prevent restenosis following uncomplicated stent implantation for *de novo* coronary artery stenosis.[86,87] In this randomized, double-blind, multicenter study, 700 patients will receive clopidogrel, aspirin, and either cilostazol or placebo after successful intracoronary stent implantation. The primary end point is MLD of the first lesion stented after 6 months; secondary end points include MLD in all lesions, mean percentage diameter stenosis, target lesion revascularization, and major angiographic end points.

D. Adverse Effects

Side effects are infrequent with cilostazol, but they include headache, palpitations, and diarrhea. Cilostazol is contraindicated in patients with congestive heart failure. The lack of cilostazol-induced hemorrhagic side effect in the 1095-patient CSPS study is discussed in Section VI.A.

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血小板GPⅠb/Ⅸ/Ⅴ受容体

Platelet Glycoprotein Ib / IX / V Receptor

村田　満

GPⅠb/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(GPⅠbα，GPⅠbβ，GPⅨ，GPⅤ)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，GPⅠb/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

GPⅠb/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GPⅠb/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GPⅥ)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-GPⅠb/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-GPⅠb/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-GPⅠb/Ⅸ/Ⅴ複合体反応をブロックすると逆に減少する．

GPⅠb/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，GPⅠbα，GPⅠbβ，GPⅨ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GPⅠb/Ⅸ/Ⅴ複合体を結合させる．検査室でVWF活性やGPⅠb/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．

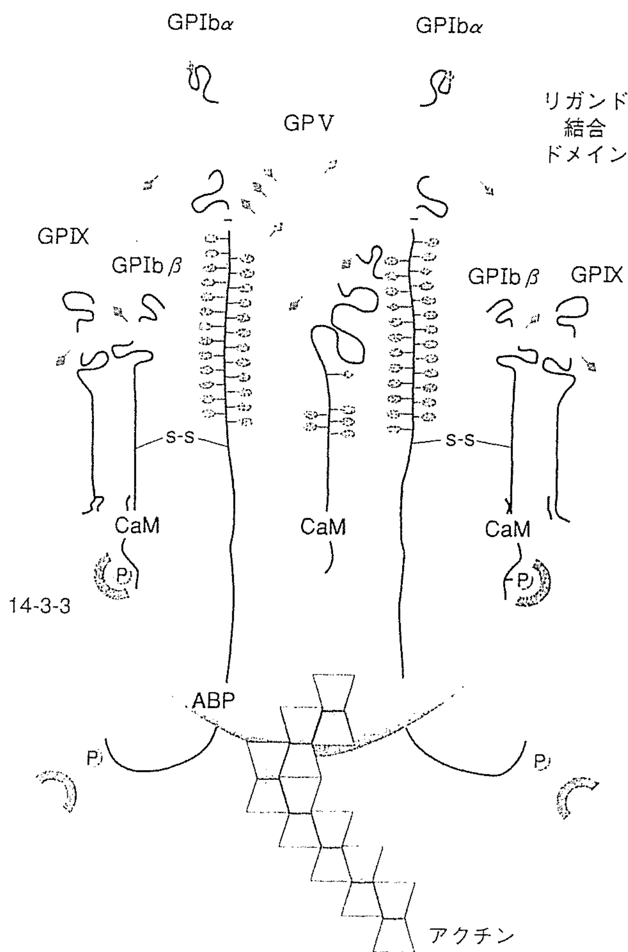

図　GP Ⅰb/Ⅸ/Ⅴ複合体の構造
（文献4より引用）

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．GPⅠbα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．GPⅠb/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

GPⅠb/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，GPⅠb/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，GPⅠb/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GPⅠb/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GPⅠb/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GPⅠb/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GPⅠb/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

GPⅠb/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GPⅠb/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．GPⅠb/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GPⅠb/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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血栓症と遺伝子多型

Polymorphisms in Relation to Thrombosis

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，GPⅠbα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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bjh research paper

Platelet glycoprotein Ib alpha polymorphisms affect the interaction with von Willebrand factor under flow conditions

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Summary

Interaction of platelet glycoprotein (GP) Ibα with von Willebrand factor (VWF) is essential for thrombus formation, particularly under high shear conditions. Previous case–control studies indicated that two GPIbα polymorphisms, ^{145}Thr/Met and/or variable number (1–4) tandem repeats of 13 amino-acid sequences, are associated with arterial thrombosis. The ^{145}Met-allele and the 3R- or 4R-allele is associated with increased risk. However, there is little clear experimental data to support this association. To elucidate the functional effects of these polymorphisms, we prepared recombinant GPIbα fragments and tested them *in vitro*. The dissociation constants of ristocetin-induced ^{125}I-labelled VWF binding to two forms of soluble recombinant GPIbα [^{1}His–^{302}Ala, either ^{145}Thr (145T) or ^{145}Met (145M)] were not different. Four types of Chinese hamster ovary cells expressing full-length GPIbαβ/IX, 145T with one repeat (T1R), 145M with one repeat (M1R), 145T with four repeats (T4R), and 145M with four repeats (M4R), were prepared, and cell interactions with immobilized-VWF were examined under various shear conditions. The cell rolling velocity of M4R under a shear condition of 114/s was significantly slower than that of T1R. Intermediate values were obtained with M1R and T4R. The results suggest that M4R interacts more strongly with VWF under flow conditions.

Keywords: polymorphisms, mutagenesis, platelets, glycoprotein Ib alpha, von Willebrand factor.

Glycoprotein (GP) Ib-IX-V complex is a platelet membrane receptor for von Willebrand factor (VWF) (Lopez, 1994; Clemetson, 1997; Ware, 1998; Andrews *et al*, 2003). This receptor consists of four subunits, GP Ibα, Ibβ, IX, and V. The largest subunit of the complex, GPIbα, has a VWF-binding site within the N-terminal 45-kDa extracytoplasmic domain of approximately 300 amino acids (Titani *et al*, 1987; Huizinga *et al*, 2002; Uff *et al*, 2002). Interaction of GPIbα with VWF mediates high shear-stress-dependent platelet activation, which is a critical step for thrombus formation (Ikeda *et al*, 1997; Dopheide *et al*, 2001; Ruggeri, 2003). The VWF/GPIbα interaction is not observed under static conditions *in vitro*, but only under shear conditions. Assessment under static conditions requires the presence of non-physiologic inducers, such as ristocetin or botrocetin.

In previous case–control studies, two genetic polymorphisms within the coding region of GPIbα were reportedly associated with arterial thrombosis, such as coronary artery disease and stroke (Hato *et al*, 1997; Murata *et al*, 1997; Sonoda *et al*, 2000; Simmonds *et al*, 2001; Yamada *et al*, 2002; Afshar-Kharghan *et al*, 2004). The first polymorphism is an amino acid dimorphism, Thr/Met, at residue 145 (Murata *et al*, 1992). The second polymorphism is a variable number tandem repeat [1–4 repeats (1R–4R)] of the 13-amino acid sequence, residues 399–411 (VNTR polymorphism) (Moroi *et al*, 1984; Ishida *et al*, 1991; Simsek *et al*, 1994). These two polymorphisms are in linkage disequilibrium (Ishida *et al*, 1991; Simsek *et al*, 1994). The ^{145}Met-allele, which is tightly linked to the 3R- or 4R-allele, is associated with increased risk. There is a race difference in the genotype distribution of the VNTR polymorphism; although 3R is observed in Caucasians, African-Americans (Afshar-Kharghan *et al*, 2004), Japanese, and Koreans (Ishida *et al*, 1996), 4R is observed in Japanese and Koreans (Ishida *et al*, 1996). Epidemiologic data indicate

that GPIbα polymorphisms are clinically significant. Molecular mechanisms for the association between thrombus formation and those polymorphisms, however, are not yet clearly understood. To elucidate the effects of ^{145}Thr/Met and/or VNTR polymorphisms on interactions with VWF, we performed two series of experiments; (a) ristocetin-induced ^{125}I-labelled VWF binding to two recombinant fragments containing a partial GPIbα sequence (^{1}His–^{302}Ala) with either ^{145}Thr (145T) or ^{145}Met (145M), and (b) the interaction between immobilized VWF and four types of Chinese hamster ovary (CHO) cells expressing full-length GPIbα/β/IX, ^{145}Thr/1R (T1R), ^{145}Met/1R (M1R), ^{145}Thr/4R (T4R), or ^{145}Met/4R (M4R), under flow conditions.

Methods

Preparation of recombinant GPIbα fragments

The GPIbα insert of a pBluescript KS (−) construct, which contained a cDNA encoding a partial GPIbα sequence (^{1}His–^{302}Ala) with ^{145}Thr, was cloned (Murata *et al*, 1991). The ^{145}Thr/Met substitutions on the GPIbα insert were performed using Quick Change™ (Stratagene, La Jolla, CA, USA), and each insert was ligated with a expression vector pcDNA3·1Zeo (−) (Invitrogen, Groningen, The Netherlands). Each construct was sequenced to ensure that the introduced mutation was restricted to residue 145 and then transfected into CHO cells (Dainippon Pharmaceutical Co., Osaka, Japan) using FuGENE™ 6 Transfection Reagent (Roche, Nutley, NJ, USA). Cells were cultured in the presence of 300 μg/ml of zeocine (Invitrogen) for selection of stable transfectants. Culture medium containing secreted soluble protein was collected from 145T-, 145M-, or mock-transfected cells after serum-free culture medium for 48 h.

Quantitation and immunologic evaluation of recombinant proteins

The expression of each recombinant protein was confirmed by Western blot analysis with anti-GPIbα monoclonal antibody, LJ-Ibα1 (a generous gift from Dr Z.M. Ruggeri, The Scripps Research Institute, La Jolla, CA, USA), which recognizes an epitope within the 45-kDa domain and reacts strongly with the reduced GPIbα fragment, using an enhanced chemiluminescence (ECL) Western blotting system™ (Amersham Pharmacia Biotech, Buckinghamshire, UK). The amounts were measured by dot-blot analysis with LJ-Ibα1 and ^{125}I-anti-mouse IgG (Daiichi Pure Chemicals, Tokyo, Japan). Purified recombinant GPIbα (Moriki *et al*, 1997) was used as a standard. Equivalent amounts of 145T and 145M were evaluated for their immunologic reactivity toward a panel of anti-GPIbα monoclonal antibodies, LJ-P3 (a generous gift of Dr Z.M. Ruggeri, The Scripps Research Institute), GUR83–35 (Takara Shuzo, Shiga, Japan), and GUR20–5 (Takara Shuzo), which recognize conformation-specific epitopes within the 45-kDa domain (Handa *et al*, 1986; Vicente *et al*, 1988; Kawasaki *et al*, 1995; Ikeda *et al*, 2000). ^{125}I-anti-mouse IgG was used as a secondary antibody in this quantitative analysis. A constant concentration (2 ng/μl) of 145T and 145M was used for subsequent functional analysis.

Ristocetin-induced ^{125}I-VWF binding to immobilized recombinant GPIbα fragment

Human VWF was provided by WelFide Co. (Osaka, Japan), and was radiolabelled with ^{125}I (Amersham Pharmacia Biotech) according to the IODO-GEN procedure (Fraker & Speck, 1978). The analysis of ristocetin (final concentration, 1·0 mg/ml)-induced ^{125}I-labelled soluble VWF (final concentration, 1·0 ug/ml) binding to immobilized 145T or 145M (400 ng/spot) was performed using the enzyme-linked immunofiltration assay apparatus (Pierce Chemical Co., Rockford, IL, USA). Details of this assay were described previously (Murata *et al*, 1991; Moriki *et al*, 1997). In the Scatchard plot analysis for the binding of ^{125}I-VWF (0·5–16 ug/ml) to 145T or 145M, the dissociation constant was analysed by a simple regression model. It was assumed that the same proportion of VWF multimers binds to each recombinant protein, and that the molecular weight of VWF was 220-kDa.

Establishment of CHO cells expressing the GPIbαβIX complex

A stable transfectant for GPIbβIX-expressing CHO cells was established, as described previously (Suzuki *et al*, 1999). A cDNA encoding the GPIbα sequence was cloned into a pBluescript KS (−) as described previously (Suzuki *et al*, 1999) and was subcloned into a mammalian expression vector pcDNA 3·1 Hygro (+) (Invitrogen) using the restriction sites for Kpn I (Takara Shuzo) and Not I. We prepared four types of plasmids for expression, T1R, M1R, T4R, and M4R. ^{145}Thr/Met substitution was created by polymerase chain reaction (PCR)-based site-directed mutagenesis using Quick Change™ (Stratagene), and then inserted into the 1R or 4R sequences; PCR was performed on genomic DNA with the 1R and 4R alleles, followed by subcloning using a TA cloning kit (Invitrogen). Each insert was subsequently cloned into the GPIbα-pcDNA 3·1 Hygro (+) using Xba I restriction sites and sequenced. Each plasmid was transfected into GPIbβIX-expressing CHO cells using FuGENE™ 6. These cells were grown in culture medium with 800 μg/ml of G418, 300 μg/ml of zeocine, and 400 μg/ml of hygromycine for selection of GPIX, GPIbβ, and GPIbα respectively.

Measurement for GPIbαβIX expression on CHO cells

Expression of GPIbα on CHO cells was confirmed by flowcytometry analysis with either the anti-GPIbα antibody, LJ-P3, or the anti-GPIX antibody, SZ1 (Immunotech,

Marseille, France), which reacts with the GPIbIX complex, but does not react with GPIb or GPIX alone. Four types of cells were independently sorted by fluorescence activated cell sorter (FACS) analysis using SZ1. Subsequently, quantitation of GPIbα on each cell was performed using an enzyme immunosorbent assay (EIA) with a glycocalicine EIA kit using GUR83–35 and GUR20–5, according to the supplied protocol. Purified glycocalicine of GPIbα (Lopez, 1994), which has a GPIbα extracytoplasmic domain that contains sites for the ^{145}Thr/Met and VNTR polymorphisms, was used as a standard in this assay.

Perfusion studies: analyses for the interaction of GPIbαβIX-expressing CHO cells with immobilized VWF under flow conditions

Glass cover slips were incubated with 10 μg/ml of VWF at 4°C overnight and then blocked with 0·5% bovine serum albumin (BSA) (Sigma-Aldrich, Tokyo, Japan) at room temperature for 1 h. CHO cells were harvested with 0·5 mmol/l EDTA, washed twice with phosphate buffered saline, and resuspended in 0·5 mmol/l EDTA/HEPES-Tyrode's buffer without Ca^{2+} and Mg^{2+} to a final concentration of 2×10^5/ml. The interaction between 10^6 cells expressing GPIbαβIX and immobilized VWF was examined using a recirculating flow chamber system (Nishiya *et al*, 2000). Cells interacting with the surface were monitored for a 4-min period. Data were stored on video tape. Single-frame video images were analysed using an image processor, an Argus 50 image processor (Hamamatsu Photonics, Hamamatsu, Japan), and rolling velocities of the cells were analysed using an image processor, an Argus 20 image processor (Hamamatsu Photonics). Rolling velocity was determined as the distance cells rolled per second. To confirm the specificity of the cell rolling, experiments under flow conditions were performed in the presence of 10 μg/ml of soluble-VWF or after incubation with 50 μg/ml of anti-GPIbα antibody GUR83–35 at room temperature for 15 min.

Statistics

Differences in immunologic reactivity between 145T and 145M were assessed using Student's *t*-test. Analysis of covariance (ANCOVA) was used to compare the influence of three variables, GPIbα sequence (145T vs. 145M), VWF binding, and day of the experiment, in VWF binding between 145T and 145M. Analysis of variance (ANOVA) was used for analysis of GPIbα expression on CHO cells and the perfusion studies among four types of GPIbα-expressing cells. The Bonferroni *post hoc* test for multiple comparisons was performed to compare the rolling velocity among the cells. ANCOVA was used to analyse three variables; GPIbα sequence (T1R vs M4R), rolling velocity, and day of experiment. A *P*-value of less than 0·05 was considered to be statistically significant.

Results

Comparison of immunologic reactivity of recombinant GPIbα fragments

To characterize the ^{145}Thr/Met polymorphism, we established stable cell lines that secreted recombinant GPIbα fragments (^{1}His–^{302}Ala), 145T or 145M, into the culture medium. The secretion of each fragment into the culture medium was confirmed by Western blot analysis using the LJ-Ibα1 antibody under reduced conditions. An LJ-Ibα1-positive species with a molecular mass of approximately 45-kDa was observed in 145T and 145M, but not in the culture medium from mock-transfected cells. Subsequently, we quantified each fragment by dot-blot analysis with LJ-Ibα1 under reduced conditions. There was similar immunologic reactivity to LJ-Ibα1 between 145T and 145M. An equivalent amount of each fragment was tested in dot-blot analysis for their immunologic reactivity to several anti-GPIbα monoclonal antibodies that recognize confirmation-specific epitopes within the 45-kDa domain and ^{125}I-anti mouse IgG as a secondary antibody. The binding of each antibody, as measured by counts per minute, was not significantly different between 145T and 145M (Table I), suggesting that the ^{145}Thr/Met polymorphism does not affect immunologic reactivity for LJ-P3, GUR20–5, and GUR83–35. Similar results by dot-blot analysis using the ECL detection system with anti-mouse IgG antibody as a secondary antibody were observed in seven independent experiments (data not shown).

Ristocetin-induced ^{125}I-labelled VWF binding to immobilized recombinant GPIbα fragments

To elucidate the effect of the ^{145}Thr/Met polymorphism on VWF binding, we performed an experiment using recombinant GPIbα fragments without the VNTR polymorphism site, containing residues 1–302. This binding was examined in the absence or presence of ristocetin under static conditions (Fig 1). The binding levels (pmol/well; mean ± SD) were 0·011 ± 0·005 for BSA, 0·018 ± 0·008 for 145T, and 0·016 ± 0·004 for 145M in the absence of ristocetin, indicating no specific binding in this experimental condition. In the presence of ristocetin, specific ^{125}I-VWF binding was observed in 145T and 145M, but not BSA; 0·015 ± 0·008 for BSA, 0·039 ± 0·015 for 145T, and

Table I. Immunologic reactivity (cpm count) in dot-blot analysis.

	145T	145M	*P*-value
LJ-Ibα1	2594·0 ± 17·0	2536·0 ± 130·1	0·5957
LJ-P3	2532·0 ± 93·3	2771·0 ± 168·3	0·2211
GUR20–5	2777·0 ± 75·0	2730·0 ± 435·6	0·8943
GUR83–35	2930·0 ± 45·3	2622·0 ± 260·2	0·2409

Values are mean ± SD of duplicate determinations in one experiment.

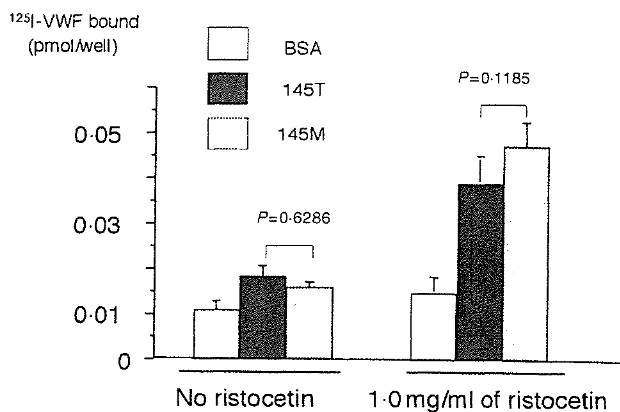

Fig 1. Binding of ristocetin-induced ^{125}I-labelled soluble VWF to immobilized recombinant GPIbα fragment was analysed on nitrocellulose membrane. Membrane-bound radioactivity was counted to assess the binding. BSA was used as a control. Results are mean value ± SD (pmol/well) of duplicates of three independent experiments. Error bars indicate SDs.

0·047 ± 0·017 for 145M, and the binding levels were not different between 145T and 145M ($P = 0{\cdot}1185$).

To evaluate the binding affinities of 145T and 145M, a Scatchard plot analysis was performed in the presence of 1·0 mg/ml of ristocetin. Both samples had saturable binding of ^{125}I-VWF, and the dissociation constants (nmol/l; mean ± SD) were 620·5 ± 176·1 for 145T and 406·0 ± 40·2 for 145M ($P = 0{\cdot}0551$). The maximum number of binding sites (B_{max}, nmol/l; mean ± SD) was 68·0 ± 11·4 for 145T and 56·1 ± 11·3 for 145M ($P = 0{\cdot}1889$). These results suggest that 145T and 145M have a similar binding affinity for VWF in the presence of ristocetin.

Quantitation of GPIbα expression on the GPIbβIX-expressing CHO cells

We prepared four types of GPIbαβIX-expressing CHO cells; two naturally occurring sequences, T1R and M4R, and two artificial or extremely rare sequences, T4R and M1R, to investigate which GPIbα polymorphisms affect the interaction with VWF under flow conditions. First, the surface density of the GPIbαβIX complex on each cell was examined by flowcytometry analysis with LJ-P3 or SZ-1, and the reactivity was not different among cells. Subsequently, four types of GPIbαβIX-expressing CHO cells were isolated using FACS to obtain cells with an equivalent amount of the GPIbαβIX complex on the surface. Moreover, quantitative determination of each sorted cell was performed by EIA using the anti-GPIbα antibodies, GUR83–35 and GUR20–5. The GPIbα expression levels, calculated by standard curves for absorbance and glycocalicine as a standard protein, are shown in Table II. There was no statistically significant difference in the GPIbα expression levels among the four types of cells, although T4R had a slightly higher expression level.

Table II. GPIbα molecule on single CHO cell.

GPIbα sequence	GPIbα molecule ($\times 10^6$)	P-value
T1R	4·26 ± 0·31	0·1279
M1R	4·46 ± 0·34	
T4R	5·26 ± 0·48	
M4R	4·36 ± 0·17	

Values are mean ± SD of duplicate determinations in three independent experiments.

Interaction between immobilized VWF and CHO cells expressing the GPIbαβIX complex under flow conditions

To determine the optimal shear condition, preliminary studies were performed using T1R cells to monitor the interaction between immobilized VWF and GPIbα-expressing CHO cells. Rolling cells per unit area (mm^2) of VWF-immobilized glass were counted under various shear conditions. The number of rolling cells was 26·91, 16·34, 8·65, 0·96, and 0 for shear conditions of 32/s, 64/s, 114/s, 214/s, and 600/s respectively. In CHO cells without GPIbα, the rolling cell number was 3·84, 0·96, 0, 0, and 0 for shear conditions of 32/s, 64/s, 114/s, 214/s, and 600/s respectively. We then examined the effect of the anti-GPIbα antibody GUR83–35 on rolling cell number per minute and rolling velocity. Under a shear condition of 32/s, the number of rolling cells per minute was 14 and 16 in the absence and presence of GUR83–35 respectively. Under a shear condition of 114/s, the number was 134 and 41 in the absence and presence of GUR83–35 respectively. The rolling velocity under a shear condition of 32/s was similar between the two experimental conditions; 240·61 ± 67·49 (μm/s, mean ± SD) in the absence of GUR83–35 and 291·98 ± 60·92 in the presence of GUR83–35. In contrast, the rolling velocity under a shear condition of 114/s was different in the absence and presence of GUR83–35; 704·74 ± 153·5 and 959·10 ± 172·1 respectively. These findings indicated that the observed interaction was specific for GPIbα under a shear condition of 114/s, but not of 32/s. Thus, we adopted the shear condition of 114/s for the subsequent experiments.

As shown in Fig 2 and Table III, the rolling velocity of M4R under shear conditions of 114/s was significantly slower than that of T1R ($P = 0{\cdot}00042$). The rolling velocities of M1R and T4R were similar, with values that were intermediate between those for T1R and M4R. Moreover, we analysed the rolling velocity between T1R and M4R by ANCOVA to test whether the difference identified by ANOVA was affected by different day of experiment. The rolling velocity was 1077·7 ± 20·9 (mean ± SD) for T1R and 918·4 ± 17·3 for M4R ($P < 0{\cdot}0001$), indicating that the significant difference between T1R and M4R was not influenced by interactions with other variables among the four independent experiments. The number of rolling cells interacting with the immobilized VWF per minute was 32·0 ± 5·6 for T1R, 34·7 ± 2·1 for M1R, 26·7 ± 3·1 for T4R, and 35·7 ± 2·5 for M4R. These values were not significantly different among the four types of cells

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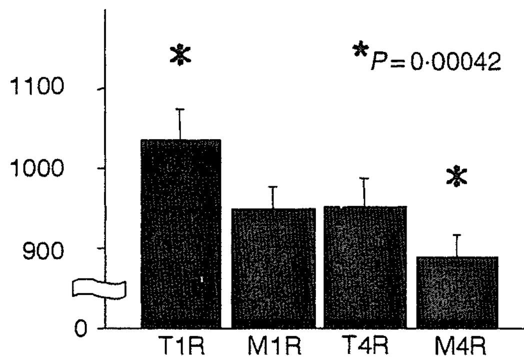

Fig 2. Interaction between immobilized VWF and CHO cells expressing GPIbαβIX complex was analysed under flow conditions (114/s). Each cell sequenced for the GPIbα polymorphism was applied to the flow chamber system with VWF-immobilized glass. Error bars indicate SDs. Results are mean value ± SD (μm/s) of four independent experiments.

(P = 0·0574). In GPIbβIX-expressing CHO cells, the rolling cell number was 5·2 ± 0·5. Under this flow condition, the cell rolling velocity was inhibited by GUR83–35; i.e. the velocities became faster and with soluble-VWF addition (Table III). Thus, the specificity of interaction between immobilized VWF and GPIbα-expressing CHO cells was confirmed under a shear condition of 114/s.

Discussion

The present study demonstrated that the ^{145}Met and 4R polymorphisms of GPIbα facilitate interaction with immobilized VWF under flow conditions, which is a highly adaptive physiologic response. To date, molecular mechanisms for the functional differences of the ^{145}Thr/Met and/or VNTR polymorphisms in GPIbα have not been fully understood whereas numerous epidemiologic data have been reported. We report the first experimental data obtained using recombinant proteins to determine the functional differences of ^{145}Thr/Met and VNTR GPIbα polymorphisms. Previously, ^{145}Met and/or 3R/4R polymorphisms were demonstrated to be associated with an increased risk for arterial thrombosis, such as coronary artery disease or stroke (Simmonds *et al*, 2001; Yamada *et al*, 2002). Because the ^{145}Thr/Met and VNTR polymorphisms are in linkage disequilibrium, focus on either ^{145}Met or 3R/4R allele was likely to be sufficient to examine the association between GPIbα polymorphisms and arterial thrombosis in the epidemiological study. We reported that the frequency of either ^{145}Met- or 4R-allele among patients with coronary artery disease was higher than that among control subjects and that the genotypes with the ^{145}Met-allele were more frequently found in the patients with cerebrovascular disease than in control subjects (Murata *et al*, 1997; Sonoda *et al*, 2000). A large case-cohort study (Afshar Kharghan *et al*, 2004) showed the relationship of the 2R/2R genotype with a lower risk of coronary heart disease in African-Americans. However, conflicting data have also been published (Hato *et al*, 1997; Simmonds *et al*, 2001). In experimental studies of these polymorphisms, Boncler *et al* (2002) demonstrated that the inhibitory effect of the VWF antagonist on ristocetin-induced agglutination was higher in ^{145}Met/3R-positive platelets than in ^{145}Met/3R-negative platelets. Ulrichts *et al* (2003) reported that platelets with ^{145}Thr or recombinant GPIbα (residues 1–289) with ^{145}Thr had a higher VWF binding affinity than ^{145}Met. These findings are not consistent with our results although the experimental conditions of the present study differed from those of previous studies: use of ristocetin or botrocetin or use of an assay system. Other studies have also used various methods with inconsistent results (Mazzucato *et al*, 1996; Li *et al*, 2000; Jilma-Stohlawetz *et al*, 2003). These reports suggest that the functional analyses of GPIbα polymorphisms seem to be easily affected by several factors in relation to platelet activation or experimental conditions. Therefore, in this study, recombinant GPIbα and purified human VWF were examined under two experimental conditions to focus on the relationship between GPIbα polymorphisms and interactions with VWF. The first study, using soluble GPIbα lacking the VNTR polymorphism site, did not show the effect of the ^{145}Thr/Met polymorphism on the major conformation because the immunoreactivity to anti-GPIbα antibodies that recognize confirmation-specific epitopes were not significantly different between these polymorphisms. The ^{145}Thr/Met polymorphism did not affect the ^{125}I-VWF binding in the presence of ristocetin under static conditions. Although ristocetin provides a

Table III. Rolling velocity for GPIbα-expressing cells interacting with VWF under 114/s flow condition

	Soluble VWF (−)				Soluble VWF (+)	
	GUR83-35 (−)	P-value	GUR83-35 (+)	P-value	GUR83-35 (−)	P-value
T1R	1035·1 ± 40·5*		1291·1 ± 61·9	NS	1385·7 ± 82·5	NS
M1R	951·1 ± 26·4**	*0·00042	1486·8 ± 109·5		1165·4 ± 62·9	
T4R	952·5 ± 36·9**	**NS	1638·9 ± 384·6		1276·6 ± 86·5	
M4R	902·2 ± 30·8*		1521·5 ± 86·6		1293·5 ± 126·4	

Values for rolling velocity (μm/s) are mean ± SD. NS, not significant.

convenient method to investigate the VWF/GPIbα interaction *in vitro*, it is not a physiologic substance. Thus, the second study was designed with an alternative approach, an *in vitro* assay for VWF/GPIbα interaction under flow conditions. Cells expressing GPIbα were prepared as a GPIbαβIX complex because expression of a full-length GPIbα alone was unstable in the cell culture system (Lopez *et al*, 1992). Two types of cells with naturally occurring sequences (T1R and M4R) and two types of cells with artificial or extremely rare sequences (T4R and M1R) were used to determine which polymorphism was more closely related to the VWF/GPIbα interaction. We carefully measured the GPIbα expression level on each cell because these levels were reported to affect the VWF/GPIbα interaction under flow conditions (Nishiya *et al*, 2000). After using FACS to obtain cells expressing similar GPIbα levels, EIA assay was performed using GUR83–35 and GUR20–5. Because these two antibodies were shown not to be influenced by the ^{145}Thr/Met polymorphism (Table I), we used these antibodies in this assay. Perfusion analyses of the quantified cells indicated that M4R, which is a risk factor for arterial thrombosis, had a high ability to interact with VWF under a flow condition of 114/s, as compared with T1R. This flow condition of 114/s may correspond to wall shear rate for large veins *in vivo* (Bevan *et al*, 1995), where VWF-dependent platelet phenomena may not take place. Compared with platelets, however, CHO cells have 2·5- to threefold larger diameters, and the GPIbα-expressing CHO cells are approximately 20-fold higher in GPIbα density. The cell size and receptor density are likely to affect the sensitivity of cells to flow conditions. Also, we were unable to determine the order of effectiveness of the polymorphisms among the four sequences, ^{145}Thr, ^{145}Met, 1R, and 4R, in VWF/GPIbα interactions because T4R and M1R had a similar ability to interact with VWF. Although the synergistic effect of the ^{145}Thr/Met and VNTR polymorphisms on GPIbα function remains unclear, the present data are compatible with previous speculations (Lopez, 1994; Murata *et al*, 1997) that GPIbα with 4R is longer in size and thus places the VWF-binding global domain further away from the platelet plasma membrane. Thus, VWF would be more easily accessible to the binding site on the receptor under high shear conditions. Functional polymorphisms of GPIbα might be responsible for the increased prevalence of arterial thrombosis. Our observations might explain the molecular basis for the previous epidemiologic studies. Further studies to examine the interactions between GPIbα polymorphisms and other ligands are necessary. The present data support a potentially new therapeutic approach to arterial thrombosis by targeting specific GPIbα polymorphisms.

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1. 総説―血栓形成の分子機構

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THROMBOSIS and Circulation

§論文のポイント

[1] 血栓形成の主な要因は，血流の変化，血液成分の変化，そして血管壁の変化である．

[2] 血栓は形成機序の違いにより，静脈で形成される「静脈血栓」と動脈で形成される「動脈血栓」に大別される．

[3] 内因系凝固，外因系凝固，線溶系，そして凝固阻止系の因子が制御している血液凝固反応の亢進は静脈血栓形成の主体となる．

[4] 動脈血栓は，動脈硬化性のプラーク破綻により露呈した血管内皮細胞と血小板の膜受容体との反応による血小板の活性化・血小板凝集により形成される．

§キーワード

静脈血栓／動脈血栓／血管内皮細胞／血小板／血液凝固・線溶因子

はじめに

1860年，Rudolf C. Virchowは血栓形成の3つの要因，❶血流の変化，❷血液成分の変化，❸血管壁の変化を提唱した．現在に至っては，この仮説を強く支持する血栓形成機序の分子レベルでの研究成果が数多く報告され，これら3つの要因が複雑に関与して血栓形成を制御している機構が解明されてきている．血栓は静脈で形成される「静脈血栓」と，動脈で形成される「動脈血栓」に大別される．これらは血管内における血流速度の違い(静脈内では遅く，動脈内では早い)により形成機序が異なる(図1)．したがって病的血栓においてはそれぞれ関係の深い疾患も異なってくる．静脈血栓は深部静脈血栓症，動脈血栓は脳梗塞や心筋梗塞の原因となっている．

静脈での血栓形成は血液のうっ滞している箇所で，血液凝固因子が主体となる凝固反応の亢進が起こり，トロンビン生成を促進させることからはじまる．血流が遅いために高濃度のトロンビンが局所に存在してフィブリン塊を形成させる．また，トロンビンは強力な血小板惹起物質であり血小板凝集塊形成も促す[1]．

一方，動脈での血栓形成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，その血管損傷部位に対して早い血流のずり応力依存性に血小板の膜受容体が司る血小板活性化→血小板凝集反応により直接血栓形成に関与する．また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も関与，すなわち正のフィードバックに働きかけている[2]．

本稿では血栓形成の分子機構，特に静脈血栓形成過程の要となる血液凝固反応の制御と動脈血栓形成の中心的役割を演じる血小板活性化の分子機構に焦点をおいて概説する．

静脈血栓の主体となる「フィブリン形成」を来す血液凝固反応

血液凝固反応は内因系凝固，外因系凝固，線溶系，そして凝固阻止系の因子が制御している[4](図2)．正常な状態の血管壁は抗血栓性に制御されているが，血管内皮細胞が傷害を受けることにより，血液凝固因子が主体となる凝固系反応の亢進と線溶系反応や阻害因子系の反応の低下が起こり，血栓形成亢進性となる．

血管傷害部位に露呈された陰性荷電物質が，凝固第XII因子の陽性荷

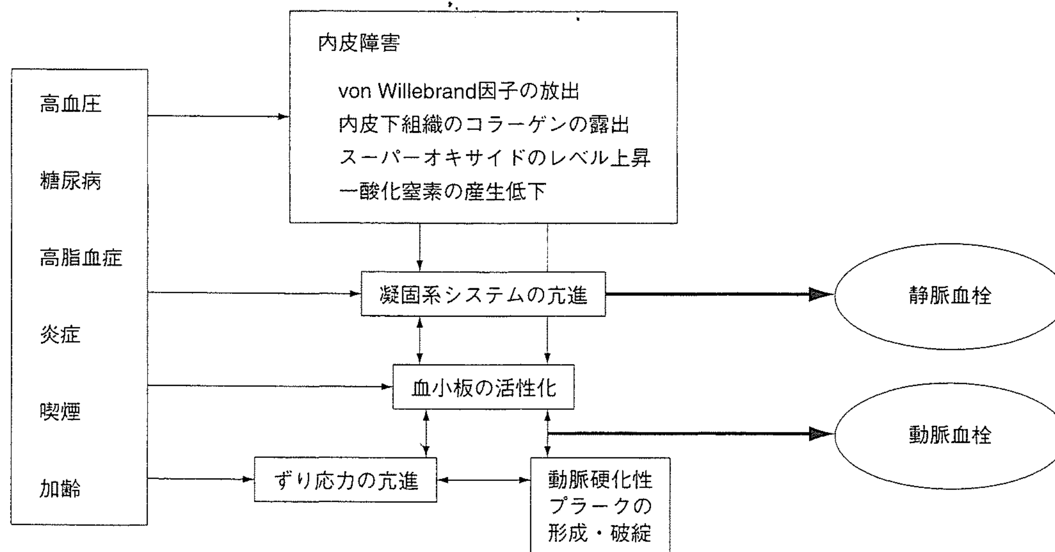
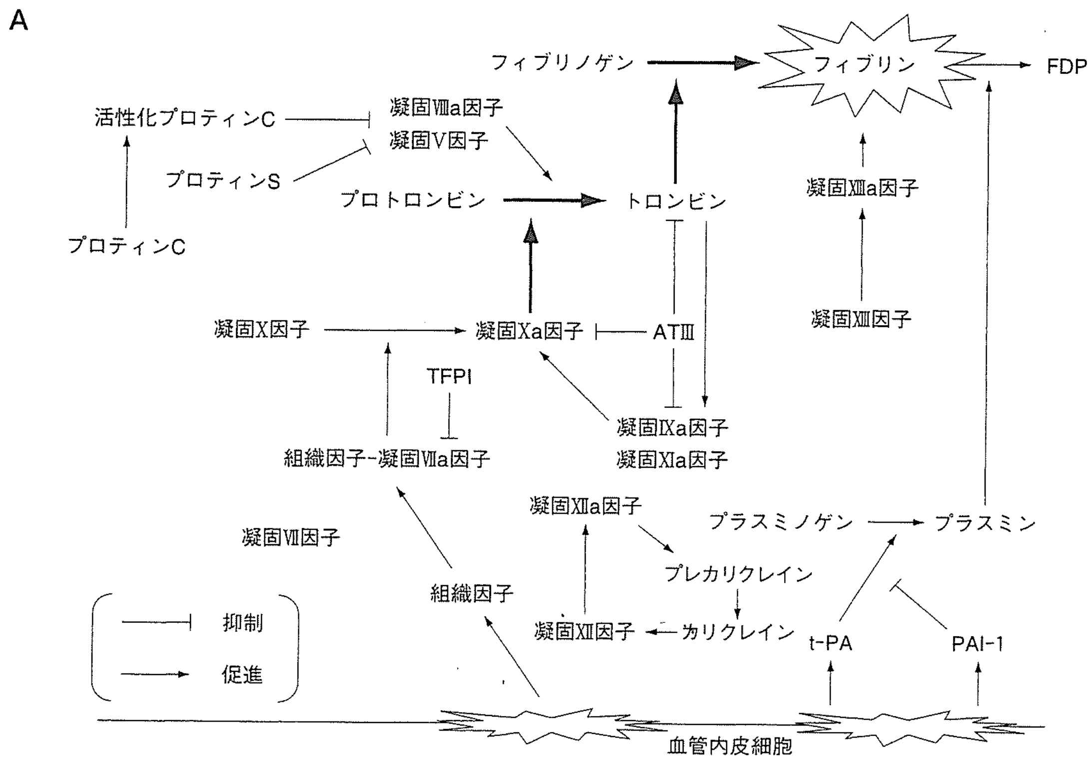


図1　血栓形成の機序

A



B

Virchow's traid

	静脈血栓	動脈血栓
血管壁の変化	傷害	傷害
血流の変化	うっ滞	早くなる
血液成分の変化	凝固因子	血小板

図2　血液凝固カスケード

A：文献2より改変引用

電領域に結合することによって凝固第XII因子の立体構造変化を引き起こし，活性化凝固第XII因子(凝固第XIIa因子)となることが内因系凝固反応スタートの主な機序と考えられている．凝固第XIIa因子はプレカリクレインをカリクレインに活性化し，このカリクレインは，さらに凝固第XII因子を活性化する正のフィードバック機構が働くとともに凝固VII因子を活性化して，外因系凝固機序とも関連する．また，凝固第XIIa因子は凝固第XI因子を活性化させる．この後，内因系凝固と外因系凝固(後述)の共通の凝固反応経路である凝固第X因子の活性化，プロトロンビンの活性化によってトロンビンが生成される．凝固第X因子やプロトロンビンは血管傷害部位に局在し各因子の活性化は亢進をたどる．

外因系凝固反応は，血管傷害を受け露呈した血管内皮下組織に多量に存在する組織因子に対する凝固第VII因子の結合からスタートする．組織因子に結合した凝固第VII因子は凝固第Xa因子，凝固第XIIa因子，トロンビンなどで活性化凝固第VII因子となる(凝固第VIIa因子)．組織因子—凝固第VIIa因子は凝固第X因子とともに凝固第IX因子を活性化す

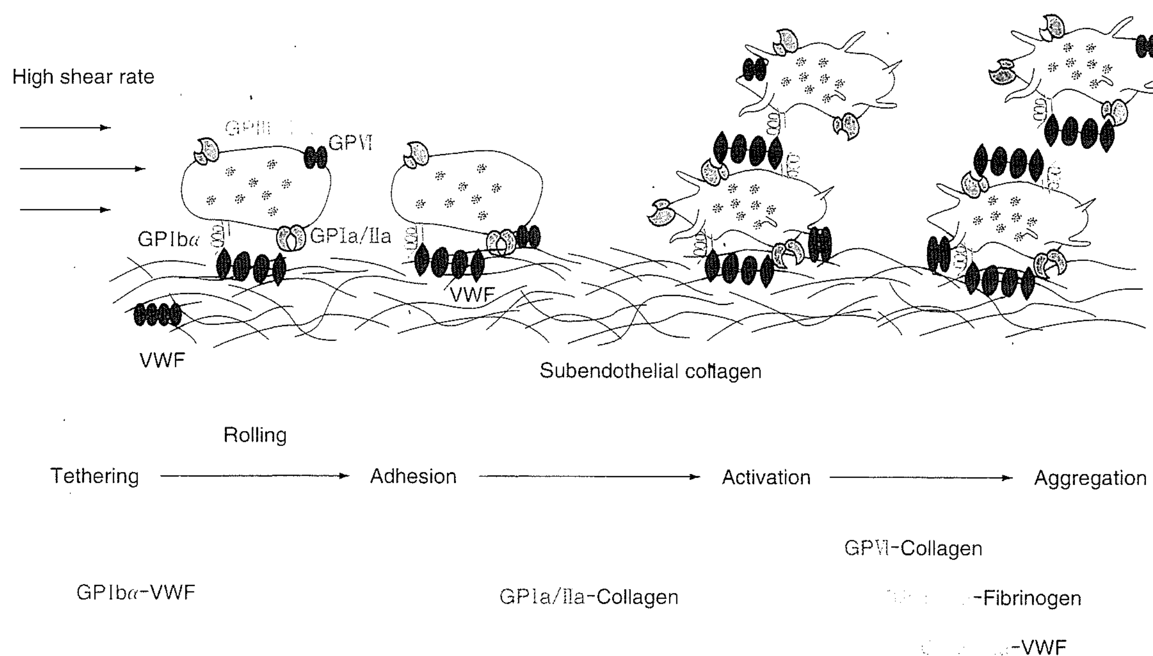


図3　動脈血栓形成過程

GP：Glycoprotein　　VWF：von Willebrand Factor　　（文献10より改変引用）

る．また，凝固第Ⅶa因子が凝固第Ⅶ因子を活性化させるという報告もある．

外因系凝固反応の阻害制御は，組織因子系凝固インヒビター(tissue factor pathway coagulation inhibitor：TFPI)が重要な働きを有している．TFPIは凝固第Xa因子，凝固第Ⅶa因子，組織因子と複合体を形成することにより凝固反応を阻害する[6]．アンチトロンビンⅢ(ATⅢ)はヘパリンを補因子として，血液凝固系の活性化の結果生じたトロンビン，凝固第Xa因子，あるいは凝固第Ⅸa因子の作用を阻害する．プロテインCはトロンビンと血管内皮細胞上のトロンボモジュリンとの結合により活性化され，活性化プロテインC(APC)となる．APCはプロテインSを補酵素としながら第Ⅴ因子や凝固第Ⅷ因子を特異的に分解，失活化することで凝固系を抑制する[7]．

凝固反応の最終段階の主要な反応はトロンビンによるフィブリノゲンからフィブリンへの反応である．フィブリンはプラスミンにより分解を受けるが，この反応における制御機序で主たる役割を有するのはプラスミノゲンアクチベータインヒビターである．血栓形成過程に深く関係している種々の炎症性の刺激や虚血刺激でPAI-1の産生亢進が起こり血栓を招来する[8]．

動脈血栓形成の主役となる血小板活性化

動脈での血栓形成は動脈硬化性のプラークの破綻が大きな引き金となる．プラーク形成の初期は脂肪敵を含有した泡沫細胞と平滑筋細胞が存在する特徴がある．ここに内皮細胞傷害とそれに引き続く，脂質と単球マクロファージの侵入，そして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湿潤→マトリックスメタロプロテアーゼの放出→コラーゲン溶解→プラーク破綻，と理解されている[5]．他にプラーク内のカルシウム蓄積や菲薄化した繊維性被膜，血小板の活性化(後述)に伴って発現する炎症性物質や，活性化した血小板から放出される顆粒内物質もプラーク破綻の機序に主要に関与している[9]．

血小板は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，あるいはそのコラーゲンに粘着したvon Willbrand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起き，動脈血栓形成がはじまる．この過程では血小板の膜受容体の膜糖蛋白(glycoprotein：GP)，GPIb/IX/V複合体やGPIa/IIa ($\alpha_2\beta_1$インテグリン)は初期過程，GPVIやGPIIb/IIIa (αIIbβ_3インテグリン)は後期過程でそれぞれ中心的役割を持つ[2)10)]．図3に示すように，内皮下組織のコラーゲンに粘着したVWFはGPIb/IX/V複合体と反応する(可逆性の粘着，tetheringと呼ばれている)．この刺激でGPIa/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GPIIb/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[11)12)]．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2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．

おわりに

血栓形成の分子機構において主たる役割を有する血液凝固反応の制御と，血小板活性化の分子機構に焦点をおいて概説した．主要な血栓形成機序は解明されてきているが，この分子制御機構は加齢や疾患といった環境因子や遺伝要因も関与しており非常に複雑である．今後，これら環境因子や遺伝因子を視野にいれた血栓形成機序の研究の進展に大きく期待がかかっている．

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