1%、女性31%)であった。
平均観察期間(男性1346日、女性1358日)で、初発心筋梗塞(男性21例、女性12例)、初発労作性狭心症(男性227例、女性12例)、初回冠動脈インターベンション(男性221例、女性6例)、初発脳卒中(男性49例、女性29例)、急性死(男女とも0例)、死亡(男性4例、女性0例)であった。 急性心筋梗塞の発症率は、男性2.01、女性1.03(千人年)、冠動脈疾患(急性心筋梗塞+労作性狭心症)男性3.40、女性2.06(千人年)、脳卒中3.47、女性2.49(千人年)であった。内服薬服用率は、降圧薬(男性17%、女性16%)、脂質異常症治療薬(男性6%、女性7%)、糖尿病治療薬(男性5%、女性2%)であった。

D. 考察

メタボリックシンドロームのコンポーネントを含めた動脈硬化性疾患リスクファクターの陽性率と心血管イベントの発生を全件調査した。 平均観察期間男性1346日、女性1358日で、急性心筋梗塞の発症率は、男性2.01、女性1.03(千人年)、冠動脈疾患(急性心筋梗塞+労作性狭心症)男性3.40、女性2.06(千人年)、脳卒中3.47、女性2.49(千人年)といずれも男性が女性に比較して、高率であった。
一方で、腹部肥満、高血圧、高血糖、高中性脂肪、低HDL血症の陽性率、喫煙率、習慣飲酒率でも、男性が女性に比較して高く、高コレステロール血症は男女で有病率に差がなかった。

E. 結論
心血管イベントの発生率には男女差があり、その発生要因も明らかな差がある。メタボリックシンドロームの診断・管理のためには、男女差を考慮にいれて個別のアプローチ基準を設定する必要があると考えられた。

F. 健康危険情報
(総括研究報告書にまとめて記入)

G. 研究発表
1. 論文発表
別紙4参照
2. 学会発表
(発表誌名巻号・頁・発行年等も記入)

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
島袋充生	心血管イベントとNASH	西原利治	NASH診療ハンドブック	中外医学社	東京	2007	72-81頁
島袋充生	メタボリックシンドロームにおける脂質代謝異常とフィブラート	山田信博	フィブラートupdate	フジメディカル出版	大阪	2008	26-29頁
島袋充生	脂肪毒性オーバービュー	日本糖尿病学会	糖尿病学の進歩第42集	診断と治療社	東京	2008	71-78頁

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Maeda Y, Inoguchi T, Tsubouchi H, Sawada F, Sasaki S, Fujii M, Saito R, Yanase T, Shimabukuro M, Nawata H, Takayanagi R.	High prevalence of peripheral arterial disease diagnosed by low ankle-brachial index in Japanese patients with diabetes: The Kyushu Prevention Study for Atherosclerosis.	Diabetes Res Clin Pract	73	27-34	2009
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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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島袋充生	脂質異常症がかかわる動脈硬化進展のメカニズム	薬局	59	9-13	2008
島袋充生	食後高血糖と血管内皮機能	動脈硬化予防	7	34-40	2008
島袋充生	インスリン分泌低下にかかわる因子 脂肪毒性	日本臨床	66 巻(増刊 3)	422-427.	2008
島袋充生	肥満を誘因とする病態	日本臨床	66(増刊 4)	621-627	2008
島袋充生	膵β細胞リポトキシシティ	The Lipid	19	242-247	2008
島袋充生	肥満と心血管不全	Life Style Medicine	2	132-138	2008
島袋充生	体質医学からみた血管病:メタボリックシンドロームの視点から 循環器(基礎)の立場から	日本体質医学会雑誌	70	50-54	2008

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

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研究要旨：J-HEALTH研究のデータを用い、日本人の高血圧患者において、降圧レベルと心血管病の関連を検討した。ロサルタンによる降圧治療を受けていた総数26,512人の高血圧患者を最長５年、平均で３年追跡調査をした。年齢、糖尿病、心血管病の既往及び喫煙が心血管病発症の独立した危険因子であった。心血管病の発症は治療中の血圧と正の相関を示し、血圧値が140/90mmHg以上では有意に心血管病のリスクが高くなっていた。また、どの年齢層をとっても、血圧が低いほうが心血管病の発症が低かった。

A．研究目的

日本における高血圧患者において降圧レベルと心血管病との関連を明らかにする。

B．研究方法

２０歳以上の未治療高血圧患者26,512人をロサルタン（25－100mg/日）で治療し、3ヶ月以降は他のクラスの降圧薬の投与を可能とした。

（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

年齢、糖尿病、心血管病の既往及び喫煙が心血管病発症の独立した危険因子であった。年齢が高くなるほど、心血管病の発症は高くなっていた。しかし、どの年代でも、血圧のレベルと心血管病の発症の間には正の相関が見られた。

D．考察

心血管病の発症には血圧レベル以外にも様々な危険因子があげられている。また、高齢者においては高圧療法を行う利益があるのかどうかは十分に解明していない。本研究では、日常診療における血圧管理と心血管病の関連を明らかにした。血圧を十分にコントロールすることの意義が明らかになった。

E．結論

どの年代層においても、血圧レベルは重要な心血管病のリスクとなる。少なくても140/90mmHg未満への降圧を達成すべきである。

G．研究発表

1．論文発表

Shimamoto K, Fujita T, Ito S, Naritomi H, Ogihara T, Shimada K, Tanaka H, Yoshiike N: Impact of Blood Pressure Control on Cardiovascular Events in 26,512 Japanese Hypertensive Patients: The Japan Hypertension Evaluation with Angiotensin Ⅱ Antagonist Losartan Therapy (J-HEALTH) Study, a Prospective Nationwide Observational Study; Hypertens Res 31: 469-478, 2008

2．学会発表

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

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。本コホートの８年次固定データベースにおいて、冠動脈疾患（狭心症と心筋梗塞）と脳卒中（脳出血、一過性脳虚血発作と脳梗塞）の発症率ならびに腹囲を中心としたリスクファクターについて解析した。患者 1000 人あたりの冠動脈疾患および脳卒中の年間発症率はそれぞれ 9.6、7.9 であった。腹囲については有意な危険因子ではなかった。

A. 研究目的

メタボリックシンドロームでは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。内臓肥満を必須項目とする IDF 基準はわが国の診断基準の基礎となっている。AHA/NHLBI の基準では病態の分子基盤が多様であることから、各構成要素の取り扱いに現状では差をつけるべきではないという立場をとり、必須項目をおいていない。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。

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

1000 人年あたり冠動脈疾患発症率は、 9.6 (男 11.2 女 7.9)、脳卒中で 7.6 (男 8.5 女 6.6) であった。本研究コホートにおいては冠動脈疾患が脳卒中より多いという特徴がみられている。これは２型糖尿病患者において、脳卒中と同等以上に冠動脈疾患に対する予防対策が必要であることを示している。これらの大血管合併症の内訳は、冠動脈疾患では狭心症と心筋梗塞が６対４であり、脳卒中では８割以上が脳梗塞であった。

腹囲とリスクファクター個数、イベントとの関係検討したところ、腹囲はリスクファクターの個数が増えるにつれて、有意なトレンドを持って、ステップワイズに増加した。しかし一方、実際にイベントを起こした者と起こさなかった者の腹囲には有意差が認められなかった。このことは、２型糖尿病患者における腹囲が、リスクファクター集積状況と良く相関していたものの、イベントとの予測には役立たなかったことを示している。

そこで実際に、腹囲をその一項目として含む現在のメタボリックシンドロームの診断基準を改変することによって、腹囲を活かしながら大血管合併症イベントを予測することが可能かどうかを検討した。腹囲閾値を日本の男性 85cm、女性 90cm からアジア人一般用の男性 90cm、女性 80cm に置換した場合、リスクが重なる部分すべてにおいて、大血管合併症発症リスクが有意に上昇していることが明らかとなった。

表１は、日本人２型糖尿病患者に適切なメタボリックシンドローム診断基準とでも言うべきものである。心血管疾患発症リスクの高い２型糖尿病患者において、さらに２つ以上心血管リスクの追加があると、有意なリスク上昇となる、という結果が得られた。

E. 結論

日本の２型糖尿病患者を対象とした前向き追跡調査により、冠動脈疾患と脳卒中の発症率やリスクファクターを解析した。日本人においても２型糖尿病患者においては、従来の日本型の動脈硬化疾病構造とは異なり、冠動脈疾患が脳卒中と同程度以上に発症しやすく、いかにこれを早期に発見し対策を立てるかが重要である。

その発症のリスク評価のためには、現在のメタボリックシンドロームの診断基準は適切でなく、腹囲の閾値を変更した上で、これを必須としない修飾を加えることが有用であることが判明した。

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ると、女性の腹囲基準についてさらに検討が必要である。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。

ちなみに、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。

F. 健康危険情報

該当事項なし

G. 研究発表

論文発表

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5.

H．知的財産権の出願、登録状況

該当事項なし

表1　JDCS 登録患者において、大血管合併症発症リスクの有意な上昇を予測していた条件（日本人2型糖尿病患者用の修飾版メタボリックシンドローム診断基準）

Suggested modification of MetS definition for Japanese T2DM patients for predicting future CVD events

Patients who possess 2 or more of the following;

1) Excess WC:
   men ≥ 90 cm, women ≥ 80 cm
2) Hypertension:
   systolic BP≥ 130 mmHg diastolic BP≥ 85 mmHg or use of agent for this condition
3) Dyslipidemia:
   TG ≥150 mg/dl and/or HDL-C < 40 mg/dl or use of agent for this condition

(Sone H, Yamada N, et al. *J Atheroscl Thromb*, in press)

平成２０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）
研究分担報告書

動脈硬化における血管平滑筋特異的分子 LR11 血中濃度の測定意義

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研究要旨　メタボリックシンドロームに伴う動脈硬化症の形成過程で血管平滑筋細胞は中膜から内膜へ遊走、増殖し多彩な機能を獲得する。したがって、メタボリックシンドロームの診療において内膜平滑筋細胞の性状をあらわす特異的バイオマーカーを樹立することが有用である。本研究の目的は内膜平滑筋細胞に特異的に発現する遺伝子 LR11 の可溶型蛋白を用いた新規の血中バイオマーカーを樹立し、動脈硬化の発症および進展における臨床的意義を確立することである。動脈硬化の指標として頚動脈内膜中膜肥厚度（IMT）を測定した 405 血液サンプルの各基本データの相関を検討したところ、IMT は年齢、BMI、収縮期および拡張期血圧値、トリグリセリド値、インスリン値、血中可溶型 LR11 濃度と有意（$P<0.05$）な正の相関を示した。また、HDL-コレステロール、LDL サイズと有意な負の相関を示した。２群間解析では有意に男性、喫煙者で高値だった。多変量解析の結果、これらの因子の中で血中可溶型 LR11 濃度は IMT を規定する独立因子だった。IMT 高値に対して血中可溶型 LR11 濃度 2.13-2.78 では 2.13 以下に比べて 1.93 倍、2.79-3.52 では 2.96 倍、3.52 以上では 7.6 倍のオッズ比を示した。以上の結果から、血中可溶型 LR11 濃度は頚動脈内膜中膜肥厚度と密接に関連し従来の動脈硬化危険因子とは独立した動脈硬化マーカーとなることが示唆された。メタボリックシンドロームにともなう動脈硬化症の新規のバイオマーカーとなる可能性がある。

A．研究目的

メタボリックシンドロームにともなう動脈硬化症の形成過程で血管平滑筋細胞は中膜から内膜へ遊走、増殖し、血管壁細胞との相互作用、細胞外マトリックスの産生と代謝など多彩な機能を獲得する。内膜平滑筋細胞の機能は糖尿病、高脂血症等の生活習慣病で修飾されプラークの性状が変化する。この変化を早期より検出するには精密な画像診断とともに血管障害をあらわす定量的な血中指標が必要だが、糖尿病、高脂血症等にともなう早期の動脈硬化の進展を特異的かつ定量的に評価する血中指標を確立するには至っていない。これまでに我々は、動脈硬化の進展における血管壁細胞の病因論的な機序解明を、とりわけ血管平滑筋細胞の病的フェノタイプという概念を提示し基盤となる分子生物学的解明を行ってきた。すなわち、平滑筋細胞は、収縮型および合成型フェノタイプの変換に加えて、動脈硬化をひき起こしやすい病態では、正常に増殖や遊走が制御されにくい形質へと変換される。この分子基盤として、動脈硬化巣の肥厚内膜に存在する平滑筋細胞に特異的に発現し、平滑筋細胞の遊走を制御する分泌蛋白である LDL 受容体スーパーファミリーである LR11 を同定した。この LR11 は分泌可溶型として存在し免疫学的に同定することが可能となった。

Lr11$^{-/-}$マウスの内皮傷害後の反応性内膜肥厚は対照マウスに比べて著しく低下する。*Lr11*$^{-/-}$血管平滑筋細胞ではウロキナーゼ受容体とインテグリンの会合が障害される結果、FAK リン酸化、ERK リン酸化、Rac-1 活性化が障害さ

れる。本研究の目的は内膜平滑筋細胞に特異的に発現する遺伝子LR11の可溶型蛋白による新規の血中バイオマーカーを樹立し、動脈硬化症の発症および進展における臨床的意義を確立することである。

B．研究方法

動脈硬化の指標として頚動脈内膜中膜肥厚度（IMT）を測定した405血液サンプルの可溶型LR11濃度をこれまでに樹立した免疫学的同定法により測定した。RAPカラムによる精製検体を電気泳動後、ナイロンメンブレンにトランスファーしてLR11特異的モノクローナル抗体を用いて反応させた。免疫学的反応をECLにより可視化した。免疫学的同定蛋白を画像処理した後に解析ソフトにより定量化した。測定結果とIMT、さらに動脈硬化に関わる危険因子との関連を検討した。

（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）IMTとヒト血中可溶型LR11濃度

405検体のIMTと各基本データとの相関を検討したところ、年齢、BMI、収縮期および拡張期血圧値、トリグリセリド値、インスリン値、ヒト血中可溶型LR11濃度と有意（$P<0.05$）な正の相関を示した。また、HDL-コレステロール、LDLサイズと有意な負の相関を示した。2群間解析では有意に男性、喫煙者で高値だった。

2）IMTと血中可溶型LR11濃度および動脈硬化危険因子

多変量解析の結果、IMTと年齢、血圧、HDL-コレステロール、血中可溶型LR11濃度の間で、血中可溶型LR11濃度はIMTを規定する独立因子であり、さらに、年齢、性別、BMI、血圧、喫煙、LDL-コレステロール、HDL-コレステロール、トリグリセリド、LDLサイズ、血糖、インスリン、血中可溶型LR11濃度の間においても、血中可溶型LR11濃度はIMTを規定する独立因子だった。

3）ヒト血中可溶型LR11濃度によるIMTリスク

血中可溶型LR11濃度4分割によるIMT高値へのオッズ比を解析すると、2.13-2.78では2.13以下に比べて1.93倍、2.79-3.52では2.96倍．3.52以上では7.6倍であった（年齢10歳増加のオッズ比は1.97倍）。

4）血中可溶型LR11濃度と動脈硬化危険因子の関係

血中可溶型LR11濃度と動脈硬化危険因子との関連を単解析検討すると、血中可溶型LR11濃度は男性で収縮期および拡張期血圧、IMTと有意な正の相関、HDL-コレステロールと負の相関を示した。また、女性でLDL-コレステロール、LDLサイズ、インスリン、IMTと有意な正の相関、HDL-コレステロールと負の相関を示した。これらの変数の間での多変量解析では男女ともにIMTが独立した規定因子だった。

DおよびE．考察および結論

動脈硬化巣の形成において平滑筋細胞が中膜から内膜に遊走し、内膜平滑筋細胞はさまざまな機能を獲得することにより動脈硬化巣の形成を修飾する。この修飾課程には、ほかの血管壁細胞である内皮細胞、浸潤細胞であるマクロファージやリンパ球との相互関係が重要である。このような位置づけにある内膜平滑筋細胞の機能変化を知る方法として新たな遺伝子発現を同定することが有用と考えられる。我々がこれまでに内膜平滑筋細胞で特異的に発現する遺伝子として同定したLR11遺伝子は、特に平滑筋細胞の遊走能の亢進に必要であり、LR11遺伝子が欠損することにより遊走能が低下する。内膜平滑筋特異的遺伝子LR11は、動脈硬化の進展における平滑筋細胞の中膜から内膜への遊走と増殖過程に重要であり、LR11機能を修飾することにより動脈硬化巣を変化させる可能性がある。*Lr11*$^{-/-}$マウス血管平滑筋細胞の解析から、LR11はウロキナーゼ受容体／インテグリンを介したとりわけAngII刺激での細胞アクチン再構成に重要であり、*Lr11*$^{-/-}$細胞ではこれが障害されることにより遊走能が減弱することが明らかになった。これらの基礎研究成果を基盤にして、本研究により血中可溶型LR11濃度が頚動脈内膜中膜肥厚度と密接に関連し、他変量解析の結果、従来の動脈硬化危険因子とは独立した動脈硬化マーカーとなることが示唆された。メタボリックシンドロームにともなう動脈硬化症の新規のバイオマーカーとなる可能性がある今後、内膜中膜肥厚度のみならず、メタボリックシンドロームを基盤にしたさまざまな動脈硬化の評価また病態における臨床的意義の解析が必要である。

F．健康危険情報

特記事項なし。

G．研究発表

論文発表

1) Jiang M, Bujo H, Ohwaki K, Unoki H, Yamazaki H, Kanaki T, Shibasaki M, Azuma K, Harigaya K, Schnerider WJ, Saito Y. AngII - stimulated migration of vascular SMC is dependent on LR11 in mice. J. Clin. Invest. 2008;118:2733-2746

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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
Jiang M, Bujo H, Ohwaki K, Unoki H. Yamazaki H, Kanaki T, Shibasaki M, Azuma K, Harigaya K, Schneider WJ, Saito Y.	AngII - stimulated migration of vascular SMC is dependent on LR11 in mice.	J. Clin. Invest.	118	2733-2746	2008
Kawamura T, Murakami K, Bujo H, Unoki H, Jiang M, Nakayama T, Saito Y.	Matrix metalloproteinase-3 enhances the free fatty acids-induced VEGF expression in adipocytes through toll-like receptor 2.	Exp. Biol. Med (Maywood).	233	1213-1221	2008
Unoki H, Bujo H, Jiang M, Kawamura T, Murakami K, Saito Y.	Macrophages regulate tumor necrosis factor-alpha expression in adipocytes through the secretion of matrix metalloproteinase-3.	Int. J. Obes.	32	902-911	2008

Ang II–stimulated migration of vascular smooth muscle cells is dependent on LR11 in mice

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Medial-to-intimal migration of SMCs is critical to atherosclerotic plaque formation and remodeling of injured arteries. Considerable amounts of the shed soluble form of the LDL receptor relative LR11 (sLR11) produced by intimal SMCs enhance SMC migration in vitro via upregulation of urokinase-type plasminogen activator receptor (uPAR) expression. Here, we show that circulating sLR11 is a novel marker of carotid intima-media thickness (IMT) and that targeted disruption of the *LR11* gene greatly reduces intimal thickening of arteries through attenuation of Ang II–induced migration of SMCs. Serum concentrations of sLR11 were positively correlated with IMT in dyslipidemic subjects, and multivariable regression analysis suggested sLR11 levels as an index of IMT, independent of classical atherosclerosis risk factors. In *Lr11*$^{-/-}$ mice, femoral artery intimal thickness after cuff placement was decreased, and Ang II–stimulated migration and attachment of SMCs from these mice were largely abolished. In isolated murine SMCs, sLR11 caused membrane ruffle formation via activation of focal adhesion kinase/ERK/Rac1 accompanied by complex formation between uPAR and integrin $\alpha v\beta 3$, a process accelerated by Ang II. Overproduction of sLR11 decreased the sensitivity of Ang II–induced activation pathways to inhibition by an Ang II type 1 receptor blocker in mice. Thus, we demonstrate a requirement for sLR11 in Ang II–induced SMC migration and propose what we believe is a novel role for sLR11 as a biomarker of carotid IMT.

Introduction

Migration of vascular SMCs from the media to the intima is a key step in the development of atherosclerosis (1, 2). Following migration, SMCs proliferate in the intima and secrete matrices and proteases to form atheromatous plaques under the influence of stimulatory cytokines. The system encompassing urokinase-type plasminogen activator (uPA) and its receptor (uPAR) is thought to be important for the migration of SMCs. For migration, cell-associated proteolysis of ECM and/or receptor-mediated signaling leading to activation of ERK and Rac1 (3, 4) play crucial roles. However, in regard to migration of SMCs relevant to the development of atherosclerosis and remodeling of injured arteries, the mechanisms for and significance of uPA/uPAR activation have not been fully elucidated yet.

Functional studies using genetically altered animals or cells have revealed that certain receptors belonging to the family of LDL receptor relatives (LRs) are important regulators of cell migration via modulating cytokine signaling and/or protease activation (5–7). Interestingly, LR-mediated regulation of migration appears to be caused at least in part by modulation of the uPA/uPAR system (6, 7). LR11 (also called *sorLA*), an unusually complex and highly conserved LR discovered and molecularly characterized by us and others (8–10), mediates uPAR's plasma membrane localization, as both the membrane-spanning form and the shed soluble form of the receptor (sLR11) bind to and colocalize with uPAR on the cell surface (11). LR11 is highly expressed in intimal SMCs at the intima-media border in the plaque area of experimental models of atherogenesis (11, 12). Furthermore, overexpression of LR11 in SMCs enhances their migration via elevated levels of uPAR (13).

In the process of atherosclerosis, the BB isoform of PDGF (PDGF-BB) is an important cytokine for migration of SMCs (1, 2). PDGF-BB performs its task through the stimulation of cell motility via accelerated cytoskeleton rearrangement and degradation of ECM components of intimal SMCs (1). Also, PDGF-BB enhances migration of intimal SMCs via activation of the LR11/uPAR-mediated intracellular pathway (14). Ang II is another potent chemoattractant for SMCs (1, 15, 16). Recent studies with Ang II type 1 receptor (AT_1R) blockers (ARBs) (17) or knockdown experiments (18) have shown that intracellular signals involving activation of Rac1 are important for the Ang II–mediated hypertrophy of SMCs and neointimal formation in injured arteries.

Here, we have studied the (patho)physiological significance of the LR11-mediated migration system of intimal SMCs in the development of intimal thickening in vivo. We found that circulating sLR11 levels reflect the degree of carotid intima-media thickness (IMT), an established atherosclerosis marker (19), independently of other risk factors of IMT in subjects with dyslipidemia. Subsequent studies using LR11-targeted mice revealed that sLR11 increases membrane

Nonstandard abbreviations used: ARB, AT_1R blocker; AT_1R, Ang II type 1 receptor; FAK, focal adhesion kinase; GST, glutathione-*S*-transferase; HDL-C, HDL cholesterol; I/M ratio; ratio of IMT; IMT, intima-media thickness; LDL-C, LDL cholesterol; LR, LDL receptor relatives; LRP1, LDL-related protein 1; NMHCII-B, nonmuscle myosin heavy chain II-B protein; OR, odds ratio; PDGF-BB, PDGF isoform with 2 B chains; RAP, receptor-associated protein; sLR11, shed soluble form of LR11; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1
Multivariate assessment of the effect of sLR11 and atherosclerotic risk factors on IMT

	OR (95% CI)	*P* values
Model 1		
Age, per 10 yr increase	2.18 (1.16–4.12)	0.02
BP, systolic, per 10 mmHg increase	0.79 (0.51–1.21)	0.27
BP, diastolic, per 10 mmHg increase	1.61 (0.80–3.21)	0.18
HDL-C, per 10 mg/dl decrease	0.89 (0.61–1.31)	0.56
sLR11, per 0.1 U increase	2.45 (1.48–4.07)	0.001
Model 2		
Age, per 10 yr increase	3.56 (1.55–8.19)	0.003
Sex	2.43 (0.46–12.85)	0.30
BMI	1.19 (0.91–1.55)	0.21
BP, systolic, per 10 mmHg increase	0.72 (0.44–1.19)	0.20
BP, diastolic, per 10 mmHg increase	1.74 (0.78–3.88)	0.18
Smoking	3.69 (0.80–17.0)	0.09
LDL-C, per 10 mg/dl increase	1.28 (0.99–1.66)	0.07
HDL-C, per 10 mg/dl decrease	1.11 (0.62–1.96)	0.73
TG, per 10 mg/dl increase	1.05 (0.95–1.16)	0.31
LDL size	0.84 (0.36–1.95)	0.68
MDA-LDL	0.96 (0.81–1.14)	0.64
Glucose, per 10 mg/dl increase	1.13 (0.67–1.93)	0.65
Insulin	1.03 (0.97–1.10)	0.36
sLR11, per 0.1 U increase	2.77 (1.56–4.90)	< 0.001
Age and sLR11 quartiles		
Age, per 10 yr increase	1.97 (1.26–3.07)	0.001
sLR11 quartile 2 vs. 1	1.93 (0.53–6.98)	0.32
sLR11 quartile 3 vs. 1	2.96 (0.86–10.21)	0.09
sLR11 quartile 4 vs. 1	7.60 (2.28–25.39)	0.001

TG, triglycerides; MDA-LDL, malondialdehyde-LDL. sLR11 ranges for quartiles 1, 2, 3, and 4 are less than 2.13, 2.13 to 2.78, 2.79 to 3.52, and more than 3.52, respectively.

ruffle formation through complex formation with uPAR and integrin αvβ3 and that the LR11/uPAR/integrin-mediated intracellular pathway is required for the Ang II–induced attachment and migration of intimal SMCs. Thus, we have identified a role for the shed form of LR11, an independent serum marker of carotid IMT, in a mechanism that involves Ang II–induced SMC migration.

Results

Circulating sLR11 is independently associated with carotid IMT. Considerable amounts of sLR11 are produced by intimal SMCs in the process of intimal thickening after cuff injury of femoral arteries in mice and enhance migration of SMCs in vitro via upregulation of uPAR expression (11, 20). We first studied immunologically detectable serum sLR11 levels in association with degrees of carotid IMT, a marker of intimal thickness of carotid arteries that predicts coronary and/or cerebral atherosclerosis (19), in 402 dyslipidemic subjects (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI32381DS1). Univariate analysis showed that sLR11 as well as age, sex, systolic and diastolic blood pressure, smoking, HDL cholesterol, triglycerides, LDL particle size, and levels of insulin were significantly correlated with IMT (Supplemental Table 2). Multiple stepwise logistic regression analysis revealed that sLR11 is associated with IMT independently of other classical risk factors of IMT (Table 1). Analysis of subject quartiles showed a gradual increase in adjusted odds ratios (ORs) depending on sLR11 levels; subjects in the highest quartile of sLR11 levels (sLR11 > 3.52 U/ml) had a significantly increased likelihood of having an IMT greater than 1.0 compared with those in the lowest quartile (sLR11 < 2.13 U/ml). Subsequent to the demonstration of a strong correlation of circulating sLR11 with carotid IMT, the univariate analysis of circulating sLR11 with the above-listed factors was performed separately in males and females (Supplemental Table 3). sLR11 levels in males and females, respectively, were significantly correlated with IMT degrees at similar *r* (correlation coefficient) values. Multivariate analysis showed that IMT is the only factor significantly correlated with sLR11. (Supplemental Table 4). Thus, circulating sLR11 levels are tightly associated with IMT of carotid arteries in dyslipidemic subjects.

Intimal thickness of injured arteries is drastically reduced in LR11-deficient mice. A close association of serum sLR11, a regulator of SMC migration, and intimal thickness of carotid arteries suggested that LR11 is involved in the process of intimal thickening of injured arteries. We studied the effect of targeted inactivation of LR11 (Figure 1, A and B) on intimal thickening in response to cuff placement of femoral arteries in mice. In the knockout mice, immunodetectable LR11 was abolished in tissues including brain and kidney, which are dominant sites of LR11 expression in WT mice. In contrast, the expression levels of LDL-related protein 1 (LRP1), a ubiquitously expressed LR (5, 6), did not change (Figure 1C). When $Lr11^{+/-}$ mice were intercrossed, the ratios of the numbers of live births of the different genotypes were according to Mendelian laws. The appearance and development of homozygous $Lr11^{-/-}$ mice were indistinguishable from those of $Lr11^{+/-}$ or $Lr11^{+/+}$ littermates. However, in $Lr11^{-/-}$ mice, the thickness of the arterial intima 4 weeks after cuff placement was drastically reduced compared with that in $Lr11^{+/+}$ mice, and the average ratio of IMT (I/M ratio) in $Lr11^{-/-}$ mice was 32% of that in $Lr11^{+/+}$ mice (Figure 1D). Immunohistochemical staining showed that the cells in the intima were predominantly SMA-positive SMCs in both $Lr11^{-/-}$ and $Lr11^{+/+}$ mice; however, nonmuscle myosin heavy chain II-B protein (NMHCII-B), an embryonic myosin isoform (14, 21), was clearly decreased in the intimal SMCs of $Lr11^{-/-}$ mice (Figure 2A). Analysis of the mRNA levels of NMHCII-B in SMCs in injured arteries demonstrated that expression in $Lr11^{-/-}$ mice was similar in the intima and media. Levels of SM1, an isoform of mature myosin fibers, were significantly increased in the intima in $Lr11^{-/-}$ mice when compared with $Lr11^{+/+}$ mice (Figure 2B). The substantial increase in SM1 expression together with the decrease of NMHCII-B expression in $Lr11^{-/-}$ mice compared with those in $Lr11^{+/+}$ mice suggests that the isoform conversion in intimal SMCs was disturbed in $Lr11^{-/-}$ mice. Thus, targeted inactivation of LR11 caused attenuation of intimal thickening in response to arterial injury accompanied by an altered expression pattern of myosin fiber isoforms in intimal SMCs.

$Lr11^{-/-}$ SMCs are characterized by grossly reduced Ang II–induced membrane ruffling. We have previously shown that cultured SMCs prepared from the intima display increased LR11 expression and PDGF-induced migration compared with medial SMCs and that inhibition of LR11 function with neutralizing antibodies reduces the migratory activity of intimal SMCs (11). Hence, we investigated the migration activity of cultured SMCs isolated from the aorta of $Lr11^{-/-}$ mice. Migration, invasion, and as shown here, attachment but not proliferation by PDGF-BB in $Lr11^{-/-}$ SMCs were decreased compared with those in WT SMCs, in agreement with previous

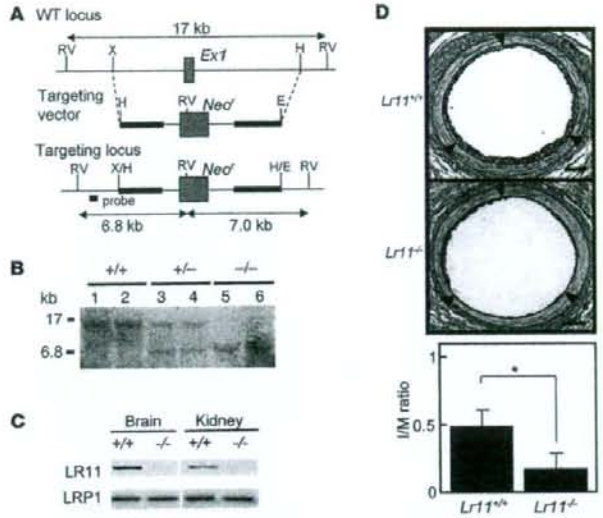

Figure 1
Intimal thickness of arteries after cuff placement in *Lr11*$^{-/-}$ mice. (**A**) Targeted disruption strategy of the murine *LR11* gene, consisting of 49 exons. The targeting vector (bold line) contains 3.3 kb (5′) and 4.4 kb (3′) of genomic DNA flanking the neomycin-resistance cassette (*Neo*^{r}). After homologous recombination, *Neo*^{r} replaced exon 1 (*Ex1*, gray box), which contained the initiation codon of the *LR11* gene. The location of the probe used for Southern blot analysis is shown. RV, *Eco*RV; X, *Xba*I; H, *Hind*III; E, *Eco*RI. (**B**) Southern blot analysis of murine-tail DNA from heterozygous intercrosses digested with *Eco*RV using the probe (see Figure 1A) that detects 17-kb and 6.8-kb fragments in the WT and knockout allele, respectively. (**C**) Immunodetection of LR11 protein. Total protein (100 μg) extracted from brain and kidney were separated by electrophoresis, blotted on a membrane, and incubated with antibody against LR11 (~250 kDa) or LRP1(~85 kDa). The samples were loaded on the same gel but not on immediately neighboring lanes. (**D**) Upper panels show sections of femoral artery of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ mouse after cuff placement, subjected to elastica van Gieson staining. Arrowheads indicate the internal elastic layers. Scale bar: 50 μm. Lower panel shows I/M ratio of arteries presented as mean ± SD (n = 15). *P < 0.05.

studies using cultured SMCs (refs. 11–14, 20, and Supplemental Figure 1). We analyzed the effects of several other chemotactic cytokines (Ang II, VEGF, bFGF, and IL-6) on the migration activity of *Lr11*$^{-/-}$ SMCs (M. Jiang and H. Bujo, unpublished observations). Among them, only Ang II did not enhance the migration of *Lr11*$^{-/-}$ SMCs in both the presence and absence of PDGF-BB but did so in WT SMCs or *Lr11*$^{-/-}$ SMCs in the presence of conditioned medium from WT SMCs (Figure 3A). There were no significant differences in levels of Ang II–induced Stat1 phosphorylation activity between WT SMCs and *Lr11*$^{-/-}$ SMCs (Figure 3B). The absence of a migration response to Ang II of LR11-deficient SMCs prompted us to further analyze their motility properties, since Ang II is a potent stimulator of cytoskeleton reorganization through intracellular signaling (15, 17). Preincubation with Ang II of *Lr11*$^{+/+}$ SMCs but not of *Lr11*$^{-/-}$ SMCs enhanced cell attachment in the presence and absence of PDGF-BB (Figure 3C). The defect in the Ang II–mediated induction of attachment activity was restored by sLR11 in *Lr11*$^{-/-}$ SMCs (Figure 3D); note that the Ang II–induced attachment activity of *Lr11*$^{+/+}$ cells was reduced by neutralizing anti-LR11 antibodies to the levels observed in *Lr11*$^{-/-}$ SMCs. We therefore studied membrane ruffling, which is related to cytoskeletal reorganization leading to enhanced cell attachment (3, 4), in *Lr11*$^{+/+}$ and *Lr11*$^{-/-}$ SMCs. Ang II appeared to increase the proportion of cells with ruffles, and this increase was attenuated by blocking uPAR with neutralizing antibody in *Lr11*$^{+/+}$ cells (Figure 4, A–C, D, and M). In *Lr11*$^{-/-}$ SMCs, Ang II's effect on ruffle formation was not significant; in addition, the proportion of cells with ruffles in the presence of PDGF-BB was lower (Figure 4, E–G, H, and M). Also, ruffling by Ang II incubation was not enhanced in *Lr11*$^{-/-}$ SMCs pretreated with sLR11 (Figure 4, I–M). Thus, as quantitated (Figure 4M), in the presence of PDGF, Ang II stimulates membrane ruffling of WT cells (Figure 4, B and C) and this stimulation is abolished in *Lr11*$^{-/-}$ SMCs (Figure 4, F and G), even in the presence of sLR11 (Figure 4, J and K). Surprisingly, sLR11 induces ruffle formation in *Lr11*$^{-/-}$ SMCs in the absence of both PDGF and Ang II (Figure 4, E and I), which is consistent with the sLR11-induced increase in attachment activity in *Lr11*$^{-/-}$ SMCs (see Figure 3D). The increased ruffling in the presence of sLR11 with PDGF and Ang II was almost abolished by blocking uPAR (Figure 4, K and L). Next, in order to study the significance of LR11 in the Ang II–mediated vascular pathology, we analyzed the effect of LR11 deficiency on intimal thickening of arteries after cuff placement in the murine Ang II infusion model. Administration of Ang II at 1 μg/kg/min for 28 days significantly increased the I/M ratio in *Lr11*$^{+/+}$ mice: note that the medial thickness in *Lr11*$^{-/-}$ mice was not significantly different from that in *Lr11*$^{+/+}$ mice (Figure 5A and Supplemental Figure 2). However, the Ang II infusion–induced increase was not observed in *Lr11*$^{-/-}$ mice. The increase by Ang II infusion in the level of NMHCII-B expression in intima versus

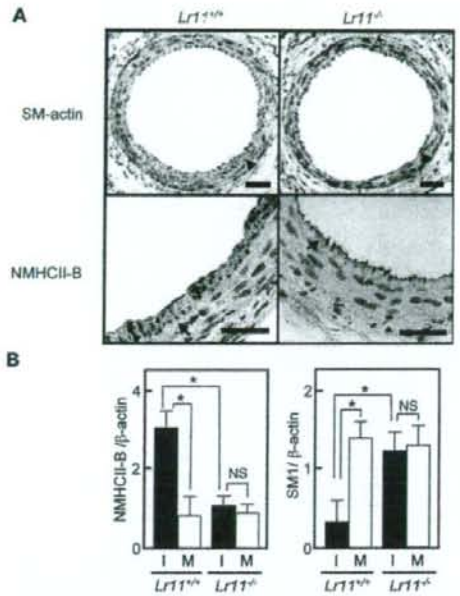

Figure 2
Myosin isoform expression pattern in injured arteries after cuff placement in *Lr11*$^{-/-}$ mice. (**A**) Sections of femoral arteries in *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ mice after cuff placement, subjected to immunohistochemistry using antibodies against SMA or NMHCII-B. Arrowheads indicate the internal elastic layers. Scale bars: 50 μm. (**B**) mRNA levels of myosin isoforms NMHCII-B and SM1 in arteries after cuff placement in *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ mice. Total RNA isolated from the thickened intima (I) or the media (M) of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ mice was reverse transcribed to cDNA and subjected to real-time PCR analysis using specific primers for NMHCII-B and SM1, respectively. The amounts of amplified products are expressed relative to the amounts of β-actin transcript. Data are presented as mean ± SD (n = 5). *P < 0.05.

media did not occur in *Lr11*$^{-/-}$ mice (Figure 5B; note that the level of LRP1 after Ang II infusion remained unchanged in both *Lr11*$^{+/+}$ and *Lr11*$^{-/-}$ mice). Thus, *Lr11*$^{-/-}$ SMCs are characterized by a failure in Ang II–mediated ruffle formation and attachment, which in turn apparently leads to a decrease in Ang II– or PDGF-BB–mediated migration activity. Furthermore, Ang II infusion–induced intimal thickening in response to arterial injury is attenuated, accompanied by an altered expression pattern of myosin fiber isoforms in intimal SMCs (see Figure 2).

LR11 activates membrane ruffling through the uPAR/integrin/focal adhesion kinase/ERK/Rac1 pathway. LR11 binds to and colocalizes with uPAR on the cell surface (11), and a fraction of cell surface–located LR11 is proteolytically shed to produce a soluble form, sLR11 (22). To gain further insight into sLR11's activity, we analyzed its effect on uPAR-mediated intracellular signals for cytoskeletal reorganization. The proportion of cells with ruffle formation was increased in dose-dependent fashion by sLR11, and this increase was clearly reduced by pretreatment with neutralizing antibodies against LR11, uPAR, or integrin αvβ3; PD98059, a specific MEK inhibitor, had the same effect (Figure 6A). The formation of a complex between LR11 and integrin αvβ3 was shown by coimmunoprecipitation, as previously demonstrated for uPAR/LR11 (11). The complex formation was inhibited by the addition of known ligands of LR11, e.g., apoE and receptor-associated protein (RAP) (Figure 6B). LR11 attenuates internalization and catabolism of uPAR, which is accelerated by LRP1 (13). RAP, a common ligand of LR11 and LRP1, reduced the membrane expression of uPAR in *Lr11*$^{+/+}$ SMCs but significantly increased the uPAR expression in *Lr11*$^{-/-}$ SMCs (Figure 6C). Thus, LR11 in association with LRP1 appears to form a complex with integrin αvβ3 via interaction with uPAR, which also binds integrin (4, 11). Accordingly, sLR11 induced the phosphorylation of focal adhesion kinase (FAK) and ERK, which act downstream of uPAR/integrin signals (Figure 7, A and B), and subsequent activation of Rac1 was detected in the presence of sLR11 (Figure 7C). Moreover, the activation of the FAK/ERK/Rac1 cascade was inhibited by neutralizing anti-integrin αvβ3 antibody (Figure 7, A–C). Likewise, in *Lr11*$^{-/-}$ cells, Rac1 activation was increased by sLR11, and this increase was diminished by neutralizing anti-integrin αvβ3 antibody (Figure 7D). In these experiments, the presence of anti–glutathione-*S*-transferase (anti-GST) antibody did not show any significant effects (data not shown). Thus, sLR11 can activate the FAK/ERK/Rac1 pathway in SMCs through complex formation with uPAR and integrin αvβ3; transduction of the signal leads to cytoskeletal reorganization associated with membrane ruffling.

Ang II induces migration of SMCs through activation of LR11-mediated cell attachment. Together with the results shown in Figures 6 and 7, the greatly diminished Ang II–induced migration in LR11-deficient SMCs (see Figure 3) suggested that it is due to a defect in Ang II's actions on the LR11/uPAR/integrin/FAK/ERK/Rac1 pathway. Ang II and PDGF-BB but not VEGF led to increased production of sLR11 in rabbit SMCs (Figure 8A). In fact, the levels of both sLR11 and membrane-bound LR11 but not LRP1 were increased by Ang II in a dose-dependent manner (Figure 8B). The Ang II–mediated increase in sLR11 levels was blocked by the ARBs, valsartan and candesartan, as well as by PD98059 (Figure 8C and Supplemental Figure 3). Ang II induced the membrane expression of uPAR in *Lr11*$^{+/+}$ SMCs, which was abolished in *Lr11*$^{-/-}$ SMCs (Figure 9A). Thus, the specific increase of sLR11 levels caused by Ang II together with the reduction in Ang II–induced migration and attachment in *Lr11*$^{-/-}$ SMCs (see Figure 3) strongly suggested that Ang II is key to enhanced migration via activation of the LR11-mediated pathway and cytoskeletal reorganization. Accordingly, Ang II–induced Rac1 activation was inhibited by neutralizing antibody against LR11 or uPAR (Figure 9B). The abolished Ang II–induced Rac1 activation in *Lr11*$^{-/-}$ SMCs was recovered by sLR11, and the sLR11-mediated activity was not inhibited by candesartan (Figure 9C). The Ang II–induced increase in cell attachment was abolished by a neutralizing antibody against LR11, and importantly, valsartan had no effect on Ang II–induced enhanced attachment in the presence of sLR11 (Figure 10A). In addition, Ang II–induced migration activity was suppressed when SMCs were pretreated with uPAR-specific siRNA (Figure 10B). Finally, the inhibitory abilities of valsartan and candesartan on migration activity were reduced in sLR11-pretreated C-1 cells (16% and 17%, respectively), an established SMC line (11), or LR11-overexpressing R-1 cells (20% and 11%, respectively) compared with untreated C-1 cells (34% and 35%, respectively), although the absolute extent of inhibition among them were similar (Figure 10C). These results indicate that Ang II

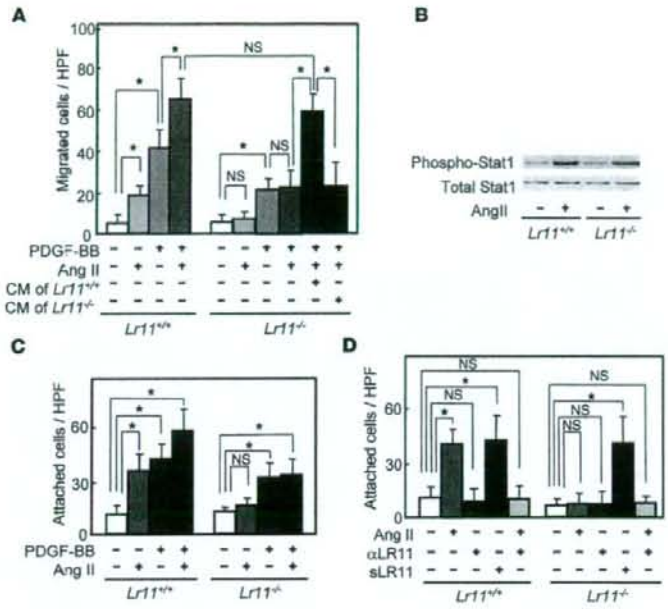

Figure 3

Ang II–induced migration and attachment of cultured SMCs derived from *Lr11*$^{-/-}$ mice. (**A**) Effect of Ang II on the PDGF-BB–induced (10 ng/ml) migration activities of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs. SMCs were incubated with 1 μM Ang II for 24 hours in the presence or absence of conditioned medium of *Lr11*$^{-/-}$ SMCs before migration analyses. Data are presented as mean ± SD (n = 6). (**B**) Effect of Ang II on Stat1 phosphorylation in *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs. SMCs were incubated with 1 μM Ang II for 10 minutes before immunoblot analysis for (phospho-) Stat1 (~90 kDa). (**C**) Effects of Ang II on cell attachment of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs in the presence or absence of 10 ng/ml PDGF-BBs. SMCs were incubated with or without Ang II (1 μM) for 24 hours before attachment analyses. Data are presented as mean ± SD (n = 6). (**D**) Effects of sLR11 on Ang II–induced attachment of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs. SMCs were incubated with or without Ang II (1 μM) for 24 hours in the presence or absence of anti-LR11 antibody (pm11, 1:5 dilution) or recombinant sLR11 (1 μg/ml) for 24 hours before attachment analyses. Data are presented as mean ± SD (n = 6). *P < 0.05.

induces cell migration through activation of LR11/uPAR-mediated cell attachment involving the Rac1 pathway.

Candesartan does not inhibit intimal thickening after arterial injury in mice overproducing sLR11. The production of sLR11 is elevated in SMCs in the course of intimal thickening after endothelial injury (20). In order to learn more about the effects of inhibition of Ang II–mediated LR11/uPAR/integrin signaling on intimal thickening, we analyzed the intimal thickness after cuff placement in LR11-overexpressing mice. Mice were implanted with R-1 cells, which are A7r5 cells stably overexpressing LR11, or with mock-transfected A7r5 cells (C-1) (13, 23). Mice implanted with R-1 cells showed a 5.2-fold increase in the serum concentration of immunodetectable sLR11 after implantation compared with those implanted with C-1 cells (4.2 ± 2.6 U versus 0.8 ± 0.1 U). Candesartan administration significantly reduced the intimal thickness (I/M ratio) 4 weeks after cuff placement in C-1 but not in R-1 mice (note that treatment of R-1 mice with neutralizing antibody against uPAR reduced the intimal thickness; Figure 11, A–J and U). Intimal versus medial expression of LR11 and NMHCII-B was significantly reduced by candesartan in C-1 mice (Figure 11, K, L, P, Q, and V). In contrast, the levels of LR11 and NMHCII-B expression in intima versus media were not significantly different in R-1 mice whether treated with candesartan or not (note that treatment of R-1 mice with neutralizing antibody against uPAR did not significantly reduce the ratio of intimal to medial expression of LR11; Figure 11, M–O, R–T, and V). Thus, the ARB candesartan, which inhibits Ang II–mediated LR11/uPAR/integrin cell migration and attachment signals, reduces intimal thickness after cuff placement in mice; this reduction is counteracted in mice that overproduce sLR11. These results, obtained using an intimal thickening model with sLR11 overproduction, show that Ang II–induced cell migration and attachment contribute to the migration of SMCs from the media to the intima after arterial injury.

Discussion

LRs play a key role in the catabolism of complexes between proteinases and their receptors (5–7). In SMCs, LRs are thought to function in the catabolism of membrane molecules that regulate intracellular signaling events important for the specific properties of these cells, particularly in regard to their motility and migration (24–33). Members of the family, such as LRP1 (34), VLDL receptor (LR8) (25), and LRP1B (32, 35), endocytose uPAR and uPA/uPAR complexes into cells for subsequent degradation

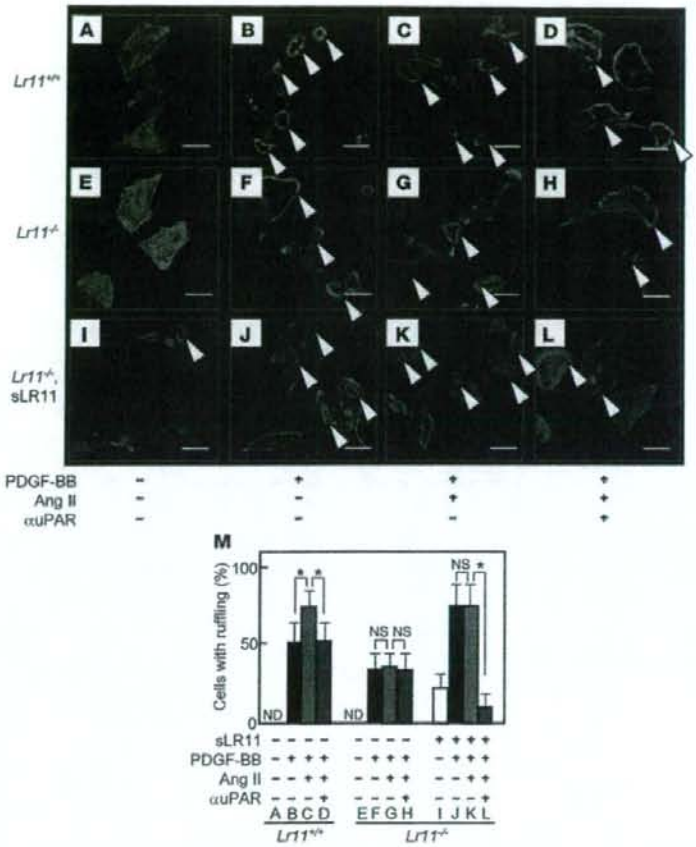

Figure 4
Ang II–induced membrane ruffle formation of cultured SMCs derived from *Lr11*$^{-/-}$ mice. (**A**–**L**) Ang II–induced membrane ruffling (arrowheads) in *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs. The indicated SMCs were incubated with or without Ang II (1 μM) for 24 hours in the presence or absence of recombinant sLR11 (1 μg/ml) with or without anti-uPAR antibody for 24 hours. PDGF-BB (10 ng/ml) was then added to the culture medium for 10 minutes before immunofluorescence analyses. Cells were then stained using Alexa Fluor 488 phalloidin. Scale bars: 10 μm. (**M**) The number of cells with membrane ruffles were countec among 500 cells in the field. Data are presented as mean ± SD (n = 3). *P < 0.05. ND, not detected.

and/or recycling. We have discovered what we believe is a novel mechanism for uPAR localization to the plasma membrane that involves LR11 (11). Recycling and degradation of uPAR on the membrane is dependent on its internalization via LRP1 (34). The extracellular sLR11 binds to and colocalizes with uPAR on the cell surface; this de facto immobilization effectively stabilizes the receptor-protease complex by inhibiting its degradation through LRP1 (11). In cultured SMCs, LR11 is highly expressed, but LRP1 expression is rather stable during the rapidly proliferating phase (32). The balance between expression levels of LR11 and LRP1 may be important for the regulation of uPAR expression in the membrane (see Figure 6C). Enhanced uPAR-mediated cell migration appears to constitute an important factor in the process of atherosclerosis and arterial modeling (3, 4). LR11 is highly expressed in atherosclerotic plaques, particularly in the intimal SMCs at the border between the intima and the media in plaques of humans and apoE-knockout mice (11, 32). Considerable amounts of sLR11 are produced by intimal SMCs in the process of intimal thickening after cuff injury of femoral arteries in mice (20). In this study, an attempt to investigate the clinical significance of circulating sLR11 levels in atherosclerosis was made in relation to dyslipidemia, since LR11 is a relative of the LDL receptor, a key receptor for maintenance of lipid homeostasis (1, 5–7). We discovered that circulating sLR11 levels are positively correlated with the degree of carotid IMT, representative of arterial intimal and medial thickness and closely associated with the development of coronary and/or cere-

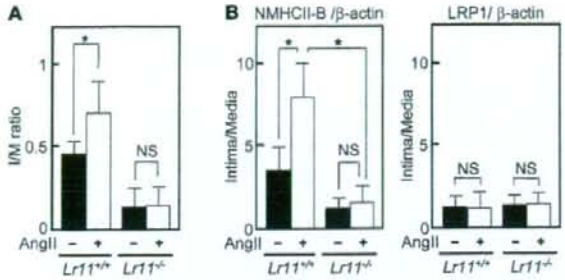

Figure 5
Intimal thickening after cuff placement in response to Ang II infusion in *Lr11*$^{-/-}$ mice. (**A**) I/M ratios of femoral arteries in *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ mice after cuff placement with saline or Ang II infusion (1 μg/kg/min for 28 days) are presented as mean ± SD (n = 15). *P < 0.05. (**B**) mRNA levels of NMHCII-B and LRP1 in injured arteries. Total RNA isolated from the intima or the media of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ mice was reverse transcribed into cDNA and subjected to real-time PCR analysis using specific primers for NMHCII-B and LRP1, respectively. The amounts of amplified products are expressed relative to the amounts of β-actin transcript, and the ratio of mRNA expression levels of intima and media are presented as mean ± SD (n = 3). *P < 0.05.

bral artery diseases (19), and that this correlation is independent of other established risk factors for IMT in subjects with dyslipidemia. Taking the results together, we propose that circulating sLR11 may be a marker reflecting the intimal and medial thickness of injured arteries. Clearly, further careful studies using subjects with different characteristics are needed for the evaluation of a (patho)physiological significance in arterial diseases. Given that sLR11 is released from other major sources, the basis for this inference remains uncertain. Considering that (a) the changes in IMT are presumably due to the evaluation of the complex of intimal thickness, medial thickness, and hypertrophy of SMCs, which can result from hypertension in the carotid arteries, and (b) LR11 is required for Ang II–induced SMC migration in culture systems and mouse models, the relation of the current results to the pathology of hypertension-induced vessel damage requires attention. sLR11 may well affect the remodeling of injured arteries through the phenotypic conversion of medial SMCs, since loss of sLR11 disturbs the myosin isoform conversion in addition to the actin rearrangement in SMCs (see Figure 2B, Figure 5B, and Supplemental Figure 1A). In this context, the pathophysiological alteration of *Lr11*$^{-/-}$ SMCs in the medial layer after Ang II infusion needs to be evaluated by a model suitable for vessel remodeling in addition to the current cuff placement model (see Figure 5A). In any case, the major sources and the plasma concentrations of sLR11 in various conditions need to be identified. In the process of atherosclerosis and vascular restenosis after coronary angioplasty, the migration of SMCs appears to play a key role in intimal thickening (1, 2). SMCs acquire or lose numerous cellular functions required for performing the above tasks in the intima, as represented by changes in myosin isoforms including NMHCII-B and SM1 (14, 21). One of these tasks is enhancing cellular mobility while interacting with components of the basement membrane and other extracellular matrix components. One of the players in matrix degradation is uPA bound to its specific receptor, uPAR, on the cell surface; its essential role in enhancing cell mobility has been intensively studied in cancer invasion and neural migration (3, 4). uPAR interacts with integrin αvβ3 (36) and subsequently activates signaling pathways such as the FAK/ERK/Rac1 cascade for cytoskeleton reor-

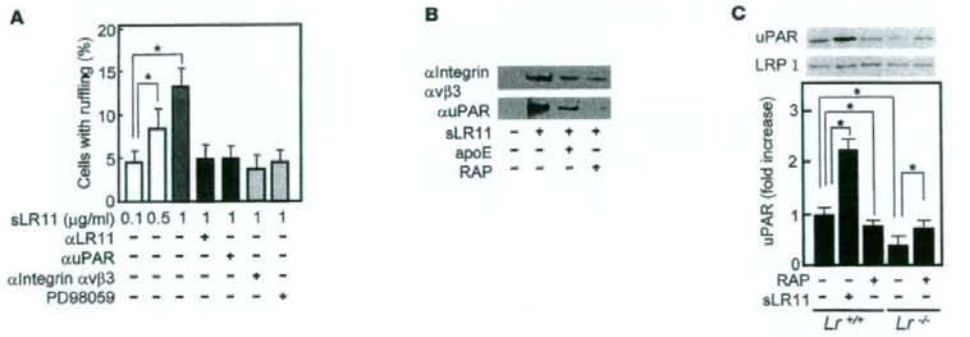

Figure 6
sLR11-mediated ruffle formation through complex formation with uPAR and integrin αvβ3. (**A**) sLR11-induced membrane ruffle formation. Rabbit SMCs were incubated with sLR11 (1 μg/ml) for 24 hours in the presence or absence of antibody against LR11, uPAR, or integrin αvβ3 (MAB1976) or of PD98059. The number of cells with membrane ruffles were counted among 500 cells in the field. Data are presented as mean ± SD (n = 5). (**B**) Coimmunoprecipitation of sLR11 (~250 kDa) with integrin αvβ3 or uPAR. Membrane extracts of rabbit SMCs were incubated with or without sLR11 in the presence or absence of apoE (50 μg/ml) or RAP (10 μg/ml), immunoprecipitated with anti-integrin αvβ3 or anti-uPAR antibody, and subjected to immunoblot analysis using anti-LR11 antibody. (**C**) uPAR expression in *Lr11*$^{-/-}$ SMCs. Membrane extracts of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs were incubated with RAP and subjected to immunoblot analysis using anti-uPAR (~50 kDa) or anti-LRP1 (~85 kDa) antibody. Blot shown is representative of 3 independent experiments. Data are presented as mean ± SD (n = 3). *P < 0.05.

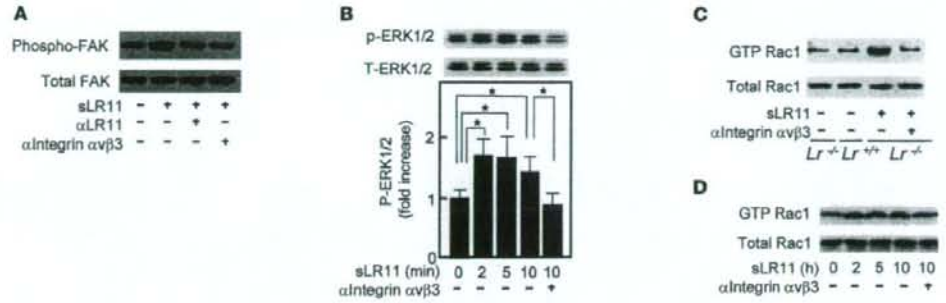

Figure 7

sLR11-mediated intracellular signals related to cytoskeleton reorganization. (**A**) sLR11-induced FAK activation. Cell lysates of rabbit SMCs were incubated with or without sLR11 (1 μg/ml) in the presence or absence of antibody against LR11 or integrin αvβ3 (MAB1976), immunoprecipitated with anti-FAK antibody, and subjected to immunoblot analysis using anti-FAK (~130 kDa) or anti–phospho-FAK (~130 kDa) antibody. (**B**) sLR11–induced phosphorylation of ERK1/2. Cell lysates (10 μg protein) of rabbit SMCs were incubated with sLR11 (1 μg/ml) for the indicated times in the presence or absence of anti–integrin αvβ3 antibody (MAB1976) and subjected to immunoblot analysis using antibody against (phospho) p42/44 MAP kinase. Upper and lower signals represent ERK1 (~44 kDa) and ERK2 (~42 kDa), respectively (13). Blot shown is representative of 3 independent experiments. Data of p-ERK1/2 are presented as mean ± SD ($n = 3$). *$P < 0.05$. (**C**) Rac1 activation in *Lr11*$^{-/-}$ SMCs. Cell lysates (60 μg protein) of *Lr11*$^{+/+}$ or *Lr11*$^{-/-}$ SMCs were incubated with sLR11 (1 μg/ml) in the presence or absence of antibody against integrin αvβ3 (RMV-7), immunoprecipitated with PAK-1 PBD Protein GST beads, and subjected to immunoblot analysis with anti-Rac1 (~21 kDa) or anti–GTP-Rac1 (~21 kDa) antibody. (**D**) sLR11-induced Rac1 activation. Cell lysates (60 μg protein) of rabbit SMCs were incubated with sLR11 (1 μg/ml) for the indicated times in the presence or absence of anti–integrin αvβ3 antibody (MAB1976) and subjected to immunoblot analysis with anti-Rac1 (Rac1 ~21 kDa and GST-Rac1 ~21 kDa) antibody with (top) or without (bottom) prior immunoprecipitation with PAK-1 PBD Protein GST beads.

ganization (3, 4). Therefore, uPAR activation contributes to the progression of vascular remodeling through enhanced migration of intimal SMCs. Based on these findings, we have now identified a regulator of uPAR–mediated activation of cytoskeletal reorganization in the process of Ang II–induced migration of SMCs (see Figure 12). Moreover, the disturbed myosin isoform conversion in *Lr11*$^{-/-}$ SMCs may indicate that the LR11/uPAR signal plays a role in the coordination of actin and myosin rearrangement. The significance of LR11-mediated cytoskeleton reorganization for the conversion from contractile to synthetic phenotype needs to be addressed in future studies.

Ang II, similar to PDGF-BB, plays an important role as a cytokine in the process of atherosclerosis and vascular remodeling (15, 16). The activation of its specific receptor, AT_1R, triggers various intracellular signals toward proliferation and migration of SMCs. The Ang II–promoted activities are greatly diminished by specific AT_1R antagonists or by inhibition of MAP signaling using synthetic inhibitors of MAP kinase (3, 4). Here, Ang II stimulated cell migration and attachment and LR11 expression through AT_1R activation; the enhancement of attachment by Ang II was more obvious in the absence than in the presence of PDGF-BB (see Figure 3). Blocking LR11 and subsequent events abolished Ang II–induced migration and attachment of SMCs (see Figure 10). The effects of LR11 ablation on Ang II–induced migration and attachment strongly suggest that Ang II–mediated enhancement of attachment depends on sLR11-mediated cytoskeleton reorganization. sLR11-induced ruffle formation and attachment activity even in the absence of Ang II or PDGF-BB (see Figure 3D, Figure 4, I and M, and Figure 10C) suggest that LR11 overproduction generally induced by such cytokines is important for the increased attachment activity through enhanced ruffle formation in the process of cell migration. ARBs prevent vascular damages in a mouse model of Marfan syndrome through amelioration of the increased activation of TGF-β signaling pathways (37). Boucher et al. recently reported on a conditional LRP1-deficient mouse model in which activation of TGF-β signaling pathways in the vascular wall was maximally increased, which is similar to what occurs in Marfan syndrome (38). LR11 seems to play an essential role in the change in cell mobility in the signals from Ang II and also in part from PDGF-BB (Figure 12).

Neutralizing antibodies against LR11 have been shown to reduce the migratory activity of intimal SMCs and intimal thickness after cuff injury (11). Here, using LR11-deficient cells, we found that sLR11 induces the complex formation of uPAR and integrin αvβ3 in SMCs (Figure 6). This finding may well explain the previous observation that incubation with aprotinin, a plasmin inhibitor, effectively reduces invasion but has much less effect on migration in LR11-overexpressing cells (ref. 13 and Supplemental Figure 1). The current study shows that overproduction of sLR11 in the absence of the membrane-bound form triggers the activation of uPAR-mediated intracellular signals via the integrin/ERK/Rac1 pathway, leading to membrane ruffling (Figures 6 and 7). Elevated sLR11 levels resulted in an exaggerated response of intimal SMCs to vascular injury and to an attenuated sensitivity to ARB treatment in mice (Figure 11). In this context, we recently observed that exogenous recombinant sLR11 interacts with macrophage uPAR and accelerates foam cell formation (20). Thus, sLR11 derived from intimal SMCs is implicated in plaque formation through its effects on SMCs and macrophages. Although our limited analysis did not show a significant relationship between sLR11 and high-sensitve C-reactive protein (hsCRP) in plasma (M. Jiang and H. Bujo, unpublished observations), the involvement of inflammation in LR11 gene expression or shedding of sLR11 in