スとは対照的であった．初代培養肝細胞へのDiI-LDLの取り込みを共焦点蛍光顕微鏡で観察しても，同様であった（図⑥C）．

autosomal recessive hypercholesterolemiaは，臨床的にはFHホモ接合体と類似しているが，皮膚線維芽細胞においてLDL受容体が正常あるいは軽度低下を示すことから，発見された病気である．今回，初代培養肝細胞を用いても同様の結果を得たことから，細胞に特異的な現象ではなく，環境によるものではないかと考えられた．

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特集 医療に役立つ実験用ウサギの展望
——その1——

食後高トリグリセリド血症家兎（PHT）の特徴

—ヒト虚血性心疾患の新しいモデル動物—

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背　景

成人病の3大死因はガン（悪性新生物），心臓病（虚血性心疾患），脳血管障害である。心臓病と脳血管障害を合わせるとガン死を越える事になる。したがって，血管病対策は21世紀の医療の重要な課題である。

血管病の原因は動脈硬化性変化である。地域住民を対象とした疫学調査によって動脈硬化症の発症と重症度を規定する危険因子が明らかとなり，その代表的なものは高脂血症，高血圧，糖尿病，喫煙，性（男性，閉経後の女性）である。高脂血症には高コレステロール血症と高トリグリセリド（中性脂肪）血症，それらの合併したものとがある。動脈硬化の危険因子としては高コレステロール血症のほうが臨床的に重大である。

しかしながら，欧米や日本の先進諸国では食事内容として年々高栄養価のものが摂取される傾向にあり，高トリグリセリド血症が問題視されるようになってきている。米国のFramingham研究によると，虚血性心疾患患者の35％は血清コレステロール（CHO）が200mg/dl以下であったと報告している（Kannel WB，1971，Ann Intern Med）。また，泰江らは心筋梗塞患者1,032例を対象として行なったJapanese Antiplatelets Myocardial Infarction Study（JAMIS）の結果において，220mg/dl以上の高コレステロール血症を有する患者の割合はたった27.1％であったと報告している（Yasue，1999，Am J Cardial)。このように虚血性心疾患を発症したすべての患者に高コレステロール血症を認めるわけではなく，むしろ他の危険因子（高トリグリセリド（TG）血症，耐糖能異常，肥満，高血圧等）を同一人が複数併せ持つ方が，虚血性心疾患を発症する頻度が高いことが示され，とくに高トリグリセリド血症と虚血性心疾患との関係が注目されている。さらに，動脈硬化症を主要所見とする臨床例の中に，食後高脂血症を示す症例が知られており，血管病進展の重篤な危険因子と考えられている。

しかし臨床例において，遺伝性の有無，脂質代謝の特徴は系統的に解析されていない。なぜ，高トリグリセリド血症が発生するのか，医学的な解明は進んでいない。その理由は，食事と高トリグリセリド血症の関係が未解決な事による。そのために，食後に高トリグリセリド血症を示す動物モデルの開発は，この問題の解明に必須と考えられる。

食後高トリグリセリド血症家兎の選抜経緯

著者らはWatanabe heritable hyperlipidemic（WHHL）rabbitの分与を受け，日本白色家兎

(JW) と戻し交配を行なった後，ホモ接合体の確立を試みた。血清コレステロールと血清トリグリセリドを酵素法で測定したところ，WHHLのトリグリセリド値（中性脂肪）は200～900mg/d*l*と幅広い分布を示す事がわかった。一方，野生型である日本白色家兎では血清トリグリセリド値は200mg/d*l*以下，多くの場合100mg/d*l*以下を示す。そこで，血清トリグリセリド値が高い個体の掛け合わせを行なった結果，世代を追う毎に血清トリグリセリド値が500mg/d*l*を超すWHHLの発現が増加した。すなわち，遺伝性高トリグリセリド血症を示す家兎の発現頻度が上昇することが観察された。1995年の第4世代では90％，第5世代以降では100％の進達率で発現したことから，遺伝性高トリグリセリド血症家兎を系統として確立できた。著者らはこの家兎を遺伝性高トリグリセリド血症家兎（TGH，CHO＝1000～1500mg/d*l*，TG＝1000～10000mg/d*l*）と命名した。

WHHLの高コレステロール血症はLDL（low density lipoprotein：低比重リポ蛋白）受容体の異常であることが明らかにされているが，遺伝性高トリグリセリド血症家兎（TGH）はWHHL家兎亜系より選抜した家兎であり，WHHLの特徴であるコレステロール高値に加えトリグリセリドの異常高値を特徴とする。

食後高トリグリセリド血症家兎の発見

著者らは，TGHの遺伝様式を検索する目的で，TGHと日本白色家兎（JW）の交配により雑種第1世代を作出した。さらに雑種第1世代〈MHF1〉の中での交配〈兄妹交配／ヘテロ接合体どうしの交配なので，メンデルの法則から1/4はホモ接合体，1/4は野生型，2/4はヘテロ接合体が産まれる〉により第二世代〈MHF2〉を作出し，遺伝形質の解析を行なったところ，ホモ接合体以外の個体に食後高トリグリセリド血症家兎を見出した。すなわち，食餌摂取と血清脂質レベルの関係を検討したところ，空腹時には脂質レベルは正常であり，食後12時間以降に血清トリグリセリド値が1000mg/d*l*以上になる特異な家兎の存在に気づいた。遺伝形質を調べたところ，食後高トリグリセリド血症は常染色体優性遺伝の形質であることがわかった。遺伝性食後高トリグリセリド血症家兎を作出した際の家系図を図1に示す。WHHLと日本白色家兎との戻し交配により，第一世代〈MHF1〉および第二世代〈MHF2〉を作出した。MHF2においてはTGH7匹，正常の脂質レベルを示す個体26匹を得た。その26匹の中で，食後に高トリグリセリド血症を示す7匹（雄4匹，雌3匹）を種動物として掛け合わせを行なった結果，第三世代〈MHF3〉を，32匹（雄20匹，雌12匹）得た。その中で，食餌に関係なく血清高トリグリセリド血症を示すTGHの性質を有する個体は9匹，空腹時の脂質レベルは正常で食後に高トリグリセリド血症を示す個体は23匹であった。従って，TGHは約25％を占め，常染色体性劣性であることが確認された。食後に高トリグリセリド血症を示す個体は23匹だったことから，本形質はTGHと別の遺伝子支配による優性遺伝である可能性が高く示唆された。そこで，本形質を示す家兎をモデル動物系として確立し，食後高トリグリセリド血症家兎（PHT：Postprandial Hyper Triglyceridemia）と命名した。食後にのみ高トリグリセリド血症を示すという遺伝形質を有するモデル動物はこれまでに例がない。PHTはコレステロール代謝異常に依存しない新しいヒト虚血性心疾患のモデル動物として有用と考えられる。また，ヒトのマルチプルリスクファクター症候群や食後高脂血症などの診断や解析に重要と考えられる。以下，食後高トリグリセリド血症家兎の特徴について述べる。

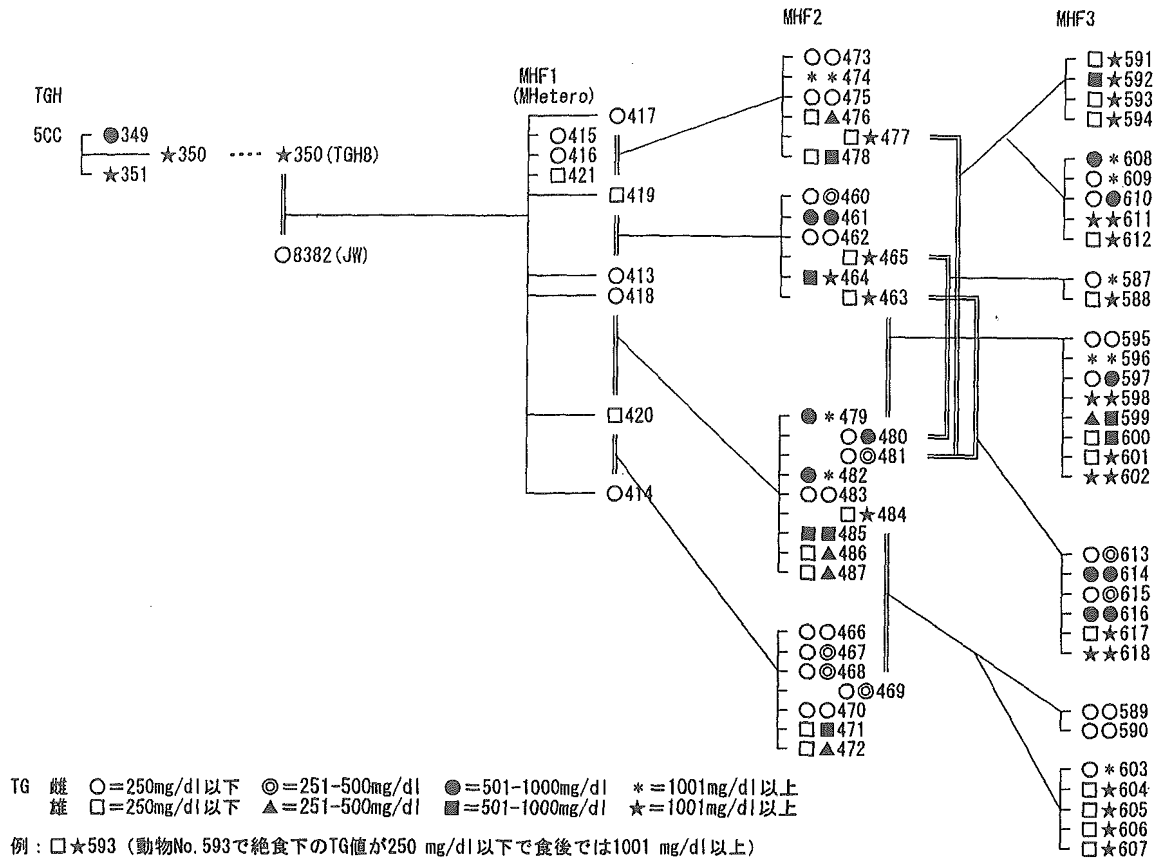

図1　食後高トリグリセリド血症家兎（PHT）の家系図

食後高トリグリセリド血症家兎の特徴

食後高トリグリセリド血症家兎（PHT）は，絶食時には10mg/dl以上200mg/dl以下の血清トリグリセリド値を示し，かつ食後12時間以後48時間以内に500mg/dl以上3000mg/dl以下の血清トリグリセリド値を示す事を特徴とする。

著者らが規定する高トリグリセリド血症とは500mg/dl以上3000mg/dl以下のトリグリセリド値を示すことを，好ましくは750mg/dl以上2500mg/dl以下のトリグリセリド値を示すことを，さらに好ましくは1000mg/dl以上2000mg/dl以下のトリグリセリド値を示すことを意味する。同様に食後とは食事開始後12～24時間後のことである。

飼育条件

各種血液脂質成分値を比較検討するためには，家兎の飼育条件，繁殖条件を一定にする必要がある。山形大学において作出維持されている家兎は温度22±2℃，湿度40～60％に管理された飼育室の中で，固型飼料（Labo R Grower，日本農産工業，東京）120gを毎日一定量給餌されている。水は自由摂取である。飼育室の明暗は午前6時から午後6時までを照明時間としている。家兎の繁殖は自家で行ない，娩出された家兎は生後30日で離乳された後に個別飼育された。生後1カ月齢から2カ月齢までの幼若家兎には80gの固型飼料を毎日定量摂取させ，2カ月齢以後は120gとした。

血清脂質の測定

家兎の耳静脈から採血し，1分間3000回転で15分間（4℃）遠心を行なって血清または血漿を分離後，Vision Analyzer（Dinabot社，日本）を用いてコレステロールとトリグリセリドの測定を行なった。

野生型家兎との比較

食後高トリグリセリド血症家兎（PHT）は，空腹時には血清トリグリセリド値は正常であるが，食後に高トリグリセリド血症を示すという特徴を有し，ヒトの高トリグリセリド血症のモデル動物として有用である。日本白色家兎では，食後のトリグリセリド増加は認められない。食後高トリグリセリド血症家兎（PHT），日本白色家兎（JW）および遺伝性高トリグリセリド血症家兎（TGH）の絶食と食後の影響について血清コレステロールと血清トリグリセリドを指標に比較検討した。コレステロールは3系統ともとくに変化を認めなかった（図2）。しかし，トリグリセリドは，日本白色家兎（JW）と遺伝性高トリグリセリド血症家兎（TGH）では絶食や食餌の影響を受けなかったのに対し，食後高トリグリセリド血症家兎（PHT）では，絶食前に約1000mg/dlあったトリグリセリド値が，絶食開始後急激に低下し，12時間で約100mg/dlを示した。絶食期間中は100～200mg/dlで推移したが，その後食餌を与えるとトリグリセリド値は急激に上昇し，食後24時間で600mg/dlを示した（図3）。このように，食後高トリグリセリド血症家兎は絶食と食餌に対して鋭敏な反応を示す。

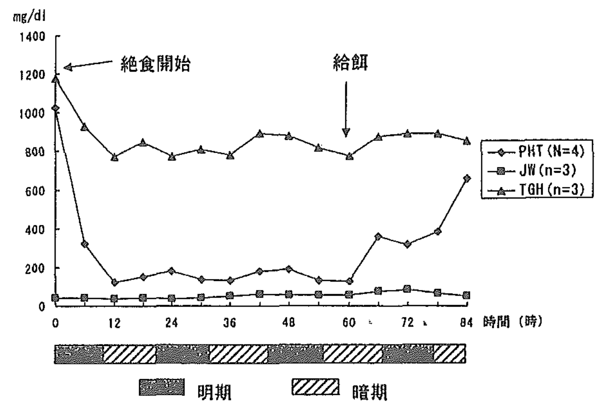

図3　PHTの食餌によるトリグリセリド変化

食餌負荷後のトリグリセリド変化

食後高トリグリセリド血症家兎の食餌投与後における血清トリグリセリド値の経時変化を詳細に測定した。結果を図4に示す。24時間絶食の後，食餌の投与を始めると食後8時間を過ぎる頃から血清トリグリセリド値は急速に上昇した。食後16～20時間で血清トリグリセリド値は1000mg/dl以

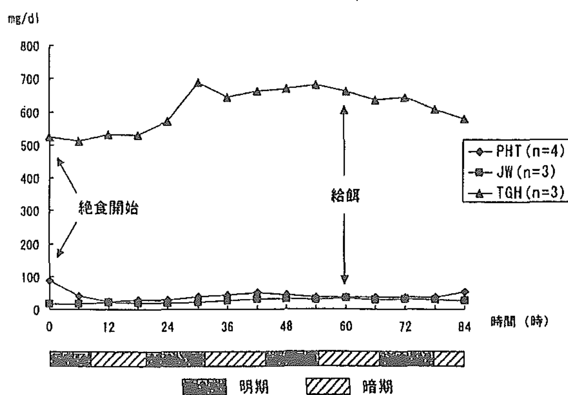

図2　PHTの食餌によるコレステロール変化

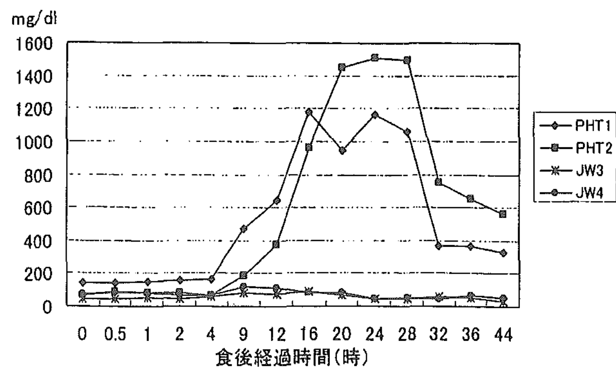

図4　PHTの食餌負荷後のトリグリセリド値
―個体ごとの測定値―

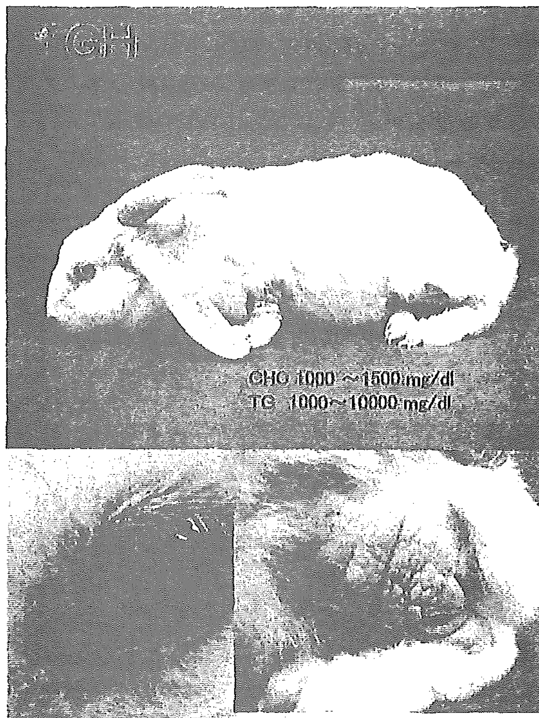

図5　遺伝性高トリグリセリド血症家兎（TGH）

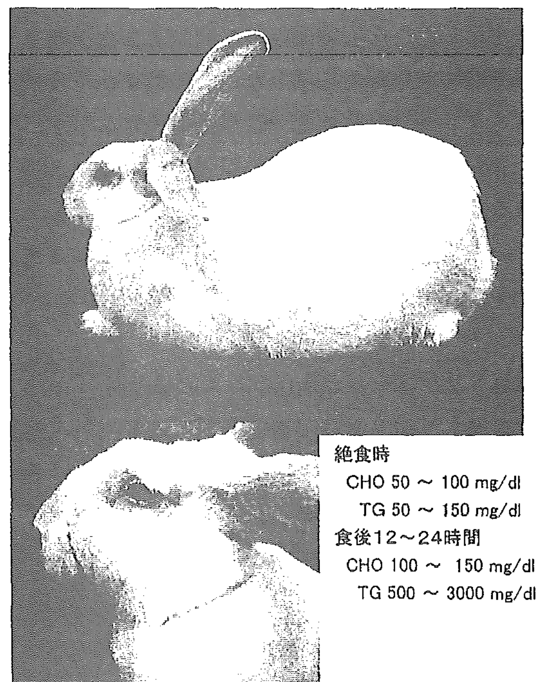

図6　食後高トリグリセリド血症家兎（PHT）

上という最高値に達し，8～10時間高いレベルを維持した後に低下した。野生型であるJW（日本白色家兎）は，摂食を開始してもトリグリセリド値の極端な上昇は認められなかった。遺伝性高トリグリセリド血症家兎（TGH）の写真を図5に，食後高トリグリセリド血症家兎（PHT）の写真を図6に示す。

食後高トリグリセリド血症の発症時期

3，6，9カ月齢の動物について，食餌と血清脂質との関係を調べた。食餌の摂取量と食後のトリグリセリドレベルの間には有意の相関は認められなかった。従って，食後高トリグリセリド血症は食物の摂取と関連するが，食餌の摂取量は血液中の濃度を規定する因子ではないことになる。絶食時と食後の血清総コレステロール値の比較において，3カ月齢では有意の差を認めないが，6カ月齢では平均44mg/dl から118mg/dl へ，9カ月齢では41mg/dl から 80mg/dlへと食餌により有意に増加した。血清トリグリセリド値を絶食時と食後で比較したところ，3カ月齢で103mg/dlから236mg/dlへ，6カ月齢で113mg/dlから 1437mg/dlへ，9カ月齢では131mg/dlから915mg/dlへと，食餌により極端に増加した（図7)。

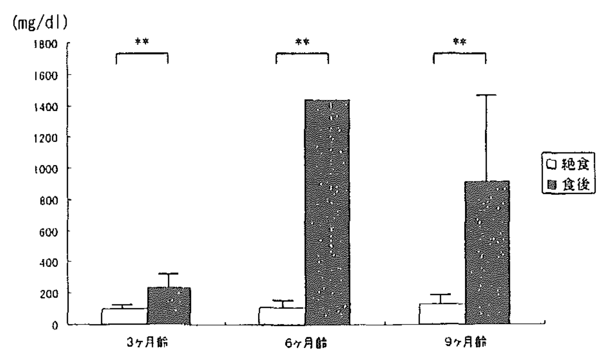

図7　月齢の違いによる食後のトリグリセリド値
—PHT第3世代（n＝23）—

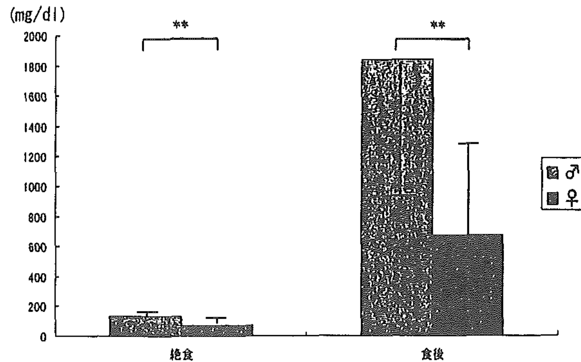

図8　食後トリグリセリド値の性差
―PHT 6カ月齢（n＝23）―

食後高トリグリセリド血症の性差

上記6カ月齢の動物に対し，トリグリセリド値の性差を検討した結果，空腹時の血清トリグリセリド値には性差を認めないが，食後のレベルは雄性が1844mg/dl，雌性が675mg/dlであり，雄性の方が顕著に高いという結果を得た（図8）。

食後高トリグリセリド血症と摂餌量

第3世代の食後高トリグリセリド血症家兎について摂餌量とコレステロール値およびトリグリセリド値の関係を検討したところ，絶食下においてコレステロール値は44±25mg/dl，トリグリセリド値は113±60mg/dlであった。そして，食後ではコレステロール値は118±50mg/dl，トリグリセリド値は1437±999mg/dlであり，食餌により12倍という顕著な増加を示した。しかし，摂餌量とコレステロール値ならびに摂餌量とトリグリセリド値との関係に相関を認めなかった。

食後のリポ蛋白分画

食後高トリグリセリド血症家兎の食後のリポ蛋白分画についてアガロースゲル電気泳動法を用いて検討した結果，他のリポ蛋白に対して食後のVLDLコレステロールとVLDLトリグリセリドが急激に増加することがわかった。VLDL（Very Low Density Lipoprotein；超低比重リポ蛋白）はカイロミクロンにつぐ大きさを持つ粒子で，アガロース電気泳動ではpre β 位に分画される。VLDLの役割は肝臓で生合成されるトリグリセリドやコレステロールを血中を介して全身の組織に運搬することにあるとされている。食後高トリグリセリド血症家兎の血中におけるVLDLの増加は，肝臓からのVLDL分泌亢進なのか，血中のVLDLの代謝が阻害されているかは不明である。また，食後に小腸上皮細胞で生成されるカイロミクロンおよびカイロミクロンレムナントの動態についても未検討である。

食後高トリグリセリド血症家兎の耐糖能

食後高トリグリセリド血症家兎にヒトの経口糖負荷試験に準じて経口糖負荷試験（1.5g OGTT）を行なった結果，日本白色家兎では，負荷後2時間値が負荷前の血糖値（140mg/dl）に戻ったのに対し，食後高トリグリセリド血症家兎では200mg/dl以上を示した。各血糖測定値の和である血糖和も統計学的有意な高値を示した。この結果から食後高トリグリセリド血症家兎は耐糖能異常を示すことがわかった。耐糖能異常の原因については不明である。また，同様の試験において，血中のインスリン濃度について検討した結果，食後高トリグリセリド血症家兎はインスリン抵抗性を示した。

食後高トリグリセリド血症家兎の内臓脂肪

食後高トリグリセリド血症家兎は日本白色家兎に比較すると，腸管膜や腎周囲などの腹腔内に多量の脂肪組織が存在する。図9に食後後高トリグリセリド血症家兎（PHT）の内蔵脂肪を示す。

食後高トリグリセリド血症家兎の寿命他

遺伝性高トリグリセリド血症家兎の体型は日本

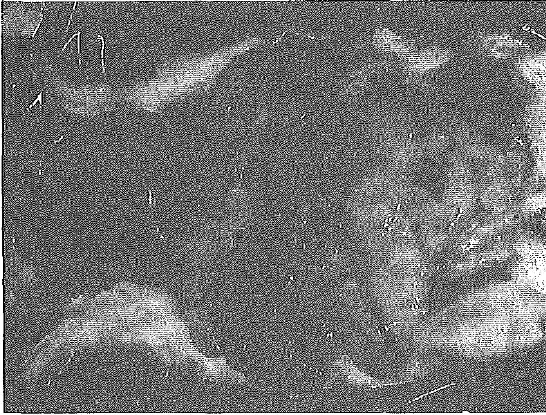

図9 食後高トリグリセリド血症家兎（PHT）の腹腔内脂肪

白色家兎と同等か若干小型であり，寿命は野生型（対照）と比較して大差を認めなかった。また，正常の生殖活動が観察された。

食後高トリグリセリド血症の遺伝様式

遺伝性高トリグリセリド血症家兎（TGH）は常染色体性劣性遺伝を示すことに対し，食後高トリグリセリド血症家兎（PHT）は常染色体性優性遺伝を示唆する遺伝を示す。しかし，表現系（食後高トリグリセリド血症）に，バラツキを認めることから複合遺伝子（ポリジーン）支配の可能性も示唆される。

引用文献

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Changes in Lipoprotein Profile after Selective LDL Apheresi

Key words: hypercholesterolemia, low-density lipoprotein, apheresis

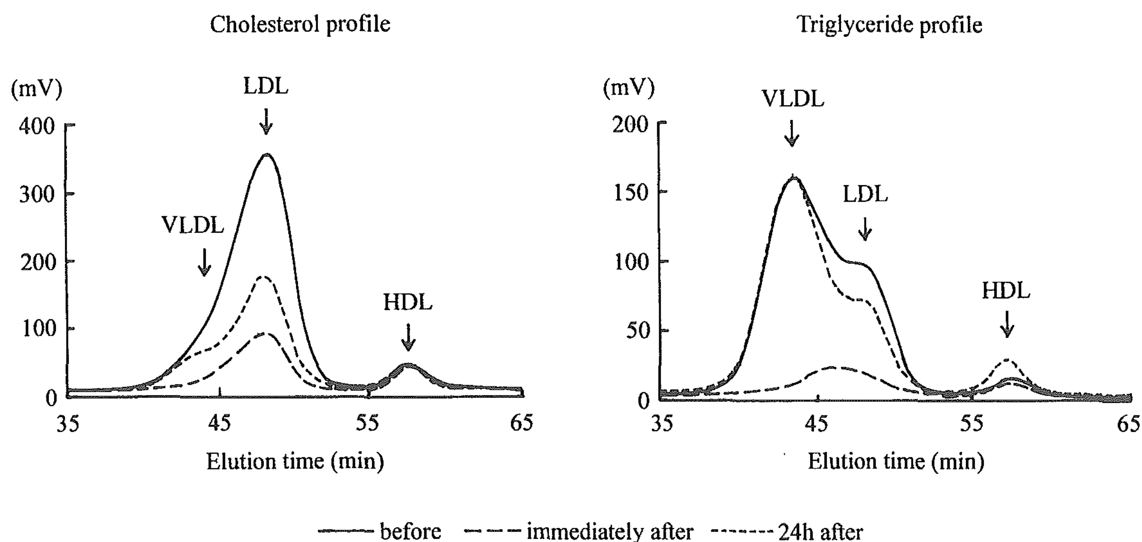

Figure 1. Left panel: Lipoprotein profile of cholesterol. Both LDL and VLDL components were prominently reduced after apheresis and half of the pre-apheresis level was noted within 24 hours, with no changes in HDL. Right panel: Lipoprotein profiles of triglyceride. Both LDL and VLDL components were prominently reduced after apheresis and the pre-apheresis level was reached within 24 hours along with doubling of the HDL component.

Low-density lipoprotein (LDL) apheresis has been used to treat patients with severe inherited forms of hypercholestei mia. This procedure selectively removes apolipoprotein B-containing particles such as LDL, very-low density lipoprc (VLDL), and lilpoprotein(a) [Lp(a)]. However, lipoprotein profiles of both cholesterol and triglyceride are less well un stood. A 33-year-old Japanese woman with homozygous familial hypercholesterolemia was treated once a week with se tive LDL filtration from the age of 28 years old. Large xanthomas occurred on the eyelid, elbow, and both sides of the k The plasma levels of total cholesterol, triglyceride, and Lp(a) were reduced from 390 to 121, from 322 to 27 and fron to 5 mg/dl immediately after apheresis (LIPOSORBER™ system) using dextran sulfate cellulose columns. HPLC anal (CCP & 8010 series, Tosoh, Tokyo) of plasma before and after apheresis clearly showed a marked reduction of LDL VLDL, not only of cholesterol but also triglyceride components (Fig. 1). A rebound phenomenon after 24 hours was r rapid for triglyceride than for cholesterol components.

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Identification of 21 single nucleotide polymorphisms in human hepatocyte growth factor gene and association with blood pressure and carotid atherosclerosis in the Japanese population

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Abstract

It has been suggested that circulating concentrations of hepatocyte growth factor (HGF) are increased in individuals with vascular endothelial damage, such as in hypertensive patients and subjects with atherosclerosis. Because the influence of genetic variation of *HGF* has not been examined, we identified single nucleotide polymorphisms (SNPs) in the *HGF* gene, and investigated the association between these SNPs and blood pressure or carotid atherosclerosis in the Japanese general population. We identified 21 SNPs in the *HGF* gene by direct sequencing in a test population of 32 Japanese subjects. Among them, considering allele frequency and linkage disequilibrium, three SNPs, *C-1652T* in the promoter, *T43839A* in intron 8, and *T44222C* in intron 9, were genotyped in 2412 members of the Japanese general population randomly selected from the residents in Suita city. None of the three SNPs were significantly associated with blood pressure. After adjusting for age, smoking habits, consumption of alcohol, and the presence of diabetes mellitus and dyslipidemia, female subjects with the T allele of *T43839A* had more severe carotid atherosclerosis compared to individuals with the A allele. This study provides the first evidence that *HGF* may be a candidate susceptibility loci that affects the progression of atherosclerosis in Japanese subjects.
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Keywords: Hepatocyte growth factor; Hypertension; Atherosclerosis; Genetics; Polymorphism

1. Introduction

Endothelial cell dysfunction has been suggested as the initiating process in the development and progression of cardiovascular disease, and it is considered to be closely related to the pathophysiology of human essential hypertension. There has been accumulating evidences that hepatocyte growth factor (HGF), mesenchyme-derived pleiotropic factor, plays an important role in endothelial cell dysfunction. Since HGF was originally identified in the plasma of rats after partial hepatectomy [1], a number of investigations have suggested that HGF is a multifunctional factor implicated in tissue regeneration and angiogenesis in not only liver but also other tissues [2,3]. A local HGF system (HGF and its receptor, c-met) is expressed in vascular cells [4], and elevated serum HGF levels have been suggested to play a cardiovascular protective role in hypertensive subjects, especially those with concomitant arteriosclerosis [5–9].

Although an association between HGF and the severity of hypertension was established, few reports have investigated the association between *HGF* gene polymorphisms and blood pressure or atherosclerosis. The *HGF* gene is composed of 18 exons encompassing 70 kb on chromosome 7q11.2-q21 [10]. In the present study, we screened for sin-

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gle nucleotide polymorphisms (SNPs) in the *HGF* gene and evaluated the significance of SNPs in high blood pressure and carotid atherosclerosis determined by an ultrasonography using a large cohort, the Suita Study, which was representative of the general Japanese population.

2. Methods

2.1. Subjects

The protocol of the Suita Study, described elsewhere [11–14], was approved by the Ethics Committee of the National Cardiovascular Center. The sample consisted of 14,200 Japanese men and women ages 30–79 years stratified by gender and 10-year age groups, selected randomly from the municipal population registry. They were all invited by letter to attend regular cycles (every 2 years) of follow-up examinations. DNA from leukocytes was collected from participants who visited the Division of Preventive Medicine, National Cardiovascular Center between May 1996 and February 1998. Subjects who gave their written informed consent for genetic analyses of the genes were included in the present study. All clinical data and genotyping results were anonymous, and all data was handled in such a way that it was/will not be possible to identify an individual.

The characteristics of the subjects analyzed in the present study are summarized in Table 1. Blood pressure was measured in the subjects after at least 10 min of rest in a sitting position. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) values are the mean of two physician-obtained measurements (recorded >3 min apart). Hypertension was defined as SBP ≥140 mm Hg or DBP ≥90 mm Hg or the current use of antihypertensive medications; diabetes mellitus was defined as fasting blood glucose ≥126 mg/dl, or the current use of insulin or oral anti-diabetic agents; and dyslipidemia was defined total cholesterol ≥220 mg/dl or the current use of antidyslipidemia medication at the time of the first examination.

Table 1
Clinical Features of Study Participants

Variables	Men n = 1158	Women n = 1254
Age, y	61.0 ± 12.2*	58.9 ± 11.7
Body mass index, kg/m2	23.1 ± 2.8*	22.3 ± 3.1
Systolic blood pressure (SBP), mmHg	129.2 ± 10.1	128.5 ± 21.1
Diastolic blood pressure (DBP), mmHg	81.0 ± 10.9*	78.8 ± 10.6
Total cholesterol (TC), mg/dL	204.6 ± 31.9	215.9 ± 32.9*
HDL cholesterol, mg/dL	54.1 ± 14.5	64.2 ± 15.6*
Current alcohol consumer (%)	38.0†	8.6
Current smoker (%)	71.5†	29.1
Hypertension (%)	39.6†	35.5
Diabetes mellitus (%)	9.1†	3.7
Dyslipidemia (%)	35.2	51.3†

Values are means ± SDs or percentages.
Hypertension indicates SBP ≥ 140 mmHg and/or DBP ≥90 mmHg or antihypertensive medication; Dyslipidemia, TC ≥ 220 mg/dL or antidyslipidemia medication; diabetes mellitus, fasting plasma glucose ≥ 126 mg/dL or antidiabetic medication.
* $P < 0.05$ between men and women by Student's t test.
† $P < 0.05$ between men and women by χ^2 test.

2.2. Evaluation of carotid atherosclerosis

Carotid ultrasonography was performed for the evaluation of atherosclerosis. Measurement methods were previously described [11,12]. Briefly, ultrasonography of both carotid arteries was performed with a high-resolution Duplex scanner (TOSHIBA SSA-250A; probe, SMA-736S mechanical sector scanner, Toshiba, Tokyo, Japan) for the B-scan. The subjects were examined in the supine position with their head slightly turned from the sonographer. All measurements were performed by two trained sonographers, who were unaware of the subjects' clinical data. The carotid arteries were carefully examined with regard to wall changes from different longitudinal (anterior oblique, lateral, and posterior oblique) and transverse views, and measurements of thickness were performed from transverse image. Intima-medial thickness (IMT) was measured at a point 10 mm proximal from the beginning of the dilatation of the carotid bulb, and Maximum-IMT (Max-IMT) was defined as the maximum thickness of intima-media including plaques from the region branching off from the brachiocephalic artery (right) or aorta (left) to the bifurcation of the common carotid artery. Plaque Score was calculated by summing the maximum thickness of all the plaques in the bilateral carotid artery in the scanning area [11,12].

2.3. Direct sequencing for the detection of polymorphisms in the HGF genes

We obtained peripheral blood samples from 32 Japanese volunteers for direct sequencing of the *HGF* gene after obtaining written informed consent. Genomic DNA was extracted with an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan). Methods of direct sequencing are described previously [15]. Briefly, all exons, a portion of introns and a region up-stream of exon 1, which included the promoter region of *HGF*, were amplified by polymerase chain reaction (PCR). The PCR products were then treated with shrimp alkaline phosphates and exonuclease I (PCR Product Pre-Sequencing Kit, USB Corporation, Cleveland, OH), and used as templates for direct single-pass sequencing using a BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The reaction products were purified with a DyeEX 96 kit (QIAGEN) and analyzed on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). The obtained sequences

Table 2
Primers and TaqMan Probes for Genotype Determination

HGF SNPs	Sequence
C(−1652)T (promoter)	
Sense	5′-GGATTAGCAATAGAAACGGGTCAT-3′
Antisense	5′-CCCTGAGGTTGTGGGATATCTAGA-3′
Probe for C(−1652)	Fam-5′-AAAATAGATCCCTCAAAAG-3′-MGB
Probe for T(−1652)	Vic-5′-AATAGATCTCTCAAAAGG-3′-MGB
A43839T (intron 8)	
Sense	5′-TTCAGTAATTTGGGCAGAGTCAGT-3′
Antisense	5′-ACGTTGGTGAAGTCAGCGCTAT-3′
Probe for A43839	Fam-5′-AGTCCAAAAAGTTAGAACT-3′-MGB
Probe for T43839	Vic-5′-AGTCCAAAATGTTAGAAC-3′-MGB
C44222T (intron 9)	
Sense	5′-GCTGGCTTGCAAACAAAATCA-3′
Antisense	5′-GGCTTAGAACTGTGGCTGTCAGT-3′
Probe for C44222	Fam-5′-TTTGAAGCTGGATTTT-3′-MGB
Probe for T44222	Vic-5′-TTGAAGTTGGATTTTT-3′-MGB

were examined for the presence of a polymorphism using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection.

2.4. Genotyping of SNPs in the HGF genes

Three SNPs were genotyped using the TaqMan system [14,16]. PCR primers and probes for the TaqMan system are shown in Table 2. Fluorescence level of the reaction products was measured by use of ABI PRISM 7700 or 7900 Sequence Detection System (Applied Biosystems).

2.5. Statistical analysis

Values are expressed as the mean ± S.D. or mean ± S.E. Multiple regression and multiple logistic analyses were performed with the covariates age, body mass index, smoking, current alcohol consumption, presence of diabetes and/or dyslipidemia using the SAS version 6.0 (SAS Institute Inc., Cary, NC). Differences in frequency among the groups were tested by χ^2 analysis. Linkage disequilibrium was evaluated by obtaining r^2 values between polymorphisms using SNPAlyze ver. 2.0 (DYNACOM Co., Ltd., Shigehara, Japan).

3. Results

3.1. Detection of genetic variants in the HGF gene

We systematically searched the sequences obtained for SNPs in 32 volunteer subjects and identified 21 SNPs including 8 SNPs in the HGF promoter, 1 SNP in exon and 12 SNPs in intron regions (Table 3). Ten of these SNPs have been deposited in the public database previously, db SNPs (http://www.ncbi.nlm.nih.gov/SNP/), but 11 of the identified SNPs were novel. Thirteen SNPs had their minor allelic frequency less than 10%, therefore no further studies of these SNPs were undertaken. Six SNPs were in tight linkage disequilibrium, therefore one of them, C44222T, was selected as a representative. Thus, three SNPs, *C(-1652)T*, *A43839T* and *C44222T* were chosen for further genotyping analysis (Table 3).

Table 3
List of 21 Polymorphisms and Alele Frequency in HGF Identified in 32 Japanese Patients by Direct Sequencing

allele 1/allele 2			allele 1		allele 2		allele frequency			
SNPs	aa info.	region	homo	hetero	homo	total	allele 1	allele 2	flanking sequence	dbSNP ID
C(−2142)A		promoter	28	4	0	32	0.938	0.063	ttggaatggggt[c/a]ttatgagctacg	
G(−1965)T		promoter	31	1	0	32	0.984	0.016	atgcctcgcctt[g/t]ggggagaatgaa	
G(−1903)A		promoter	30	1	0	31	0.984	0.016	gctgattctgag[g/a]tcttcatttggg	
C(−1652)T		promoter	10	16	5	31	0.581	0.419	ataaaatagatc[c/t]ctcaaaaggaat	rs3735520
G(−1268)C		promoter	30	1	0	31	0.984	0.016	tctctgaatcaa[g/c]tgaggggtctgg	rs3735521
-(−1215)C		promoter	27	4	0	31	0.935	0.065	taggagtccccc[-/c]atgcccatacaa	
T(−955)C		promoter	31	1	0	32	0.984	0.016	ggacaatgactg[t/c]ttcttggacttt	
T(−578)C		promoter	31	1	0	32	0.984	0.016	aactagacagat[t/c]aggagctggggc	
T40171-*		intron7	0	7	25	32	0.109	0.891	taagttttttttt[t/-]gttgttgttttt	rs5745686
A43839T		intron8	17	14	1	32	0.750	0.250	ctgagtccaaaa[a/t]gttagaactcta	rs2286194
C44222T*		intron9	0	7	25	32	0.109	0.891	ccaagtttgaag[c/t]tggatttttctt	rs2887069
C49065T*		intron9	0	7	25	32	0.109	0.891	acttgtaaaaaa[c/t]ctttttgtttta	
T49080C*		intron9	0	7	25	32	0.109	0.891	ttttgttttatc[t/c]gccttgatattc	
A52603G*		intron10	0	6	26	32	0.094	0.906	cctgttgtttcc[a/g]cagtcatatctt	rs1800793
G58294A		intron11	31	1	0	32	0.984	0.016	gcctgggtgaca[g/a]aatgagactctg	
T59941A		intron13	31	1	0	32	0.984	0.016	agggcacctggg[t/a]gaggcagtaaaa	rs5745739
T59984G*		intron13	26	6	0	32	0.906	0.094	tgcttgccagac[t/g]gtaagctctgga	rs2074725
G62753T		intron14	31	1	0	32	0.984	0.016	tttgctcttaag[g/t]ttataatatgta	rs5745745
G63555T	Asp543Tyr	exon15	31	1	0	32	0.984	0.016	agagacttgaaa[g/t]attatgaagctt	
A64588G		intron17	31	1	0	32	0.984	0.016	atgtgaggtaaa[a/g]aggaagttcttt	
T67183G		intron17	31	1	0	32	0.984	0.016	tttaattcctaa[t/g]aatactttgttt	rs5745767

Three SNPs underlined had the minor allele frequency over 10% and were selected for genotyping in this study.
* Six polymorphisms are in strong linkage disequilibrium (r-square > 0.5).

Table 4
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by C(−1652)T Genotypes

Male	CC	CT	TT	*P*
n (%)	519(47.1%)	438(39.7%)	146(13.2%)	
SBP (mmHg)	129.3 ± 0.7	128.8 ± 0.8	129.3 ± 1.4	0.850
DBP (mmHg)	81.4 ± 0.5	80.6 ± 0.5	80.8 ± 0.9	0.355
%Hypertension	41.4	39.0	35.6	0.419
IMT (mm)	0.898 ± 0.005	0.903 ± 0.005	0.892 ± 0.009	0.896
Max-IMT (mm)	1.729 ± 0.035	1.745 ± 0.038	1.650 ± 0.067	0.492
Plaque Score	4.6 ± 0.2	4.9 ± 0.2	4.5 ± 0.3	0.981
Female	**CC**	**CT**	**TT**	***P***
n (%)	579(48.0%)	481(39.9%)	146(12.1%)	
SBP (mmHg)	129.1 ± 0.7	128.8 ± 0.8	127.5 ± 1.5	0.405
DBP (mmHg)	79.0 ± 0.4	79.1 ± 0.5	78.5 ± 0.8	0.727
% Hypertension	35.6	36.0	34.9	0.973
IMT (mm)	0.846 ± 0.004	0.852 ± 0.004	0.848 ± 0.008	0.601
Max-IMT (mm)	1.332 ± 0.021	1.368 ± 0.023	1.371 ± 0.042	0.257
Plaque Score	2.4 ± 0.1	2.6 ± 0.1	2.7 ± 0.2	0.143

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

3.2. Study population

Table 1 shows the clinical characteristics of the present subjects by sex. Most variables (i.e., age, body mass index, diastolic blood pressure, percentage of current alcohol drinking, smoking, diabetes, and hypertension) were significantly higher in men than in women, but percentage of dyslipidemia, serum total cholesterol and HDL cholesterol levels were significantly higher in women than in men. There were no significant differences in systolic blood pressure.

3.3. Association of three polymorphisms with blood pressure and carotid arterioscleorsis

We investigated the possible association of three SNPs in the human *HGF* gene with blood pressure and carotid atherosclerosis in a population-based sample (the Suita Study) that consisted of 2412 participants. The frequencies of each genotype are described in Tables 4-6. The genotype frequencies of all analyzed polymorphisms were consistent with Hardy-Weinberg equilibrium. There were no significant differences in the genotype frequencies of polymorphisms for either sex. Tables also show systolic and diastolic blood pressure levels, and carotid IMT and Plaque Scores in each genotype of the three polymorphisms.

After full adjustment of all confounding factors (age, body mass index, current smoking status, alcohol consumption, presence of diabetes mellitus and dyslipidemia), there was no significant association between the three genotypes and blood pressure levels or the prevalence of hypertension in all subjects and in each sex.

Table 5
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by A43839T Genotypes

Male	AA	AT	TT	*P*
n(%)	555(59.8%)	304(32.8%)	69(7.4%)	
SBP (mmHg)	129.2 ± 0.7	128.4 ± 0.9	130.6 ± 2.0	0.981
DBP (mmHg)	81.3 ± 0.4	80.2 ± 0.6	81.7 ± 1.2	0.550
%Hypertension	38.0	41.1	34.8	0.522
IMT(mm)	0.897 ± 0.005	0.896 ± 0.006	0.888 ± 0.013	0.643
Max-IMT (mm)	1.733 ± 0.034	1.691 ± 0.046	1.743 ± 0.098	0.703
Plaque Score	4.8 ± 0.2	4.4 ± 0.2	4.5 ± 0.5	0.200
Female	**AA**	**AT**	**TT**	***P***
n(%)	636(61.5%)	340(32.9%)	59(5.7%)	
SBP (mmHg)	129.3 ± 0.7	127.4 ± 1.0	131.2 ± 2.3	0.578
DBP (mmHg)	79.0 ± 0.4	78.7 ± 0.5	78.9 ± 1.3	0.790
%Hypertension	36.6	32.1	44.1	0.135
IMT (mm)	0.854 ± 0.004	0.842 ± 0.005	0.837 ± 0.012	0.039
Max-IMT (mm)	1.390 ± 0.021	1.310 ± 0.028	1.232 ± 0.065	0.003
Plaque Score	2.7 ± 0.1	2.3 ± 0.2	1.8 ± 0.4	0.002

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

Table 6
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by C644222T Genotypes

Male	CC	CT	TT	*P*
n (%)	7(0.7%)	194(19.1%)	817(80.3%)	
SBP (mmHg)	125.7 ± 6.2	128.7 ± 1.2	129.3 ± 0.6	0.547
DBP (mmHg)	80.0 ± 3.9	81.2 ± 0.7	80.9 ± 0.4	0.780
%Hypertension	14.2	38.1	40.3	0.334
IMT (mm)	0.895 ± 0.039	0.885 ± 0.008	0.900 ± 0.004	0.096
Max-IMT (mm)	2.066 ± 0.287	1.690 ± 0.057	1.732 ± 0.028	0.856
Plaque Score	2.4 ± 0.9	2.4 ± 0.2	2.5 ± 0.1	0.414
Female	**CC**	**CT**	**TT**	***P***
n (%)	11(1.0%)	207(18.6%)	897(80.5%)	
SBP (mmHg)	130.2 ± 5.3	128.2 ± 1.2	129.1 ± 0.6	0.616
DBP (mmHg)	80.0 ± 3.0	78.2 ± 0.7	79.0 ± 0.3	0.439
%Hypertension	36.4	31.9	37.0	0.383
IMT (mm)	0.825 ± 0.030	0.841 ± 0.007	0.850 ± 0.003	0.161
Max-IMT (mm)	1.317 ± 0.162	1.362 ± 0.036	1.349 ± 0.017	0.844
Plaque Score	6.2 ± 1.5	4.4 ± 0.3	4.8 ± 0.1	0.414

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

Although no association was found between carotid IMT, Plaque Scores and *A43839T* genotype in male subjects, women with the A allele showed significantly thicker IMT and greater Plaque Scores than those with the T allele (Table 5). There was no association between carotid IMT, Plaque Scores and genotypes of the two SNPs, *C(-1652)T* and *C44222T*, in both male and female subjects (Tables 4 and 6).

4. Discussion

Although a number of reports have suggested a strong association between the severity of hypertension and serum HGF levels, there have been few reports that investigated the association between cardiovascular disease and *HGF* gene polymorphisms by direct sequencing. Of those identified single nucleotide polymorphisms, 11 were not deposited in the public database. We have performed a large genetic epidemiological study of the Japanese general population regarding three candidate SNPs in the promoter and intron of the *HGF* gene. There was no significant association between the three *HGF* SNPs and blood pressure or the prevalence of hypertension. Interestingly, female subjects with the A allele of *A43839T* in intron 8 had more severe carotid atherosclerosis than those with the T allele.

4.1. HGF and hypertension, atherosclerosis

Clinical studies have demonstrated a positive correlation between serum HGF concentrations and blood pressure. These studies have gone on to show that serum HGF concentrations in hypertensive patients were significantly higher than those seen in normotensive control subjects [5,6]. In an experimental setting, serum HGF concentrations were significantly increased in spontaneous hypertensive rats compared to Wistar-Kyoto rats at any age, and there was a positive association between serum HGF concentration and blood pressure level [17].

A number of reports have also suggested that serum HGF concentrations are increased in proportion to the development of hypertensive target organ damage. The circulating level of HGF was elevated in patients with myocardial infarction [18] and peripheral arterial disease [9,19,20]. Furthermore, serum HGF concentrations were significantly correlated with the hyperemic response of forearm blood flow and pulse wave velocity [21]. Alternatively, it is apparent that endothelial cell dysfunction may promote abnormal vascular growth, and this vascular remodeling clearly plays an important role in the pathophysiology of atherosclerosis. In normotensive subjects, serum HGF was suggested to maintain the vascular structure and stimulate tissue regeneration in an autocrine-paracrine manner [19,22]. In patients with hypertension or diabetes mellitus, this local HGF system was disturbed by transforming growth factor-β or angiotensin II with the resultant development of abnormal vascular smooth muscle cell growth [17,23].

4.2. HGF SNPs, hypertension, and atherosclerosis

Polymorphisms of several growth factor genes have been investigated because they may potentially play a key role in the maturation of atheromatous lesions. Among these growth factors, HGF was of particular interest because it could have cardiovascular protective effects in several disorders including hypertension, diabetes mellitus, and cardiovascular diseases.

In the present study, we identified 21 SNPs in the *HGF* gene, and determined the genotype of three of these polymorphisms in more than 2000 individuals. Our results showed that there were no significant associations between *HGF* genotypes and blood pressure levels or the prevalence of hypertension. However, the A allele of *A43839T* in intron 8 was significantly associated with increased severity

of carotid atherosclerosis in females. The reason for this sex-specific effect in our study subjects is unclear. The interaction of HGF with angiotensin II and/or transforming growth factor β could contribute to some of the difference seen in our patients population. Although still controversial [24], renin-angiotensin system related genetic variation tends to appear in male individuals in Japanese [13] and Caucasians [25,26]. Additionally, a T → C transition at nucleotide 869 of the *transforming growth factor β* gene has been reported to be one of the candidate susceptibility loci for hypertension only in the female Japanese population [27]. We assume that there might exist some link or interaction between HGF and the genetic variation of these two growth factors.

Our results suggest that the HGF gene located at chromosome 7q11.2-q21 is a candidate susceptibility locus for atherosclerosis in Japanese women. The polymorphism conferring increased susceptibility for atherosclerosis was located in the intron region without amino acid substitution. Thus, it is possible that the *A43839T* polymorphism in intron 8 of the *HGF* gene is in linkage disequilibrium with some other polymorphisms which are actually responsible for the development of atherosclerosis. To elucidate the exact mechanisms and clinical implications of the association, further functional and linkage disequilibrium analyses are required.

In conclusion, we identified 21 SNPs in the *HGF* gene including 11 SNPs that have never been reported. The present study provides the first evidence that *HGF* may be a candidate susceptibility loci that affects the progression of atherosclerosis in Japanese subjects.

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Chronic hypertriglyceridemia in young watanabe heritable hyperlipidemic rabbits impairs endothelial and medial smooth muscle function

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Abstract

Several studies have suggested that hypertriglyceridemia is a common risk factor for coronary heart disease. Although increasing serum levels of triglyceride correlate with hypercoagulability, little is known about the contribution of hypertriglyceridemia to vascular function. We successfully segregated two lines of rabbits with genetically-determined severely high (TGH; 2764 ± 413 mg/dl) and moderately high (TGL; 191 ± 12 mg/dl) levels of triglyceride, but with comparable levels of total cholesterol, from Watanabe heritable hyperlipidemic rabbits. To determine whether hypertriglyceridemia was involved in alterations of vascular function, we conducted isometric tension studies and analyzed protein expression on thoracic aortic rings isolated from young (3–4 month) TGH, TGL and Japanese White rabbit (JW). No difference in percentage of plaque area in the thoracic aorta was found between TGH and TGL. Relaxing responses, evoked by sodium nitroprusside were similar in JW, TGL and TGH, but endothelium-dependent relaxation to acetylcholine was impaired in TGH compared with JW or TGL (maximal relaxation in JW; 83.5 ± 2.7%, TGL; 79.9 ± 5.3%, TGH; 59.1 ± 5.7%, $p < 0.05$). Relaxation to A23187 was also attenuated in TGH compared with JW, but not significantly different between TGL and JW. Endothelium-independent relaxation elicited by isoproterenol in TGH was significantly decreased compared with JW or TGL (maximal relaxation in JW; 95.2 ± 2.6% TGL; 91.0 ± 4.9%, TGH; 75.1 ± 5.2%, $p < 0.05$). Protein expression of angiotensin II type-1 receptor was increased in TGH and that of nitric

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oxide synthases-3 was attenuated in TGH compared with TGL. This is the first study showing that endothelium-dependent and -independent vascular relaxation under the condition of combined hyperlipidemia was severely impaired as compared to that under only hypercholesterolemia. These results suggest that hypertriglyceridemia aggravates functional impairment induced by hypercholesterolemia in endothelial and smooth muscle cells.
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Keywords: Hypertriglyceridemial; Combined hyperlipidemia; EDRF; Isoproterenol; Vascular relaxation; Endothelial dysfunction; WHHL; NOS3; Angiotensin II

Introduction

Hyperlipidemia has been implicated as a common risk factor for cardiovascular disease (Gordon et al., 1977; Hulley et al., 1980). Although elevated TG levels are predictive of subsequent occurrence of coronary heart disease and mortality in healthy persons, adjustment for HDL cholesterol and fasting serum glucose eliminates the independent relationship between serum TG levels and cardiovascular events (Castelli et al., 1977). However, recent epidemiological studies have demonstrated that hypertriglyceridemia is identified as one of the potential risk factors for coronary heart disease (Egger et al., 1999; Rosenson et al., 1999). Lines of evidence have shown that hypercholesterolemia induces endothelial dysfunction, smooth muscle cell proliferation, enhanced vascular reactivity to some contractile agonists, infiltration of monocytes into vessel walls, and production of reactive oxygen species (Ross, 1993). Studies using Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model with deficient low-density lipoprotein (LDL) receptors, have demonstrated that hypercholesterolemia causes progression of atherosclerosis and fatty streak formation in the vascular wall, which correlates with reduced endothelium-dependent relaxation (Tagawa et al., 1991, 1993). However the contribution of chronic hypertriglyceridemia to endothelial and medial smooth muscle function is largely undefined (de Man et al., 2000; Inoue et al., 1998). Several studies have demonstrated that flow-mediated vasodilatation is impaired in patients with hypertriglyceridemia (Lundman et al., 2001a; Lupattelli et al., 2000). It has been reported that aortic rings pre-incubated with triglyceride-rich fat emulsion attenuates endothelial-dependent vasodilation (Lundman et al., 2001b). However, little is known about the potential mechanisms for vascular dysfunction induced by chronic hypertriglyceridemia.

In order to elucidate the different roles of cholesterol and triglyceride in vascular dysfunction, we successfully segregated two lines of heritable hypercholesterolemic rabbit models with high (TGH) and low (TGL) levels of triglyceride (Takasaki et al., 1999). The aim of the present study was to use these animal models to clarify the role of hypertriglyceridemia in the alteration of vascular function. We examined differences in vasoconstrictor and relaxing function of thoracic aortic rings isolated from TGH and TGL rabbits.

Methods

Materials

The pharmacological agents used in the present study were acetylcholine chloride, calcium ionophore A23187, isoproterenol, sodium nitroprusside, angiotensin II, and phenylephrine hydrochloride. Krebs-

Henseleit solution contained (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_2$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1, and ascorbic acid 0.057. All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals

The experimental procedures conform to the guiding principles of the animal core and use in Yamagata University School of Medicine. Male WHHL rabbits, which were segregated into two lines of severely high triglyceride (TGH, n = 12) and moderately high TG level (TGL, n = 11), and Japanese White rabbits (JW, n = 19) aged 3–4 months were used in the present study. All animals were housed individually in a controlled environment with unlimited access to water and were fed standard rabbit chow (120 g/day, Labo R Grower, Nihon Nosan Kogyo, Ltd., Tokyo, Japan). The animals were anesthetized with an intravenous administration of 30 mg/kg sodium pentobarbital, and segments of the descending thoracic aorta were carefully removed and immediately immersed in ice-cold Krebs-Henseleit solution for isometric tension studies.

Isometric tension recording

After the connective tissue was carefully removed, the rings from the lower thoracic aorta (3 mm width) were mounted on two stainless steel triangular clips and placed into organ baths filled with 10 ml oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution maintained at 37 °C. The endothelium was gently removed in some rings by rubbing with a small wooden stick in order that the endothelium-independent relaxation and contractile responses in denuded rings could be studied. Isometric tension was monitored continuously using transducers (T7-15-240, Orientec Co, Tokyo, Japan) connected to a data acquisition system (RJ1000, San-ei, Tokyo, Japan). The rings were equilibrated under a resting tension of 2 g for 60–90 minutes, and the buffer was changed every 15 minutes (Tagawa et al., 1991, 1993). The rings were pre-contracted with phenylephrine (1 μM) and relaxed with acetylcholine (1 μM) to test endothelial integrity (Dam et al., 1997). After 60 minutes of equilibration, high potassium solution (66.7 mM) was added with intervals during which the resting tension was re-adjusted to 2 g (Hirata et al., 1992). High potassium solution was prepared by replacing NaCl with equimolar KCl. The cumulative contractile responses of the aortic ring to angiotensin II and phenylephrine were studied in the absence and presence of endothelium. Contraction was expressed as percentage of the maximal contraction by high potassium solution. To assess relaxation, cumulative concentration response curves were obtained with endothelium-dependent vasodilators, acetylcholine and calcium ionophore A23187, and endothelium-independent vasodilators, sodium nitroprusside and isoproterenol. Endothelium denuded rings were used to study endothelium-independent relaxation. Relaxation was expressed as a percentage of the pre-contractile tension induced by phenylephrine at levels of 30–50% of maximal contraction by high potassium solution (Kolodgie et al., 1990; Tagawa et al., 1991). EC_{50} was defined as the concentration of agonist at which 50% of the maximal response was obtained.

After completion of each experiment, some rings were washed with Krebs-Henseleit solution and removed from the apparatus, cut open, and were stained with Sudan III. Finally the stained strips were

photographed, and the total surface and atherosclerotic plaque areas were assessed quantitatively using NIH Image (version 1.55). Results were expressed as the percentage of the arterial surface covered by atherosclerotic lesions (Tagawa et al., 1993).

Microscopic analysis of atherosclerotic lesion

Descending thoracic aortas obtained from TGL and TGH (n = 5 in each) were fixed in 10% formaldehyde, and embedded in paraffin. Quantitative analysis of lesion composition was performed as previously described (Shiomi et al., 1994). Elastica-Goldner staining was performed to determine intimal thickening. Cell components of the plaque that were quantitatively evaluated included macrophages, smooth muscle cells, and collagen. Immunohistochemical staining was performed using RAM-11 (Dako A/S, Glastrup, Denmark) and monoclonal antibody against a-smooth muscle actin (Dako A/S, Glastrup, Denmark). Immunostaining was carried out with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions. The area of macrophages and smooth muscle were expressed as the percentage of the total intimal lesion area in each section reacting positively with diaminobenzidine (Shiomi et al., 1994). Collagen was defined as fibers staining cobalt blue with Azan-Mallory stain. The medial thickening, total cell number, and percentage of fractional fibrosis in the aortic media of vessel sections without atheroma were also determined (Fujii et al., 1999).

Lipid analysis

Blood samples were taken from a marginal ear artery 18 hr after the last feeding. All blood samples were centrifuged at 4 °C, and the plasma was stored at − 80 °C until assay. Plasma concentrations of total cholesterol, triglyceride and glucose were measured by enzymatic methods using a SPOTCHEM-EZ (ARKRAY Co, Kyoto, Japan).

Protein extraction and assay

Aortic samples were homogenized in a lysis buffer containing 25 mM Tris-HCl, 50 mM NaCl, 2% NP-40, 0.5% Na-deoxycholate, 0.2% SDS, 200 μM $NaVO_3$, 100 mM NaF and protease inhibitor mixture, and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatant was collected, and the protein concentration of each sample was measured using BCA protein assay reagents (Pierce Chemical Co., Rockford, IL, USA). For SDS–PAGE, equal amounts of protein were added on 8–12% polyacrylamide gel, electrophoretically separated, and transferred to PVDF membrane. After blocking with a buffer containing 5% non-fat milk and 0.2% Tween-20 for 1 hr at room temperature, the membrane was incubated with either anti-nitric oxide synthase (NOS)-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-angiotensin II type-1 receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibody. After washing three times with PBS, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Promega Co., Madison, WI, USA) for 1 hr at room temperature. The membrane was washed again and the signals were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ, USA).