨：糖尿病性腎症の進展抑制には、血圧、血糖、脂質の管理とレニン・アンジオテンシン系(RAS)の抑制が重要である。尿中アルブミン/クレアチン（ACR）が100-300の2型糖尿病患者をCa拮抗薬またはRAS抑制薬の初期用量で1年間、増量してさらに1年間治療したときの、到達血圧レベル、投与薬剤、ACRの変化を検討した。同じ到達血圧レベルではRAS抑制薬のACR減少効果が大きかった。Ca拮抗薬でもRAS抑制薬でもACRの減少は降圧に依存しており、ACRが正常化した群の収縮期血圧は120mmHg前後であった。糖尿病性腎症の治療では微量アルブミン尿期から至適血圧(120/80mmHg未満)にコントロールすることが重要と考えられる。

A．研究目的

糖尿病性腎症の進行抑制や病期の改善に必要な降圧レベルを明らかにする。

B．研究方法

ACRが100-300の2型糖尿病患者をCa拮抗薬またはRAS抑制薬の初期用量で1年間、増量してさらに1年間治療したときの、到達血圧レベル、投与薬剤、ACRの変化を検討した。

（倫理面への配慮）

倫理委員会の承認を得ている。

C．研究結果

同じ到達血圧レベルではRAS抑制薬のACR減少効果が大きかった。Ca拮抗薬でもRAS抑制薬でもACRの減少は降圧に依存しており、ACRが正常化した群の収縮期血圧は120mmHg前後であった。

D．考察

糖尿病性腎症の進展抑制には、血圧、血糖、資質の管理とRASの抑制が重要である。RAS抑制薬の腎保護効果は降圧作用とは独立したものであると考えられていたが、本研究の結果は、RAS抑制でも降圧が極めて重要であることを示す。微量アルブミンから正常アルブミンへの病期改善には至適血圧までの降圧が推奨される。

E．結論

糖尿病性腎症の治療においては微量アルブミン尿期から至適血圧(120/80mmHg未満)にコントロールすることが重要と考えられる。

G．研究発表

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H．知的財産権の出願・登録状況
（予定を含む。）

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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小川晋、森建文、伊藤貞嘉	糖尿病の合併症と対策　血圧管理	渡邊有三、羽田勝計、馬場園哲也	新しい糖尿病学と透析医療	日本メディカルセンター	東京	2007	141－145
角田一男、伊藤貞嘉	心腎連関からみた管理	羽田勝計、槇野博史	糖尿病性腎症基礎と臨床の最前線	中外医学社	東京	2007	157－165

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Naritomi H, Fujita T, Ito S, Ogihara T, Shimada K, Shimamoto K, Tanaka H, Yoshiike N.	Efficacy and Safety of Long-Term Losartan Therapy Demonstrated by a Prospective Observational Study in Japanese Patients with Hypertension: The Japan Hypertension Evaluation with Angiotensin II Antagonist Losartan Therapy (J-HEALTH) Study.	Hypertens Res.	31(2)	295-304	2008
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厚生科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
分担研究報告書

脂質に関する診断のエビデンス

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研究要旨

わが国の２型糖尿病患者コホートの中間解析により、日本人２型糖尿病患者のメタボリックシンドロームの特徴を検討した。脂質の管理ではnonHDL コレステロールの意義について検討し、高中性脂肪血症における有用なリスクマーカーであることを示した。わが国におけるメタボリックシンドロームの脂質の基準については国際的なガイドラインとは明らかに異なり、その科学的根拠を明らかにするべきである。

A. 研究目的

メタボリックシンドロームは生活習慣の欧米化を背景に心筋梗塞、脳卒中や糖尿病を起こしやすい病態として、俄かに注目されている。メタボリックシンドロームでは肥満（なかでも動脈硬化をおこしやすい内臓脂肪蓄積型肥満：内臓肥満）やインスリン抵抗性とともに高血圧、高血糖、高脂血症（高中性脂肪血症あるいは低HDL コレステロール血症）を重複しやすいことが特徴であり、重複することにより心筋梗塞、脳卒中や糖尿病を生じやすいハイリスク病態となる。

メタボリックシンドロームの診断基準は病態を反映するのみならず、実用的であるべきだが、診断基準のみが一人歩きして、科学的な病態概念・基盤を見失わないように留意すべきである。脂質の診断基準については国際的な基準と異なることから、科学的な検証を行う必要がある。

B．研究方法

JDCS 研究対象者におけるメタボリックシンドロームについて解析した。登録患者は、全国59 ケ所の糖尿病専門施設外来に通院中の、日本糖尿病学会の診断基準に合致する45-70 歳の２型糖尿病患者で、開始時に HbA_{1c} 6.5%以上であった 2200 名である。全登録患者の開始時平均年齢は59 歳、平均糖尿病罹患年数は11 年、平均 HbA_{1c} 値は約 7.7%であった。各患者の血糖、血清脂質、血圧などのコントロール状態、および細小血管（網膜症・腎症・神経障害）・動脈硬化合併症などが平成８年度から毎年調査されてきた。動脈硬化合併症の主要なエンドポイントは、虚血性心疾患（狭心症、心筋梗塞）または脳卒中（脳梗塞、脳出血）の発症である。

（倫理面の配慮）

本研究のプロトコールは、各施設の倫理委員会によって承認されており、本研究へ参加する患者には、本研究の目的や実施方法について説明がなされ、informed consent を取得している。

C&D. 研究結果と考察

腹囲に関してわが国の基準（男性及び女性の腹囲基準を各々85cm, 90cm 以上）と IDF 基準（男性及び女性の腹囲基準を各々90cm, 80cm 以上）について比較検討した。2 型糖尿病においてわが国の基準ではメタボリックシンドロームの頻度は男性 32.1%、女性 9.1%であった。女性の頻度が極めて少ないことが特徴であり、糖尿病女性のハイリスク者を確実にスクリーニングすることが困難であることを示唆した。男性の脂質代謝異常を有するメタボリックシンドローム群が有意に虚血性心疾患のハイリスク群であった。一方、IDF 基準を用いた場合、メタボリックシンドロームの頻度は男性 16.1%、女性 31.7%であった。女性の頻度が増加し、男性より頻度が高くなった。男女ともに、IDF 基準ではメタボリックシンドロームは有意に虚血性心疾患のハイリスク群であった。従って、女性のハイリスク者をスクリーニングする観点から、わが国の腹囲基準を再検討すべきと考える。

原発性高脂血症調査研究班において、nonHDL コレステロールを指標とする高中性脂肪血症の診療指針を示した。nonHDL コレステロールは LDL コレステロールに加えて、レムナント、IDL, small dense LDL、HDL の要素を包含するのみならず、食事の影響も最小であることや計算が容易であることが大きなメッリトである。一方、計算式によるは LDL コレステロール値は高中性脂肪血症（>400mg/dl）では不正確であることや、直接法による LDL コレステロール値は標準化が不十分であるなどの限界が指摘されている。

nonHDL コレステロール値の目安は LDL コレステロール+３０mg/dl である。その診療指針はリスク評価などの病態評価も含めて、現在の LDL コレステロールの診療指針（動脈硬化性疾患予防ガイドライン）に準拠しながら、nonHDL コレステロールの是正を目標とすればよい。2 型糖尿病患者の虚血性心疾患のリスクについて解析したところ、nonHDL コレステロールは LDL コレステロール及び中性脂肪を包含する指標であることが明らかとなった。

E．結論

内臓肥満を共通の病態として位置づける IDF(International Diabetes Foundation)基準はわが国の診断基準の基礎となっている。内臓肥満は国際的な基準の統一がなされていないことや男女差が大きいことが大きな課題であり、女性の肥満は皮下脂肪型であることに留意すべきである。

アメリカ心臓病学会を中心に用いられている AHA/NHLBI 基準では、病態の分子基盤が十分に解明されていないことから、一つの項目のみを必須項目としてウェイトをおくべきではないという立場とっている。高血糖、高血圧、TG, HDL, 肥満の中で 3 つが揃えばメタボリックシンドロームとするという見解を堅持しており、この診断基準は国際的にも汎用されている。IDF 基準、AHA/NHLBI 基準の何れも、わが国の基準と異なり、高中性脂肪血症および低 HDL-コレステロール血症を別個のリスク要因として評価することが特徴であり、HDL 基準値に男女差を設定している。この脂質基準について、今後検証されるべきである。

動脈硬化性疾患の男女差、脂質代謝の男女差、肥満の男女差は明らかであり、新たな診断基準作成においては男女差を十分に評価するべきである。

F． 健康危険情報

該当事項なし

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H. 知的財産権の出願、登録状況

該当事項なし

平成１９年度厚生労働科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
分担研究報告書

メタボリックシンドロームの減量治療における血中リポ蛋白リパーゼ量の意義

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研究要旨　メタボリックシンドロームと密接に関わる肥満症の減量治療は食事療法と運動療法であり、その実践には行動修正療法が必要である。メタボリックシンドロームの病態をあらわす臨床指標として内臓脂肪蓄積が重要であり、内臓脂肪蓄積は合併症の病因と考えられるインスリン抵抗性と密接に関わる。本研究はメタボリックシンドロームの健康管理で重要な位置を占める減量治療における食事療法および運動療法による血中リポ蛋白リパーゼ（LPL）蛋白量の臨床的意義を明らかにする。対象は減量治療中の肥満症症例である。肥満症患者の食事療法を行うにあたり、REEはインスリン抵抗性と密接に関与し、血中LPL蛋白量は肥満症患者の減量治療中のREEの変動を反映した。このように、LPLは、肥満症治療経過における安静時エネルギー代謝量の変化を密接に関わる。また、肥満症の運動療法おける血中HDL-C反応に血中LPL蛋白の変動が関与する。運動前の血中LPL値によるΔHDL-C量の変動を解析したところ、LPL高値群でΔHDL-C量が低下するのに対しLPL低値群で有意に上昇した。

以上の結果から、メタボリックシンドロームの健康管理および減量治療を行うにあたり、食事療法にともなうインスリン抵抗性の変化を把握するにはREEの変化と合わせてLPL測定を行うことが有用であり、運動療法により血中LPL値を増加させることが低HDL-C血症を改善することにつながる。

A．研究目的

メタボリックシンドロームと密接に関わる肥満症の減量治療は食事療法と運動療法であり、その実践には行動修正療法が必要である。メタボリックシンドロームの病態をあらわす臨床指標として内臓脂肪蓄積が重要であり、内臓脂肪蓄積は合併症の病因と考えられるインスリン抵抗性と密接に関わる。これまでに我々は、減量早期より内臓脂肪が減少するとともにインスリン抵抗性が改善し、インスリン作用により制御されることが知られるリポ蛋白リパーゼ（LPL）蛋白および活性が上昇し高トリグリセリド（TG）血症が改善することを明らかにした。細胞移植法を用いた血中 LPL 蛋白過剰発現モデルマウスではインスリン感受性が亢進する。このように、血中 LPL 蛋白量は内臓脂肪蓄積およびインスリン抵抗性と密接に関連する。これらの成績から、血中 LPL 蛋白量の測定は一般住民におけるインスリン抵抗性およびメタボリックシンドロームの病態をあらわす日常指標として有用な可能性がある。本研究は減量治療における食事療法および運動療法による血中LPL蛋白量の臨床的意義を明らかにする。

B．研究方法

1）安静時エネルギー代謝量（REE）とLPLの関係

対象は肥満症24症例(平均年齢42歳、BMI 33.2kg/m²)。REE は簡易熱量計により換気量および酸素消費量を測定

しハリスベネディクト式による予想値との差異であるΔREEを算出した。血中LPL蛋白量（45 mg/ml）により2群に分けて比較した。血中LPL蛋白量はヘパリン静注前血清を用いてELISA法により測定した。

2）運動療法中のKDL-C変動とLPLの関係

対象は肥満症20症例（平均年齢45歳、BMI 35.6）である。有酸素運動（歩行6000歩以上）、ストレッチ、筋力運動を6ヶ月間外来指導し、指導前後における血中脂質、LPL蛋白量、HOMA指数を測定した。

（倫理面への配慮）

研究解析に関しては研究実施機関における倫理委員会の承認の上、施行した。

C．研究結果

1）安静時エネルギー代謝量（REE）とLPLの関係

LPL低値群（12症例、LPL 32±7 mg/ml）はLPL高値群（12症例、LPL 62±17 mg/ml）に比べて、血中インスリン、TGが有意（$p<0.05$）に高値、HDL-Cが有意（$p<0.05$）に低値だった。REEとハリス予想値の差異であるΔREE／BMIはLPL低値群がLPL高値群に比べて有意（$p<0.05$）に高値だった。全症例のΔREE／BMIは血中LPL蛋白量と負の相関（$r=0.41$, $p<0.05$）を示した。そこで、同一症例における減量過程でのREEとLPLの推移を解析したところ、肥満症の減量過程においてLPLが上昇する症例でREEが低下した。

2）運動療法中のKDL-C変動とLPLの関係

運動療法前後の血中TG変化およびHDL-C変化は症例により異なり増加する症例、減少する症例また変わらない症例が存在した。しかしながら、運動前後の血中TG変化量とHDL- C変動量は有意な負の相関（$r=0.69$, $p<0.01$）を示した。HDL-C増加群（10症例）と非増加群（10症例）における血中LPL増加量はHDL-C増加群で有意に高値だった（$p<0.05$）。全症例のΔHDL-C量とΔLPL量は有意な正の相関を示した（$r=0.60$、$p<0.05$）。そこで、運動前の血中LPL値（40 ng/ml）によるΔHDL-C量の変動を解析したところ、LPL高値群が低下するのに対しLPL低値群で有意に上昇した。

DおよびE．考察および結論

肥満症患者の食事療法を行うにあたり、REEとインスリン抵抗性が密接に関与し、血中LPL蛋白量は肥満症患者のREEの変動を反映すると考えられる。LPLは、肥満症治療経過における安静時エネルギー代謝量の変化を密接に反映する。また、肥満症の運動療法おける血中HDL-C反応に血中LPL蛋白の変動が関与する。

以上の結果から、メタボリックシンドロームの健康管理および減量治療を行うにあたり、食事療法にともなうインスリン抵抗性の変化を把握するにはREEの変化と合わせてLPL測定を行うことが有用であり、運動療法により血中LPL値を増加させることが低HDL-C血症を改善することにつながる。

F．健康危険情報

特記事項なし

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H. 知的財産権の出願、登録状況

特になし。

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ohwaki K, Bujo H, Jiang M, Yamzaki H, Schneider WJ, Saito Y.	A secreted soluble form of LR11, specifically expressed in intimal smooth muscle cells, accelerates a formation of lipid-accumulated macrophages.	Arterioscler Thromb Vasc Biol.	27(5)	1050-6	2007
Kubota Y, Unoki H, Bujo H, Rikihisa N, Udagawa A, Yoshimoto S, Ichinose M, Saito Y.	Low-dose GH supplementation reduces the TLR2 and TNF-alpha expressions in visceral fat.	Biochem Biophys Res Commun.	368	81-87	2008
Murakami K, Bujo H, Unoki H. Saito Y.	Effect of PPARalpha activation of macrophages on the secretion of inflammatory cytokines in cultured adipocytes.	Eur. J. Pharmacol.	561(1-3)	206-13	2007

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-

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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

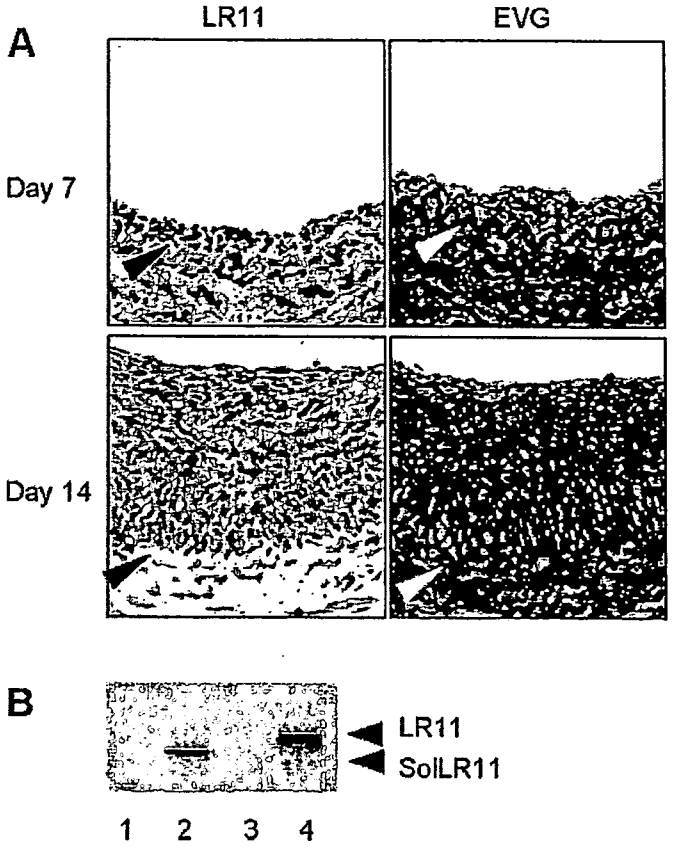

Figure 1. SolLR11 expression in intimal SMCs in balloon-denuded rat artery. A, Sections of balloon-denuded carotid artery were subjected to histological analysis using elastica van Gieson staining (EVG), and to immunohistochemistry with anti-LR11 antibody (pm11) at day 7 (top) and day 14 (bottom) after 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.

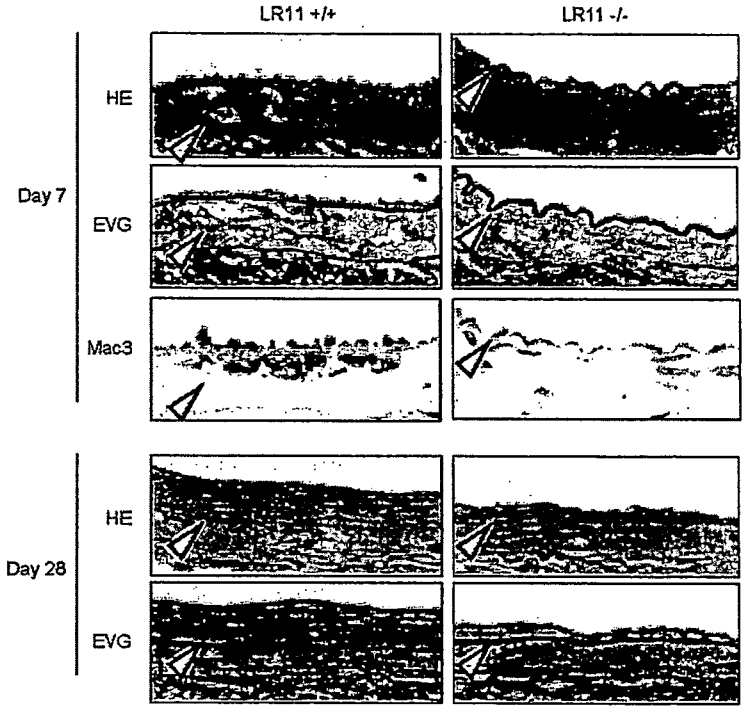

Figure 2. Reduced macrophage infiltration into intima of cuff-injured artery in LR11$^{-/-}$ mice. Sections of femoral artery obtained at day 7 and day 28 after cuff injury in LR11$^{+/+}$ and LR11$^{-/-}$ mice on a high-fat diet were subjected to histological analysis using hematoxylin & eosin (HE) 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

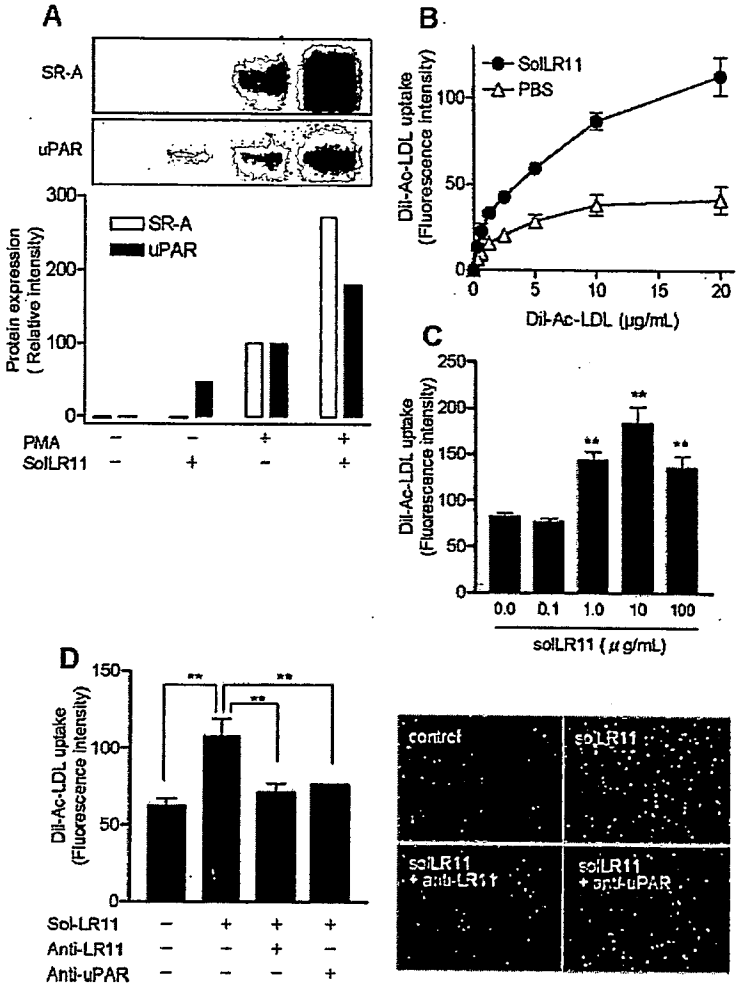

Figure 3. SolLR11 enhances cell-surface expression of SR-A and uPAR, and the uptake of modified LDL by THP-1 macrophages. A, Membranes of THP-1 monocytes or macrophages, prepared from cells obtained under the conditions indicated in the bottom panel, were subjected to Western blotting with anti-SR-A 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 DiI-AcLDL in the presence or absence of excess amount of Ac-LDL. C, Dose-dependent effect of solLR11 on DiI-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 DiI-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-