緒　　言

動脈硬化症は、虚血性心疾患や脳血管障害、閉塞性動脈硬化症などの虚血性血管障害の主要な原因である。動脈硬化症の発症・進展には多くの因子が関与しており、なかでも高コレステロール血症に代表される血中脂質異常は、現在までに立証されている危険因子の一つである[1,2]。さまざまな疫学調査[1]や高脂血症患者[2]および疾患モデル動物の解析[3]などにより、その粥状動脈硬化発症・進展への関与が証明されている。しかしながら、血中脂質の中でも中性脂肪の上昇と血管壁での脂質の蓄積との関連は、必ずしも明解ではなく[4]、高中性脂肪血症による動脈硬化症の発症・進展機序に関しては、未だ確固たる結論は得られていない。

粥状動脈硬化病変は、組織学的にみると、脂肪線条（fatty streak）、線維性硬斑（fibrous plaque）を経て、石灰化や血栓付着を伴った進行性病変へと進展し血管内腔の閉塞をきたす。これは、単球がスカベンジャー受容体を発現するマクロファージに分化し、酸化 LDL などの変性 LDL を取り込んで泡沫細胞となり、線条脂質沈着巣を形成することによる[5,6]。コレステロールは、最終的には低比重リポ蛋白（LDL）として血液中に運搬されるので、脂質の中でもコレステロールが、動脈硬化発症の初期において重要な役割を担うと考えられている。さらに、高脂血症では、組織学的な動脈硬化発症に加えて、血管機能の低下がおこる。これは、酸化 LDL、β VLDL のようなリポ蛋白が内皮依存性弛緩反応を低下させることによる[7]。すなわち、血管内で停滞した LDL は、局所の酸化ストレスにより変性して酸化 LDL となり、血管内皮細胞機能障害をもたらす[7]。血管内皮依存性弛緩反応は、内皮細胞に存在する一酸化窒素合成酵素（eNOS）から産生される NO により生じる[8]。動脈硬化症や高脂血症では、酸化 LDL による血管内皮障害の結果、eNOS から産生される NO が減少することにより、内皮依存性血管弛緩反応が低下する[7,8]と考えられる。

コレステロールの重要性に比較して、中性脂肪の動脈硬化危険因子としての役割は、これまで十分な評価がなされてこなかった。その理由は、①中性脂肪それ自体は粥状動脈硬化病変に蓄積されないこと[9]、②著しい高中性脂肪血症を呈する高脂血症においても、粥状動脈硬化は軽度だったとの報告があること[9]、などによる。一方、中性脂肪含有リポ蛋白であるいわゆるレムナントリポ蛋白は、加水分解後に容易に血管内皮細胞下に侵入して粥状動脈硬化に関係する[10]。その際、レムナントリポ蛋白は、LDL とは異なり変性を受けることなくマクロファージの受容体（LRP，VLDL 受容体，アポ B48 受容体など）を介して取り込まれ、マクロファージの泡沫細胞化に働く。しかしながら、中性脂肪は加水分解されて蓄積されないまま消失することも報告されている[9]。

山形大学医学部器官病態統御学講座、循環・呼吸・腎臓内科学分野と山形大学医学部動物実験施設では、遺伝性高コレステロール血症家兎を中性脂肪値にしたがって繰り返し選抜交配し、中性脂肪が高値（TG＞500 mg/dl）の群（high-triglyceride group; TGH）と低値（TG＜250 mg/dl）の群（low-triglyceride group; TGL）の２系統の遺伝疾患モデルの樹立に成功した[11]。この遺伝疾患モデル動物の基礎的循環動態については明らかにされていない。そこで本研究では、高中性脂肪血症家兎（TGH）の動脈硬化病変の分布および循環動態について検討した。

材料と方法

実験には、24 ヶ月齢の Japanese White rabbits（JW）（♂, 体重 3.7±0.1 kg, n＝5）、TGH rabbits（TGH）（♂, 体重 2.5±0.02 kg, n＝6）を用いた。本実験は、山形大学医学部動物実験指針[12]、動物の愛護及び管理に関する法律（昭和

48年法律第105号)、実験動物の使用及び保管等に関する基準（昭和55年総理府告示第６号）を遵守して行った。

１．**組織学的検討**：大動脈を摘出した後、Ⅰ：心臓起始部から総頸動脈分岐部までの部位（大動脈弓部）、Ⅱ：総頸動脈分岐部から肋間動脈分岐部までの部位（胸部大動脈上部)、Ⅲ：肋間動脈分岐部付近の部位（胸部大動脈中部)、Ⅳ：横隔膜付近の部位（胸部大動脈下部）の４カ所に分け（図1，右)、24時間10%ホルマリン溶液で固定した。固定後の大動脈は、Hematoxylin-Eosin 染色および Elastica-Masson trichrome 染色を行い、それらの組織像を Adobe Photoshop を用いて Film Scanner (Nikon; LS 1000）で読み込んだ。画像解析には NIH Image を用いて、動脈硬化面積〔(肥厚内膜面積 / 平滑筋内膜で囲まれた面積) ×100〕を計算した。

２．**血圧測定**：ketamine (0.11mg/kg/min, i.v.) と xylazine (0.02mg/kg/min, i.v.）の静脈内投与により麻酔を行った。さらに、大腿動脈にカニューレを挿入し、圧トランスデューサーを用いて持続的に血圧を測定し、Thermal Array Recorder (NIHON KOHDEN; RTA 1200M) に記録した。また、大腿静脈に留置したカニューレより NO 合成酵素阻害薬である N^G-nitro-L-arginine methyl ester (L-NAME; 26mg/kg) を投与し、それによる血圧の経時的変化を測定した。

使用薬物

実験には、ketamine hydrochloride（三共，東京)、xylazine（バイエル，東京)、N^G-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Co., St. Louis, MO, USA) を用いた。

統計処理

得られたデータは、正規分布の検定を行い、正規分布の確認後に、対応のない Student's *t*-test（等分散の場合）あるいは Welch's *t*-test（等分散でない場合）を用いて検定した。また、血圧変化率の比較には、ノンパラメトリック検定である Mann-Whitney's U test を用いた。それぞれの値はすべて平均値±標準誤差で表示した。$P<0.05$ をもって有意差があると判定した。

結　果

１．**大動脈における動脈硬化病変**（図１，図2)

図１は、JW と TGH の大動脈における動脈硬化病変の顕微鏡写真を示している。JW では、検討したいずれの部位においても動脈硬化病変は認められなかった。一方、TGH では、検討したほとんどの部位において動脈硬化病変を認め、特に大動脈弓部において病変が顕著であった（図１)。動脈硬化病変の分布率を NIH Image を用いて解析したところ、JW では検討した部位に硬化巣は全く見られなかったのに対し、TGH の動脈硬化分布率は、大動脈弓部 52.9±6.8%、胸部大動脈上部 48.7±6.3%、胸部大動脈中部 31.3±7.5%、胸部大動脈下部 20.9±9.2%であった（図２)。

JW では、検討したいずれの部位においても、単層の内皮細胞が脱落せずに血管内腔を覆っていた。その直下に整然と内弾性板があり内膜肥厚は全く生じていなかった。また、マクロファージや泡沫細胞は観察されなかった。一方 TGH では、内皮細胞が一部で脱落しているのが観察された。粥状硬化部では、膠原線維の豊富な線維性内膜肥厚の進行が見られ、内弾性板が途切れて断絶している箇所も認められた。さらに、中膜平滑筋細胞が内弾性板を越えて内膜へ遊走、侵入して増殖している像も観察された。TGH では、動脈硬化部位内膜においてマクロファージおよび大型化した泡沫細胞が多数見られた。

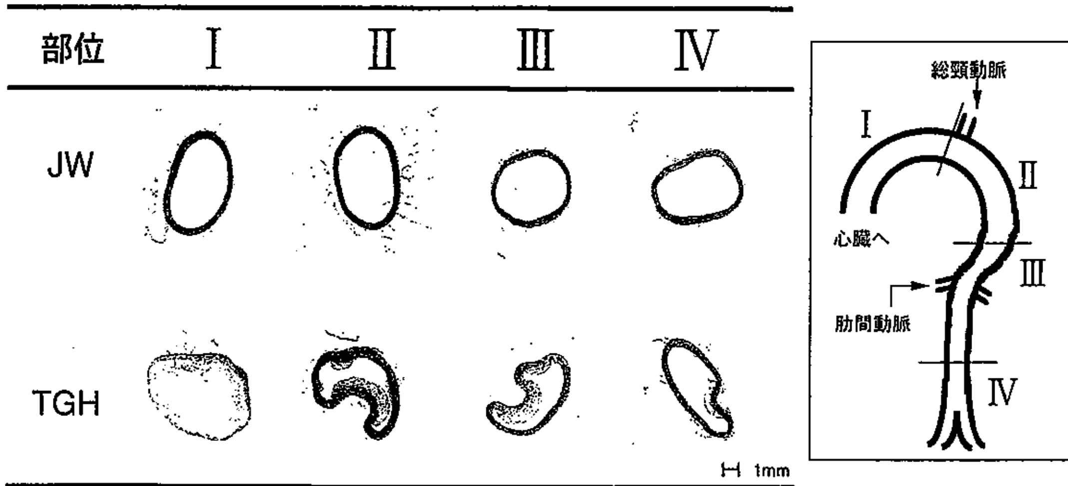

図 1．大動脈における動脈硬化病変の比較
左図の上段は Japanese White rabbits（JW）、下段は TGH rabbits（TGH）の大動脈における動脈硬化病変の顕微鏡写真を示したものである。 I：大動脈弓部（心臓起始部から総頸動脈分岐部までの部位）、II：胸部大動脈上部（総頸動脈分岐部から肋間動脈分岐部までの部位）、III：胸部大動脈中部（肋間動脈分岐部付近の部位）、IV：胸部大動脈下部（横隔膜付近の部位）を示している。右図はI～IVの各部位を示している。

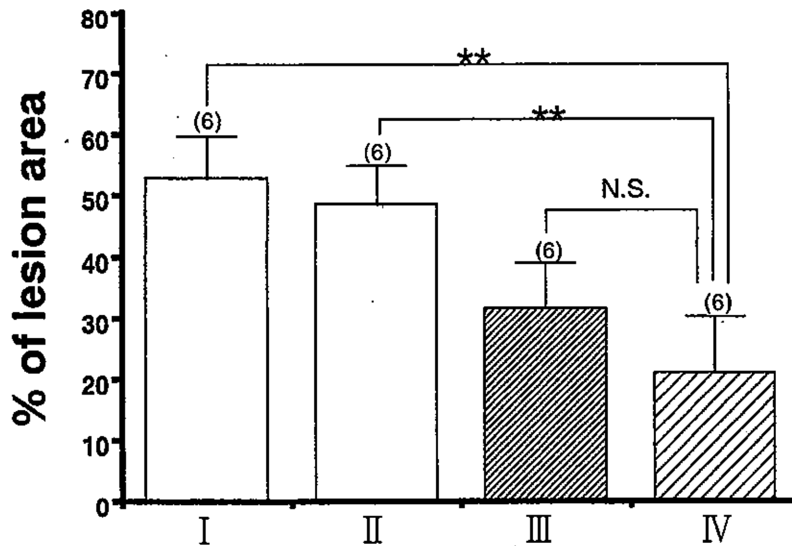

図 2．動脈硬化病変の NIH Image による解析
グラフの値は、% of lesion area=（肥厚内膜面積 / 血管平滑内膜で囲まれた面積）× 100 で示したものである。この解析には Image editing software Adobe Photoshop と NIH Image を用い、値は平均±標準誤差で表している。
I：大動脈弓部（心臓起始部から総頸動脈分岐部までに部位））
II：胸部大動脈上部（総頸動脈分岐部から肋間動脈分岐部までの部位）
III：胸部大動脈中部（肋間動脈分岐部付近の部位）
IV：胸部大動脈下部（横隔膜付近の部位）
$^{**}P < 0.01$（vs. 胸部大動脈下部）N.S.: not significant　（　）内は実験例数。

2．大腿動脈圧

(1) 定常時血圧（図3）

JWとTGHの定常時血圧は、それぞれ拡張期血圧69.0±6.9mmHg、63.3±2.8mmHg、収縮期血圧88.5±6.4mmHg、105.8±4.8mmHg、平均血圧75.5±6.5mmHg、77.5±2.6mmHgであった。これらの値には、JWとTGH間で有意差はみられなかった。しかし、JWの脈圧は19.6±3.5mmHgだったのに対し、TGHでは40.5±5.6mmHgであり、JWよりもTGHのほうが有意に増大した。

(2) L-NAMEによる血圧の変化（図4）

NOの血圧への関与を検討するためにL-NAMEによる平均血圧の変化をみた。L-NAME投与前の平均血圧はJWでは85.5±6.3mmHg、TGHでは88.6±4.8mmHgであったのに対し、L-NAME投与10分後の平均血圧は、JWでは101.6±2.4mmHg、TGHでは110.6±6.9mmHgであった。このようにL-NAMEによって、JWとTGHいずれにおいても血圧が有意に上昇した。また、その血圧上昇率は、JWでは121.5±8.4%、TGHでは125.1±3.5%であり、両群間で変化率に差はみられなかった。

考　　察

高コレステロール血症に代表される血中脂質異常は、動脈硬化発症の危険因子の一つである。さらに、高中性脂肪血症が冠動脈疾患の発症にどのように関与するのかについて、欧米およびわが国において研究が進められてきた[1),2)]。しかし、中性脂肪の上昇と血管壁で生じる脂質の蓄積との関連は明確ではなく[9)]、高中性脂肪血症がどのような機序で動脈硬化を発症するのか、また動脈硬化の進展にどの程度関与するのかは不明である。

本研究では、新しく樹立された遺伝性高中性脂肪血症家兎を用いて、その基礎的特性を明らかにする目的で、①大動脈における動脈硬化病変の分布、②遺伝性高中性脂肪血症家兎の循環動態について検討した。

<u>大動脈における動脈硬化病変</u>

JWでは、検討したいずれの部位においてもマクロファージ・泡沫細胞は見られず、単層の内皮細胞が血管内腔を覆っていた。TGHでは、

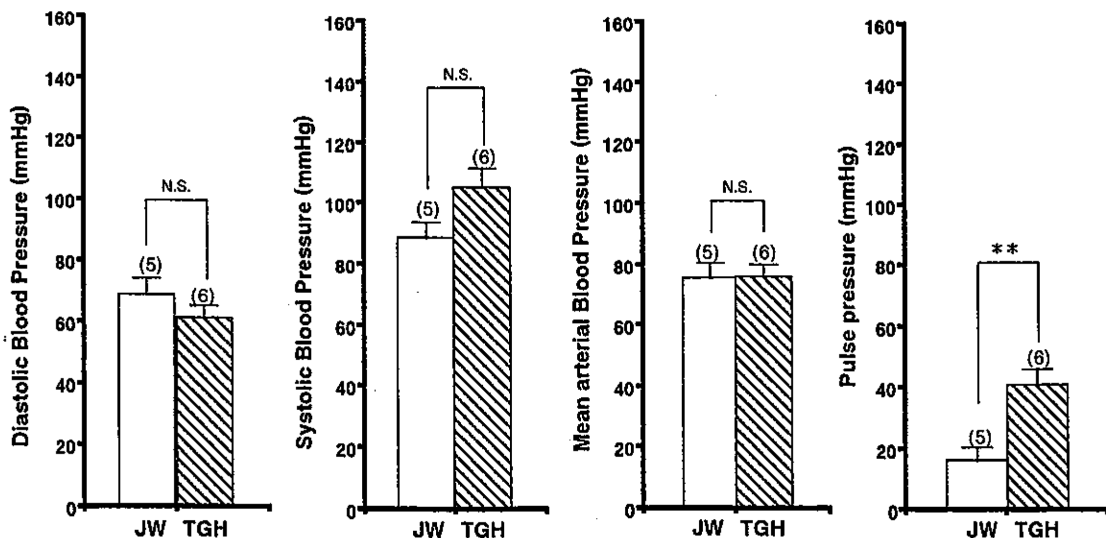

図3．定常時血圧
グラフの値は、左から拡張期血圧、収縮期血圧、平均血圧、脈圧の実数値を示し、値は平均値±標準誤差で表している。**$P<0.01$（JW vs.TGH） N.S.: not significant （ ）内は実験例数。

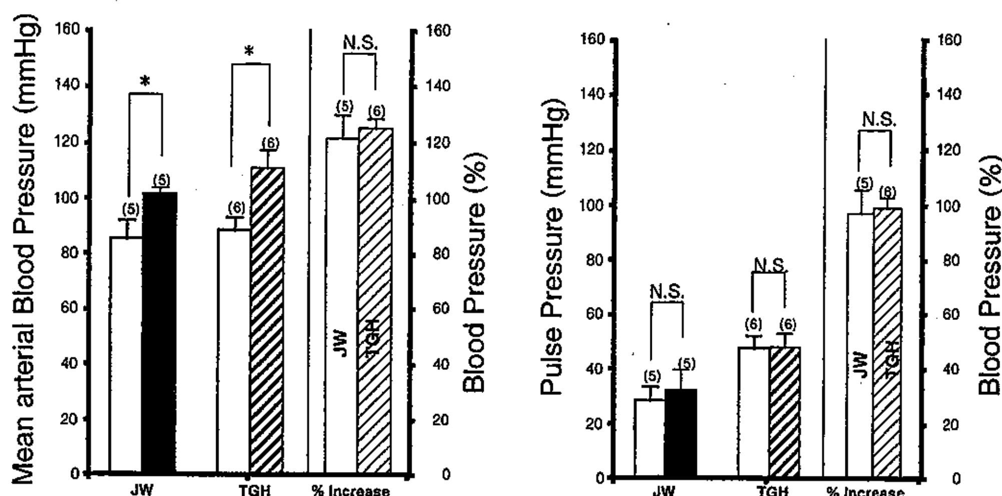

図4. L-NAME による血圧の変化
グラフの値は、左から平均血圧と脈圧の実数値と血圧変化率を表している。血圧変化率とは、L-NAME 投与前の血圧を100%とし、L-NAME 投与10分後の血圧変化率を示した。値は平均値±標準誤差で表している。
*$P<0.05$（vs. L-NAME 投与10分後の血圧） N.S.: not significant （ ）内は実験例数。
□：JW の L-NAME 投与前の血圧 □：TGH の L-NAME 投与前の血圧
■：JW の L-NAME 投与10分後の血圧 ▨：TGH の L-NAME 投与10分後の血圧
□：JW rabbits の血圧変化率
▨：TGH rabbits の血圧変化率

内皮細胞の脱落、線維性内膜肥厚の進行、中膜平滑筋細胞の内膜下への遊走・増殖、多数の泡沫細胞が見られた。以上の結果は、TGH においては、内皮細胞の機能異常に伴って血液中の単球・マクロファージが内膜下に侵入し、変性脂質等を貪食して泡沫細胞化し、さらに種々の増殖因子やサイトカインを産生して中膜平滑筋の遊走・増殖を促進し、粥状硬化病変を形成するという一連の過程が進行していることを示唆するものと考えている。

本研究の TGH（24ヶ月齢）で得られた結果は、22ヶ月齢の WHHL の大動脈で見られた動脈硬化病変の進展[13]と類似していた。また、TGH では、動脈硬化病変は大動脈弓部から胸部大動脈にかけて広範囲にみられたが、大動脈弓部にもっとも強く認められた。WHHL においても、動脈硬化病変は大動脈弓部と細動脈分岐部に形成される傾向がある[13]。その理由のひとつとして、動脈分岐部付近での血液の流れに、乱流が生じやすいことが挙げられる。

定常時における大腿動脈圧

持続麻酔下の大腿動脈圧を測定した結果、定常時の収縮期・拡張期・平均血圧のいずれにおいても、JW と TGH 間に有意差は認められなかった。これらの結果は、TGH と同様に粥状動脈硬化の進展している WHHL と JW の定常時血圧に有意差を認めなかったという報告[14]と一致している。このように各パラメータには有意差がなかったものの、TGH の脈圧は、JW に比較して有意に増大していた。同様に、WHHL においても加齢に伴い脈圧が増大するという報告がある[18]。さらに、LDL 受容体欠損により高脂血症を引き起こす Kurosawa and Kusanagi-Hypercholesterolemic (KHC) rabbits においても、24ヶ月齢の大動脈弓部に動脈硬化病変を認め、10ヶ月齢の KHC と比較して24ヶ月齢では脈圧が増大したとの報告がある[17]。したがって、本研究で明らかになった TGH における脈

圧増加は、動脈硬化によって大血管の弾力性が低下していることが原因であろう。動脈硬化が進展すれば大血管の弾性低下をきたし、さらに脈圧を増大させることが報告されている[15),16)]。脈圧増加は、動脈壁に大きな負荷をあたえることにより血管構造変化を引き起こす可能性が高い。そのため、動脈硬化が進展するとさらに大動脈弾性が低下する。このような悪循環の結果、収縮期血圧が上昇し、拡張期血圧が低下してくるものと考えられる。

大腿動脈圧の調節におけるNOの関与

血管内皮細胞は、NOを介して循環調節および血管壁の恒常性維持に寄与し、その機能障害は動脈硬化の発症・進展に深く関与する[7)]。高脂血症では、組織学的に動脈硬化性変化がほとんど認められない初期段階でも、血管内皮依存性弛緩反応の障害がおこる[19)]。これは、酸化LDL等のリポ蛋白が血管内皮傷害を発生する結果、血管内皮依存性弛緩反応が低下するからである[19)]。

動脈硬化症の進展とともに内皮依存性血管弛緩反応が低下する[7),8)]。実際にWHHLでは、血漿NO値がJWよりも有意に低下していること[14)]や内皮依存性弛緩反応の減弱が報告されている[21)]。内皮依存性弛緩反応の減弱は、内皮細胞障害によりNOの産生が減少することが原因のひとつと考えられる。定常状態での内皮由来NO生成を抑制することによって、血管の緊張が亢進し、血圧が上昇する可能性がある。そこで本研究では、TGHおよびJWにおいて、NO合成酵素阻害薬であるL-NAMEの血圧に対する影響を調べることにより、定常状態でのNO生成状態を比較検討した。その結果、L-NAMEを投与することによって、JWの収縮期血圧は約16mmHg上昇した。一方、定常状態におけるNO生成が少ないために、L-NAME投与による血管収縮反応は減弱するだろうと予想されたTGHにおいても、収縮期血圧は約22mmHg上昇した。このL-NAME投与による血圧上昇には、JWとTGH間で有意差はなかった。本研究では、血中NO濃度の測定は行っていないが、L-NAME投与による血圧上昇反応に差が認められなかったことから、TGHにおいても十分な量のNOが産生されている可能性がある。生体内でNOを生成するNO合成酵素には、3タイプのアイソフォーム、すなわち神経型NO合成酵素（nNOS; NOS1)、誘導型NO合成酵素（iNOS; NOS2)、内皮型NO合成酵素（eNOS; NOS3)が知られている。nNOSは、中枢神経細胞、末梢神経細胞、膵臓β細胞などに存在し、細胞内カルシウム濃度依存性に活性調節される。iNOSは、サイトカインやリポ多糖類などにより発現誘導される酵素で、肝細胞、マクロファージ、消化管上皮、血管平滑筋細胞、グリア細胞等に存在し、カルシウム濃度非依存性である。eNOSは、おもに血管内皮細胞に存在し、その活性は、細胞内カルシウム濃度あるいは酵素のリン酸化によって調節される[22)]。Shishidoら[23)]の報告によると、TGHの大動脈におけるeNOS蛋白およびmRNA発現はJWと同程度であるが、TGHの摘出血管標本ではアセチルコリンに対する内皮依存性弛緩反応が減弱している。このことから、TGHでは、eNOSの量とeNOSから産生されるNOの量は相関していないことが示唆される。TGHでは、動脈硬化病変の形成に関与するマクロファージや増殖した平滑筋細胞においてiNOSが誘導、活性化されているため、それらが局所におけるNOの産生・放出を増加させている可能性[13)]が挙げられる。したがってTGHにおいては、内皮細胞のNO産生能の変化のみならずマクロファージや血管平滑筋から産生されるNOの関与を検討することも重要であろう。

本研究で用いた動物の血中コレステロール値を測定した結果、JWでは58.2±5.1mg/dl、TGHでは442.7±27.7mg/dlであった。また、中性脂肪値は、JWでは63.1±8.0mg/dl、TGHでは446.0±35.2mg/dlであった。このようにTGHでは、血中コレステロールおよび中性脂肪のいずれも、JWよりも数倍高値を示した。

同様に、若齢（3－4ヶ月齢）のウサギで比較したShishidoら[23]の報告でも、TGHの血中コレステロール値と中性脂肪値はJWよりも高値であった。このことから、TGHで観察された動脈硬化病変が高中性脂肪血症によるのか、それとも高コレステロール血症に合併した高中性脂肪血症によるのかを明らかにするには、24ヶ月齢のTGHとTGLを比較検討する必要がある。この問題については、今後TGLを用いて明らかにしていく予定である。

本研究の結果から、TGHでは動脈硬化病変が著明に進行しており、TGHで見られる血中の脂質異常が動脈硬化症の発症・進展における重要な危険要因であることが示唆された。本学で新しく樹立された遺伝性高中性脂肪血症モデル家兎であるTGHは、脂質代謝異常、特に中性脂肪と動脈硬化発症との関連を研究するうえで、今後益々有用な動物モデルとなると考えられる。

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Progression of Atherosclerosis and Femoral Arterial Blood Pressure in Heritable Hypertriglyceridemic Rabbits

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ABSTRACT

[Objective] It has been reported that hypercholesterolemia plays a role in progression of atherosclerosis. Watanabe heritable hyperlipidemic rabbits have been used widely as a model of familial hypercholesterolemia with atherosclerosis, while the emerging data raised a possibility of an important role of hypertriglyceridemia in the pathogenesis of atherosclerosis. We have recently segregated a new line with severely high (TGH) and moderately high (TGL) levels of plasma triglyceride. The hemodynamic parameters of TGH and TGL are not defined. The aim of present study was to examine the progression of atherosclerosis and hemodynamic parameters of TGH.

[Methods] Japanese White rabbits (JW) and TGH were anesthetized with ketamine and xylazine. BP was measured by a catheter implanted in the femoral artery. Histological examination was carried out with Elastica-Masson trichrome staining to detect atherosclerotic lesions.

[Results] JW had no atherosclerotic lesions. In TGH, severe atherosclerotic lesions were observed in the aortic arch. The basal femoral arterial pressure was not significantly different between JW and TGH. However, the basal pulse pressure in TGH was significantly greater than that of JW. Intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME) increased the blood pressure of TGH as well as JW. There was no difference in the response to L-NAME. The greater pulse pressure in TGH may be due to the increased vascular stiffness with atherosclerosis.

Key words : hyperlipidemia, triglyceride, atherosclerosis , blood pressure, NO

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Probucol Inactivates ABCA1 in the Plasma Membrane with Respect to Its Mediation of Apolipoprotein Binding and High Density Lipoprotein Assembly and to Its Proteolytic Degradation*

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Probucol has been shown to inhibit the release of cellular lipid by helical apolipoprotein and thereby to reduce plasma high density lipoprotein. We attempted to explore the underlying mechanism for this effect in human fibroblast WI-38. Probucol inhibited the apoA-I-mediated cellular lipid release and binding of apoA-I to the cells in a dose-dependent manner. It did not influence cellular uptake of low density lipoprotein, transport of cholesterol to the cell surface whether *de novo* synthesized or delivered as low density lipoprotein, and overall cellular content of cholesterol, although biosynthesis of lipids from acetate was somewhat increased. Probucol did not affect the mRNA level of ABCA1, and ABCA1 was recovered along with marker proteins for plasma membrane regardless of the presence of probucol. However, the protein level of ABCA1 increased, and the rate of its decay in the presence of cycloheximide was slower in the probucol-treated cells. ABCA1 in the probucol-treated cells was resistant to digestion by calpain but not by trypsin. We concluded that probucol inactivates ABCA1 in the plasma membrane with respect to its function in mediating binding of and lipid release by apolipoprotein and with respect to proteolytic degradation by calpain.

Cholesterol is an essential molecule for animal cells to maintain and regulate function and structure of the biomembrane. It is synthesized in most somatic cells, whereas its catabolic site is limited to the liver and to the steroidogenic cells except for partial hydroxylation in some somatic cells. Accordingly, cholesterol is removed from the cells and transported to the liver for its conversion to bile acids, and this is one of the essential events in cholesterol homeostasis for the body and for the cells (1). High density lipoprotein (HDL)[1] is believed to play a central role in this system, and this is thought to be one of the antiatherogenic characteristics of HDL. This reaction takes place through at least two distinct mechanisms: 1) physicochemical release of cholesterol from the cell surface, which is driven by cholesterol esterification on HDL, and 2) the apolipoprotein-mediated pathway to remove cellular cholesterol and phospholipid to generate new HDL particles (2). HDL thus plays a central role in both mechanisms.

Apolipoprotein-dependent cellular cholesterol release is absent in fibroblasts from patients with Tangier disease (3, 4), and mutations in the gene encoding the ATP-binding cassette transporter A1 (ABCA1) are the underlying cause of this disease (5–9). On the other hand, *in vitro* overexpression of functional ABCA1 in the cells (10, 11) and induction of ABCA1 expression by cyclic AMP analogues (12, 13) or by the ligands for the liver X receptor or retinoid X receptor (14, 15) enhanced the release of cellular cholesterol and phospholipid by apolipoprotein. The transgenic mice for ABCA1 had a significant increase in plasma HDL (16, 17). These results indicate that this protein is a regulating factor for the plasma HDL level through generation of HDL by the apolipoprotein-cell interaction.

Probucol has been clinically used as an antiatherogenic compound, not only because of its lipid-lowering effect but also because of the hypothesis that its antioxidative nature prevents atherogenic oxidative modification of low density lipoprotein (LDL) shown by *in vitro* (18, 19) and *in vivo* models (20, 21). However, probucol substantially reduces plasma HDL (22). We reported that probucol causes dramatic selective inhibition of the apolipoprotein-mediated cellular lipid release and its binding to cells (23) to cause reduction of HDL (24), which is analogous to the finding with Tangier disease (3, 4). Thus, this is another piece of evidence that the apolipoprotein-cell interaction to generate HDL is a major source of plasma HDL. Probucol can therefore be considered an inhibitor of the function of ABCA1.

Cellular ABCA1 undergoes both transcriptional and post-transcriptional regulations (25). Transcriptional regulation of ABCA1 is carried out by oxysterol through the liver X receptor/retinoid X receptor system, which seems relevant to the function of ABCA1 to expel an excess amount of cell cholesterol (25). Up-regulation of ABCA1 is also mediated by other factors such as cyclic AMP (13), of which the exact mechanism is unknown. On the other hand, stabilization of ABCA1 can be an alternative mechanism to regulate ABCA1 activity, such as protection of ABCA1 protein by helical apolipoprotein (26–28) and enhancement of its degradation by unsaturated fatty acid (29) or overloaded cholesterol (30). Previous studies have suggested that ABCA1 phosphorylation is involved in its stabilization (31, 32). A PDZ adaptor protein α1-syntrophin was shown to stabilize ABCA1 (33).

In the present study, we attempted to examine the underlying mechanism for inhibition of ABCA1 activity by probucol. Probucol did not influence the transcription and intracellular

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[1] The abbreviations used are: HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; LDL, low density lipoprotein; PBS, phosphate-buffered saline; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; CE, cholesteryl ester; MEM, modified Eagle's essential medium; PMSF, phenylmethylsulfonyl fluoride.

This paper is available on line at http://www.jbc.org

distribution of ABCA1 but suppressed its degradation by calpain to increase inactive ABCA1 in the plasma membrane.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Probucol (4,4′-(isopropylidenedithio)bis [2,6-di-*tert*-butylphenol]) was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Calpain and calpeptin were from Calbiochem. [1,2-^{14}C]Cholesteryl oleate and [^{3}H]acetic acid were obtained from Amersham Biosciences, and iodine-125 (^{125}I) was from PerkinElmer Life Sciences.

Lipoprotein and Apolipoprotein—Lipoproteins were isolated from fresh human plasma by sequential ultracentrifugation in sodium bromide at a density of 1.006–1.063 g/ml for LDL and 1.125–1.21 g/ml for HDL. Lipoprotein-free plasma protein fraction was collected as a bottom fraction with a density of 1.21 g/ml. All plasma fractions were thoroughly dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS). Apolipoprotein A-I (apoA-I) was isolated from the human HDL fraction by delipidation followed by anion exchange column chromatography in 6 M urea as described previously (34). ApoA-I was dissolved in PBS before use in experiments according to the methods described previously (34).

Preparation of Probucol-containing LDL and Probucol-carrying Bovine Serum Albumin (BSA)—To deliver probucol to the cells, probucol-containing LDL was prepared according to the method described previously (23, 35). Lipid microemulsion was prepared by sonicating egg phosphatidylcholine (Avanti) and triolein (Wako Pure Chemicals) with and without probucol. LDL (15 mg of protein) was incubated at 37 °C for 48 h with the microemulsion containing or not containing probucol in the presence of lipid-free plasma fraction (1.5 g of protein), dithionitrobenzoic acid (2 mM), aprotinin (20 units/ml), gentamycin (0.1 mg/ml), EDTA (0.5 mM), and NaN_3 (0.1% w/v). The mixture was applied to a dextran sulfate-cellulose column to recover LDL as the bound fraction eluted with 0.5 M NaCl. LDL was further purified by ultracentrifugal floatation at a density of 1.063 g/ml and thoroughly dialyzed against PBS. To label cholesteryl ester (CE) in LDL, the emulsion was prepared with [^{14}C]cholesteryl oleate (30 μCi), and the same procedure was applied to prepare the labeled LDL containing probucol. Alternatively, probucol was conjugated with BSA (fatty acid-free, Sigma). 250 μg of probucol was solubilized in 125 μl of methanol and incubated with 10 ml of 10% BSA (w/v) for 1 h at 37 °C.

Cell Culture and Loading with LDL—WI-38 human fibroblast cells (RIKEN Cell Bank) (36) were grown at 37 °C in Eagle's minimum essential medium (Sigma) with 10% fetal calf serum (HyClone Laboratories, Inc.), 5 units/ml penicillin, and 5 μg/ml streptomycin (Invitrogen). Cells were seeded into a 35-, 60-, or 100-mm dish at a density of 1.5×10^5 cells/ml. When the cells were grown to 80% of a confluent stage, the probucol-containing LDL (0–50 μg as LDL protein/ml) or probucol-carrying BSA (0.2%, w/v) was added for 24-h incubation to load probucol in the cells.

Cellular Lipid Release Assay—The probucol-loaded cells prepared as described above were washed and maintained in the lipoprotein-free medium for the next 24 h prior to any further experiments. The cells were incubated for 24 h with various amounts of apoA-I in the medium containing 0.02% BSA. Lipid was extracted from the medium with chloroform:methanol (2:1, v/v) and cells with *n*-hexane:2-propanol (3:2, v/v), and total cholesterol, free cholesterol, and choline-phospholipid were determined by colorimetric enzymatic assay system (Kyowa Medics) (13).

Determination of Probucol in the Cells—Probucol in the cell lipid extracts was measured by reverse-phase high performance liquid chromatography according to the method described by Satonin and Coutant (37). The cell extracts from the medium of a 60-mm dish were dissolved in acetonitrile, hexane, 0.1 M ammonium acetate (90:6.5:3.5, v/v/v) and injected into a Deltapak C_{18} reversed-phase column (150 × 3.9 mm, 300 Å; Waters). The mobile phase was acetonitrile:water (85:15, v/v) with the flow rate at 1.5 ml/min, and the probucol was detected by absorbance at 240 nm.

Evaluation of the Synthesis of Lipids—WI-38 cells at 80% of confluent stage in 35-mm dishes were washed with PBS and cultured in 1 ml of modified Eagle's essential medium (MEM) containing 0.2% BSA with or without probucol. After replacement with 1 ml of fresh medium, the cellular lipid was labeled by incubating for 2 h with 20 μCi/ml [^{3}H]acetic acid. Lipid was extracted with *n*-hexane:2-propanol (3:2, v/v), and radioactivity was determined in cholesterol, phosphatidylcholine, sphingomyelin, and cholesteryl ester after separation by thin layer chromatography.

Determination of Cholesterol Distribution to the Plasma Membrane—To determine traffic of newly synthesized cellular cholesterol moved to the cell surface, the cells were treated with 0.2% BSA carrying and not carrying probucol for 24 h and were labeled with [^{3}H]acetic acid for 2 h. To determine distribution of LDL-derived cholesterol to the cell surface, the cells were incubated for 24 h with [^{14}C]CE-LDL (50 μg of protein/ml) containing or not containing probucol, washed with PBS, and incubated with MEM containing heparin (1400 IU/ml) for 45 min at 4 °C to remove surface-bound LDL. The labeled cells were quickly washed with PBS, fixed with 1% glutaldehyde at room temperature for 10 min, and washed with PBS to remove the fixative reagent. The cells were then incubated with cholesterol oxidase (1 unit/ml) in MEM for 1 h to allow the conversion of cholesterol to cholestenone in the plasma membrane (38). After the cells were washed with PBS, cellular lipids were extracted, and radioactivity was determined for cholesterol, cholestenone, and cholesteryl ester separated by thin layer chromatography.

Subcellular Membrane Fractionation—Bulk membrane fraction was prepared as follows. Cells in 100-mm dishes were harvested and treated with 5 mM Tris-HCl (pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM benzamidine) for 30 min on ice with vortexing every 10 min. The cell debris and nuclei were removed by centrifugation at 650 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 444,000 × *g* for 40 min at 4 °C. Subcellular membrane subfractions were prepared as follows. The cell pellet after centrifugation at 600 × *g* for 10 min was lysed with cold extract solution (0.02 M boric acid, 0.3 mM EDTA, 1 mM PMSF, 1 mM benzamidine, and protease inhibitor cocktails (Sigma), pH 10) for 15 min on ice with vortexing every 5 min. The cell debris and nuclei were discarded by centrifugation at 650 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 12,000 × *g* for 1 h at 4 °C. The pellet was harvested, and the supernatant was further centrifuged at 290,000 × *g* for 30 min at 4 °C. The second pellet was harvested, and the supernatant was treated with 10% trichloroacetic acid to precipitate the protein.

Western Blotting—The membrane fraction and cellular subfractions were resuspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 10 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1% Triton X-100, and 1% protease inhibitor cocktails (Sigma) and were sonicated for 5 s. After determination of the protein content by a BCA method (Pierce), the fractions were dissolved in 9 M urea, 2% Triton X-100, 1% dithiothreitol and were developed in 6 or 15% (w/v) polyacrylamide gel electrophoresis in the presence of 10% SDS, respectively, and the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) by semidry blotter in blotting buffer (25 mM Tris-HCl, 0.2 M glycine, and 10% methanol (v/v)) for 3.5 h. The membrane was blocked with 5% skim milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 and was probed with the rabbit antiserum against the C-terminal peptide of human ABCA1 (26, 39), rabbit anticaveolin-1 (N-20) (Santa Cruz Biotechnology), anti-GLUT-1, mouse anti-integrin β_1 (CHEMICON International, Inc.), anti-GM130, and anti-Bip/GRP78 (BD Transduction Laboratories, BD Biosciences), respectively. The immunoreactive proteins were visualized by ECL or the ECL Plus system (Amersham Biosciences).

ABCA1 Degradation Rate—WI-38 cells in 100-mm dishes were incubated in 6 ml of the medium with LDL (300 μg of protein) containing or not containing probucol as described above. Cells were washed three times with PBS and incubated at 37 °C in MEM containing 20 ng/ml cycloheximide (Wako Pure Chemicals). ABCA1 in the cell membrane fraction was detected by immunoblot analysis as described above. For studying the calpain-mediated proteolysis of ABCA1, the experiment was performed according to the method described by Wang *et al.* (27). Cells were washed three times with PBS and placed on ice for 10 min. Then cells were permeabilized by incubating on ice for 15 min with 80 μg/ml digitonin in MEM. The cells were washed twice with PBS and incubated for 20 min at room temperature with μ-calpain (0.1 μM) in MEM containing 2 mM $CaCl_2$. The cells were lysed with 3 ml of buffer (5 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 1 mM benzamidine) containing 40 μg/ml calpeptin. The ABCA1 in the cell membrane fraction was analyzed by using immunoblot analysis.

RNA Extraction and Real Time Quantitative PCR—Total RNA was extracted from cells by using RNA extraction reagent (Isogen, Nippon Gene). After contaminated genomic DNA was digested with DNase I (Takara Shuzo Co.), first standard cDNA was synthesized by a SuperScript™ preamplification system (Invitrogen) from 2 μg of the total RNA. PCR was performed by using primers (sense and antisense) for cDNA 5′-GAA CTG GCT GTG TTC CAT GAT-3′ and 5′-GAT GAG CCA GAC TTC TGT TGC-3′ (for ABCA1) and 5′-ATG GTG GGA ATG GGT CAG AAG-3′ and 5′-CAC GCA GCT CAT TGT AGA AGG-3′ (for β-actin)

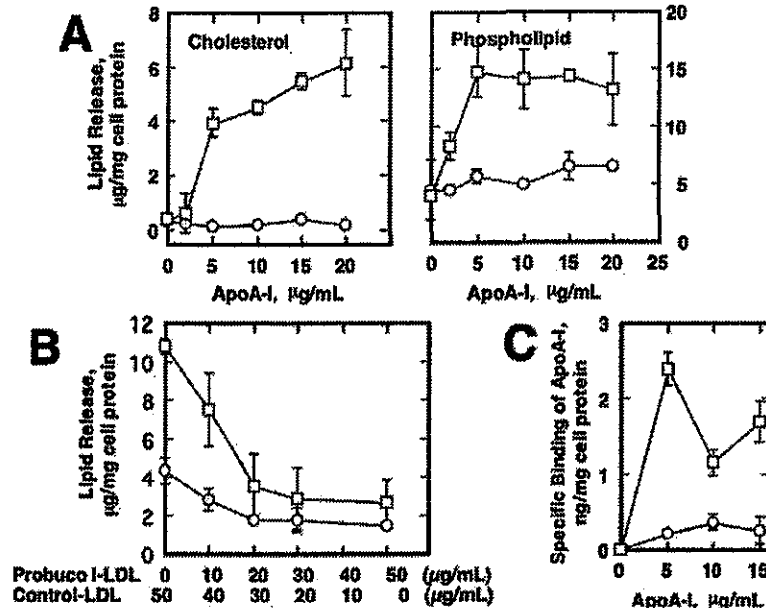

FIG. 1. **Effect of probucol on the apoA-I-mediated cellular lipid release.** WI-38 cells were incubated with LDL containing probucol or not containing probucol (50 µg of LDL protein/35 mm-dish) at 37 °C for 24 h and equilibrated for distribution of cholesterol and probucol by further incubation in 0.2% BSA for 24 h. Lipid-free apoA-I was added to the cells and incubated for 24 h. *A*, the *left-hand panel* shows release of cholesterol into the medium from the control cells (*squares*) and the probucol-treated cells (*circles*). The *right-hand panel* shows the release of choline-phospholipid from the control and probucol-treated cells (*squares* and *circles*, respectively). *B*, dose-dependent effect of probucol. The dose of probucol-containing LDL was increased (0, 10, 20, 30, 40, and 50 µg of LDL protein/35-mm dish) as the control LDL was decreased to maintain the total LDL protein at 50 µg. The release of cholesterol (*circles*) and choline-phospholipid (*squares*) was measured as described above. *C*, the ^{125}I-labeled apoA-I was incubated at 0 °C for 2 h with the WI-38 cells pretreated with LDL containing probucol or not as described above. Specific binding of ^{125}I-apoA-I was calculated by subtracting the binding after displacement with cold apoA-I from the total binding of the labeled apoA-I as described in the text for the control-LDL-treated (*squares*) and probucol-LDL-treated (*circles*) cells. The *data points* represent the average ± S.E. of an assay performed in triplicate.

(synthesized by Sawady Technology Co., Ltd.). The quantification of ABCA1 and β-actin mRNA was achieved using SYBR Green PCR master mix reagent in an ABI PRISM 7700 sequence detection system (Applied Biosystems Japan).

^{125}I-Apolipoprotein A-I Binding to the Cell Surface—Specific binding of ^{125}I-labeled apoA-I was estimated as displaceable binding by excessive cold apoA-I (23). ApoA-I was dissolved as 1 mg in 20 ml of the 0.1 M PBS (pH 6.5) and incubated at 37 °C for 1 h. ^{125}I (2 mCi) and 15 beads of IODO-BEAD® iodination reagent (Piece) were added to the solution, and the labeling reaction was carried out with stirring for 15 min at room temperature. The solution was concentrated by using the Ultrafree-15 centrifugal filter device (Millipore) with centrifugation at 700 × *g* for 1.5 h at 4 °C. The analysis of product by polyacrylamide gel electrophoresis showed that 94% of the total radioactivity was recovered in apoA-I and that the specific radioactivity was 2367, 608 cpm/µg of protein. After treatment with LDL containing or not containing probucol, cells in 35-mm dishes were incubated with various concentrations of ^{125}I-apoA-I at 0 °C for 2 h in 500 µl of MEM (pH 7.4, containing 25 mM HEPES, 0.2% BSA). The cells were chased twice by the medium with or without 50 µg/ml non-labeled apoA-I at 0 °C for 4 h and washed with cold PBS. The cell-bound radioactivity was recovered in 1 ml of 0.5 M NaOH.

RESULTS

Consistent with our previous reports (23, 24), probucol inhibited the apoA-I-mediated cellular lipid release (Fig. 1*A*). This effect was dose-dependent with respect to the dose of probucol-containing LDL (Fig. 1*B*). Probucol also inhibited displaceable binding of ^{125}I-apoA-I (Fig. 1*C*). Dose-dependent increase of cellular probucol content was observed within the range of the dosage of the probucol-containing LDL employed in the present experiment (Fig. 2*A*). Loading of cholesterol to the cells via LDL was not influenced by the presence of probucol in LDL (Fig. 2*B*).

The effect of probucol on lipid biosynthesis was examined. To avoid the effect of lipid loading via LDL, probucol was given to the cells as a BSA-probucol conjugate. Probucol also inhibited the apoA-I-mediated lipid release even when it was given directly by this procedure (Fig. 3*A*). In this condition, incorporation of acetate was somewhat increased by probucol in all of the lipid fractions tested, including free cholesterol, cholesteryl ester, phosphatidylcholine, and sphingomyelin (Fig. 3*B*).

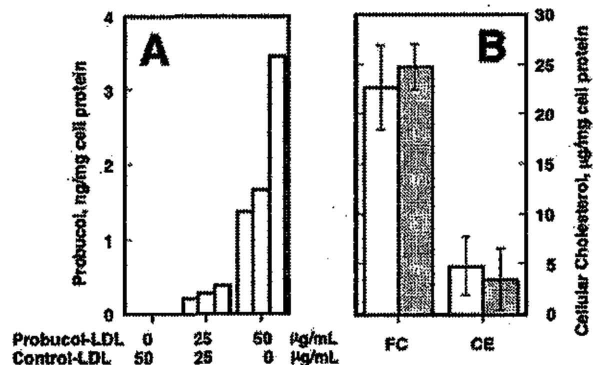

FIG. 2. **Accumulation of LDL-derived cholesterol and probucol in WI-38 cells.** *A*, WI-38 cells were incubated with probucol-free or probucol-containing LDL (total of 50 µg of protein) in 3 ml of medium within a 60-mm dish for 24 h. Probucol accumulated in the cells was measured using reverse-phase high pressure liquid chromatography as described under "Experimental Procedures." Each *bar* represents an individual experimental data point. *B*, the accumulation of cholesterol in WI-38 by incubating with probucol-free LDL (*open bar*) and probucol-carrying LDL (*dot-filled bar*) was measured. LDL (50 µg of LDL protein/35-mm dish) was incubated with cells at 37 °C for 24 h. The cellular cholesterol was determined as described under "Experimental Procedures." The values are the average and S.E. of triplicate assays. *FC*, free cholesterol.

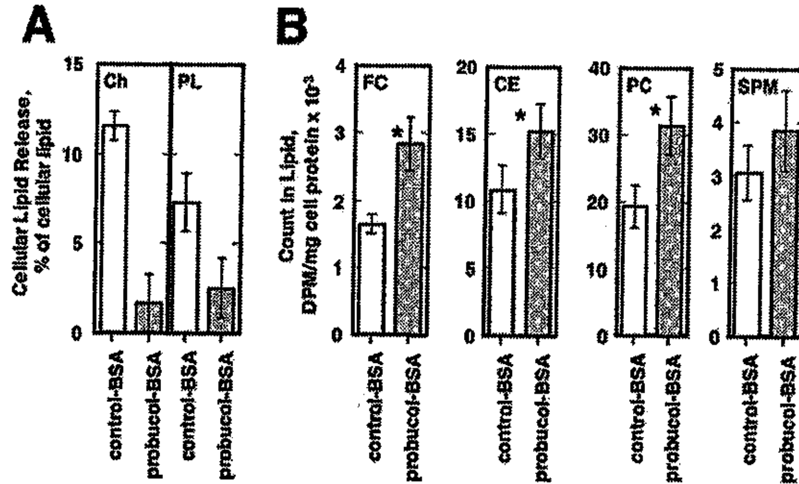

FIG. 3. **Effect of probucol on cellular lipid synthesis.** *A*, probucol-carrying or probucol-free 10% BSA was added to the medium to make the final BSA concentration 0.2% and incubated for 24 h at 37 °C. ApoA-I was added to the cells and incubated for another 24 h. The cholesterol (*Ch*) and phospholipid (*PL*) in the medium were measured as described under "Experimental Procedures." *B*, WI-38 cells were loaded with probucol as described above, followed by labeling with [^{3}H]acetic acid (20 μCi/ml) for 2 h. The cellular lipids were extracted and separated by thin layer chromatography, and radioactivity was determined in the fractions of free cholesterol (*FC*), cholesteryl ester (*CE*), phosphatidylcholine (*PC*), and sphingomyelin (*SPM*). Each *bar* represents the average and S.E. of three data points. *Dot-filled columns* and *open columns* indicate the data for probucol-treated and probucol-free cells, respectively. *Asterisks* indicate a significant increase from the control ($p < 0.05$).

Cellular cholesterol is distributed throughout 60–80% of the plasma membrane (40), and cholesterol accounts for as much as 30–40% of lipid molecules in the plasma membrane (41). Transport of cholesterol to the plasma membrane is differentially regulated for the newly synthesized and the LDL-derived cholesterol molecules. Therefore, distribution of cholesterol from these different sources was examined with respect to the effect of probucol. Cellular cholesterol was labeled by incubating with [^{3}H]acetic acid or by incorporating [^{14}C]CE-LDL, and cell surface cholesterol was probed by extracellular cholesterol oxidase. As shown in Fig. 4, probucol caused a small decrease in relative distribution of *de novo* synthesized cholesterol to the surface, probably because of the apparent increase of the synthesis shown in Fig. 3*B*, but did not cause any significant difference in the LDL-derived cholesterol. Based on the results above, it is unlikely that the inhibitory effect of probucol on the HDL assembly reaction is related to alteration of cellular cholesterol metabolism.

We analyzed the expression of ABCA1. The message of ABCA1 was not influenced by probucol at all (Fig. 5*A*). However, probucol increased ABCA1 as analyzed by immunoblotting of the membrane fraction, whereas there was no change in integrin β_1 and GLUT-1 (Fig. 5*B*). The initial immunocytochemical studies suggested that endogenously expressed human ABCA1 was localized in the plasma membrane (7, 42, 43), so the effect of probucol on intracellular distribution of ABCA1 was investigated. The cell membrane was fractionated as described under "Experimental Procedures," and each fraction was analyzed by immunoblotting for ABCA1 and membrane marker proteins. As demonstrated in Fig. 5*C*, ABCA1 was detected along with a plasma membrane marker, integrin β_1, predominantly in the pellet fraction that was centrifuged at 12,000 × *g* for 60 min (*lanes 1* and *5*). Caveolin-1 was also recovered mainly in this fraction. In contrast, a significant amount of a marker for endoplasmic reticulum, Bip/GRP78, was found in the supernatant fraction centrifuged at 290,000 × *g* for 30 min (Fig. 5*C*, *lanes 3* and *4* and *lanes 7* and *8*). A Golgi marker, GM130, was also distributed to this fraction. Probucol did not influence this pattern of distribution of ABCA1 and marker proteins. Thus, ABCA1 is predominantly present in the plasma membrane, and probucol does not significantly alter

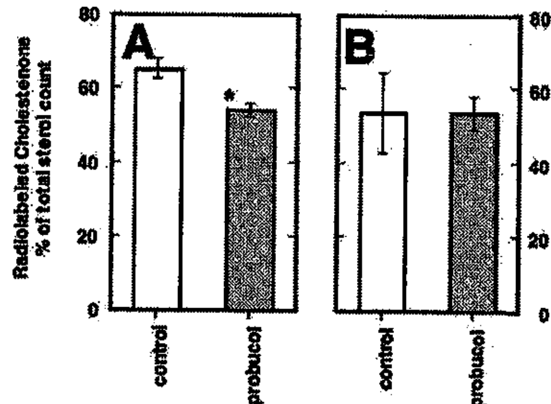

FIG. 4. **Determination of cholesterol content in plasma membrane by cholesterol oxidase.** WI-38 cells were incubated with 0.2% probucol-free or probucol-carrying BSA for 24 h and then were radiolabeled with [^{3}H]acetic acid (20 μCi/ml) for 2 h (*A*) or were incubated with LDL (50 μg protein/ml) containing [^{14}C]cholesteryl ester and probucol for 24 h at 37 °C (*B*). The cholesterol on the cell surface was probed by extracellular cholesterol oxidase as described under "Experimental Procedures." The results are displayed as the percentage of radioactive cholestenone within total cellular sterol. Results represent the mean ± S.D. of triplicate determinations of a representative experiment. An *asterisk* indicates a significant difference from the control ($p < 0.05$).

the traffic of ABCA1. ABCA1 and caveolin-1 may also be somewhat recovered with the Golgi-endoplasmic reticulum fractions, which is consistent with previous findings (44, 45).

To study the underlying mechanism for the increase of ABCA1 without changing its message, degradation of ABCA1 in the cell was examined. ABCA1 was analyzed by immunoblotting in the presence of cycloheximide. A decrease in ABCA1 was apparent at 30 min, and ABCA1 decayed throughout the incubation up to 120 min (Figs. 6*A* and 7*A*). Probucol apparently slowed the rate of the decay of ABCA1 (Figs. 6*A* and 7*A*).

To examine the effect of probucol on proteolytic degradation of ABCA1, the susceptibility of ABCA1 to protease was observed after the cells were permeabilized by digitonin. To exclude a possibility that probucol may inhibit the membrane

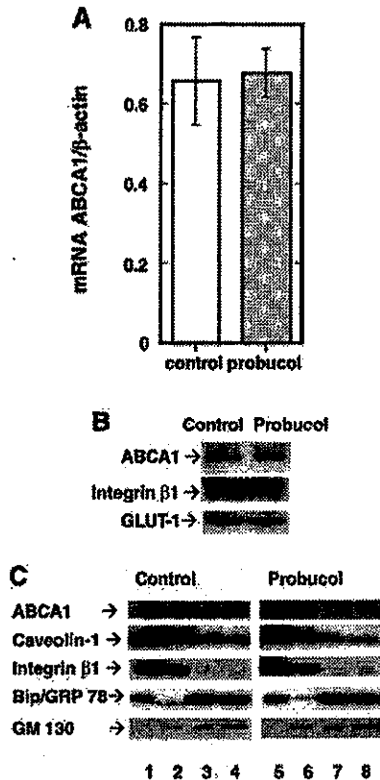

FIG. 5. **Effect of probucol on ABCA1.** WI-38 cells were incubated with LDL containing probucol or not for 24 h. *A*, specific messages of ABCA1 and β-actin were quantified by real time PCR. The ABCA1 message is standardized for the β-actin mRNA in the figure. The data represent the average ± S.E. of an assay performed in triplicate. *B*, total membrane fraction was prepared from the probucol-treated and control cells as described in the text. Fifty μg of the membrane protein was analyzed by immunoblotting for ABCA1, GLUT-1, and integrin β_1. *C*, the subcellular membrane fractions were prepared as described under "Experimental Procedures" as the pellet of the first centrifugation (12,000 × *g* for 60 min), the pellet of the second centrifugation (290,000 × *g* for 30 min), the supernatant of the first centrifugation, and the supernatant of the second centrifugation. Each fraction was analyzed by immunoblotting for ABCA1, caveolin-1, integrin β_1 (plasma membrane marker), Bip/GRP78 (endoplasmic reticulum marker), and GM130 (Golgi marker). *Lanes 1* and *5*, 55 μg of protein of the pellet fraction of the first centrifugation. *Lanes 2* and *6*, 55 μg of protein of the pellet fraction of the second centrifugation. *Lanes 3* and *4* and *lanes 7* and *8*, 55 μg of protein precipitated with 10% trichloroacetic acid from the supernatant of the second centrifugation.

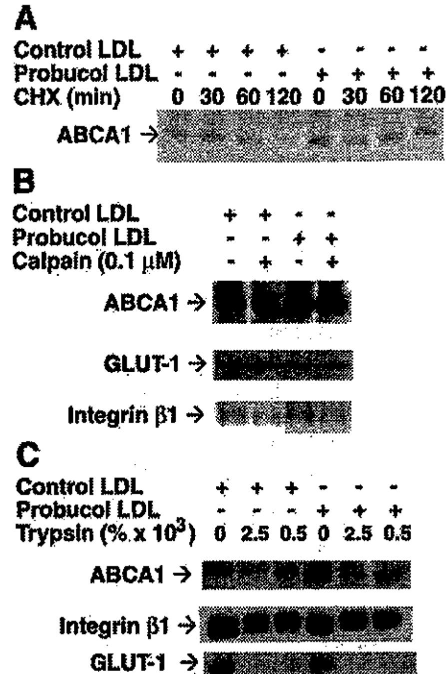

FIG. 6. **Effect of probucol on ABCA1 degradation.** *A*, WI-38 cells were pretreated with control or probucol-containing LDL. The cells were then incubated in the presence of cycloheximide (*CHX*) (20 ng/ml) for the indicated times, and membrane ABCA1 was analyzed by immunoblotting. 55 μg of the membrane protein was applied to each *lane*. *B* and *C*, WI-38 cells pretreated with control or probucol-containing LDL were permeabilized by incubating with 80 μg/ml digitonin on ice for 15 min. The cells were then incubated with μ-calpain (0.1 μM) in MEM containing 2 mM $CaCl_2$ for 20 min at room temperature (*B*) or with trypsin at the indicated concentration for 3 min at room temperature (*C*). Membrane ABCA1, GLUT-1, and integrin β_1 were analyzed by immunoblotting assay.

permeabilization by digitonin, cells were examined with immunostaining for β-tubulin after the permeabilization treatment. There was no difference between the cells treated with LDL containing probucol and no probucol (data not shown). ABCA1 in the cells treated with probucol became resistant to μ-calpain (a ubiquitously expressed subtype of calpain) (Figs. 6*B* and 7*B*). In contrast, probucol did not influence degradation of ABCA1 by trypsin (Figs. 6*C* and 7*C*). GLUT-1 was insensitive to calpain, whereas integrin β_1 was susceptible (Figs. 6*B* and 7*B*). Probucol did not influence digestion of integrin β_1 and GLUT-1, either by calpain or by trypsin (Fig. 6, *B* and *C*, and Fig. 7, *B* and *C*). The data provided direct evidence that physiological degradation of ABCA1 by calpain is inhibited by probucol.

DISCUSSION

The underlying mechanism for the inhibitory effect of probucol on the function of ABCA1 was investigated. The results of the study are summarized as follows. 1) Probucol inhibited the events mediated by ABCA1 such as apolipoprotein-mediated cellular lipid release and apolipoprotein binding to the cells. 2) Probucol did not interfere with transcription and intracellular trafficking of ABCA1, and ABCA1 was predominantly found in the plasma membrane even in the probucol-treated cells as judged by biochemical parameters. 3) Probucol made ABCA1 resistant to calpain-mediated degradation and consequently increased its cellular level. 4) Probucol did not influence cellular lipid accumulation via LDL and cholesterol distribution to the cell surface but rather enhanced lipid biosynthesis. We thereby concluded that probucol inactivated ABCA1 in the plasma membrane with respect to its functions and its susceptibility to proteolysis.

Probucol was used as a lipid-lowering drug for years before statins became available. This drug was known for its apparent clinical effect of regression of cutaneous and tendinous xanthomas more than expected from the reduction of plasma LDL (46). Because probucol is characterized for its strong antioxidative nature, it was expected to work as an antioxidant against oxidative modification of plasma lipoprotein to prevent development of atherosclerotic vascular lesions and even to cure them. This hypothesis was indeed strongly supported by several experimental approaches using animal models for atherosclerosis (20, 21). However, this drug has also been known for its strong effect of lowering plasma HDL. What is more puzzling is that reduction of HDL by probucol is sometimes seriously aggravated by fibrates, which are otherwise expected to raise HDL in plasma (22). Thus, probucol has been a very

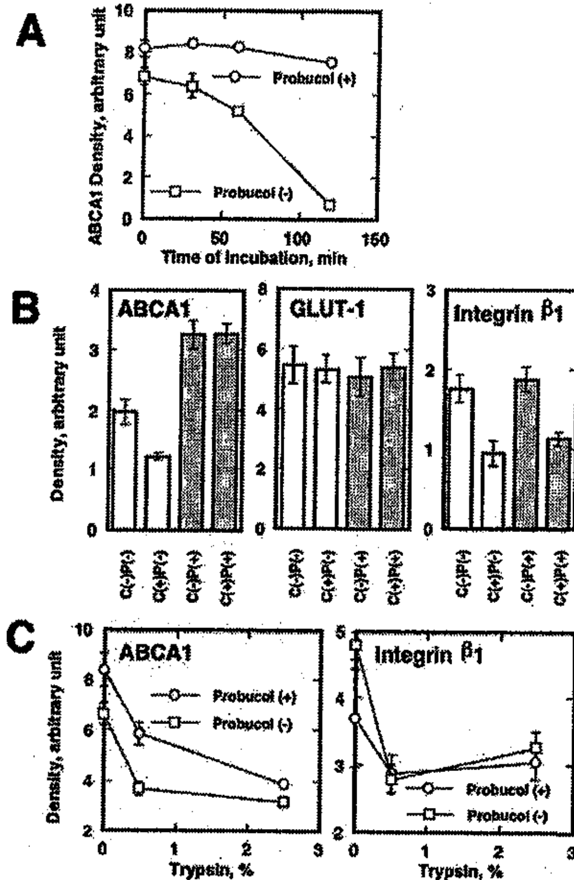

FIG. 7. **The results of density scanning of the Western blotting bands in the experiments represented by Fig. 6.** *Panels A, B,* and *C* correspond with *A, B,* and *C* of Fig. 6, respectively. In *B, C* and *P* indicate calpain and probucol, respectively. The density of each band was quantitated by digital scanning in an Epson GT9500. Data points represent mean ± S.E. of three experiments.

controversial drug, and it lost its popularity in the market as statins took over.

We discovered that probucol inhibits the apolipoprotein-mediated cellular lipid removal and generation of HDL as well as the binding of apolipoprotein to the cells (23). This finding is very similar to the observation with the cells of patients with Tangier disease, a familial HDL deficiency (3, 4). We thereby speculated that this is a reason for probucol to reduce HDL, and accordingly the apolipoprotein-mediated generation of HDL with cellular lipid is a main source of plasma HDL. We further investigated an *in vivo* effect of probucol by using mouse models. Probucol inhibited the production of HDL in mice, and the kinetic analysis of plasma HDL revealed the enhanced clearance of HDL apoprotein by probucol but no difference in the HDL-lipid clearance (24), an exact analogy to the behavior of HDL in Tangier disease patients (47), indicating that probucol inhibits the reaction to generate HDL that is lacking in patients with Tangier disease. The use of probucol in the lecithin:cholesterol acyltransferase-deficient mice showed that cholesterol accumulated only in the liver, suggesting that the liver is the major organ where generation of HDL by this mechanism takes place (48). ABCA1 was identified as a protein essentially responsible for this reaction, and the action of probucol on ABCA1 has become a subject of study.

In the current study, we focused on the effects of probucol on ABCA1 with respect to its transcription and trafficking as well as its role in mediating apoA-I binding and cellular lipid release by apolipoprotein. We also investigated its effect on proteolytic degradation of ABCA1, another main mechanism for regulating the level of ABCA1 in cells. Interestingly, there was no effect of probucol either on transcription or on trafficking of ABCA1 as judged by biochemical analysis. The cellular ABCA1 was rather significantly increased, and this was apparently because of the decrease of susceptibility of ABCA1 to proteolysis by calpain, a physiological regulation pathway for ABCA1. Thus, probucol inactivated not only functional aspects of ABCA1 but also its clearance system. Probucol is a very hydrophobic compound, and accordingly it is almost water-insoluble. Therefore, it is almost exclusively carried by lipoprotein in plasma and distributes in the membrane when cleaved to cells (37). Because almost all ABCA1 is recovered in plasma membrane, probucol is likely to act on ABCA1 in the plasma membrane. The decrease of protease susceptibility is not observed for trypsin, so the effects of probucol seem to induce a certain specific conformational alteration of ABCA1 to inactivate this protein against calpain, presumably at the membrane-spanning regions.

Probucol did not significantly influence intracellular lipid metabolism except that incorporation of [^{3}H]acetic acid into various lipid fractions increased somewhat, which may require further investigation. Thus, the effect of probucol on ABCA1 is unlikely to be a secondary phenomenon to its effect on cellular lipid metabolism.

There are no previous reports of any chemical compound acting in such a manner to inactivate membrane proteins. Further studies are required to elucidate the detail of the mechanism by which probucol inactivates ABCA1 in the membrane. This would provide important information about the reaction mechanism of ABCA1, cellular cholesterol homeostasis, and generation of HDL.

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Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease

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Abstract

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease (AD) and HIV encephalitis. The FGF1 gene is therefore a strong candidate gene for AD. Using the promoter polymorphism of the FGF1 gene, we examined the relationship between AD and the FGF1 and apolipoprotein E (APOE) genes in 100 Japanese autopsy-confirmed late-onset AD patients and 106 age-matched non-demented controls. The promoter polymorphism (−1385 A/G) was significantly associated with AD risk. The odds ratio for AD associated with the GG vs non-GG genotype was 2.02 (95% CI = 1.16–3.52), while that of ε4 vs non-ε4 in APOE4 gene was 5.19 (95% CI = 2.68–10.1). The odds ratio for APOEε4 and FGF1 GG carriers was 20.5 (95% CI = 6.88–60.9). The results showed that the FGF1 gene is associated with autopsy-confirmed AD.
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Keywords: Definite Alzheimer's disease; Fibroblast growth factor 1 gene; Promoter polymorphism; Association study; APOE; Risk factor

Alzheimer's disease (AD; MIM#104300) is the most common cause of dementia in mid- to late-life. Studying the factors that influence the risk of developing AD may lead to the identification of those at high risk for developing it, strategies for prevention or intervention, and clues to the cause of the disease. Both genetic and environmental factors have been implicated in the development of AD [1], but the cause of AD remains unknown, and no cure or universally effective treatment has yet been developed [2]. Even the diagnosis is difficult. A definitive diagnosis depends on analysis of neuritic plaques and neurofibrillary tangles found in brain tissue [3]. Given the recognition that AD constitutes a heterogeneous disorder, identification of established risk factors would be difficult using conventional methods.

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) is a member of the fibroblast growth factor family that possesses broad mitogenic and cell survival activities and is involved in a variety of biological processes [4]. FGF1 protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease [5,6] and HIV encephalitis [7]. Immunohistochemical examination of postmortem brain tissue of AD revealed that FGF1 was specifically expressed in a subpopulation of reactive astrocytes surrounding senile

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plaques. Such upregulation of FGF1 expression might be related to the presence of reactive astrocytes rather than β-amyloid protein deposition [8,9]. Recent studies suggest that FGF1 upregulates APOE synthesis and subsequently HDL production in reactive astrocytes in an autocrine or paracrine manner, and exerts its effect after central nervous system (CNS) damage through APOE secretion [10,11]. Besides, the fact that FGF1 expression is lower in the hippocampal formation than in motoneurons suggests that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [6,12]. The FGF1 gene is therefore a strong candidate gene for AD. However, there are no reports regarding the association of FGF1 gene polymorphism with AD. Therefore, we investigated whether FGF1 gene polymorphism could contribute to risk in a limited subgroup of AD (autopsy-confirmed AD).

Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected based on the NINCDS-ADRDA criteria for definite AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination [3,13]. Brain and blood samples were obtained with informed consent from subjects in the Chubu and Kansai areas of Japan. A total of 100 unrelated late-onset AD (LOAD) patients had been diagnosed previously, and 106 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence of the patients as described elsewhere [14,15]. The mean age ± SD (years) at the time of this study was as follows: 85.3 ± 6.0 for LOAD, 83.0 ± 4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol–chloroform method [16].

During screening for FGF1 gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of −1385 G/A (C/T) (rs34011) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *HhaI*, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-TCAAGC AATTCTCCTGCCTT-3′) and FGF1-R (5′-CCACTTCAAGGGATT ATGGTG-3′). PCR was carried out in a 25-μl reaction volume containing standard reaction buffer (1.5mM $MgCl_2$, 50mM KCl, and 10mM Tris-HCl, pH 8.3), 200μM each dNTP, 5μM each primer, 0.5U *Taq* DNA polymerase and 50ng genomic DNA as a template with 35 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 1min. PCR product size was 355bp, and the G allele was digested by *HhaI* to 53 + 141 + 161bp, and the A allele to 53 + 302bp. DNA was electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under UV light (Fig. 1). To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and normal control subjects. Because APOEε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously. Allelic and genotypic distribution were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p < 0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals.

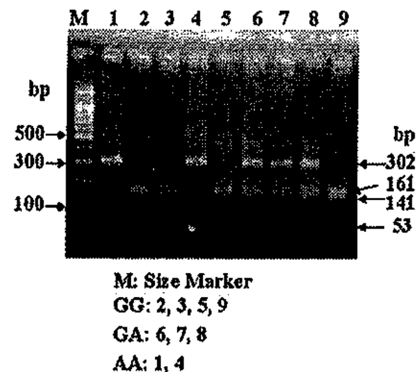

Fig. 1. Promoter polymorphism of FGF1. After amplification, PCR products were digested with *HhaI* and DNA was detected after electrophoresis on 2% agarose gels. Three genotypes of −1385 G/A (*HhaI* polymorphism) are shown: genotypes GG (lanes 2, 3, 5, and 9), GA (lanes 6–8), and AA (lanes 1 and 4).

Results

The PCR results were scored by two independent investigators who did not know whether each sample was from a case patient or a control. No intraobserver variability was found on repeated readings of the same gel, and the interobserver variability was less than 1%. All ambiguous samples were analyzed a second time.

The distribution of the three genotypes (GG, GA, and AA) reached Hardy–Weinberg equilibrium. The G allele was found in 75% of the 100 LOAD patients and 63% of the 106 control subjects. A significant association was observed between the −1385 G/A polymorphism and LOAD ($p < 0.03$; Table 1). We then examined the GG genotype as a risk factor for AD, considering the APOE status. As expected, APOEε4 conferred an increased risk for AD [odds ratio (OR) = 5.19]. OR in homozygotes for the G allele was 2.02 [95% confidence interval (CI) = 1.16–3.52]. However, the risk-increasing effect was smaller for −1385 G than for APOEε4 (Table 2). Four categories were defined by the presence (+) or absence (−) of a ε4 or GG genotype. The GG genotype alone showed an increased risk (95% CI: 1.81–7.69), and OR for APOEε4 and the GG genotype was 20.5 (95% CI: 6.88–60.9).

Discussion

To date, some polymorphisms of the FGF1 gene have been reported to associate with intracranial aneurysm [17]. However, functional role of the haplotype in its pathophysiology remains unclear. As the FGF1 gene contains alternative 5′-untranslated exons, the transcription is controlled by at least four distinct promoters in a tissue-specific manner [18–20]. Payson et al. [19] have reported that the sequence from −1614

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in promoter of FGF1

Group	Genotype (frequency)				Allele (frequency)	
n	AA	GA	GG	AA + GA	G	A
LOAD (100)	6 (0.06)	38 (0.38)	56 (0.56)*	44 (0.44)**	150 (0.75)	50 (0.25)***
Control (106)	14 (0.13)	51 (0.48)	41 (0.39)	65 (0.61)	133 (0.63)	79 (0.37)

LOAD, late-onset AD.
* $p < 0.03$.
** $p < 0.02$.
*** $p < 0.01$.

Table 2
Relative risk for interaction between APOEε4 and −1385 GG

		LOAD cases	Controls	Odds ratio	95% CI
	−1385 G/A				
	non-GG	44	65	Reference	
	GG	56	41	2.02	1.16–3.52
APOEε4					
−		52	90	Reference	
+		48	16	5.19	2.68–10.1
APOEε4	−1385 GG				
−	−	17	58	Reference	
−	+	35	32	3.73	1.81–7.69
+	−	18	11	5.58	2.21–14.1
+	+	30	5	20.5	6.88–60.9

APOEε4 (+), one or two copies of ε4: APOEε4 (−), no copies of ε4, 95% CI, confidence interval at 95% level.

to the FGF1 start site is sufficient to stimulate promoter activity. Therefore, it is reasonable to think that −1385 G/A polymorphism in the FGF1 promoter region can contribute the promoter activity. We performed an association study of the promoter polymorphism of the FGF1 gene.

We have evaluated definite LOAD as a relatively homogeneous case group. Our preliminary data suggest that the FGF1 gene, or a nearby gene, is an additional risk factor, independent of the APOE gene. Association studies often produce conflicting results. There are three possible reasons. First, this might be due to a type I statistical error, where there is a weak association between the polymorphism and the disease. Second, it might arise from the difference in genetic background between the American, French, Asian, and Japanese populations. In some studies, the AD group was made up of a mixture of familial and sporadic patients. We therefore tried to choose homogeneous subjects (autopsy-confirmed and late-onset AD) as much as possible. A third possibility could be linkage disequilibrium with other causative polymorphisms.

Patients with the GG genotype in this study had a higher risk of AD than those with the A allele. This indicates that the GG genotype in the promoter may influence the expression of FGF1 and could be involved in the selective vulnerability of neurons in AD. The results of this study support the hypothesis that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [11,6,12]. This hypothesis should be further examined by functional analysis of FGF1 polymorphisms.

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