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血小板GP Ib/IX/V受容体

村田　満

GP Ib/IX/V複合体は血小板のvon Willebrand因子受容体である．本受容体は速い流れの血液中で，血小板が内皮下組織に接触し活性化を起こす際に必須である．先天的に欠損する疾患はBernard-Soulier症候群と呼ばれる．止血だけでなく病的血栓と関連し，この分子を標的とした抗血小板薬が考案されている．

概念

糖蛋白(glycoprotein；GP) Ib/IX/Vは血小板膜上に存在し，血小板粘着反応に関係する主要な受容体蛋白の1つである．生理的止血のみならず病的血栓形成に深く関与しており，その機能解明は出血性疾患や血栓性疾患の病態理解や治療に多大な情報をもたらすと考えられる[1)2)]．近年は血栓症，とくに動脈血栓症にかかわる重要な分子として着目されており，抗血小板療法の標的分子の1つとなる可能性も示唆されている[3)]．本受容体の構造を図に示す[4)]．4つのサブユニット(GP Ibα，GP Ibβ，GP IX，GP V)からなる．

本受容体は血漿中や内皮下組織に存在するvon Willebrand因子(VWF)を認識する．とくに流速が速い血液中では，GP Ib/IX/V複合体は血小板の内皮下組織への接触と，その後の血小板活性化を引き起こすtethering moleculeとして必須であり，血小板血栓形成の初期段階を制御する分子として重要と考えられている．

関与する因子

GP Ib/IX/V複合体は，そのリガンドであるVWFとは流血中で常に接触しているが，自然には結合しないと考えられている．両者の結合を*in vitro*で誘発するには，**リストセチン**やボトロセチンといった非生理的な物質が必要である．一方，*in vivo*では狭窄血管などで生じるずり応力下での血小板活性化にGP Ib/IX/V複合体とVWFの結合が重要な役割を演じることが知られている．両分子がずり応力によりいかに変化し，結合が起こるかは十分解明されていないが，一旦VWFが血小板に結合すると，血小板は活性化され引き続いて血小板上のほかの受容体に変化が起こり，凝集が起こると考えられている．内皮下への粘着にはほかにコラーゲン受容体($\alpha_2\beta_1$インテグリンやGP VI)も重要であるが，コラーゲン受容体との決定的な違いは，VWF-GP Ib/IX/V複合体相互作用はとくに高ずり速度下での粘着に決定的な役割を果たす点である．*in vitro*のフローチャンバーで血小板を灌流すると，VWF-GP Ib/IX/V複合体が正常に機能する場合にはずり速度の上昇に従って粘着血小板数が増加するが，VWF-GP Ib/IX/V複合体反応をブロックすると逆に減少する．

GP Ib/IX/V複合体は血小板の高親和性トロンビン結合部位でもある．低濃度トロンビンに対する血小板反応に関与していると考えられているが，その生理学的意義はいまだ明らかではない．

疾患との関連

この複合体を先天的に欠損する疾患はBernard-Soulier症候群(BSS)と呼ばれる常染色体劣性遺伝形式の先天性血小板機能異常症である．これまでに数十種類の遺伝子異常が同定されており，GP Ibα，GP Ibβ，GP IXいずれの異常も報告されている．一方，この複合体の“gain-of-function

用語解説――リストセチン
血小板凝集惹起物質として使用される抗生物質で，VWFとGP Ib/IX/V複合体を結合させる．検査室でVWF活性やGP Ib/IX/V複合体の機能測定に用いられる．

用語解説――Bernard-Soulier症候群
巨大血小板，血小板減少，VWF依存性血小板機能の異常(出血時間延長，リストセチン凝集欠如，血小板粘着障害，ずり依存性血小板凝集欠如など)がみられる．

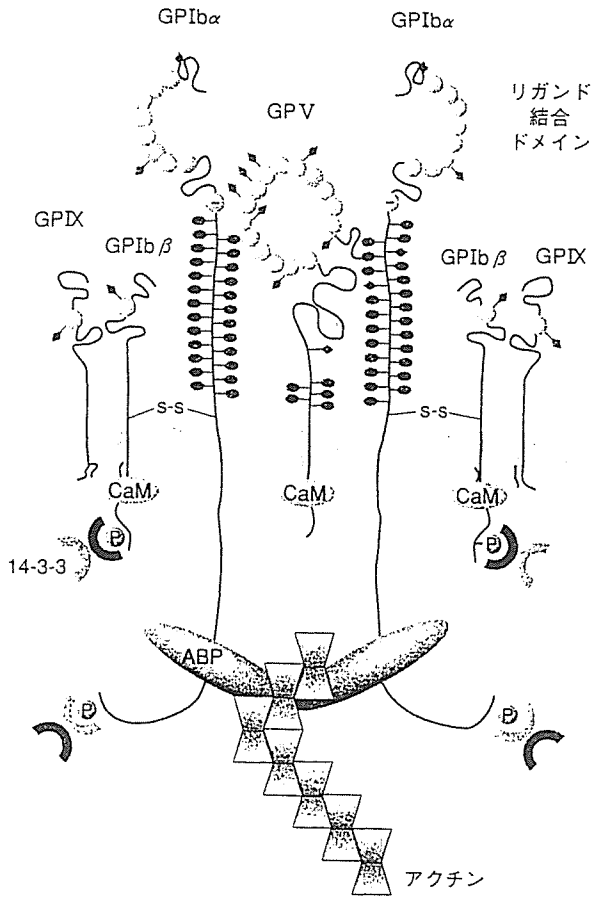

図　GP Ⅰb/Ⅸ/Ⅴ複合体の構造
(文献4より引用)

mutation" としては，VWF との自然結合がみられる血小板型 von Willebrand 病が知られている．GP Ⅰbα 鎖に少なくとも2カ所の変異がこれまでに同定されている．

動脈血栓の発症には血流，とくに血液に負荷されるずり応力の関与が大きい．GP Ⅰb/Ⅸ/Ⅴ複合体の機能は動脈硬化，動脈血栓症と関連すると考えられる[5]．

現在のトピックス

GP Ⅰb/Ⅸ/Ⅴ複合体は，速い血流のなかでいかに VWF を感知し血小板に一過性の接着を起こさせるか，GP Ⅰb/Ⅸ/Ⅴ複合体がどのように血小板内にシグナルを伝えるか，GP Ⅰb/Ⅸ/Ⅴ複合体は止血のみならず本当に病的血栓(動脈血栓)と関連するのか，するならば GP Ⅰb/Ⅸ/Ⅴ複合体を標的とした抗血小板薬は画期的な薬剤となりうるのか，などがホットな話題である．動物モデルでは VWF の拮抗物質(抗 VWF 抗体や，VWF と GP Ⅰb/Ⅸ/Ⅴの結合を抑制する薬物)を *in vivo* に投与すると末梢動脈や冠状動脈の血栓の発生が抑制されている．同様の効果は GP Ⅰb/Ⅸ/Ⅴ複合体をブロックした場合(抗体や可溶性 VWF フラグメント)にも認められている．血中 VWF の増加は不安定狭心症，心筋梗塞や血管障害のある糖尿病などにおいて認められ，血中 VWF 濃度の増加は狭心症患者における危険因子であると考えられている．これらは動脈血栓における VWF の意義を明確に示すものであり，また動脈血栓の予防薬の標的として GP Ⅰb/Ⅸ/Ⅴ複合体を選択するアプローチを正当化することになる．

今後の展望

GP Ⅰb/Ⅸ/Ⅴ複合体はこれまで生理的止血に必要と考えられ，出血性疾患との関連で研究されてきた分子であるが，最近は血栓症の原因や治療のターゲットとして注目されている．とくに GP Ⅰb/Ⅸ/Ⅴ複合体の遺伝的個体差(多型)が心筋梗塞や脳梗塞などの易罹病性と関連するとの報告がみられ，この受容体の血栓症における意義は大きいものとなっている．GP Ⅰb/Ⅸ/Ⅴ複合体を標的とすることで強力かつ出血の副作用の少ない抗血栓療法の開発が可能か，大変興味深いところである．また，巨核球や血小板系に特異的に発現する GP Ⅰb/Ⅸ/Ⅴ複合体が欠損する BSS ではなぜ巨大血小板が生成されるのか，そのメカニズムの解明は巨核球—血小板造血の機序解明の一助となるに違いない．

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関連事項

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血栓症と遺伝子多型

松原由美子

血栓症の発症や進展に関与する遺伝子多型が報告されている．現在，血栓症の日常診療に用いられている遺伝子多型はないが，今後，個々の遺伝子多型情報に基づいたテーラーメイド医療が期待されている．

検査の目的

個人の遺伝子情報に基づいて，血栓症のマネージメントを行う際の最適ターゲット因子を個別に把握することが本検査の目的である．

血栓症は静脈血栓症と動脈血栓症に大別される．これらの形成過程は異なるので，疾患に対する重要な因子も異なっている．さらに多因子性疾患である血栓症において，その発症，進展の過程に関与する因子は個人により多くの差があり，その個人差には遺伝的要因の関与があると考えられている．血栓に関連の強い因子，すなわち脂質代謝，血管調節，凝固線溶や血小板の遺伝子多型が，それら遺伝子産物の濃度や機能の個体差に影響を及ぼすことによる関与や，血栓症の薬物療法の際の薬剤の代謝や薬物受容体に関与する遺伝子多型も疾患のマネージメントに影響を及ぼす可能性は大きい．このような遺伝的多様性が血栓症に影響を与えていると考えられ，遺伝子多型，とりわけ解析が容易なSNP(single nucleotide polymorphism)解析による血栓症の遺伝子診断は注目されている．現在，先天性血栓傾向として臨床的に有用と考えられ，議論されている遺伝子多型は凝固第Ⅴ因子のLeiden変異のみである[1]．これは静脈血栓症のおもな原因であり，動脈血栓とも関係する．しかしこの変異は欧米では認められているが日本人には存在しない[2]．したがって，血栓症のマネージメントを目的とした遺伝子診断として，日本人を対象として日常診療に有用と考えられるものは存在せず，現在は遺伝子多型の臨床的意義を確立するために多くのデータの蓄積，解析が進行中である[3][4](表)．

検査法の実際

遺伝子多型には，SNPのほかにもマイクロサテライト，塩基欠損，塩基挿入，繰り返し配列の回数の違いがある．これら遺伝子多型について，血栓症のマネージメントを目的とした遺伝子診断の際，遺伝子型を決定するための検査のサンプルは白血球より抽出したgenomic DNA，あるいは全血である．サンプル量は解析方法やその候補因子数により異なるが，全血10 μL～5 mLで必要量を満たす．解析方法はサンプルをPCRにて検査目的部位を含む遺伝子を増幅→直接塩基配列を解読するシークエンス法，特定の塩基配列を認識して切断する制限酵素を用いてその切断の有無による方法，特定の塩基配列に蛍光色素を取り込ませる方法など数多いが，最近の研究では遺伝子多型の解析にマイクロアレイ技術を用いる試みが行われており注目されている．この方法は，候補遺伝子多型の何種類かをスポットしたアレイを用いて一度に候補遺伝子多型の遺伝子型を決定することができるため，将来の日常診療への応用使用に期待されている．

診断的意義

血栓症のマネージメントのための遺伝子診断は，個々の遺伝的素因を考慮した疾患の予防や治療法選択を行うテーラーメイド医療に貢献できる．この遺伝子診断は短時間で検査可能であり，検査結果

用語解説――SNP(single nucleotide polymorphism)
(一塩基変異多型)の各頭文字で集団の1%以上にみられる一塩基の置換．

用語解説――Leiden変異
深部静脈血栓症の主要原因である活性化プロテインC(凝固ⅤやⅧ因子の不活性化による抗凝固能を有する)レジスタンスの原因遺伝子．

表　血栓症に関連するおもな遺伝子多型

因　子	遺伝子多型	血栓性疾患との関連
アポリポ蛋白 (a)	クリングル 4 の反復数 ; a, b, c, d	冠動脈疾患，虚血性脳血管障害
アポリポ蛋白 E	エクソン 4; E2/E3/E4	動脈硬化病変発生率，冠動脈疾患，虚血性脳血管障害
CETP	イントロン 1; B1/B2	冠動脈硬化進展，心筋梗塞
パラオキソネース	Gln192Arg	冠動脈疾患
ACE	insertion/deletion; イントロン 16	冠動脈疾患，高血圧を伴う脳血管障害，ラクナ梗塞
アンジオテンシンⅡタイプⅠ受容体	1166A/C	心筋梗塞
ecNOS	4-/5- リピート ; イントロン 4	冠動脈疾患，ラクナ梗塞
	Glu298Asp	冠動脈疾患，ラクナ梗塞
プロトロンビン	20210G/A	深部静脈血栓症，冠動脈疾患
凝固第Ⅴ因子	Arg506Gln	深部静脈血栓症
凝固第Ⅶ因子	Arg353Gln	心筋梗塞
	H5, H6, H7; hypervariable region 4 (イントロン 7)	心筋梗塞
凝固第 XII因子	46C/T	心筋梗塞
凝固第 XIII因子	Val34Leu	心筋梗塞，虚血性脳血管障害
フィブリノーゲン		心筋梗塞，虚血性脳血管障害
PAI-1	プロモーター ; 4G/5G	心筋梗塞
トロンボモジュリン	Ala25Thr，Ala455Val	心筋梗塞
血小板膜 GPIbα	Thr145Met	冠動脈疾患，虚血性脳血管障害
	399-411 の 1，2，3，4 リピート	冠動脈疾患
血小板膜 GPIα	807T/C	心筋梗塞，虚血性脳血管障害
血小板膜 GPⅢα	Leu33Pro	冠動脈疾患，虚血性脳血管障害

CETP : cholesteryl ester transfer protein, ecNOS : endothelial constitutive nitric oxide synthase,
ACE : angiotensin converting enzyme, PAI-1 : plasminogen activator inhibitor-1 , GP : glycoprotein.

に日内変動や検査日による違いはない．さらにサンプルに用いる DNA は比較的安定して保存できる．すなわち，安定な検査結果に基づいての遺伝子診断による疾患へのアプローチが期待できる．さらに，疾患の細分類化や予防医学への多大な貢献が予測される．

その他注意すべき点

遺伝子多型には民族差が報告されている．民族により異なる特徴をもつように，遺伝子多型も異なるものがある．現在，先天性血栓傾向として臨床的に確立されている凝固第Ⅴ因子の Leiden 変異は，欧米では認められているが日本人には存在しない(「検査の目的」参照)．また，プロトロンビンや GPⅢa の多型も日本人ではほとんど存在しない．一方，GP Ⅰbα の 4 リピートは欧米人ではきわめてまれに存在するが，日本人では 5.7 %(コントロール群)，17.7 %(冠状動脈疾患患者群)に認められている[5]．したがって，今後，候補遺伝子の選択において民族を考慮することは非常に重要である．

遺伝子診断の施行において最も注意すべき点は，倫理面の管理である．その法的関与を含む管理システムの整備が重要である．

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関連事項

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Coronary artery disease and a functional polymorphism of hTERT

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Abstract

Genetic variation, a $^{-1327}$T/C polymorphism, of human telomerase reverse transcriptase (hTERT) is associated with leukocyte telomere length in healthy subjects, but clinical significances of this functional polymorphism are not clear. Recently, the relationship between the telomere system and coronary artery disease (CAD) was reported. We investigated the association between the $^{-1327}$T/C polymorphism and (a) susceptibility to CAD and (b) telomere length in CAD patients. In a case-control study, 104 patients confirmed by coronary angiography and 115 age- and sex-matched controls were enrolled. There was a higher frequency of the $^{-1327}$C/C genotype in CAD patients (51.9%) compared with controls (36.5%, $p = 0.0218$). Among the 104 CAD patients, leukocyte telomere length in the $^{-1327}$C/C genotype (7.62 ± 2.19 kb, mean ± SD) was shorter than that in the $^{-1327}$T/C and $^{-1327}$T/T genotypes (8.74 ± 2.92, $p = 0.0287$). These findings suggest that the $^{-1327}$C/C genotype is a genetic risk factor for CAD and relates to shorter telomere length among CAD patients.

Keywords: Human telomerase reverse transcriptase; Polymorphism; Coronary artery disease

Telomeres at the ends of mammalian chromosomes have a critical role in chromosome stability, and telomerase is a ribonucleoprotein enzyme that elongates chromosomes by adding telomere repeat sequences to the telomere ends [1,2]. Telomerase activity, which stabilizes telomeres, is sometimes incomplete, and telomere length decreases with cell divisions. Thus, telomere shortening occurs after repeated cell divisions and has an essential role in cellular senescence [1–3]. Telomere length is regulated by telomerase activity, and the expression level of human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is a major determinant of telomerase activity [4–6]. hTERT might have a key role in regulation for telomere length.

Coronary artery disease (CAD) is related to significant morbidity and mortality in developed countries, particularly in older populations, despite significant advances in its management. Therefore, new approaches for the prevention and treatment of CAD have been highlighted [7]. A large number of studies indicate that cellular senescence is associated with the occurrence and development of CAD [8]. There is accumulating evidence of a relationship between the telomere system and CAD [9–14], although the molecular mechanism by which telomere shortening causes vascular dysfunction is not fully understood. In *ex vivo* studies, Ogami et al. demonstrated that the telomere length of coronary endothelial cells was shorter in patients with CAD than in age-matched patients without CAD [9]. Sanami et al. and Brouilette et al. reported that leukocyte

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telomere length in CAD patients is shorter than that in healthy subjects, and there is a relationship between patients with a strong family history of CAD and shorter telomere length [10,11]. In *in vitro* studies, senescence-associated telomere phenotypes were observed in the atherosclerotic regions of coronary artery endothelial cells from autopsied individuals with CAD, and functional alterations in cultured senescent endothelial cells were reversed by the introduction of hTERT [12]. Also, increased hTERT expression is closely related to decreased cellular senescence in normal vascular endothelial cells [13,14]. These reports suggest that shorter telomere length is associated with the higher prevalence of CAD and that hTERT has a critical role in the protection against endothelial dysfunction.

We recently reported that an hTERT $^{-1327}$T/C polymorphism within the promoter region, a T to C transition 1327 bp upstream of the transcription-starting site (nucleotide numbering according to Horikawa et al. [15]), is associated with hTERT promoter activity: the $^{-1327}$C sequence had lower activity in human umbilical vein endothelial cells [16]. We also reported that the $^{-1327}$C/C genotype had lower telomerase activity and a shorter telomere length in leukocytes from healthy individuals [16]. The clinical significance of this functional polymorphism, however, remains unclear. In this study, the hypothesis that the ^{1327}T/C polymorphism represents a potential risk factor for CAD was tested, and we examined the association between the $^{-1327}$T/C polymorphism and (a) the prevalence and severity of CAD and (b) telomere length among CAD patients.

Methods

Genetic analysis. The present study was approved by the Ethics Committee of the School of Medicine, Keio University, and written informed consent was obtained from all subjects that entered the study. The study subjects were genetically unrelated Japanese subjects. Genotyping of the $^{-1327}$T/C polymorphism was performed using Mega-base 1000 (General Electric, Fairfield, CT), according to the manufacturer's protocol for the single nucleotide primer extension-based method.

Study populations for case-control study (CAD patients vs. controls). We studied patients ($n = 104$) who recruited at Keio University Hospital with a diagnosis of myocardial infarction or angina pectoris. Only patients whose coronary lesions were confirmed by coronary angiography (identified stenosis >75%) were eligible for the study. Affected vessel number, classifying the number of vessels as one, two, or three, was used to determine CAD severity. To match patients in age at diagnosis of CAD, healthy control subjects ($n = 115$) were selected from personnel working at Keio University Hospital who visited for their regular checkups. They had no clinical or laboratory evidence of past vascular disorders. Only male study subjects were enrolled into this case-control study. Hyperlipidemia was defined as a total cholesterol level greater than 220 mg/dl or a triglyceride level greater than 150 mg/dl at presentation, or under treatment. Hypertension was defined as systolic blood pressure greater than 140 mm Hg and diastolic blood pressure greater than 90 mm Hg at presentation, or under treatment. Diabetes mellitus was defined by the World Health Organization criteria. These data were collected from patient's medical records and regular checkup records of control subjects.

Assay for telomere length in CAD patients. We examined the association between the $^{-1327}$T/C polymorphism and telomere length in CAD patients ($n = 104$) from the case-control study. To measure telomere length of leukocyte DNA, as assessed by mean length of terminal restriction fragments (TRF), we used Southern hybridization of telomeric DNA [17] and real time kinetics quantitative polymerase chain reaction [18]. Previous report showed that the results of these two methods were correlated [18]. After confirming the correlation between these two methods for measuring telomeres of our samples, we calculated telomere length.

Statistics. Mean values of the two groups in this study were compared by Student's t test. A χ^2 test was performed to compare two proportions in the case-control study. Multiple logistic regression analysis was performed to evaluate the relationship between subjects with and without CAD (categorical variable, yes or no) and other variables. Independent variables included in the analysis were $^{-1327}$T/C genotype (categorical variable), body mass index (quantitative value), diabetes mellitus (categorical variable, yes or no), hyperlipidemia (categorical variable, yes or no), hypertension (categorical variable, yes or no), and smoking (categorical variable, yes or no). The Cochran–Armitage trend test was used to examine the association between the $^{-1327}$T/C polymorphism and CAD severity. Statistical analyses other than the Cochran–Armitage trend test were performed using StatView (ver 5.0, for Macintosh, SAS, Cary, NC). A p value less than 0.05 was considered to be statistically significant.

Results

In the case-control study, we investigated the association between the $^{-1327}$T/C polymorphism and the presence and severity of CAD. Table 1 shows the number of study subjects and their characteristics. There were no significant differences in mean age or prevalence of hypertension. The CAD patient group had a higher body mass index and a higher prevalence of diabetes mellitus, hyperlipidemia, and smoking. The frequency of the $^{-1327}$C/C genotype in CAD patients (51.9%) was significantly higher than that in controls (36.5%, $p = 0.0218$), and there was a dose-effect of the $^{-1327}$C-allele on the prevalence of CAD: the odds ratios in a comparison of the $^{-1327}$TT genotype (9.6% for CAD patients, 17.4% for controls) were 1.51 (95% CI, 0.64–3.58, $p = 0.3481$) for the $^{-1327}$T/C genotype (38.5% for CAD patients, 46.1% for controls) and 2.57 (95% CI, 1.09–6.07, $p = 0.0284$) for the $^{-1327}$C/C genotype (51.9% for CAD patients, 36.5% for controls, shown in Table 2).

Association of the $^{-1327}$T/C polymorphism with the prevalence of CAD, adjusted for other risk factors (body mass index, smoking, hypertension, hyperlipidemia, and diabetes mellitus), was analyzed by a multiple logistic regression model (Table 3), and an adjusted odds ratio of 1.83 (95% CI, 1.00–3.34, $p = 0.0487$) for the relation between CAD and the $^{-1327}$C/C genotype. This analysis

Table 1
Characteristics of CAD patients and controls

	CAD patients ($n = 104$)	Controls ($n = 115$)	p value
Age (y, mean ± SD)	57.4 ± 4.5	58.2 ± 2.9	0.1170
BMI[a] (kg/m^2, mean ± SD)	24.1 ± 2.6	23.0 ± 2.7	0.0025
Diabetes mellitus (%)	31.1	5.2	< 0.0001
Hyperlipidemia (%)	66.3	40.4	< 0.0001
Hypertension (%)	51.5	44.3	0.2941
Smoking (%)	58.8	44.7	0.0386

[a] Body mass index.

Table 2
Genotype frequency of the $^{-1327}$TC polymorphism

	CAD patients ($n = 104$) n (%)	Controls ($n = 115$) n (%)	p value
$^{-1327}$TT and $^{-1327}$TC genotypes	50 (48.1)	73 (63.5)	0.0218
$^{-1327}$CC genotype	54 (51.9)	42 (36.5)	

Table 3
Logistic regression analysis for risk factors of CAD

Independent variables	p value	Odds ratio (95% CI)
hTERT genotype	0.0487	1.83 (1.00–3.34)
BMI[a]	0.0911	1.11 (0.98–1.25)
Smoking	0.1263	1.60 (0.88–2.91)
Hypertension	0.1081	1.69 (0.89–3.18)
Hyperlipidemia	0.0056	2.36 (1.29–4.33)
Diabetes mellitus	0.0247	2.30 (1.11–4.74)

[a] Body mass index.

indicated that the $^{-1327}$C/C genotype is independently associated with a higher prevalence of CAD.

To examine association between the $^{-1327}$T/C polymorphism and severity of CAD, classified by affected vessel number, we analyzed the genotype distribution of this polymorphism in patients with one-, two-, and three-vessel disease. The greater the affected vessel number in CAD, the greater the frequency of patients with the $^{-1327}$C/C genotype (45.8%, 50.0%, and 68.2% among one-, two-, and three-vessel disease populations, respectively). These differences were marginally significant in CAD patients ($p = 0.0688$).

Next, we measured leukocyte TRF length to examine the association between the $^{-1327}$T/C polymorphism and telomere length, which is closely related to the final stages of the telomere system, in the 104 CAD patients from the case-control study. The TRF length in the $^{-1327}$C/C genotype was significantly shorter than that in the $^{-1327}$T/C and $^{-1327}$T/T genotypes: 7.62 ± 2.19 (kb, mean $\pm$ SD) for the $^{-1327}$C/C genotype and 8.74 ± 2.92 for the $^{-1327}$T/C and $^{-1327}$T/T genotypes ($p = 0.0287$; Fig. 1). Mean age was not significantly different in each group: 57.74 ± 4.89 (year, mean $\pm$ SD) for the $^{-1327}$C/C genotype and 57.08 ± 7.74 for the $^{-1327}$T/C and $^{-1327}$T/T genotypes ($p = 0.4619$). These findings suggest that the $^{-1327}$T/C polymorphism is associated with leukocyte telomere length among CAD patients.

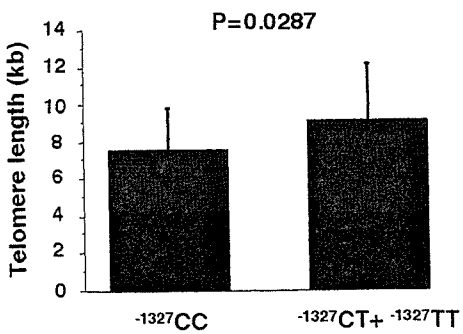

Fig. 1. Relationship between telomere length and the $^{-1327}$T/C polymorphism in CAD patients. Bars show mean TRF length (i.e., telomere length) (mean $\pm$ SD) between two groups of the $^{-1327}$T/C genotypes.

Discussion

In the present study, we report for the first time that the $^{-1327}$T/C polymorphism of hTERT is associated with susceptibility to CAD and telomere length among CAD patients; patients with the $^{-1327}$CC genotype have a higher prevalence of CAD and shorter telomere length.

For the $^{-1327}$T/C polymorphism, a $^{-1327}$T to $^{-1327}$C transition is reported (rs 2735940) in the Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/index.html). There are, however, no reports on the genotype distribution or functional data for this substitution. We recently demonstrated that this substitution is a polymorphism by gene screening within the promoter region of hTERT and analysis of genotype distribution in healthy Japanese subjects [16]. We also reported a functional effect of the $^{-1327}$T/C polymorphism on the telomere system in normal cells: the $^{-1327}$T sequence has higher hTERT promoter activity in cultured human umbilical vein endothelial cells, and healthy individuals with the $^{-1327}$T/T genotype have higher telomerase activity and longer telomere length in leukocytes. Subjects with the $^{-1327}$T/C or $^{-1327}$T/T genotypes, but not the $^{-1327}$C/C genotype, have decreased age-related telomere shortening [16], although the age-related decrease in leukocyte telomere length is approximately 30 to 40 bp/year in healthy subjects [19–21]. Thus, we postulated that the $^{-1327}$T-sequence with higher hTERT transcriptional activity is associated with more effective extension of the telomeric end during cell division.

Recently, experimental studies suggested a protective effect of hTERT on vascular cells [12–14], and clinical studies demonstrated that telomere length in leukocytes and coronary endothelial cells among CAD patients was shorter than that among age-matched controls [9–11]. Telomere length in both leukocytes and endothelial cells is inversely correlated with age [19–22], and endothelial cells and leukocytes are exposed to the same hemodynamic stress; thus, their turnover rate is considered to be related [22].

Together, these findings prompted us to test the association between the functional $^{-1327}$T/C polymorphism of hTERT and CAD. This study focused on the effects of this polymorphism on the susceptibility to CAD and leukocyte telomere length among CAD patients, and we performed a case-control study and an experimental study, measurement of telomere length. Results of the case-control study suggest that the $^{-1327}$C/C genotype is a genetic risk factor for CAD. Oxidative stress or homocysteine induces telomerase inactivation or telomere shortening *in vitro* [23–25], which causes vascular dysfunction, although the details of the molecular mechanisms are not known. When vascular cells are exposed to oxidative stress or homocysteine, cells with the $^{-1327}$C-sequence are likely to have lower

replicative capacity, which enhances vascular dysfunction. For the association between the $^{-1327}$T/C polymorphism and telomere length, we found that telomere length in the $^{-1327}$C/C genotype was shorter than that in the $^{-1327}$T/C and $^{-1327}$T/T genotypes, and this association was found in both healthy controls in our previous study [16] and CAD patients in the present study. The difference in the telomere length between the $^{-1327}$C/C and $^{-1327}$T/C + $^{-1327}$T/T genotypes was 670 bp for the controls [16] and 1120 bp for the patients. Although these data from two populations with different mean age were not directly compared, it is likely that the $^{-1327}$T/C polymorphism has more impact on telomere shortening under pathogenic conditions and CAD progression, as compared with non-pathologic conditions.

We measured leukocyte telomere length, but not that of coronary endothelial cells. It is possible that leukocyte telomere length reflects systemic condition. On the contrary, coronary atherosclerosis is a local disorder, and telomere shortening during development of CAD occurs in coronary endothelial cells. Further studies need to analyze the telomere length of coronary endothelial cells in coronary atherosclerosis.

In conclusion, the results suggest that the $^{-1327}$T/C polymorphism is strongly associated with the prevalence of CAD and telomere length among CAD patients. The present results contribute to provide a novel target for the management of vascular diseases.

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Identification of ADRA2A polymorphisms related to shear-mediated platelet function

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Abstract

α2A adrenergic receptor (ADRA2A) on platelets interacts with epinephrine, which has a key role in regulating platelet functions. There is familial clustering of inter-individual variations in the epinephrine-induced platelet aggregation, the molecular basis of which, however, has not been fully understood. In this study, we screened the sequence variations in the transcriptional region of ADRA2A gene and analyzed the relationship between the two common polymorphisms and platelet function using epinephrine/collagen cartridge in the platelet function analyzer-100 system®, in a healthy Japanese male population ($n = 211$). Among the identified 16 sequence variations including five novel variations, 1780GG genotype was associated with longer closure time which represents low platelet function under high shear-stress conditions ($p = 0.0478$). We also observed enhanced effect of the combination of 1780GG and 2372AA genotypes on longer closure time ($p = 0.0319$). These findings suggest that 1780A/G and 2372A/G polymorphisms are associated with platelet function in interactions with collagen/epinephrine.

Keywords: α2A adrenergic receptor; Polymorphism; Platelet

The α2A adrenergic receptor (ADRA2A), a member of the G protein-coupled receptor superfamily, is a membrane receptor for epinephrine [1]. ADRA2A is expressed on presynaptic neurons, blood vessels, adipocytes, kidney, pancreas, and platelets [2]. This receptor has a key role in regulating neurotransmitter release, blood pressure, lipolysis, insulin secretion, and platelet aggregation [3–6].

Interactions between platelets and epinephrine cause platelet activation and aggregation via the Gi-coupled signaling pathway, and this epinephrine-induced signaling pathway is mainly mediated by ADRA2A on platelets [7–9]. The ADRA2A/epinephrine interaction is an essential step in physiologic hemostasis and thrombus formation [10]. The ADRA2A/epinephrine-induced Gi-dependent pathway was reportedly involved in ERK activation by collagen-stimulation [11]. *In vitro*, epinephrine acts as an agonist for platelet activation and subsequent aggregation. The measurement of epinephrine-induced platelet aggregation is widely used to evaluate platelet function in basic research and clinical laboratories [12]. Previous studies demonstrated inter-individual variations in the levels of ADRA2A expression on platelets and epinephrine-induced

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platelet aggregation, with familial clustering [13,14]. These reports suggest that a genetic factor would be associated with epinephrine-induced platelet function mediated by ADRA2A.

The gene encoding ADRA2A contains no introns. For genetic polymorphisms in the ADRA2A gene, an Entrez SNP database search (www.ncbi.nlm.nih.gov/SNP/) and previous reports showed 25 nucleotide substitutions, including the MspI restriction-fragment length polymorphism (RFLP), the HhaI RFLP, and the DraI RFLP [15–18]. These polymorphisms were reportedly related to athletic endurance, subcutaneous fat patterns, glucose metabolism, lipid metabolism, hypertension, baroreceptor sensitivity, salt excretion, and childhood attention deficit hyperactivity disorder [16,19–24]. One report demonstrated an association between the DraI polymorphism and epinephrine-induced platelet aggregation [24]. Because there are a number of ADRA2A polymorphisms, other polymorphisms of the gene might have functional and/or clinical significance. In this study, to elucidate the relationship between polymorphism(s) of the ADRA2A gene and individual variability in epinephrine-mediated platelet function, we performed sequence screening of the ADRA2A gene in healthy individuals to identify single nucleotide polymorphisms (SNPs), and then the relationship between the polymorphisms and epinephrine-mediated platelet function was examined using the platelet function analyzer (PFA)-100® system.

Methods

Study subjects. Genetically unrelated Japanese males ($n = 255$) were recruited at their regular checkups, and the study subjects were enrolled between August 2003 and March 2005. Of the 255 subjects, an initial screening for the sequence variation(s) in the ADRA2A transcriptional region was performed on 44 subjects enrolled in 2003. Subsequently, relationship between ADRA2A polymorphisms and platelet function was investigated in 211 subjects who were enrolled between 2004 and 2005. The subjects had no apparent hematologic or vascular disease and were not taking any medications that affect platelet function. Written informed consent was obtained from all subjects enrolled in the study. Our protocol was approved by the Ethics Committee of the School of Medicine, Keio University.

Genetic analyses. Genomic DNA was isolated from peripheral blood leukocytes, as described previously [25]. To screen the sequence variations in the ADRA2A transcriptional domain, a direct sequencing analysis for the 44 DNA samples was performed with a DYEnamic ET dye terminator kit (Amersham Biosciences, Piscataway, NJ) using the MegaBACE1000 model (Amersham Biosciences) according to the manufacturer's protocol. The sequence of the ADRA2A gene sequence from nucleotide No. 31585521 to nucleotide No. 31589121 was analyzed according to GenBank Accession No. NT_030059. After screening the sequence variations, genotyping of the target polymorphisms was performed on 211 DNA samples, using the MegaBACE1000 (Amersham Biosciences) according to the manufacturer's protocol for the single nucleotide primer extension-based method.

Assay for platelet function. Whole blood samples were drawn into evacuated tubes containing 3.8% (0.129 M) buffered sodium citrate solution. Platelet function was measured with the PFA-100® (Dade-Behring, Liederbach, Germany). This is a relatively new device for assessing platelet function *in vitro* under high shear flow conditions (5000–6000/s) with citrated whole blood [26]. Blood samples were aspirated through a capillary in the instrument collagen-epinephrine (CEPI) cartridge. The membrane of the CEPI cartridge was coated with collagen fibrils and epinephrine. The membrane triggered platelet adhesion, activation, and aggregate formation, induced by collagen and epinephrine, leading to the occlusion of the 150 μm central aperture and blood flow cessation. This time interval was defined as the closure time (CT), and a longer CT indicates lower platelet function. In the present study, CT was measured with a CEPI cartridge, and the device and cartridges were handled in accordance with the manufacturer's instructions.

Measurements for von Willebrand factor levels. After centrifugation of blood samples at 2000*g* for 10 min, plasma samples were stored at −80 °C until assayed. The von Willebrand factor (VWF): ristocetin-cofactor activity (RCo) levels were assessed by ristocetin-induced platelet aggregation (Dade-Behring, Liederbach, Germany) [27].

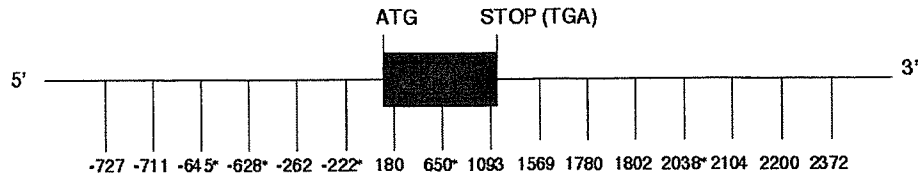
Statistical analysis. The χ^2 test was used to evaluate the relationship between the ADRA2A genotype and CEPI-CT. A multiple logistic regression analysis was performed to evaluate the relationship between CEPI-CT and other variables, and the independent variables were VWF:RCo levels, platelet count, hematocrit value, and ADRA2A genotype. Mean values of the two groups in this study were compared by Student's *t* test. Statistical analysis was performed using StatView (ver 5.0, for Macintosh, SAS Institute Inc., Cary, NC). Odds ratio (OR) and 95% confidence intervals (CI) were used to estimate the strength of the association between independent variables and CEPI-CT. A p value of less than 0.05 was considered to be statistically significant.

Results and discussion

Identification of ADRA2A polymorphisms

We analyzed the ADRA2A gene sequence (from the transcription initiation site at nucleotide No. 31585521 to the termination site at nucleotide No. 31589121, according to GenBank Accession No. NT_030059), and 16 single nucleotide substitutions were detected (Fig. 1a and b). In this report, the first nucleotide of the open reading frame is nucleotide No. 1, which corresponds to nucleotide No. 31586326 of the reference sequence, with the 5′ untranslated region beginning at nucleotide No. −1 and continuing in the negative direction. Of the 16 substitutions, 5 were novel, and 11 were previously reported [15–17] and entered in the NCBI SNP database. Among the 44 samples, none of the subjects had the homozygote genotype for the reference sequence in the −727G/C and −711T/C polymorphisms, and strong linkage between the 1093C/A and 2372A/G polymorphisms was observed. The 650T/A polymorphism would result in a Val to Glu amino-acid change at residue 217. The reported HhaI and DraI polymorphisms were identical with the −262G/A and 1780A/G polymorphisms, respectively. Previously, Small et al. [17] reported the allele-frequencies of the −727G/C and 1780A/G polymorphisms in an Asian population, and those frequencies were markedly different from our results in Japanese population. Although the race details were not described in the previous report, it is possible that there are ethnic differences in the genotype distribution of ADRA2A polymorphisms among Asian subjects. The genotype distributions of the 1093C/A, 1569C/A, 1802C/A, and 2372A/G polymorphisms in this study were compatible with previous reports in Japanese population [16] and in Asian population [17]. For the ADRA2A haplotype, we observed differences in the

a



b

position	reference	substitution	genotype			frequency(%) (n=44)		
-727	G	C	GG	GC	CC	0	4.5	95.5
-711	T	C	TT	TC	CC	0	0	100
-645	G	T	GG	GT	TT	90.9	9.1	0
-628	C	A	CC	CA	AA	97.7	2.3	0
-262	G	A	GG	GA	AA	59.1	36.4	4.5
-222	G	C	GG	GC	CC	97.7	2.3	0
180	G	C	GG	GC	CC	95.5	4.5	0
650	T	A	TT	TA	AA	97.7	2.3	0
1093	C	A	CC	CA	AA	47.7	38.6	13.7
1569	C	A	CC	CA	AA	62.8	32.5	4.7
1780	A	G	AA	AG	GG	18.2	40.9	40.9
1802*	C	A	CC	CA	AA	61.4	34.1	4.5
2038	C	T	CC	CT	TT	97.7	2.3	0
2104	A	G	AA	AG	GG	97.7	2.3	0
2200	G	A	GG	GA	AA	90.9	9.1	0
2372	A	G	AA	AG	GG	45.5	40.9	13.6

Fig. 1. Screening of the sequence variations within transcriptional region of human ADRA2A gene. (a) Gene structure and positions of nucleotide substitutions are shown. The first nucleotide of the open reading frame was numbered as nucleotide No. 1, which corresponds to nucleotide No. 31586325 of the reference sequence NT_030059. *Novel single nucleotide substitutions found in the present study. (b) Genotype distribution of the ADRA2A single nucleotide substitutions is shown. *Only 43 samples were analyzed for this position.

frequency and haplotype sequence between the present study on Japanese and previously reported Asian populations [17], although the sample number in both studies was small. Notably, the GGCCACA haplotype for the −727G/C, −262G/A, 1093C/A, 1569C/A, 1780A/G, 1802C/A, and 2372A/G polymorphisms had different frequencies between these two populations: 1.2% for the present data and 22.5% for the Asian population [17].

We first examined the polymorphic status of the 650T/A. Because it would result in a Val to Glu amino-acid change at residue 217, a functional change was speculated in the protein. In the analyses of the sequence variations in the 44 samples, only one sample had this substitution. In a subsequent analysis of another 211 samples (for study on platelet function as described below), however, we did not find this variant, reinforcing the suspicion that this is rare mutation.

Association between the CEPI-CT and ADRA2A polymorphisms

We next investigated the effect of ADRA2A polymorphisms on platelet function among 211 healthy controls. The study subjects were genetically unrelated Japanese males, whose mean age was 46.6 ± 5.1 years. Platelet function was assessed using the PFA-100® system with CEPI, and a longer CEPI-CT indicates lower platelet function in the interaction with collagen and epinephrine. Because the manufacturer's instructions for the PFA-100® report a mean CEPI-CT value of 132 s, the study subjects were divided in two groups: a higher function group with a CEPI-CT < 132 s ($n = 90$) and a lower function group with a CEPI-CT ⩾ 132 s ($n = 121$). In this study, we focused on two polymorphisms, the 1780A/G and 2372A/G polymorphisms, because a pilot study indicated that among the 16 substitutions these SNPs would most likely affect the CEPI-CT (data not shown). Also, there was a strong link between the 1780A/G and 2372A/G polymorphisms (Table 1). Therefore, we examined the relationship between CEPI-CT and (a) the 1780A/G polymorphism, (b) the 2372A/G polymorphism, and (c) the combination of the 1780A/G and 2372A/G polymorphisms. The frequency of the 1780GG genotype in the lower function group was

Table 1
The ADRA2A haplotypes of the 1780A/G and 2372A/G polymorphisms

	1780AA ($n = 34$)	1780AG ($n = 100$)	1780GG ($n = 77$)
2372AA ($n = 107$)	2	34	71
2372AG ($n = 87$)	16	65	6
2372GG ($n = 17$)	16	1	0

Table 2
Association between the ADRA2A polymorphisms and PFA-100® closure time (CT) using the collagen/epinephrine cartridge

	High platelet function group (CT < 132 s)	Low platelet function group (CT ⩾ 132 s)	p value
1780GG, *n* (%)	26 (28.9)	51 (42.1)	0.0478
1780GA + AA, *n* (%)	64 (71.1)	70 (57.9)	
2372AA, *n* (%)	40 (44.4)	67 (55.4)	0.1164
2372AG + GG, *n* (%)	50 (55.6)	54 (44.6)	
Combination			
1780GG + 2372AA, *n* (%)	23 (25.6)	48 (39.7)	0.0319
Others, *n* (%)	67 (74.4)	73 (60.3)	

significantly higher than that in the higher function group ($p = 0.0478$; Table 2). The frequency of the 2372AA genotype was not significantly different between the higher and lower function groups ($p = 0.1164$). Analysis of the association between CEPI-CT and the genotypic combination of the 1780A/G and 2372A/G polymorphisms indicated a significantly higher frequency of the 1780GG + 2372AA genotypes in the lower function group than in the higher function group ($p = 0.0319$). The CEPI-CT showed an enhanced effect of the genotypic combination of the 1780A/G and 2372A/G polymorphisms as compared with the 1780A/G alone: the odds ratios (95% CI) comparing the 1780GG genotype and the 1780GG + 2372AA genotypes were 1.79 (1.00–3.23) and 1.92 (1.05–3.45), respectively. These findings suggest that the 1780GG genotype and the combination of the 1780GG + 2372AA genotypes are associated with lower platelet function in the interaction with collagen and epinephrine.

The association of the genotypic combination of the 1780A/G and 2372A/G polymorphisms with CEPI-CT was analyzed to adjust for other confounding factors (VWF: RCo, platelet count, and hematocrit) by a multiple logistic regression model because the manufacturer's instructions for the PFA-100® show the possibility that these factors affect the CT. An adjusted odds ratio was 2.10 (95% CI, 1.01–4.06, $p = 0.0274$) for the relation between CEPI-CT and the combination of 1780A/G and 2372A/G polymorphisms (Table 3), suggesting that the 1780GG + 2372AA genotypes are an independent predictor for longer CEPI-CT value. Plasma VWF:RCo level was also an independent predictor for CEPI-CT. Also, analysis of the association between the 1780A/G alone and CEPI-CT in this multiple logistic model showed an adjusted odds ratio of 1.82 (95% CI, 0.94–3.52, $p = 0.0757$). Observation suggests the enhanced effect of the genotypic combination of the 1780A/G and 2372A/G polymorphisms as compared with the 1780A/G alone in the multiple logistic models.

A report by Freeman et al. demonstrated a relationship between epinephrine-induced platelet aggregation and the DraI polymorphism (6.3/6.7 genotypes according to DraI±) [24]. The present 1780A/G polymorphism would correspond to the DraI polymorphism. The 1780G-sequence is not digested by the DraI, thus genotype with this sequence would correspond the DraI 6.7 genotype [24] associated with lower function in epinephrine-induced platelet aggregation. Thus, our findings confirm previous data. We showed additional evidence that the effect of the ADRA2A haplotype on platelet function is stronger, and these data point out the importance of haplotype analysis, but not single specific polymorphisms, to examine the association between platelet function and ADRA2A polymorphisms.

The present study reports for the first time the relationship between the 1780A/G and 2372A/G polymorphisms and platelet function assessed by the PFA-100® system using collagen–epinephrine cartridge. Because the signaling pathway induced by ADRA2A/epinephrine interactions is involved in the collagen-induced ERK activation for regulation of platelet function, this study used both epinephrine and collagen as inducers for platelet activation. Meanwhile, using the other cartridge of the PFA-100® system, collagen-ADP cartridge, we did not observe the association between the ADRA2A polymorphisms and platelet function (data not shown). This PFA-100® system has highlighted as a screening tool for congenital and acquired platelet disorders or evaluation of anti-platelet therapies [26]. The sensitivity of this system, however, has not been completely determined. It is possible that individual differences in the ADRA2A genotypes affect the CEPI-CT as determined using the PFA-100® system, and this might interfere with the results obtained using the PFA-100® system.

Table 3
Possible confounding factors for the closure time of the collagen/epinephrine cartridge

Factors	Multiple logistic regression analysis		Students' *t* test		
	Odds ratio (95% CI)	p value	High platelet function group	Low platelet function group	p value
Genotype of the ADRA2A polymorphism[a]	2.10 (1.09–4.06)	0.0274			
VWF levels (ristocetin-cofactor activity)[b] (%)	1.02 (1.01–1.03)	<0.0001	106.03 ± 39.23	84.16 ± 38.16	<0.0001
Platelet count[b] ($\times 10^4/\mu l$)	0.98 (0.92–1.05)	0.5704	23.79 ± 4.63	23.69 ± 5.03	0.8719
Hematocrit[b] (%)	1.09 (0.97–1.23)	0.1538	44.95 ± 2.42	44.51 ± 3.03	0.2224

[a] Categorical variable, the 1780GG + 2372AA genotypes vs. others.
[b] Quantitative variable.

As the molecular mechanism underlying the relationship between epinephrine-mediated platelet function and the 1780A/G and 2372A/G polymorphisms, we speculate that these polymorphisms located in the 3′ untranslated region would affect ADRA2A mRNA stability and protein expression. This is supported by a previous report that the polymorphism corresponding to the 1780A/G polymorphism affects transcript levels of the ADRA2A, using the chloramphenicol acetyl transferase assay with Chinese hamster ovary cells [18]. Small et al. also demonstrated the effects of recombinant sequences of the ADRA2A haplotype on ADRA2A mRNA levels and protein expression in the human neuroblastoma cell lines [17]. Further studies are needed to elucidate the roles of the 1780A/G and/or the 2372A/G polymorphisms in the mRNA level, protein expression, and function of ADRA2A.

In conclusion, we demonstrated 16 sequence variations of the transcriptional domain of the human ADRA2A gene by direct sequencing. The 1780A/G polymorphism and the combination of the 1780A/G and 2372A/G polymorphism were associated with epinephrine/collagen-mediated platelet function. The results of the present study might contribute to a better understanding of the genetic variations of the ADRA2A gene in Japanese subjects and the critical role of this receptor in epinephrine-mediated platelet function.

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Gorog Thrombosis Test: analysis of factors influencing occlusive thrombus formation

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We used the Gorog Thrombosis Test to analyze the factors influencing the occlusion time, which represents platelet activation and subsequent occlusive thrombus formation, in 132 healthy Japanese volunteers (116 men, 16 women; mean age, 45.0 ± 12.0 years). The Gorog Thrombosis Test was designed to evaluate platelet aggregation and thrombolytic activity under a high shear stress condition (175 dynes/cm^2) in a native blood sample *in vitro*. The mean ± SD occlusion time was 154.8 ± 64.7 s (men, 153.4 ± 64.2 s and women, 165.4 ± 56.5 s). The occlusion time was inversely correlated with von Willebrand factor ristocetin cofactor activity (VWF:Rco) ($r = -0.242$, $P = 0.0055$) and von Willebrand factor antigen ($r = -0.230$, $P = 0.0080$). The mean occlusion time in the group with VWF:Rco of at least 170% (137 s) was significantly shorter than that in the group with VWF:Rco less than 170% (156 s, $P < 0.05$). Platelet counts, other coagulation markers and smoking showed no significant correlations with occlusion time. Red blood cells ($r = -0.177$, $P = 0.0365$), hemoglobin ($r = -0.191$, $P = 0.0245$) and hematocrit ($r = -0.182$, $P = 0.0329$) also showed inverse correlations with the occlusion time. This report is the first to clearly demonstrate the role of von Willebrand factor in the formation of occlusive thrombi in the Gorog Thrombosis Test. *Blood Coagul Fibrinolysis* 17:203–207 © 2006 Lippincott Williams & Wilkins.

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Introduction

Thrombotic diseases are the main cause of mortality and morbidity in many developed countries. In most cases, these disorders result from atherosclerosis, which narrows blood vessels and reduces blood flow. Shear stress is very high at the throat of the stenosis and very low in the area of recirculation immediately after the stenosis. Generally, after platelets have been exposed to shear stress in the absence of exogenous agonists, such as ADP, collagen, thromboxane and epinephrine, platelets are activated and, under certain conditions, are aggregated. The shear-induced platelet activation and aggregation is initiated by the plasma von Willebrand factor (VWF) interacting with the platelet GPIb/IX/V complex and platelet crosslinking through integrin-αIIbβ3 [1–4].

For many years, the skin bleeding time test (Mielke, Ivy, and Duke) was the only test available to confirm hemostatic abnormalities, but this test is not reliable and is associated with skin pain [5]. In the 1980s, the Thrombostat-4000 (PFA-100; Dade Behring, Marburg, Germany) and the haemostatmeter appeared [6–8]. The PFA-100 is a shear-inducing test; that is, platelet activation under shear stress in the capillaries is likely to act synergistically with the agonists to cause aperture occlusion. In the PFA-100, the small aperture is obstructed by a platelet plug, not a fibrin plug. Activated platelets contribute significantly to occlusion.

The Clot Signature Analyzer (Xylum Corporation, New York, USA) and the Thrombotic Status Analyzer (B. Iren, D. Gorog, *et al.*, London, UK) were also designed in the late 1990s. Recently, the Gorog Thrombosis Test (GTT), an in-vitro test using a new instrument designed to evaluate both platelet function and thrombolytic activity from a native blood sample, was devised by Montrose Diagnostics Ltd (London, UK). This test assesses the main physiological factors in haemostasis, such as shear-induced platelet activation, thrombin generation and thrombolysis [9–11].

The objective of this study was to analyze the factors that influence the platelet aggregation and subsequent thrombus formation induced by shear stress using the GTT in normal healthy Japanese volunteers.

Materials and methods

Study population

One hundred and thirty-two healthy Japanese volunteers (116 men, 16 women) with a mean age of 45.0 ± 12.0 years (range, 19–61 years) were enrolled in this study in October 2003 (Table 1). This study was

0957-5235

Table 1 Clinical characteristics of subjects undergoing the Gorog Thrombosis Test (GTT) and the association between occlusion time and serum parameters in univariate correlation analysis

Parameter	Mean ± SD	*R*	*P* value
Number of participants	132		
Number of men (%)	116 (88)		
Age (years)	45.0 ± 12	−0.037	0.6714
Body mass index (kg/gm^2)	23.1 ± 3.2	0.158	0.0707
Systolic blood pressure (mmHg)	132 ± 18	−0.050	0.5682
Diastolic blood pressure (mmHg)	80 ± 13	−0.059	0.5019
Waist–hip ratio	0. 87 ± 0.07	0.040	0.6508
Ankle body index	3.577 ± 1.849	0.019	0.8264
Pulse wave velocity	1362 ± 211	0.023	0.7953
White blood cells (× 10^3/μl)	6.3 ± 1.5	−0.055	0.5285
Red blood cells (× 10^4/μl)	**468 ± 37**	**−0.177**	**0.0365**
Hemoglobin (g/dl)	**14.6 ± 1.1**	**−0.191**	**0.0245**
Hematocrit (%)	**44.2 ± 3.0**	**−0.182**	**0.0329**
Platelet (× 10^4/μl)	25 ± 4	−0.074	0.3991
γ-Glutamyl transferase (IU/l)	44 ± 42	0.059	0.4990
Creatinine (mg/dl)	0.8 ± 0.1	0.030	0.7342
Total cholesterol (mg/dl)	206 ± 38	−0.036	0.6798
Triglyceride (mg/dl)	128 ± 93	0.037	0.6746
High-density lipoprotein-cholesterol (mg/dl)	55 ± 12	−0.074	0.3984
Uric acid (mg/dl)	5. 5 ± 1.2	0.031	0.7229
Glucose (mg/dl)	95 ± 20	0.127	0.1477
Hemoglobin A1c (%)	4. 9 ± 0.4	−0.089	0.3085
Homocysteine (mg/dl)	9. 3 ± 2.3	−0.163	0.0657
Fibrinogen (mg/dl)	278 ± 56	−0.002	0.9793
VWF:Ag (%)	**145 ± 47**	**−0.230**	**0.0080**
VWF:Rco (%)	**126 ± 41**	**−0.242**	**0.0055**
Thrombomodulin (U/ml)	14.0 ± 3.7	0.156	0.0760
Antithrombin III (%)	96.0 ± 9.2	−0.201	0.0770
Protein C (%)	122 ± 22	−0.069	0.5455
Protein S (%)	86 ± 15	0.016	0.8927
t-PA/PAI-1 (ng/ml)	15 ± 10	−0.079	0.4913
Plasminogen activator inhibitor-1 (ng/ml)	10.8 ± 9.5	−0.060	0.4971
Soluble P-selectin (ng/ml)	44.8 ± 26.8	0.157	0.0746
Brinkman Index	511 ± 311	−0.053	0.5660

VWF:Ag, von Willebrand factor antigen; VWF:Rco, von Willebrand factor ristocetin cofactor; t-PA/PAI-1, tissue-plasminogen activator/plasminogen activator inhibitor complex; Brinkman Index = smoking (/day) × smoking duration (months).

approved by the institutional ethics committee of the School of Medicine, Keio University, and written informed consent was obtained from each participant. None of the participants was taking antiplatelet drugs.

Blood samples

First, blood was drawn for routine analysis of regular checkups. Blood was then drawn from all of volunteers into plastic tubes without using any anticoagulants. These samples were not considered to be 'activated specimens' for use in the GTT analysis. The GTT analysis was started within 15 s after the blood samples were acquired. In addition to these samples, serum and platelet-poor plasma were also prepared to evaluate other laboratory parameters including coagulation/fibrinolysis factors (Table 1).

Measurement of the occlusion time using the Gorog Thrombosis Test

The GTT was designed to evaluate platelet aggregation and thrombolytic activity from a native blood sample *in vitro*. Yamamoto *et al.* recently introduced the principle and features of this test [9]. In this instrument, native blood samples flow at 37°C by gravity through a narrow gap between the larger ball bearing under a high shear stress condition (175 dyne/cm^2), which activates the platelets but prevents platelet aggregation. Next, between the ball bearings, the generation of thrombin and blood coagulation by the activated platelets begins. Fibrin-coated platelet aggregates flow through a narrow gap between the side wall and the smaller ball, and are then captured. The results for the occlusion of the gap and the arrest of the flow and occlusion time are then evaluated.

Other assays

Platelet-poor plasma was used to evaluate coagulation/fibrinolysis parameters such as fibrinogen, von Willebrand factor antigen (VWF:Ag), von Willebrand factor ristocetin cofactor (VWF:Rco), thrombomodulin, antithrombin III, protein C, protein S, tissue-plasminogen activator, tissue-plasminogen activator/plasminogen activator inhibitor complex, plasminogen activator inhibitor-1, and soluble P-selectin. Serum samples were also used for γ-glutamyl transferase, creatinine, total cholesterol, triglyceride, high-density lipoprotein-cholesterol, uric acid, glucose, hemoglobin A1c and homocysteine.

Statistical analysis

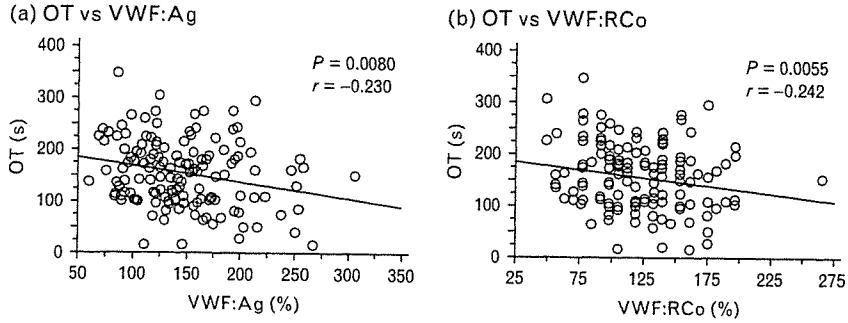
The Student *t*-test was used to compare the continuous variables, presented as the mean ± SD. The influence of known factors on the occlusion time was determined by univariate and multivariate correlation analysis, using the Stat View program (version 5.0J; SAS Institute, Cary, North Carolina, USA). All data are expressed as the mean ± SD. *P* values less than 0.05 were considered statistically significant.

Results

One hundred and thirty-two healthy Japanese volunteers were enrolled in this study. The mean occlusion time measured by the GTT was 154.8 ± 64.7 s (153.4 ± 64.2 s in men and 165.4 ± 56.5 s in women, $P > 0.05$). In univariate correlation analysis, a weak but significant negative correlation was observed between the occlusion time and VWF:Rco and VWF:Ag (VWF:Rco, $r = -0.242$, $P = 0.0055$ and VWF:Ag, $r = -0.0230$, $P = 0.0080$) (Table 1 and Fig. 1). Since the number of women was small, the effects of gender on the relationship between the occlusion time and VWF:Rco and VWF:Ag were not analyzed. When only male subjects were analyzed, however, significant negative correlation was still observed between the occlusion time and VWF:Rco and VWF:Ag (VWF:Rco, $r = -0.324$, $P = 0.0004$ and VWF:Ag, $r = -0.286$, $P = 0.0018$).

Platelet counts and other coagulation markers showed no significant correlation with the occlusion time. In our analysis, 72 subjects were smokers, but no significant

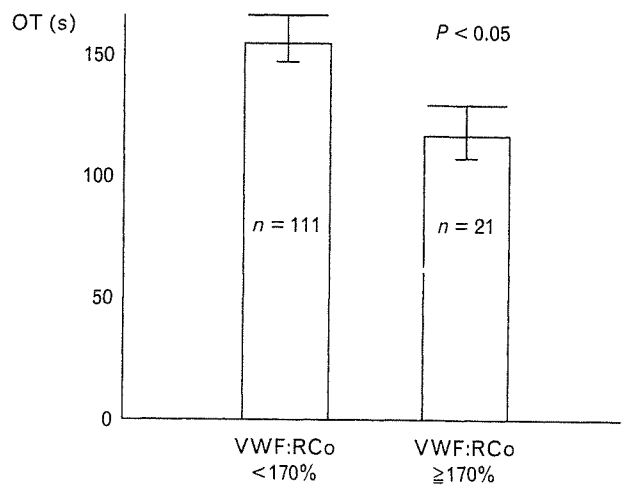
Fig. 1



Correlation between von Willebrand factor ristocetin cofactor activity (VWF:Rco), von Willebrand factor antigen (VWF:Ag) and occlusion time (OT) in the healthy group as a whole.

difference was observed between the occlusion time and smoking habits. A comparison of the occlusion time between plasma VWF:Rco less than 170% and VWF:Rco of at least 170% showed that the occlusion time was significantly shorter in the group with VWF:Rco of at least 170% (137 s) than in the group with VWF:Rco less than 170% (156 s) ($P < 0.05$) (Fig. 2). Those with VWF:Rco of at least 170% had significantly higher levels of pulse wave velocity, blood pressure, blood glucose, and hemoglobin A1c (data not shown). In this study, red blood cell (RBC) counts ($r = -0.177$, $P = 0.0365$), hemoglobin ($r = -0.191$, $P = 0.0245$) and hematocrit ($r = -0.182$, $P = 0.0329$) were within the normal range, and they were inversely correlated with the occlusion time by univariate correlation analysis (Table 1 and Fig. 3). In multivariate correlation analysis, hematocrit ($r = -0.244$, $P = 0.0380$), VWF:Rco ($r = -0.250$, $P = 0.0333$) and soluble P-selectin ($r = 0.224$, $P = 0.0421$) showed significant negative correlations with the occlusion time (Table 2).

Fig. 2



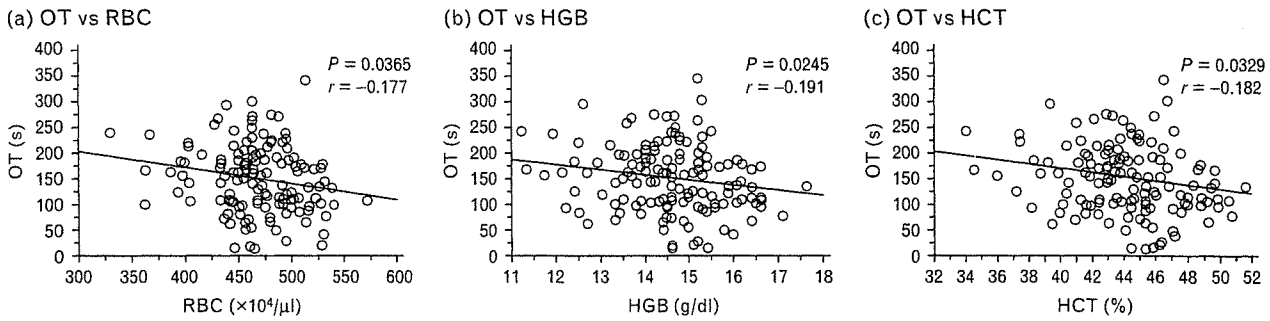
Comparison of the occlusion time (OT) between those with plasma von Willebrand factor ristocetin cofactor activity (VWF:Rco) less than 170% and VWF:Rco of at least 170%. The OT was shorter in the group with VWF:Rco of at least 170% (137 s) than in the group with VWF:Rco less than 170% (156 s), $P < 0.05$.

Discussion

Shear stress generated in stenosed arteries promotes platelet thrombus formation by accelerating the binding of VWF to platelets. The relationship between shear stress and platelet aggregation has been thoroughly discussed. It was initially believed that shear stress did not directly cause platelet activation, but instead caused platelet lysis [12]. The extracellular release of platelet agonists, such as ADP, activates platelets in excess of 250 dynes/cm^2 [13]. This shear-induced platelet activation and aggregation are initiated by plasma VWF interacting with platelet GPIb/IX/V and GPIIb/IIIa, and are independent of plasma platelet fibrinogen. The fibrinogen interaction with the platelet GPIIb/IIIa occurs under low fluid shear stress [3,4,14].

In-vitro tests of shear-induced platelet aggregation, platelet-rich plasma, and anticoagulated or thrombin-inhibited whole blood were previously used for platelet preparation. For a thrombosis to form in an artery, RBCs, leukocytes, endothelial cells and plasma calcium ions (Ca^{2+}) are required [15]. The main problem with the in-vitro platelet function test is that platelet aggregation is dependent on the extracellular Ca^{2+} concentrations. The GTT is the first laboratory evaluation of a new instrument to evaluate whether shear-induced platelet activation results in thrombin generation and thrombus formation; namely, platelet aggregation and coagulation from a native sample, *in vitro*, more relevant than those requiring anticoagulation. In this test, the shear stress in the first gap reached 175 dynes/cm^2. In various normal vessels, the shear stress ranged from less than 2 dyne/cm^2 (at the venous level), to 20–30 dynes/cm^2 (at the arterial level), to greater than 200 dynes/cm^2 (in stenosed coronary, peripheral or cerebral arteries) [1]. In this test, therefore, the exposure to a high shear stress showed

Fig. 3



Correlation between red blood cell (RBC) counts, hemoglobin (HGB), hematocrit (HCT) and occlusion time (OT) in the healthy group as a whole.

that in the areas of stenosed coronary arteries, platelets are activated and this activation causes platelet aggregation and thrombin generation and arrests the flow (occlusion time). Our data revealed that the occlusion time was inversely correlated with plasma VWF:Ag and VWF:RCo, which showed that VWF is essential for thrombosis formation in this system. There is an increased frequency of arterial thrombosis in cigarette smokers [16]. The changes in blood coagulation in smokers have been studied. In our analysis, 72 of all volunteers were smokers, but, as reported by Ikarugi *et al.*, the occlusion time had no correlations with smoking [11].

In addition, the occlusion time was shown to be inversely correlated with RBCs, hemoglobin and hematocrit. Ikarugi *et al.* have already reported an inverse correlation between hematocrit and the occlusion time [11]. The correlation between the platelet thrombus volume and hematocrit has been thoroughly discussed. Thrombus formation did not occur at a high shear stress in platelet-rich plasma or in whole blood with reduced hematocrit [17–22].

RBCs, by concentrating in the center of the blood stream and colliding with platelets, may facilitate transport of platelets toward the vessel wall (physical mechanism) [17–19]. RBCs may, as a result of shear-induced lytic or sublytic injury, liberate ADP, which may activate platelets and promote platelet–platelet, as well as platelet–surface, interaction (chemical mechanism) [17–19]. Both RBC-derived ADP and RBC-mediated platelet surface transport are involved in the potentiation by RBCs of platelet aggregation by shear stress [17]. Hematocrit is one of the main factors influencing platelet adherence. RBC size and deformability will also influence platelet transport [20,21,23,24]. The occlusion time is influenced by the consequent platelet activation and subsequent thrombus formation by high shear stress, and may have a potential to become a test for platelet hyper-reactivity. RBC deformability may be correlated with occlusion time, although in our case it was not analyzed.

Platelets, when stimulated, secrete specific proteins such as ADP, β-thromboglobulin, platelet factor 4 and serotonin [19,25]. These secreted compounds are proaggregatory and stimulate other platelets in the microenvironment [25]. Further studies are required to evaluate the relationship between the occlusion time and platelet aggregability, adhesiveness, and plasma markers of platelet activation such as β-thromboglobulin and platelet factor 4.

A global in-vitro test of hemostasis and thrombosis that measures both aggregation and the procoagulant activity of platelets requires the use of unaltered native blood. Testing native blood is far less convenient, mainly because it requires the patient to be present. Tests using native blood such as hemostatometry and the GTT allow the combined, comprehensive assessment of both aggregation and procoagulation.

Table 2 Multivariate correlation coefficients between the occlusion time and serum parameters

Parameter	*r*	*P* value
Age (years)	0.088	0.5583
Systolic blood pressure(mmHg)	−0.010	0.9337
Waist–hip ratio	−0.080	0.5667
Body mass index	0.164	0.1768
White blood cells (× 10^3/μl)	−0.067	0.6071
Hematocrit (%)	**−0.244**	**0.0380**
Platelet (× 10^4/μl)	−0.099	0.3988
γ-Glutamyl transferase (IU/l)	0.146	0.2131
Creatinine (mg/dl)	0.233	0.0527
Total cholesterol (mg/dl)	0.034	0.7888
Uric acid (mg/dl)	−0.007	0.9532
Hemoglobin A1c (%)	−0.167	0.1582
Homocysteine (mg/dl)	−0.106	0.3283
Fibrinogen (mg/dl)	0.132	0.3178
VWF:Rco (%)	**−0.250**	**0.0333**
Plasminogen activator inhibitor-1 (ng/ml)	−0.005	0.6300
Soluble P-selectin (ng/ml)	**0.224**	**0.0421**
Brinkman Index	−0.027	0.8223

VWF:Rco, von Willebrand factor ristocetin cofactor; Brinkman Index = smoking(/day) × smoking duration (months).

In conclusion, inverse correlations between occlusion time and VWF:Ag, VWF:RCo, RBCs, hemoglobin and hematocrit were detected in the GTT analysis; especially, relations between VWF and the occlusion time in the GTT was reported for the first time. Further diagnostic values in the GTT should be determined in patients with atherosclerotic disorders.

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Short Communication

A561C polymorphism of E-selectin is associated with ischemic cerebrovascular disease in the Japanese population without diabetes mellitus and hypercholesterolemia

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ABSTRACT

E-selectin, which is a member of the selectin superfamily of adhesion molecules, contributes to the leukocyte–endothelial cell attachments and is involved in the pathogenesis of thrombovascular diseases as a consequence. We investigated the A561C mutation in the E-selectin gene in 235 Japanese patients with ischemic cerebrovascular disease (CVD) and 301 age- and sex-matched healthy controls. Excluding the subjects with diabetes mellitus and hypercholesterolemia, the AC genotype frequencies of patients with ischemic CVD were higher than those of controls: 12.7% vs. 5.8% ($P=0.04$). Our results show that E-selectin gene polymorphisms represent an increased risk for ischemic CVD in the Japanese population without diabetes mellitus and hypercholesterolemia.

Leukocyte–endothelial attachments contribute to acute and chronic inflammation and atherosclerosis (Yoshida et al., 2003). In the normal physiological state, endothelial cells have low adhesiveness for leukocytes. However, inflammation or atherosclerosis activates both leukocytes and endothelial cells (Fassbender et al., 1999). E-selectin, L-selectin, and P-selectin are members of the selectin superfamily of adhesion molecules. Selectins are expressed on activated endothelial cells (E-selectin and P-selectin), leukocytes (L-selectin), and activated platelets (P-selectin) (Haring et al., 1996). They have in common an epidermal growth factor (EGF)-like domain connected with variable repeats of amino acid units to a membrane and cytoplasmic domain, and they bind to specific carbohydrate molecules on leukocyte molecules (Bevilacqua, 1993). These molecules, especially E-selectin, were revealed to facilitate leukocyte–endothelial cell attachments and contribute to the pathogenesis of thrombovascular diseases as a consequence (Cherian et al., 2003).

Recent studies showed that E-selectin plasma levels in homozygous C561C subjects and heterozygous A561C subjects were statistically higher than in wild-type A561A subjects (Mlekusch et al., 2004) and the A561C allele enhanced thrombin generation and fibrin formation significantly (Jilma et al., 2005). Positive results for C561 were associated with myocardial infarction in Japanese patients (Yoshida et al., 2003).

Interestingly, the E-selectin and P-selectin serum levels in patients with acute ischemic stroke were significantly higher than in controls, suggesting that these selectins are directly associated with the development of stroke (Cherian et al.,

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