ていたことであるが，「インスリン抵抗性」という明確な概念で認識されるようになったのは比較的最近である．

TNFαとインスリン抵抗性

われわれは，生体侵襲時にはマクロファージが代謝異常を誘発するペプチドを分泌することを示し，このペプチドをカケクチンと命名した．これが今日でいうTNFαである．ここで名前の由来について解説する紙数はないが，TNFα/カケクチンがインスリン抵抗性を惹起することをわれわれが報告したのは20年以上も前のことである．しかし，TNFαは主としてマクロファージが産生するサイトカインであることから，インスリン抵抗性におけるTNFαの役割は主として感染症など特殊な状況に限られると考えられていた．ところが，1993年にSpiegelmanらは脂肪細胞がこのTNFαを分泌することを明らかにし，さらに脂肪の蓄積量が多くなるほど，すなわち肥満になるほどその分泌量は多くなることから，肥満者におけるインスリン抵抗性の原因は脂肪細胞由来のTNFαであると提唱した．

脂肪細胞による炎症性サイトカインの産生

現在では，脂肪細胞はTNFα以外にもIL-6，単球走化性タンパク質（MCP-1），アディポネクチン，レジスチン，レプチン，PAI-1，アンジオテンシノーゲン，ビスファチン，レチノール結合蛋白4（RBP-4），SAAなど，さまざまなサイトカインや生物活性物質を産生分泌することが知られている（アディポサイトカインの項参照）．肥満，すなわち脂肪蓄積の増加によりインスリン抵抗性や動脈硬化を促進するTNFα，IL-6，MCP-1の産生が増加し，反対にこれらを抑制するアディポネクチンの産生が減少する．

炎症と動脈硬化

動脈硬化は血管壁にコレステロールが蓄積する病変であるが，その発症と進展の過程は慢性炎症反応ととらえることができる（図2）．初期病変として血管内皮細胞に単球やリンパ球が接着し，次いでこれらの細胞が血管壁の内膜内に移入することが重要

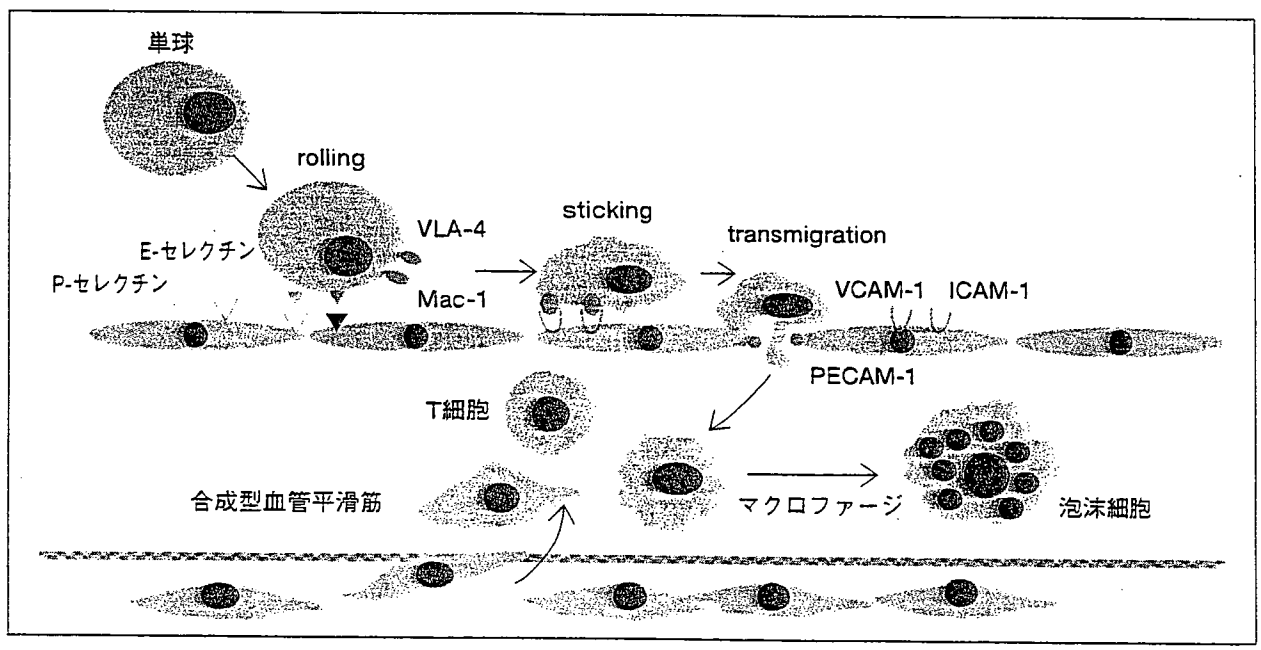

図2　動脈硬化の発症機序

であり，この過程には多くの炎症性サイトカインや接着分子，血液凝固因子，細胞増殖因子が関与している．血管の内膜内で単球はマクロファージに成熟するが，TNFα/カケクチンやIL-6などのサイトカインはマクロファージや血管平滑筋を活性化し，これらの細胞自身による炎症性サイトカインの産生を刺激する．さらに，血管壁基質のコラーゲンを分解するマトリックスプロテイナーゼの分泌も促進し，これによりプラークは脆弱になる．プラークの破綻は急速な血栓形成をもたらし，いわゆる急性冠動脈症候群を起こす．このように，動脈硬化の形成にはその初期病変から最終的なイベントに至るまで，多くの炎症性サイトカインが関与している．血管壁の細胞自体もTNFαやIL-6を含む炎症性サイトカインを産生するが，メタボリックシンドロームにおいてはその発端として脂肪組織由来の炎症惹起物質による全身性炎症反応が重要な意義をもつとされている．

脂肪組織と炎症反応

上述のように脂肪細胞はTNFα，IL-6，レジスチン，MCP-1，PAI-1やアンジオテンシノーゲンなどを産生する．これらのうち，レプチン，アディポネクチン，レジスチンなどは脂肪細胞のみで産生されるとされているが，TNFα，IL-6などの主たる産生細胞はマクロファージである．脂肪組織にはマクロファージが多数存在し，また，肥満になると脂肪組織中のマクロファージが増加することから，脂肪組織におけるこれらの炎症性サイトカインは主として脂肪細胞が産生しているのか浸潤している免疫細胞が産生しているのかは不明である．遺伝子工学的手法により，脂肪細胞によるサイトカインの産生は阻害せずに選択的に骨髄細胞による炎症性サイトカインの産生を阻害すると，肥満に伴うインスリン抵抗性が軽減することから，脂肪組織におけるサイトカインの産生はマクロファージをはじめとする免疫細胞の役割が主であるとの報告もあるが，この事実だけでは，脂肪の蓄積が脂肪組織のマクロファージを増やして炎症性サイトカインの産生を促進する機序は説明できない．しかし，脂肪細胞（特に内臓脂肪組織の）に多量の脂肪が蓄積することが肥満におけるインスリン抵抗性の原因であることは確かであるので，脂肪細胞が炎症反応を惹起してマクロファージがこれを増幅すると推測されている．

単球（マクロファージ），樹状細胞およびT細胞に対する走化因子としてはMCP-1が知られるが，脂肪細胞は細胞内の脂肪蓄積量に応じてMCP-1の産生も増加させることが知られており，肥満に伴う一連の炎症反応は脂肪細胞によるMCP-1の産生が始まりであるとの説がある（図3）．実際にMCP-1受容体をノックアウトしたマウスでは高脂肪食により誘発されるインスリン抵抗性が軽減することが示されている．ただ，完全には阻止できないことからMCP-1以外の要因も関与することは確かである．

肥満と肝臓

肥満に伴い肝臓でも炎症関連遺伝子の発現が増加する．これが，脂肪細胞と同様に肝細胞内の脂肪の蓄積によるものであるのか，あるいは内臓脂肪で作られた起炎性物質が門脈を介して肝臓に働き炎症を起こすのかは不明であるが，いずれにせよ，脂肪肝ではTNFα，IL-6，IL-1の過剰産生がみられる．肝臓にはもともとマクロファージ由来のKupffer細胞がある．脂肪組織と違って脂肪蓄積に伴ってKupffer細胞の数が増加するということはないが，活性化されることが観察されている．肝臓には

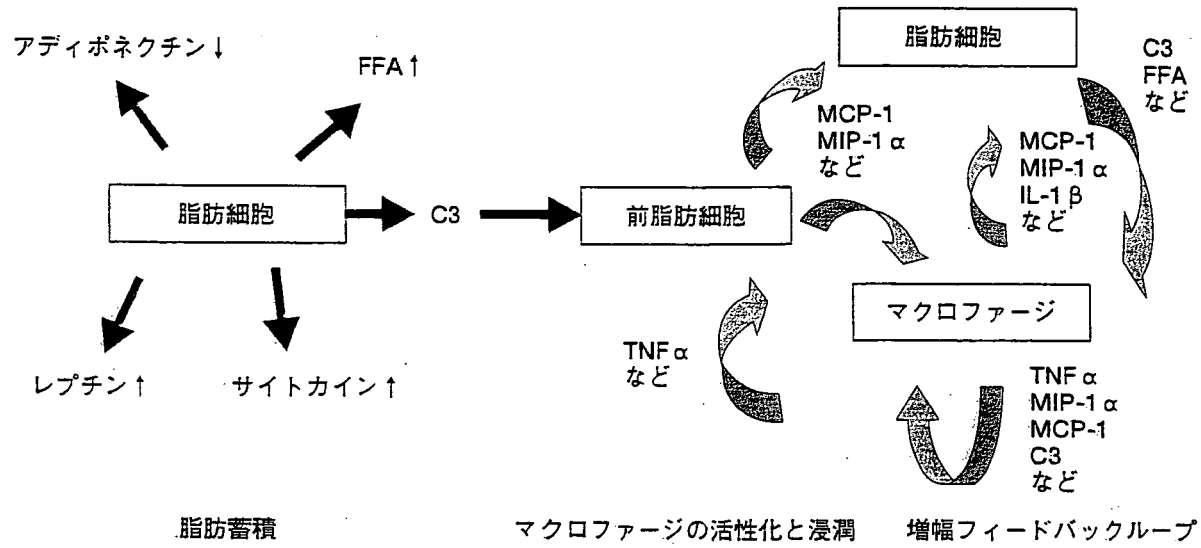

図3　脂肪組織と炎症
(Xu H, *et al*: *J Clin Invest* 2003; 112: 1821)

Kupffer細胞以外にもT，Bリンパ球，樹状細胞，NK細胞などの免疫担当細胞が存在する．脂肪肝ではNK細胞が減少することが知られ，NKT細胞を外部から補充するとNASH（非アルコール性脂肪肝炎）や耐糖能異常の改善することが観察されている．

抗炎症治療によるインスリン抵抗性の治療

肥満に伴うインスリン抵抗性にTNFαが重要な役割を演じているとされることから，TNFαの阻害によりインスリン抵抗性の改善することが期待されるが，これまでのところ抗TNFα抗体による治療が期待されたほどの効果を示したという報告はない．その理由として，肥満者では上述のようにTNFα以外にも多くの起炎性物質が増加するということがあげられている．このような観点からより広範に炎症性サイトカインを抑制する治療法が望まれる．そのような作用を示す薬物として，チアゾリジン誘導体とHMG-CoA還元酵素阻害薬（スタチン）の有用性が検討されている．ピオグリタゾンやロシグリタゾンは糖代謝や脂質代謝に対する作用に加えてマクロファージによる炎症性サイトカインの産生を抑制する作用も示す．これらの薬物のインスリン抵抗性改善作用の一部はこの抗炎症作用も関与すると考えられる．

スタチンも炎症性サイトカイン産生を抑制するとされ，臨床的にもスタチンの服用によるCRPや炎症性サイトカインの低下が観察されているが，これによりインスリン抵抗性が改善することは明らかにされていない．

前述のようにアスピリンの大量療法が血糖改善作用を示すことが古くから知られ，今日ではその作用機序の少なくとも一部はNF-κBの抑制を介するものであることが明らかにされている．糖代謝だけでなく，脂質代謝も改善することが示されているが，消化器系の副作用のためアスピリン自体の臨床応用は現実的ではない．現在，抗血液凝固作用や消化管刺激作用をもたないサルチル酸誘導体のメタボリックシンドロームに対する有効性が検討されている．

[文　献]

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教育講演

5. 動脈硬化の危険因子としての糖尿病

川上　正舒

Key words：糖尿病，動脈硬化，NO（一酸化窒素），TNFα，ポリオール経路，食後高血糖

はじめに

生活習慣の欧米化と人口の高齢化に伴い，動脈硬化性疾患は増加の一途をたどっており，虚血性心疾患と脳血管障害をあわせるとわが国の死因の約30%を占め悪性腫瘍とともに第一位となっている．

動脈硬化の危険因子としてはLDL(low density lipoprotein)コレステロールをはじめとした高脂血症，高血圧，肥満，喫煙，ホモシステインなど多くが知られており，近年は高コレステロール血症が重視されているが，糖尿病も非常に重要な危険因子である．最近の報告によると，高血糖が心血管病による死亡の主要な原因となっていることは，わが国のような先進国に留まらず，南アジアやアフリカなどの経済的発展途上国においても同様であり，全世界で虚血性心疾患死の21%，脳卒中死の13%が高血糖に起因すると言われている[1]．

1．動脈硬化の危険因子としての糖尿病

われわれの施設で，虚血性心疾患の疑いで冠動脈造影を行い有意狭窄を示した人と有意狭窄の無かった人につき，既知の危険因子につき多変量解析をした横断的研究では，糖尿病，喫煙，ヘリコバクターピロリ感染が有意な危険因子として同定され，なかでも糖尿病が最も強い因子であることが示された[2]．

糖尿病では虚血性心疾患による死亡が非糖尿病の3倍から7倍とも言われているが，Haffnerらのフィンランドの健康保険のデータをもとにした研究によると，7年間における糖尿病患者での初発の心筋梗塞の発症頻度（20.2%）は非糖尿病者の再発の頻度（18.8%）に匹敵し，再発の頻度（45.0%）は初発の頻度の2倍以上であったとしている[3]．

わが国では，糖尿病患者を対象とした前向きの（prospective）疫学調査は多くないが，筑波大学の山田らを中心として現在も進行中のJapan Diabetes Complications Study（JDCS）の中間報告によると，糖尿病患者における虚血性心疾患の発症頻度は1年間で1000人当たり6.7人，脳血管障害が6.5人であったとされている[4]．これは，九州大学の久山町研究の報告ともほぼ一致しており[5]，UKPDS(United Kingdom Prospective Diabetes Study)の報告でみられるイギリスでの頻度の約3分の1である[6]．JDCSでは非糖尿病についてのデータはないが，久山町研究では非糖尿病は虚血性心疾患が1.6人，脳血管障害が1.9～2.3人とされているので，糖尿病ではどちらも約3倍の危険度となる．

高コレステロール血症など他の危険因子と糖

かわかみ　まさのぶ：自治医科大学大宮医療センター総合医学第一講座

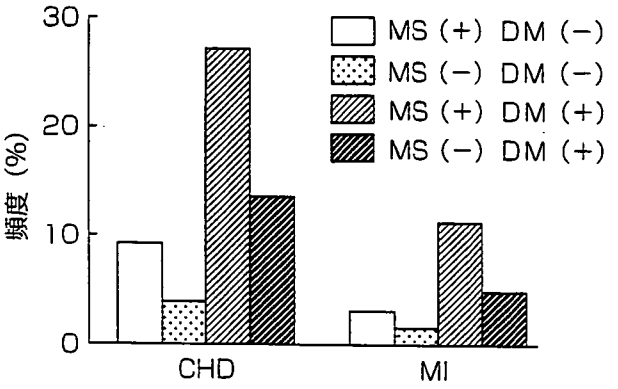

図1．メタボリックシンドロームと心血管疾患
—The Botnia Study—（文献7より改変）
MS：metabolic syndrome
DM：diabetes mellitus
CHD：coronary heart disease
MI：myocardial infarction

尿病のどちらが重要であるかという比較は，後述するようにどちらも厳重に管理するべきものなのであまり意味のないことではあるが，図1にスウェーデンの一般人口での心血管死の頻度に関する調査を示す．これによると，非糖尿病でもコレステロール，高血圧，肥満が危険因子であることは確かであるが，例えこれらが正常範囲であっても糖尿病があるということだけで，非糖尿病で高コレステロール血症や高血圧がある人よりもむしろ危険度は高いということが示されている[7]．

2．糖尿病患者の虚血性心疾患

糖尿病患者の動脈硬化のもう1つの特徴は，発見時においてすでに病変が広範囲にわたっているということである．私たちの施設でのカテーテル検査のデータでも糖尿病では多枝病変が多く，これは，外国の多くの研究でも指摘されていることである．

私どもの施設では，急性心筋梗塞で救急搬送されて来る人は年々増加しており，2006年は1年間で200人を超えたが，その中で糖尿病あるいは耐糖能異常を示す症例の割合も増加しており，1990年91年では42%であったが，2002年の時点では実に65%の方が糖尿病ないしは境界型であり[8]，2006年は救急入院時点でHbA1cが6.5%を超える人あるいは糖尿病の治療を受けている人は32%に達していた．

3．境界型及び食後高血糖の重要性

さらに重要なことは，診断基準からは糖尿病には至らない境界型も有意の危険因子であり，特に食後高血糖が問題であるということである．すなわち，例え食前血糖が正常でも食後血糖の高いひとは動脈硬化を起こしやすいということが多くの研究により示されている．

富永らによる山形県舟形町のコホート研究でも，境界型で有意に心血管死が増加することが明らかにされたが，しかし，境界型でも空腹時血糖だけが高く，食後血糖が正常範囲にある人（IFG：impaired fasting glucose）は正常耐糖能の人と有意差が無く，従って，食後高血糖が問題であるとしている[9]．このことは，日本を含むアジア諸国を対照としたDECODA研究でもより一層明確にされており，空腹時血糖を基準に診断された境界型及び糖尿病は心血管死の有意な危険因子とされるが，それを2時間血糖で補正すると例え糖尿病群でも正常血糖者との有意差はなくなり，一方，2時間値を基準に診断されたものは，空腹時血糖値で補正してもその危険度は変わらないということが明らかにされている．

4．糖尿病における動脈硬化の成因

糖尿病では高脂血症，高血圧の合併が多いことが知られている．我々の施設で急性心筋梗塞患者につき90年，91年と2002年を比較したところ，糖尿病で3つ以上の危険因子を有する人は90年，91年の時点では46%であったが，10年後には68%になっている[8]（図2）．高血糖自体もさまざまな影響を示し，また，炎症性サイト

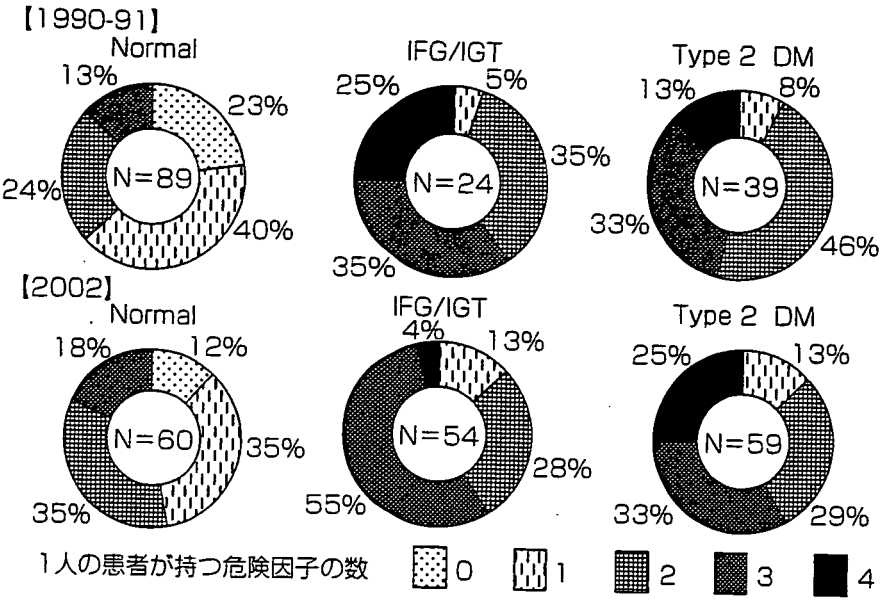

図 2. 急性心筋梗塞患者の危険因子の数（文献 8）

表 1. 糖尿病の動脈硬化促進機序

高脂血症	高血糖
VLDL 増加	PKC 亢進
LDL 増加	ポリオール経路亢進
脂質代謝異常	AGE
レムナントの増加	酸化ストレス亢進
HDL-C の低下	サイトカイン増加
Small Dense LDL	レニン・アンジオテンシン系亢進
LDL の易酸化性	
高血圧	
凝固能亢進	

PKC : protein kinase C
AGE : advanced glycation endproduct

カインの増加や，レニンアンジオテンシン系の亢進も知られている（表 1）.

脂質代謝についてみると，糖尿病ではコレステロールやトリグリセリドの増加だけでなく，リポ蛋白の質的変化も重要である．コレステロールやトリグリセリド，HDLコレステロールの値がほとんど同じ集団において，糖尿病患者では電気泳動上ミッドバンドとして検出されるリポ蛋白が 73% に存在し，非糖尿病ではこれを持つものは 37% であった．一方，糖尿病患者でこのミッドバンドが検出された人とされなかった人を比べると，コレステロールやトリグリセリドやHDL-コレステロールの値がほぼ同じでも，検出された人では虚血性心疾患の頻度が 44% で検出されなかった人（11%）の 4 倍であり，糖尿病では血清脂質値だけでなくリポ蛋白の質の変化も重要であると考えられる．

高血糖自体の影響についても多くの機序が知られているが，われわれはポリオール経路の亢進と内皮細胞の一酸化窒素の産生について検討した．血管の正常な機能の維持には血管内皮細胞の産生する一酸化窒素NOが非常に重要である．このNOは血流によるずり応力が刺激となって産生されるが，高血糖状態ではその産生が低下する．培養内皮細胞でもずり応力を加えるとNOの産生は著しく増加するが，高濃度のブドウ糖により低下する．これにポリオール経路の律速酵素であるアルドース還元酵素の阻害薬を加えるとNOの産生が回復した．内皮細胞のNO合成酵素（eNOS）はNOの産生にNADPHを必要とするが，高濃度のブドウ糖によりポリオール経路が亢進するとアルトース還元酵素による反応でNADPHを消費され，eNOSがNADPHを十分に利用することができずにNO合成が阻害される．アルドース還元酵素を阻害するとNADPHの供給が回復してNO産生も回復したと考えられる．

表2. CRPによるTUNEL陽性細胞とカスパーゼ-3活性の増加（文献11）

	対照	CRP	p値
TUNEL陽性細胞（%）	0.47±0.10	0.77±0.10	＜0.01
カスパーゼ-3活性（対照比）	1.0±0	1.16±0.07	＜0.05

また，糖尿病ではインスリン抵抗性が問題となる．我々は83年にマクロファージがインスリン抵抗性誘発物質を産生すると報告したが[10]，これが今日TNFα（tumor necrosis factor-α）と呼ばれているものである．93年にこのTNFαが脂肪組織でも産生されることが明らかになり糖尿病におけるインスリン抵抗性の原因物質として非常に注目されるようになってきた．脂肪組織に脂肪が蓄積して肥満状態になるとTNFαの産生が増加し，インスリン抵抗性と軽度の炎症状態をもたらすと考えられている．このような状態では炎症性蛋白であるCRP(C-reactive protein)の濃度が軽度上昇することが知られており，糖尿病でもCRPが上昇しやすいと言われている．

近年，CRPは動脈硬化の危険因子として注目されている．しかし，CRPが動脈硬化を起こしやすい状況を反映する指標であるのか，それ自体が動脈硬化を誘発するのかについては不明であった．そこで，我々は内皮細胞の増殖およびアポトーシスに対するCRPの影響について検討した．CRPは血管内皮細胞の増殖を有意に抑制しアポトーシスを促進した[11]（表2）．また，CRPは単球・マクロファージに働きTNF，IL-1（interleukin-1）およびメタルプロテネース9（MMP9）の産生も刺激した．従って，CRPは動脈硬化の危険因子としての単なる指標ではなく，それ自体が内皮細胞を傷害し，また，血管壁のコラーゲンを分解するMMP9の産生を刺激して血管壁を脆弱化することにより心血管イベントの発症を促進する作用を持つと推測される．

5. 動脈硬化の予防を目的とした糖尿病の管理

これまで述べたように糖尿病では動脈硬化を起こし易いことから，糖尿病の治療は動脈硬化を予防するということを視野に管理する必要がある．今日，さまざまな機序の血糖降下薬が用いられているが，インスリン抵抗性改善薬であるチアゾリジン誘導体は血管内皮細胞の機能改善作用を示し，虚血性心疾患を予防することが大規模臨床試験でも示されている．

そこで，我々は耐糖能異常のある狭心症患者のステント留置後の再狭窄に対するピオグリタゾンの影響を検討した．ステント留置時と6カ月後にIVUS（intravascular ultrasound）を用いて内膜の厚さを測定し，ステント内の面積（stentCSA）から内腔面積（lumen CSA）を引いた面積を内膜面積（intimal area）とした．6カ月後に内膜面積とステント内面積との比であるintimal indexで評価したところ，ピオグリタゾンは有意に内膜肥厚を抑制することが確認された[12]．

前述のように，内皮細胞の機能維持にはNOが非常に重要であり，糖尿病ではこれが減少するが，SU（sulfonylurea）薬のうちグリメピリドは内皮細胞のNO産生を増加させる．この効果はPI3（phosphatidylinositol 3）カイネースを阻害すると無くなることから，グリメピリドは内皮細胞における生理的なNO産生系を刺激すると推測している[13]．

これらのことは血糖降下薬の選択に当たっては考慮されるべきことと考えるが，繰り返し述べたように糖尿病では動脈硬化のさまざまな他

の危険因子が合併することから，血糖のコントロール以外の管理も重要である．

JDCSの中間解析によると，糖尿病患者で大血管症の発症に特に強く影響するのは，当然のことながら血糖コントロールすなわち，HbA_{1c}が挙げられるが，その他に心疾患ではLDLコレステロールとTG (triglyceride) と喫煙，脳血管では血圧が重要であるとされている[4]．従って，血圧や血清脂質などを含めた多面的な管理が非常に重要であると考えられる．

デンマークのステノ研究グループは血糖，血圧，血清脂質などを多面的に非常に厳密に管理することを目標とした強化療法群とそれよりもややゆるい目標で治療した従来療法群で心血管イベント予防の効果の違いを検討し，約8年間で，心筋梗塞，脳卒中，末梢血管障害のすべてを合わせた動脈硬化性疾患の発症は，強化療法により約半分に抑制されることを示している[14]．興味深いことに，強化療法群ではコレステロールや血圧については50～70%が目標値に達したが，血糖のコントロールはHbA_{1c}が6.5%未満という目標を満たした人は強化療法群でも2割に満たなかったと報告されている．このことは，血圧や脂質に比べて血糖のコントロールがいかに難しいかということを示すと同時に，例え，血糖のコントロールが完璧でなくても血圧や高脂血症などが管理できるものについては厳格に管理することにより動脈硬化性疾患はある程度抑制できるということ，換言すれば，血圧や高脂血症の厳重な管理が血糖コントロールにまさるともおとらず非常に重要であるということを示すものと考える．

6. 動脈硬化性疾患の予防を目的とした糖尿病治療に関する臨床研究

わが国においても同様の調査研究がいくつか行われているが，その1つに元国立循環器病センターの原納らが中心となって行われている

表3. 糖尿病の動脈硬化診療ガイドライン

日本糖尿病学会・日本動脈硬化学会合同委員会

①高血糖の是正
：HbA_{1c}の正常化：5.8%以下を目指し，6.5%未満を上限とする
②高脂血症
：日本動脈硬化学会のカテゴリーCに準ずる
LDL-C < 100mg/dl (TC < 180mg/dl)
TG < 150mg/dl
HDL-C ≧ 40mg/dl
③高血圧の正常化
：130/85mmHg未満を目標とする
④肥満の解消
：BMI 25kg/m²未満になるよう努め，BMI 22kg/m²を目標とする
⑤禁煙

及川眞一，他，糖尿病 44：777，2001

MRFACTがある．40歳から70歳の2型の糖尿病を対象にして，血糖，コレステロール，TG，血圧，体重の目標値の違いにより従来療法群と強化療法群にわけて，虚血性心疾患，脳血管障害，末梢血管障害にどのような違いが出るのかを検討しようという多施設研究である．もう1つ最近，厚生労働省の大きな事業として同様な検討を目的にJ-DOIT3研究というものが，東京大学の門脇をリーダーとして始められている．これも従来療法と強化療法の二群に分けての比較検討であるが，それぞれの指標が目標値に達するように薬物の種類を含めた具体的な指針が示されており，それにそった治療の効果を検討するものである．

7. 動脈硬化予防を目標とした糖尿病治療のガイドライン

将来的には上述の臨床試験などの結果を踏まえた治療指針が提案されることも予想されるが，糖尿病学会と動脈硬化学会の合同委員会では，現在のところ動脈硬化の予防を目標とした表3に示すガイドラインを提示している[15]．血糖のコントロールはHbA_{1c}で5.8%以下，悪くても6.5%

未満，LDLコレステロールは100未満，TGは150未満，HDLコレステロールは40以上，血圧は130/85未満，BMIは22，悪くても25未満，そして禁煙とされている（表3）．

おわりに

以上をまとめると，糖尿病は動脈硬化を起こしやすく，たとえ食前血糖が正常でも食後血糖が高いだけで動脈硬化の危険因子となること．また，高血圧や高脂血症も非常に厳格に管理する必要があるということになる．

謝辞：われわれの施設のデータは，総合診療科，内分泌代謝科，循環器科の共同により検討されたものであり，関係諸氏に感謝する．

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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-

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From the Departments of Clinical Cell Biology (K.O., Y.S.) and Genome Research and Clinical Application (H.B., M.J.), Chiba University Graduate School of Medicine, Chiba, Japan; Kowa Research Institute (H.Y.), Kowa Co Ltd, Higashimurayama, Japan; Department of Medical Biochemistry (W.J.S.), Max F. Perutz Laboratories, Medical University of Vienna, Vienna, Austria.

Correspondence to Hideaki Bujo, Departments of Genome Research and Clinical Application Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. E-mail hbujo@faculty.chiba-u.jp

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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

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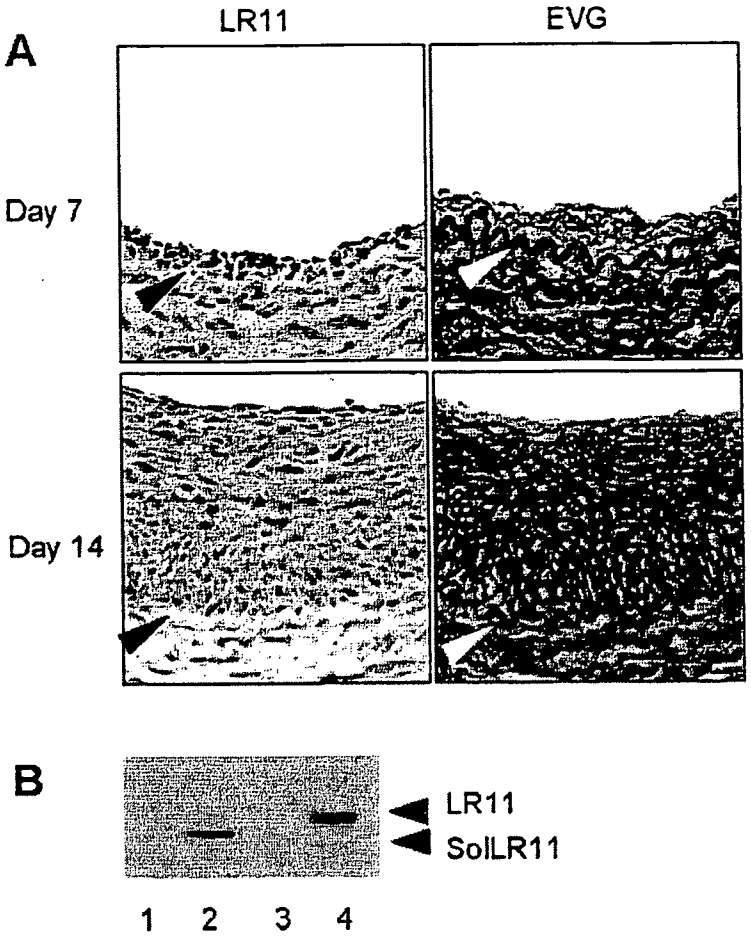

Figure 1. SolLR11 expression in intimal SMCs in balloon-denuded rat artery. A, Sections of balloon-denuded carotid artery were subjected to histological analysis using elastica van Gieson staining (EVG), and to immunohistochemistry with anti-LR11 antibody (pm11) at day 7 (top) and day 14 (bottom) after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

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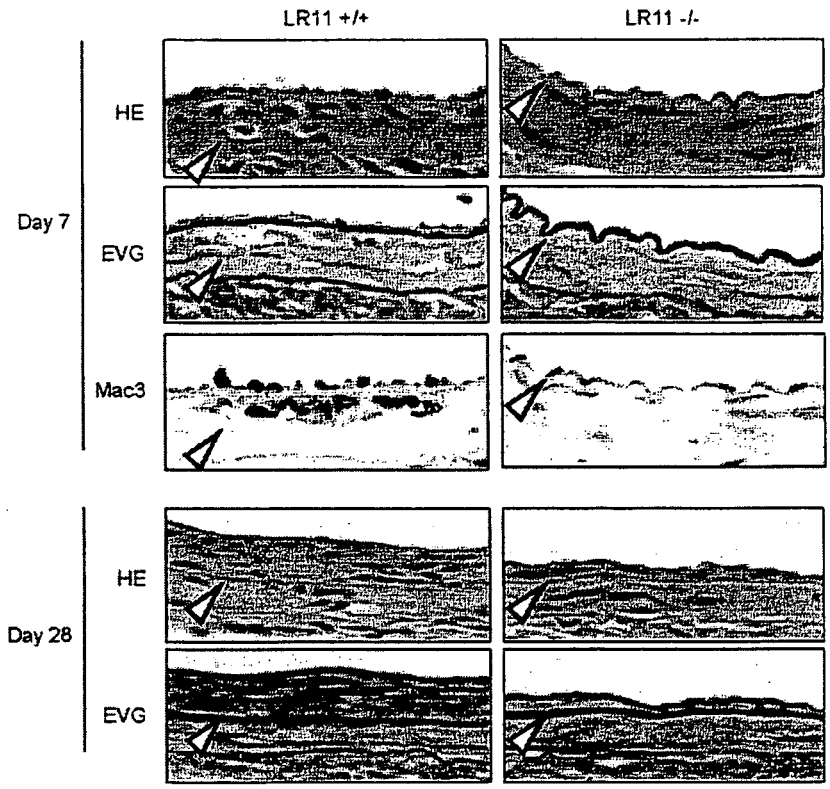

Figure 2. Reduced macrophage infiltration into intima of cuff-injured artery in LR11$^{-/-}$ mice. Sections of femoral artery obtained at day 7 and day 28 after cuff injury in LR11$^{+/+}$ and LR11$^{-/-}$ mice on a high-fat diet were subjected to histological analysis using hematoxylin & eosin (HE)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

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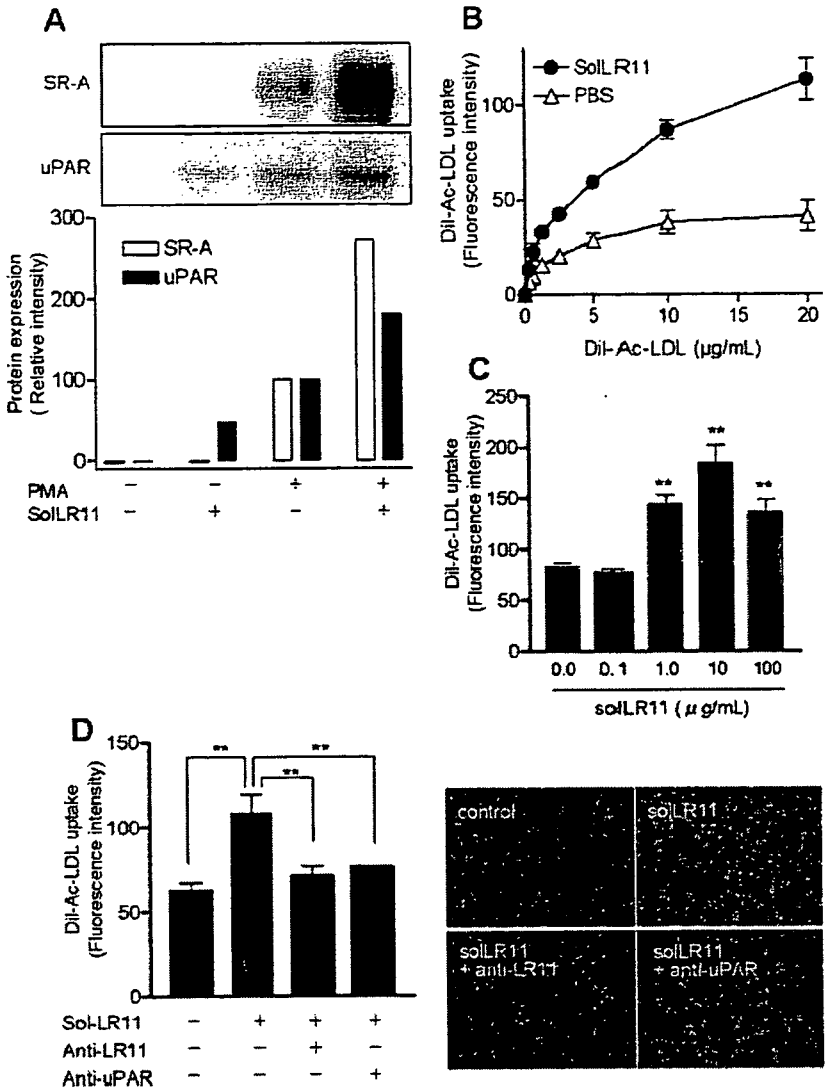

Figure 3. SolLR11 enhances cell-surface expression of SR-A and uPAR, and the uptake of modified LDL by THP-1 macrophages. A, Membranes of THP-1 monocytes or macrophages, prepared from cells obtained under the conditions indicated in the bottom panel, were subjected to Western blotting with anti-SR-A 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-

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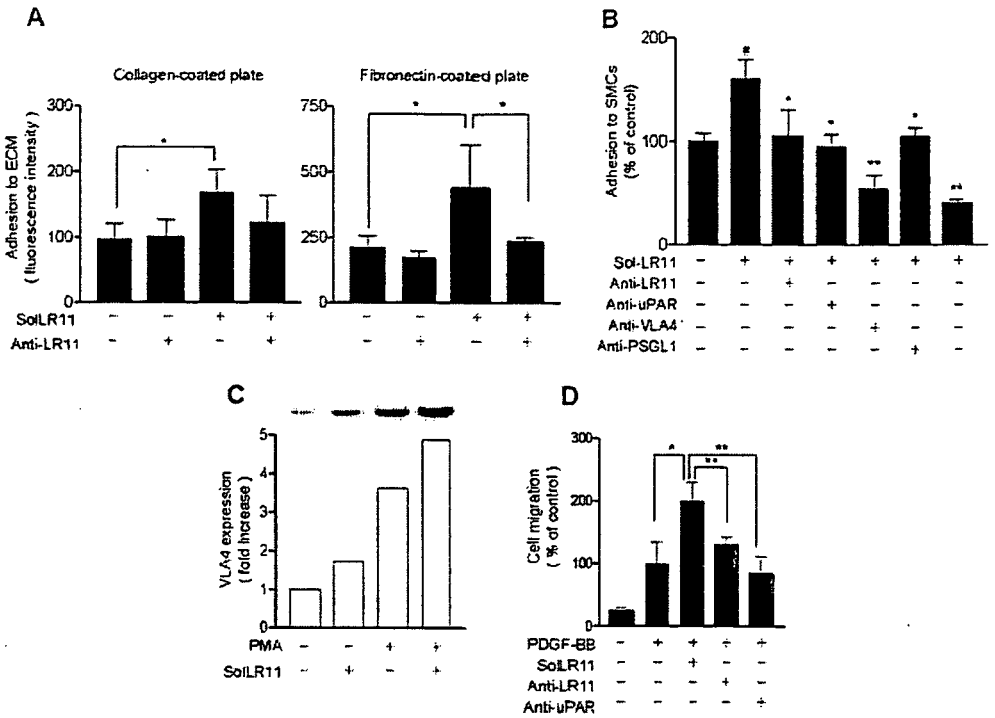

Figure 4. SolLR11 enhances adhesion and migration of THP-1 macrophages. A, THP-1 macrophages were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-uPAR antibodies. The cells were washed and then incubated on collagen- or fibronectin-coated plates. B, THP-1 macrophages 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,

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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.

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Disclosures

None.

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

Yoshitaka Kubota [a], Hiroyuki Unoki [b], Hideaki Bujo [c,*], Naoaki Rikihisa [a], Akikazu Udagawa [a], Shinya Yoshimoto [a], Masaharu Ichinose [a], Yasushi Saito [d]

[a] *Department of Plastic and Reconstructive Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[b] *Department of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[c] *Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[d] *Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*

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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.
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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

* Corresponding author. Fax: +81 43 226 2095.
E-mail address: hbujo@faculty.chiba-u.jp (H. Bujo).

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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)-1 (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

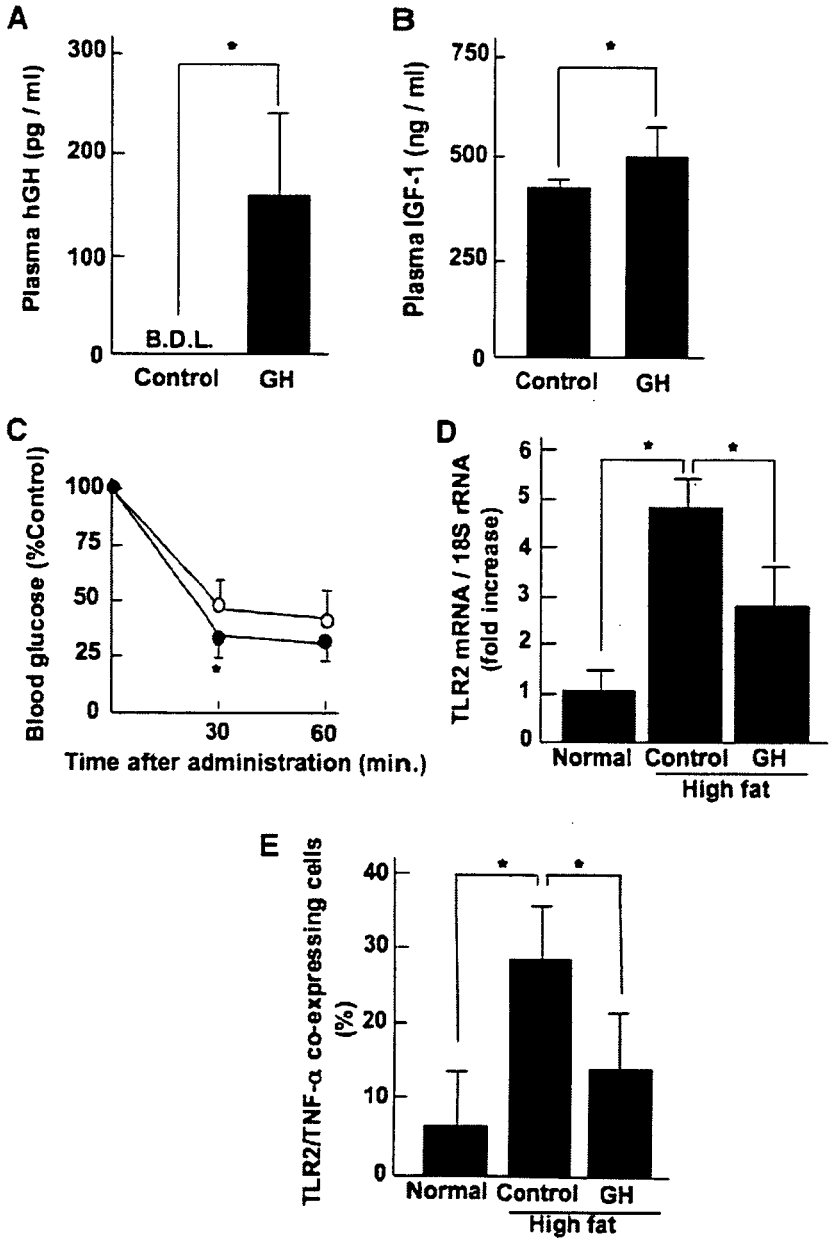

Fig. 1. The effects of low-dose hGH supplementation on reduced insulin sensitivity in high-fat fed mice. (A) The plasma hGH concentration in mice supplemented with hGH (GH) or PBS (control). Male C57BL/6J mice, which have been fed with a high-fat diet (60% fat) for 6 months, were supplemented with hGH (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.

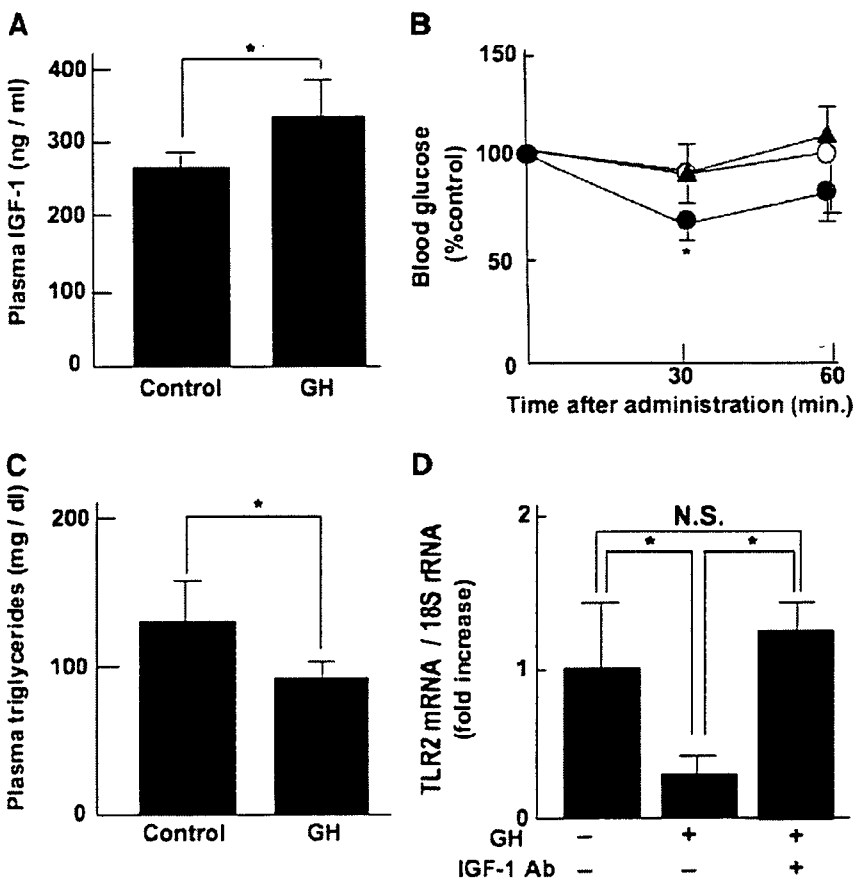

Fig. 2. Effects of IGF-1 neutralization on the actions of low-dose GH supplementation using cell transplantation models. Male BALB/c nude mouse was subcutaneously injected with 10^6 cells of hGH-overexpressing 3T3-L1 preadipocytes. A high-fat diet was started at a week after cell implantation, and continued 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.