アテローム破綻に血小板はどこまで関与しているのか？

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アテローム血栓形成のプラーク形成→進展→破綻→血栓形成の過程において，プラーク破綻後に血小板が主要な役割を演じていることは広く理解されている．最近では，アテローム血栓の引き金である"プラーク破綻"は炎症反応の強い関与が示唆されているが，活性化血小板に伴って発現する炎症性物質がこのプラーク破綻の機序に影響を与えていることが示され，注目されている．また活性化血小板はプラーク破綻に関連する因子を血小板顆粒より放出する．これらの知見は，"血小板"がアテローム血栓形成過程の中期～後期のみならず，その初期であるプラーク破綻に対するアプローチの標的にもなりうる可能性を示している．

KEY WORDS

血小板　動脈硬化　血栓症　プラーク　炎症

はじめに

アテローム血栓とは動脈硬化性のプラーク（粥腫）を基盤とし，プラークの進展・破綻とともに血管内皮が露呈，その内皮に血小板が主体となる血栓が形成された状態を示す．プラークの破綻は冠循環系，脳循環系，末梢循環系のさまざまな血管に発生する可能性があり，形成された血栓は動脈を閉塞するため，冠動脈では急性冠症候群の原因に，脳動脈では一過性脳虚血発作や脳梗塞の原因に，末梢動脈では間歇性跛行や虚血性壊死の原因となる．また，血栓形成途中の血小板の塊が血流により剝がれ，微小血管の閉塞に至ることもある．したがってアテローム血栓を基盤とする血栓症の有効なマネージメントのためには，その引き金となるプラークの破綻に対するアプローチが重要視されている．

血小板が血栓症において重要な役割を演じていることは，疾患に対する抗血小板薬の有用性が大規模臨床試験により示されていることからも明らかである．抗血小板薬はおもにプラーク破綻後の血栓形成の進展機序に対して作用し，血栓症の治療や予防に効果を有していると考えられている．プラーク破綻後における血小板の血栓形成への関与はよく知られているが（後述），最近ではプ

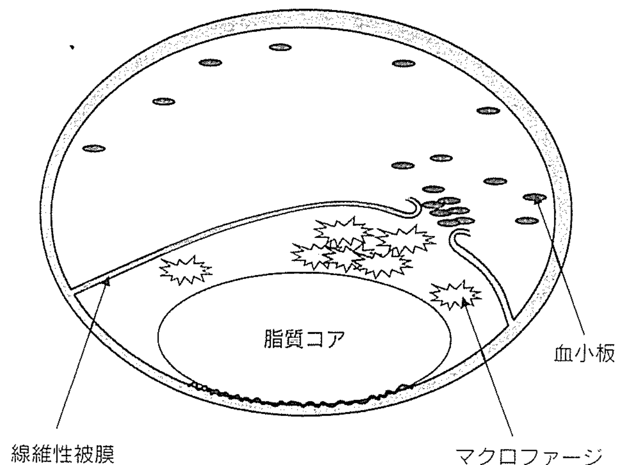
ラークの破綻を引き起こす過程にも血小板が影響を与えることを示唆する報告がある．つまり血小板はアテローム血栓の初期過程，プラークの破綻に対するアプローチの標的にもなる可能性がある．

本稿ではこの血小板のプラーク破綻への関与に着目し，最近の知見について述べる．

○ プラーク破綻の機序とは？

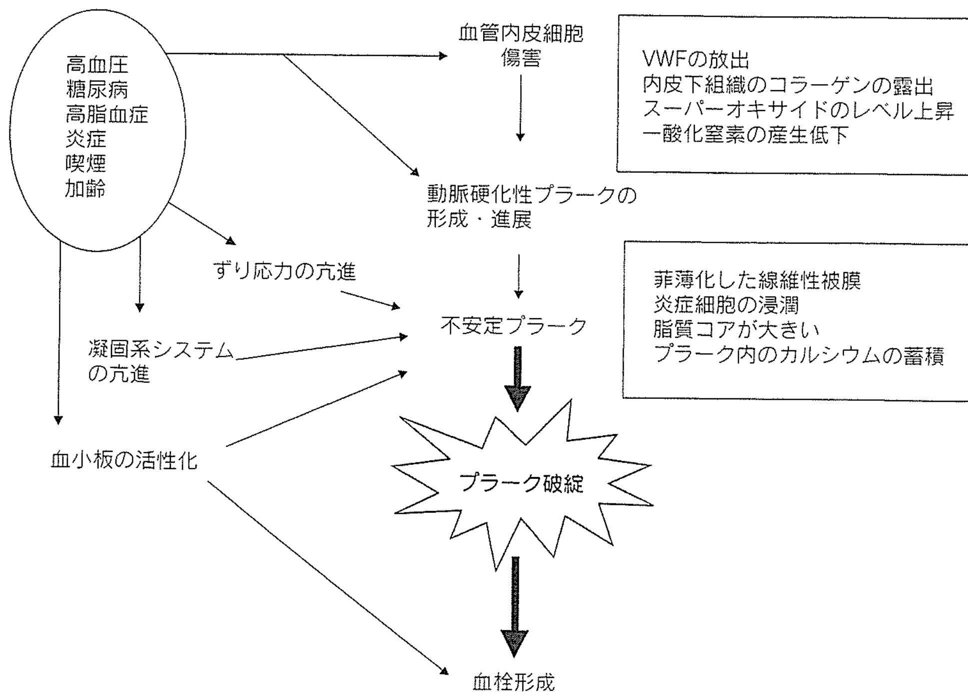
プラークはコレステロールとそのエステルで満たされた脂質コアと，細胞外マトリックスとよばれるプラークを安定化させる線維性被膜で構成されている（図❶）．マクロファージが酸化 LDL を取り込んで細胞内脂質が増大すると細胞が泡沫化し，さらに細胞死により脂質が蓄積して脂質コアの基盤となる．脂質コア周囲にマクロファージあるいは T リンパ球などの炎症細胞が集簇し，CD40-CD40 リガンド（CD40L），組織因子，マトリックスメタロプロテアーゼ（MMP）などが分泌される．細胞外マトリックスを分解する MMP が過剰産生されると，線維性被膜が分解してプラークの破綻を生じる[1]．

図❷に示すようにプラーク形成→進展→破綻の機序は多様であるが，高血圧，高脂血症，糖尿病，炎症，喫煙や加齢に伴う血管内皮細胞傷害がその初期形成に深く関与している（これら血管内皮細胞傷害に影響を与えるものはプラーク進展・破綻のリスクとしても広く知られて



図❶ 動脈硬化性のプラーク

（筆者作成）



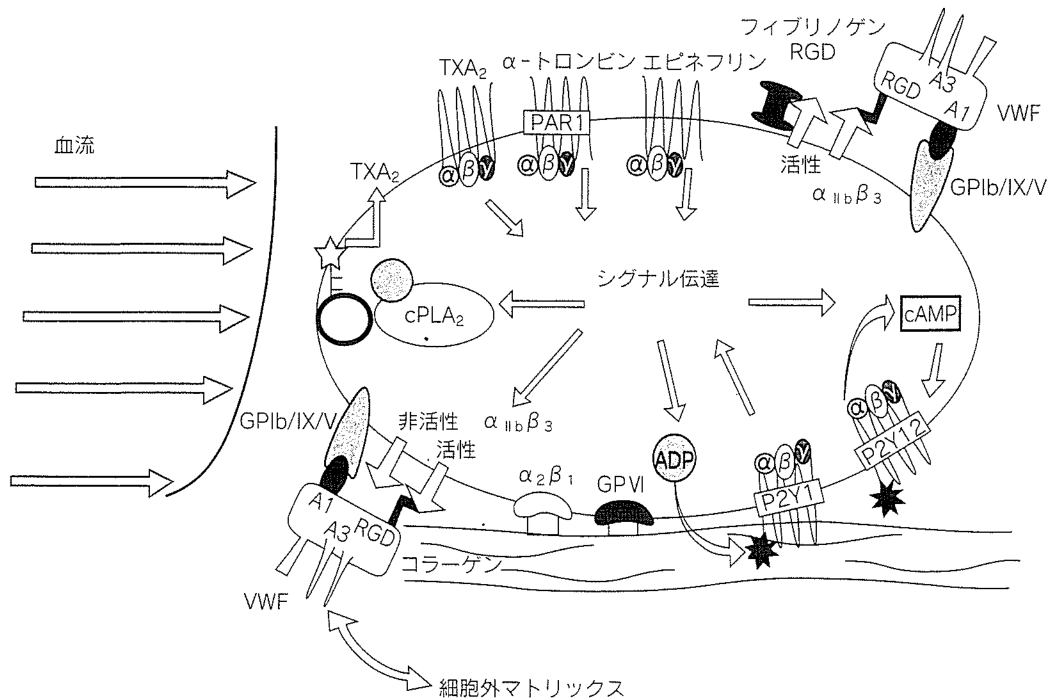
図❷ プラーク破綻機序

（筆者作成）

いる)．プラーク形成の初期は脂肪滴を含有した泡沫細胞と血管平滑筋細胞が存在する特徴がある．ここに血管内皮細胞傷害とそれに引きつづく，脂質と単球マクロファージの侵入，そして血管平滑筋細胞の遊走と増殖の結果プラーク進展が起こる．プラーク破綻の主要な機序は血液中の脂質レベルの代謝亢進・低下→脂質コアの増大→プラーク破綻，また炎症によるマクロファージやTリンパ球のプラークへの浸潤→MMP の放出→コラーゲン溶解→プラーク破綻，と理解されている．ほかにプラーク内のカルシウム蓄積や，菲薄化した線維性被膜がプラーク破綻の機序に主要に関与している．これらプラーク破綻の主要機序に関与する因子は数多いと考えられるが，注目すべきはこれらの因子のなかには血小板と密接に関係するものがいくつか存在していることである．

○ 血小板は活性化により顆粒内物質を放出する

血小板は，損傷し露呈した内皮下組織のコラーゲン，あるいはそのコラーゲンに粘着した von Willebrand 因子（VWF）と血小板膜受容体の反応により活性化のシグナル伝達がおきる．また，高いずり応力や高い脂質レベル，トロンビンやアデノシン二リン酸（ADP）も血小板活性化を引き起こし，その結果血小板機能を亢進させる（**図❸**）[2]．活性化血小板は血小板の3つの主要な顆粒（α顆粒，濃染顆粒，リソソーム）からの顆粒内物質（**表❶**）を放出する．これは血小板機能発現に必須と考えられている．さらに血小板から放出された顆粒内物質は，血小板活性化のポジティブフィードバックを司っている．これら顆粒内物質のなかにプラーク破綻の主要機序に関与する因子が報告されていることから，血小板はその顆粒内物質放出を介してプラーク破綻に関与していると考えられる．



TXA_2：トロンボキサン A_2，RGD：RGD ペプチド配列，$cPLA_2$：細胞型ホスホリパーゼ，A3：VWF A3 ドメイン，A1：VWF A1 ドメイン

図❸　血小板の活性化シグナル

（Ruggeri ZM, 2002[2]より改変引用）

表❶ 血小板のおもな顆粒内物質

α顆粒	VWF, 血小板第4因子, β-トロンボグロブリン, トロンボスポンジン, アルブミン, IgA, IgG, IgM, フィブリノゲン, フィブロネクチン, 凝固第V因子, オステオネクチン, P-セレクチン, α-アクチニン, 血小板由来増殖因子 (PDGF), 血管内皮増殖因子 (VEGF), インスリン様成長因子, クラスリン, β-アミロイド前駆蛋白, プラスミノゲンアクチベータインヒビター1 (PAI-1), Rab, Rap1
濃染顆粒	アドレナリン, ノルアドレナリン, セロトニン, アデノシン二リン酸 (ADP), アデノシン三リン酸 (ATP), Ca^{2+}
リソソーム	カテプシンD, カテプシンE, β-ガラクトシダーゼ, β-グルクロニダーゼ, 乳酸脱水素酵素

(筆者作成)

○ 活性化血小板由来の炎症メディエーターがプラーク破綻に関与する

CD40はCD40Lの受容体で, B細胞における恒常的発現が報告されている. また, 樹状細胞や単球に発現し, これら細胞の抗原提示細胞への分化過程に必要とされている. CD40Lは非活性化T細胞には発現せず, 活性化CD40陽性T細胞に発現している. そのほかに免疫系細胞, そして活性化血小板表面におけるCD40L発現の報告がある. 免疫系におけるCD40/CD40L系の重要な役割は明らかにされているが, 近年では動脈硬化病変の進展においてもCD40/CD40L系は主要なはたらきを担っていることが明らかにされてきている.

CD40Lは血管内皮細胞のCD40を介して血管内皮細胞を活性化し, その表面上に血管細胞接着因子のVCAM-1, 細胞接着因子のICAM-1, あるいはE-セレクチンといった粘着蛋白の発現を誘導し, 炎症を引き起こすことが血小板と培養血管内皮細胞を用いた実験により示唆されている[3]. さらに抗CD40L抗体を用いた*in vivo*での実験の結果や, CD40Lノックアウト (KO) マウスを用いた検討, CD40L/アポリポプロテインEダブルKOマウスを用いた検討の結果は, CD40/CD40L系の脂質コアの形成やプラークの不安定化, あるいは血栓の安定化に対する重要性を示している[4)5)].

CD40Lは可溶型と膜結合型の2種類が存在し, 活性化血小板における膜結合型CD40Lは血管内皮細胞の炎症反応を直接誘導する. 一方, 可溶型CD40L (活性化血小板からのCD40Lが他の血小板CD40と反応することにより切断され血中に放出されるもの) はMMPの誘導を介して組織因子を発現させる[6)7)].

またCD40/CD40L反応は, インターロイキン (IL)-1βの生成を血管平滑筋細胞で促進することが報告されている[8]. このようにCD40/CD40L系が動脈硬化病変において, その初期の脂質コアの形成, それにつづくプラークの破綻と血栓形成のいずれの段階においても重要であり, しかもCD40Lを発現している活性化血小板がこれらの過程において主たる役割を有することを裏付けする報告が集積されている.

P-セレクチンは血小板のα顆粒と血管内皮細胞に発現している. 活性化血小板にその発現は認められ, 活性化血管内皮細胞への白血球の粘着や白血球のrollingを司る. 活性化血小板と単球への粘着は, 炎症性の転写因子であるNF-κBのp65が細胞質から単球核内へ移行することが示されている[9]. また, T細胞によって発現, 分泌されるケモカインであるRANTES (regulated on activation, normal T-cell expressed and secreted) は血管内皮細胞に反応し沈着する. この沈着は単球との反応を誘導する. そしてこのRANTESの血管内皮細胞への沈着にP-セレクチンが関与しているとの報告がある[10].

プロテアーゼによる細胞外マトリックス(コラーゲン, ラミニン, プロテオグリカン) 破壊は, アテローム性動脈硬化のプラーク破綻においても重要な役割を有している. アテロームでマクロファージが集積している病巣ではマトリックスの破壊が著しく, これはマクロファージから分泌されるプロテアーゼの作用と考えられている. マクロファージに富んだ領域では組織因子やMMP-1, 2, 9など種々のプロテアーゼが発現し, プラーク破綻に密接に関与している可能性が示された. MMP-2の発現が強い領域では膜型 (membrane type : MT) 1-MMPの発現の増強, 酸化LDL濃度の増加に伴うMMP-9の発現の増強が確認されている. 血小板はMMP-1, 2, 3, 9, 14を有することから, このMMPとプラーク破綻の

機序においても血小板の関与が考えられている[11].

活性化血小板由来のリゾリン脂質性メディエーターがプラーク破綻に関与する

スフィンゴシン一リン酸は新規リゾリン脂質性メディエーターで血漿中にも存在するが，活性化血小板から放出される．さらに，これらが血小板活性化をきたすことが示されている[12]．血小板は特有のスフィンゴ脂質代謝の経路を有している．血小板はスフィンゴシンを取り込み，代謝経路によりスフィンゴシン一リン酸とし，血小板活性化に伴って放出する．

このスフィンゴシン一リン酸は血管内皮細胞や血管平滑筋細胞に豊富に発現しているG蛋白結合リゾリン脂質受容体を介して，細胞増殖亢進作用，細胞骨格形成作用，強い遊走反応の誘導を示す．すなわち，スフィンゴシン一リン酸は血管新生因子と考えられている．血管内皮細胞もスフィンゴシン一リン酸を有しているが，その発現レベルは低く，刺激によるスフィンゴシン一リン酸の放出がない．したがって，血管内皮細胞と相互反応を示すスフィンゴシン一リン酸は，活性化血小板から放出されたものと考えられている．スフィンゴシン一リン酸のキャリアがHDLであることから，血管内皮細胞に対して防御的に作用していることを示唆する報告や，スフィンゴシン一リン酸に誘導されたリンパ球のMMPの放出がプラーク破綻を亢進するという考えも示されており，今後の研究が注目されている．

細胞死をきたした血小板はマクロファージの進展に関与するのか？

血小板は約7〜14日間で細胞死をきたす．その細胞死した血小板はCD36やスカベンジャー受容体クラスA，あるいはホスファチジルセリン受容体を介してマクロファージに貪食される．マクロファージ内ではアミロイド前駆蛋白がβ-アミロイドペプチドに切断されるが，この反応の促進に細胞死した血小板が関与していることが示されている．さらに細胞死した血小板はプラーク内のマクロファージの活性化を誘導することを示唆する報告[13]がある．これは血小板が細胞死をきたすことによりプラークの形成，ひいてはプラークの破綻に関与している可能性を示唆しているが，“血小板の細胞死”は生理的反応としてとらえられているため，どのような“血小板の細胞死”がよりプラークの形成や破綻に関与するのかについての詳細な検討が今後期待されている．

血小板のプラーク破綻後の血栓形成過程への関与の機序とは？

アテローム血栓の形成過程は，その初期過程のプラーク形成・進展・破綻と破綻後の血栓形成過程に分けて考えられることが多い．これまでに血小板はプラーク破綻後の主たるはたらきが報告されている[2]．この過程では抗血小板薬の標的にもなっている血小板の膜受容体である膜糖蛋白（グリコプロテイン：GP），GP Ib/IX/V複合体やGP Ia/IIa（$\alpha_2\beta_1$インテグリン）は初期過程，GP VIやGP IIb/IIIa（$\alpha_{IIb}\beta_3$インテグリン）は後期過程でそれぞれ中心的役割をもつ．**図❹**に示すように，動脈硬化プラークの破綻に伴って露呈する内皮下組織のコラーゲンに粘着したVWFは，GP Ib/IX/V複合体と反応する（可逆性の粘着，tethering）[14]．この刺激でGP Ia/IIaが活性化してコラーゲンと反応する（血小板粘着）．粘着後活性化する血小板の膜上には活性型GP IIb/IIIaが発現して（血小板活性化），フィブリノゲンやVWFと結合する．その結合を介して，あるいは活性化した血小板から放出される強力な血小板凝集惹起物質であるトロンボキサンA_2をはじめとする種々の因子を血小板外へ放出し，正のフィードバック機構により血小板同士が強固に凝集（血小板凝集）して安定した動脈血栓をもたらす．

おわりに：血小板はプラーク破綻と破綻後の血栓形成過程に深く関与する

アテローム血栓形成過程の各段階，とくにプラーク破

図❹ 動脈血栓形成過程
（村田満ほか，2001[14]より改変引用）

図❺ 血小板とプラーク破綻・血栓形成
（筆者作成）

綻に関与する機序に着目して血小板の役割を述べた．図❺に示すように，血小板はその活性化に伴って発現する炎症性物質や活性化血小板から放出される顆粒内物質により，プラーク破綻の機序に直接関与する．またプラーク破綻後は，血小板の膜受容体が司る血小板凝集反応により血栓形成に直接関与する．ここで血栓形成反応に関与している血小板は活性化状態にあるため，さらにプラーク破綻に対しても正のシグナルをはたらきかけている．

プラーク破綻後の血栓形成の進展機序における血小板の関与はよく知られているが，プラーク破綻にはどのように関与しているかは，現在まだデータの集積中ではある．しかしながら，血小板がアテローム血栓の形成過程の各段階において重要なはたらきを有していることは明らかと考えられる．そして，その各段階の血小板の役割を理解することは疾患に対するより有効なアプローチの設計につながるため，今後“疾患のリスクを低下させる

ためのプラーク破綻の回避や，プラーク破綻を臨床的に予想するための標的としての血小板の可能性”の研究の進展に期待したい．

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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.
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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.

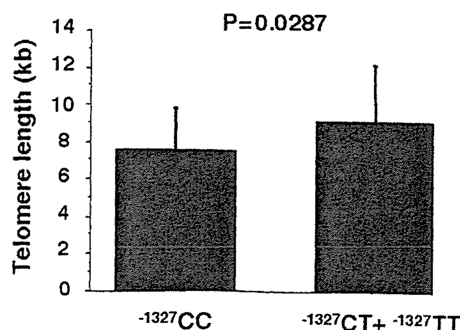

Fig. 1. Relationship between telomere length and the $^{-1327}$T/C polymorphism in CAD patients. Bars show mean TRF length (i.e., telomere length) (mean $\pm$ SD) between two groups of the $^{-1327}$T/C genotypes.

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.
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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.

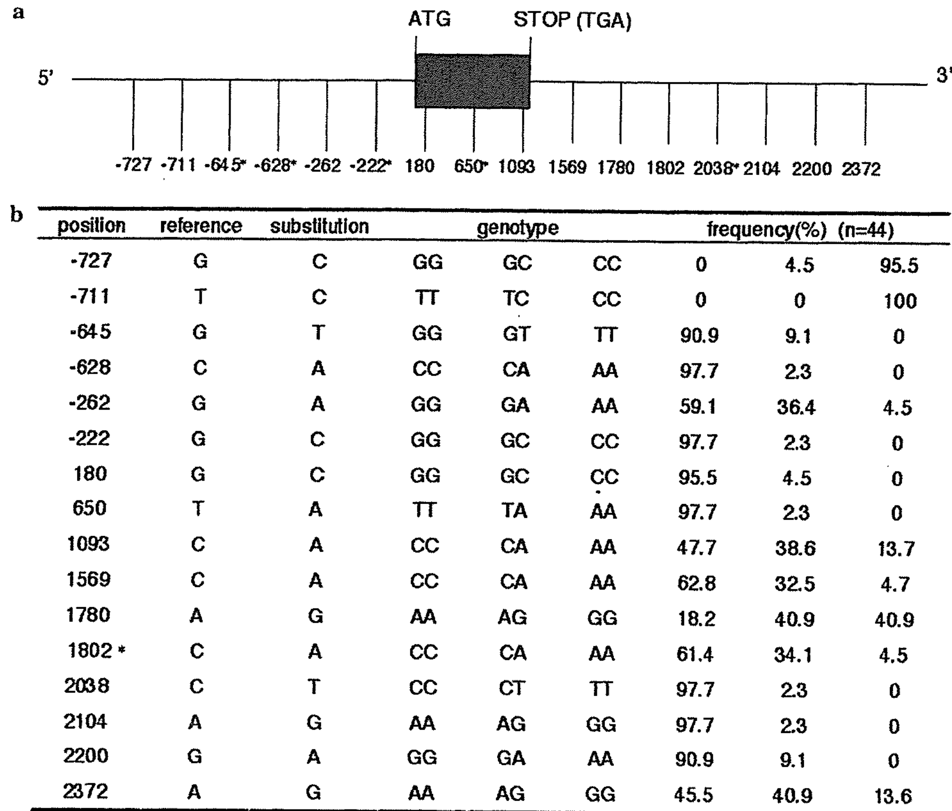
Measurements for von Willebrand factor levels. After centrifugation of blood samples at 2000*g* for 10 min, plasma samples were stored at −80 °C until assayed. The von Willebrand factor (VWF): ristocetin-cofactor activity (RCo) levels were assessed by ristocetin-induced platelet aggregation (Dade-Behring,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



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

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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

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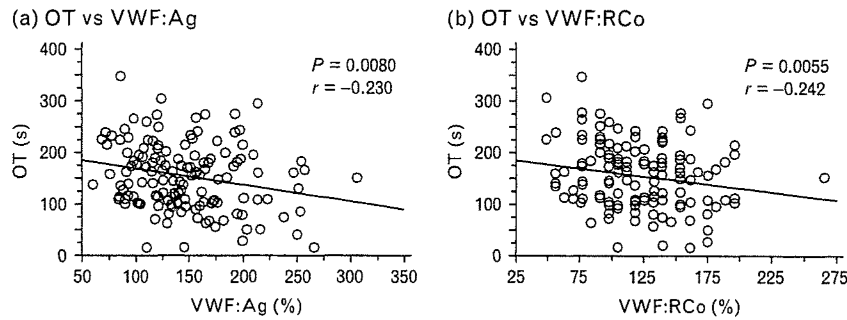
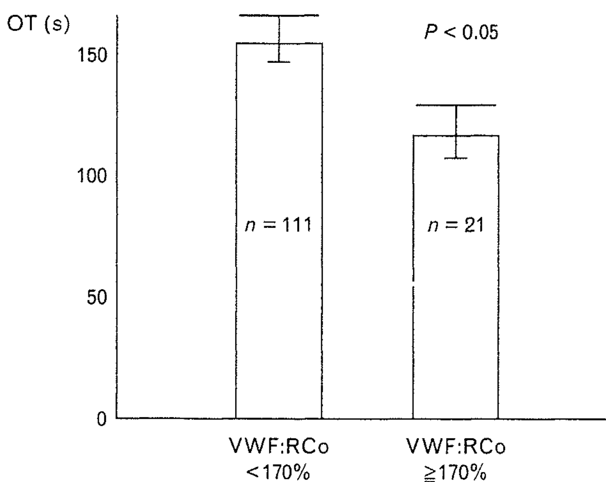
Fig. 1



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

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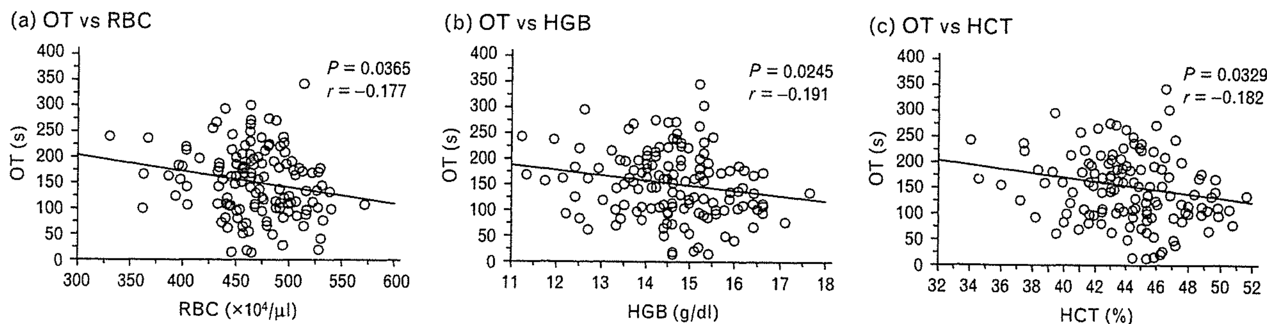
Fig. 3



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].

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 ($\times 10^3/\mu l$)	−0.067	0.6071
Hematocrit (%)	**−0.244**	**0.0380**
Platelet ($\times 10^4/\mu l$)	−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).

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.

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