アスピリンの抱える問題点　1
アスピリン抵抗性とそのメカニズム

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● はじめに

アスピリンの二次予防に対する有効性については，ほぼ確立されたといっても過言ではない。血栓塞栓症のハイリスク患者（脳梗塞や一過性脳虚血発作の既往をもつ患者，心筋梗塞患者，その既往をもつ患者など）をランダムに割り付けた試験のメタ解析[1]において，アスピリン投与群（29,652名）では，追跡2年間でのイベント（心筋梗塞，脳梗塞，致死血管障害）の発生割合は12.9%であり，コントロール群（29,743名）では16.0%であったと報告されている。したがって，これら患者群に対する二次予防として，アスピリンは23%のオッズ減少，19%の相対リスク減少，3.1%の絶対リスク減少が認められることとなる。

しかしながら，これらの事実を裏返しにみてみると，アスピリンは，血栓塞栓症のハイリスク患者でのイベント再発を4/5以上（81%）抑えることができない。また，血栓塞栓症のハイリスク患者の8人に1人（12.9%）は，2年間にアスピリンを飲んでいるにもかかわらずイベントを発症したこととなる。このような事実関係から，「なぜ，アスピリンでイベントの再発を防ぐことができない患者群が存在するのか」と疑問をもつことから始まり，アスピリンの抗血小板効果には個人差が認められるとの報告[2~4]をもとに，アスピリン抵抗性（aspirin resistance）という疾患概念が生まれてきた。ではアスピリン抵抗性とは，どのように定義され，どのような特徴をもつ疾患なのであろうか。

● アスピリンの効果判定

抗凝固薬であるワルファリンは，その効果を患者個々でPT-INRを測定することで判定し，投与量を調節して用いられる。また，未分画ヘパリンはaPTTもしくはACTを測定し，その結果にて投与量が調整されることが多い。ヘパリンに対しては，アンチトロンビン，ヘパリンコファクターII，TFPIの低下や，凝固第VIII因子，顆粒球エラスターゼ，血小板第4因子の増加などが原因となり，ヘパリンの効果が減弱するヘパリン抵抗性（heparin resistance）の存在が知られている。ワルファリンに関しても，近年その効果に対する責任遺伝子が明らかにされ，そのSNPがワルファリンの効果と相関するとの報告がなされている[5]。したがって，アスピリンについても，その効果に個人差があり，責任候補遺伝子が関与している可能性があると考えることは自然な流れであろう。現在，アスピリンは体重や患者の状態にかかわらず一定量（たとえば，バイアスピリン® 100 mg 1錠投与）で治療がなされることが多いが，患者個人に適した量ならびに抗血小板薬の投与を行うことで，さらに抗血小板治療効果が増加する可能性があると考えることは不自然ではない。

ここでの大きな問題は，抗血小板薬の効果に

ついて現時点で有効にモニタリングする方法が存在しないことにある。過去にときとしてその効果判定に用いられた出血時間は，出血のリスクを予知する指標とはなり得ないと報告されており[6]，実際，出血時間を検査項目から削除している施設も多い。また，吸光度法による血小板凝集能検査は，①血小板富血漿（PRP）を作製する必要がある，②ADP（アデノシン二リン酸）やコラーゲンなど，アゴニストに依存した部分的な血小板機能を測定しているにすぎない，③血小板がわずかに活性化されて小さい凝集塊ができている段階では検出できず，大きな凝集塊になってはじめて検出される，などの問題が存在し，本来，先天性血小板機能異常症など血小板機能が極度に低下しているような疾患の定性的診断のために開発されたものであり，抗血小板薬の効果の評価などの定量的評価には向いていないとの指摘[7]もある。したがって，現時点で抗血小板効果をモニタリングできる適切な評価方法が存在しないことが，アスピリン抵抗性という疾患概念を複雑にしている。

● アスピリン抵抗性の臨床に与えるインパクト

最近，次々とアスピリン抵抗性の患者予後への関与を指摘した報告がなされている。

アスピリン治療（325 mg/日を7日以上）を受け，ほかの抗血小板薬を服用していない症状が安定した循環器疾患患者325名を前向きに検討した研究報告がある[8]。冠動脈造影で60%以上の狭窄が証明されている患者，心筋梗塞，脳梗塞の既往がある患者，血行再建術を受けている患者を対象に，外来もしくは心臓カテーテル検査目的で来院した患者を連続的に登録し，追跡（追跡期間は679±185日）した報告である。アスピリン抵抗性は，血小板凝集計を用い，10 μM のADP凝集で70%以上ならびに0.5 mg/mLのアラキドン酸凝集で20%以上の凝集を認めた患者として定義され，5.2%の患者がアスピリン抵抗性と診断された。アスピリン抵抗性の危険因子として，女性である，喫煙していない，高齢であることが指摘されている[9]。この試験の主要評価項目として，死亡，心筋梗塞，24時間以上続く症候性脳血管障害の複合エンドポイントが設定され，アスピリン抵抗性群は，アスピリン感受性群と比較して，上記複合エンドポイントに達した割合が高かった（24% vs 10%，ハザード比3.12　95%信頼区間1.10～8.90）。また，層別多変量解析により血小板数，年齢，心不全，アスピリン抵抗性が独立した危険因子（アスピリン抵抗性のハザード比4.14，95%信頼区間1.42～12.06）であったと報告されている。

カナダにおけるHOPE研究（The Heart Outcomes Prevention Evaluation Study）のコホート内ケースコントロール研究[10]として，5年間のフォローアップ期間中に，アスピリン投与を受けているにもかかわらず，心筋梗塞，脳梗塞，致死血管障害を起こした患者488症例の尿中11-デヒドロトロンボキサン B_2（11-$DTXB_2$）を測定し，アスピリン抵抗性を定義した報告がある。488名の年齢と性別をマッチさせたコントロール（アスピリンを服用し，心筋梗塞，脳梗塞，致死血管障害を起こさなかった患者）を抽出した。患者背景の調整後に，尿中11-$DTXB_2$値を四分位（quartile）に分けた場合，心筋梗塞，脳梗塞，致死血管障害の複合エンドポイントに達した患者は，尿中11-$DTXB_2$値が高くなるにつれて，その割合が増加した。また，尿中11-$DTXB_2$値を四分位に分けていちばん高い群は，いちばん低い群と比較して複合エンドポイントに対するオッズ比は1.8（95%信頼区間1.2～2.7）であり，心筋梗塞のオッズ比2.0（95%信頼区間1.2～2.7），致死血管障害のオッズ比3.5（95%信頼区間1.7～7.4）であったと報告されている。

経皮的カテーテルインターベンション（PCI）施行患者に対するアスピリン抵抗性の関与を指摘した報告も存在する[11]。アスピリン（80～325 mg/日）を7日間以上服薬している緊急でないPCI施行予定患者151名を対象とした報告である。すべての患者に，PCI施行12時間から24時間前に導入量としてクロピドグレル300 mgが投与され，維持量として75 mgがPCI施行日の朝に投与された。アスピリンの反応性は，

Ultra Rapid Platelet Function Assay-ASA を用いて評価され，アスピリン反応単位（aspirin reaction unit）550 以上をアスピリン抵抗性と定義し，550 以下のアスピリン感受性群と比較検討した。アスピリン抵抗性は 29 名（19.2%）で，女性に多い傾向がみられた。PCI 後に CK-MB 上昇（16 U/L 以上）を示した症例は，アスピリン抵抗性群では 51.7%，アスピリン感受性を示した患者では 24.6%であった（$p=0.006$）。PCI 後にトロポニン I の上昇（2.0 ng/mL 以上）を示した症例は，アスピリン抵抗性群で 65.5%，アスピリン感受性群では 38.5%であった（$p=0.012$）。多変量解析の結果，CK-MB 上昇の独立危険因子は，アスピリン抵抗性（オッズ比 2.9，95%信頼区間 1.2〜6.9），分岐部病変（オッズ比 2.8，95%信頼区間 1.3〜6.0）の二つであったと報告されている。

心臓外科周術期へのアスピリン抵抗性の関与を指摘した報告もある[12]。冠動脈バイパス術（CABG）95 名を対象とした試験である。患者は，CABG の 1 週間から 10 日前にアスピリン投与を中止し，術後 1 日目から再開（100 mg）された。術後 1 日，5 日，10 日目に血小板機能（アラキドン酸凝集ならびに P-セレクチンの発現を観察）が測定された。CABG 術前と比較して，術後には，アスピリンのアラキドン酸凝集，P-セレクチン発現に対する抑制効果が，有意に減弱していた。10 日目でもその回復は十分ではなかったと報告されている。この事実は，CABG においては抗血小板薬の効果が十分でないというメタ解析の結果[1]を反映しており，CABG によるシクロオキシゲナーゼ（COX）-1 活性への影響によるアスピリン抵抗性によって説明できる可能性が示唆された。

● アスピリン抵抗性のメカニズム

しかし，上述した臨床的アウトカムとアスピリン抵抗性を検討した報告では，さまざまな測定方法を用いてアスピリン抵抗性を定義しており，いまだ普遍的な定義，評価方法が定められていない。また，アスピリン抵抗性のメカニズムについては，いまだ明らかにされていない。アスピリン抵抗性のメカニズムについて現時点で指摘されている主要な要因を**表 1** に示す[13]。

遺伝子多型とアスピリン抵抗性との関係を指摘した報告がある。アスピリンは，COX-1（prostaglandin endoperoxide G/H synthase）の Ser530 をアセチル化することにより，アラキドン酸のプロスタグランジン H_2（PGH_2）への変換を阻害し，その結果，強力な血小板活性化アゴニストであるトロンボキサン A_2（TXA_2）産生を抑制する。したがって，アスピリン抵抗性に影響を与える候補遺伝子は COX-1 となる。

COX-1 遺伝子の多型のうち，−842A>G と 50C>T（P17L 変異）は連鎖不平衡しており，このヘテロ接合体者の血小板は，アスピリンによるアラキドン酸から PGH_2 生成の抑制率が高かったとの報告がある[14]。この多型のアレル頻度は欧米人 38 人では 11%であった。次いでアイルランドから，アスピリン服用者 144 名を対象に行った研究が報告された[15]。この研究では，アラキドン酸による血小板凝集能が 20%以上残存している人は，−842A>G ヘテロ体に多いと報告されている。アイルランドでの G アレル頻度は 6%であった。−842A>G と P17L で構成されるハプロタイプがアスピリン抵抗性にどの程度寄与するかを明らかにする大規模研究の結果がまたれる。

アスピリンの投与量について，副作用やアスピリンジレンマの観点からできるだけ低用量が望ましいということになり，最近では 100 mg 前後の投与量が推奨されている。EBM の観点からみても，前述のメタ解析[1]で 75〜150 mg のアスピリンがもっとも効果的であることが示されている。しかしアスピリン抵抗性の存在が問題になるにつれて，投与量についても再考の余地があることが指摘されている。アスピリンを服用するとごく少量から COX 阻害作用が出現することが指摘されているが，用量依存性についての検討もなされている。Lee ら[16]は VerifyNow Aspirin という測定計を用いて血小板機能の評価を行い，アスピリン投与量と血小板機能を調べたところ，80〜100 mg の投与量ではアスピリン抵抗性を示す症例が 30%程度存在し

表 1　アスピリン抵抗性のメカニズム

1．アスピリンのバイオアベイラビリティ
- 服薬不全
- アスピリン量の不足
- サリチル酸の蓄積によるアスピリンの COX-1 結合部位への結合の妨害
- 非ステロイド性抗炎症薬の同時服用によるアスピリンの効果の妨害
- プロトンポンプ阻害薬によるアスピリンのバイオアベイラビリティの減少

2．血小板機能
- 血小板の代謝によるアスピリンにさらされていない新しい血小板の血中への出現
- 新しく形成された血小板中の COX-2 の発現量の違い
- ADP とコラーゲンへの血小板感受性の増強

3．遺伝子多型
- 血小板コラーゲン受容体の遺伝子多型
- COX-1，COX-2，TXA_2合成酵素，アラキドン酸代謝酵素の遺伝子多型
- 血小板フィブリノーゲン受容体 GPIIb/IIIa 遺伝子多型
- 低用量アスピリンによる FXIII 活性化の阻害の差異につながる FXIII Val34Leu 遺伝子多型

4．他の血球細胞や細胞由来産物と血小板の相互作用
- 赤血球による血小板活性化の不十分な阻害
- アスピリン処理された血小板と血管細胞の間に起こるアラキドン酸代謝の細胞間の移動
- 単球-マクロファージ由来 TXA_2
- 血小板 TXA_2 の制御因子として，COX-1/COX-2 が生成する血管内皮細胞から放出される PGI_2，もしくは血管内皮からの tPA の放出

5．他の因子
- 過度の運動や心理的ストレスによるノルエピネフリン量の増加
- 喫煙
- 酸化ストレスとアラキドン酸非酵素的な過酸化によって生成する活性をもつ 8-iso-$PGF_{2\alpha}$ の生成
- アスピリンとアセチルコリンによって生じる一酸化窒素（NO）の抗血小板作用と血管拡張作用の相互作用

文献 13 から引用

たが，150 mg では 17%，300 mg では 0%と用量依存性にアスピリン抵抗性を示す症例が少なくなっていることを報告している。この報告は断面調査であり，用量依存性に個人差があるのかどうかを調べる必要があるが，アスピリン抵抗性の原因を考えるうえで興味深い報告である。

● アスピリン抵抗性は本当に存在する？

しかしながら，アスピリン抵抗性に関して，その存在を疑う報告もある[7]。その大きな理由として，*in vivo* における血小板機能を反映する *in vitro* もしくは *ex vivo* 血小板機能測定系はいまだ存在しないため，普遍的に認められた診断上のカットオフ値が存在しない。すなわち，アスピリン抵抗性の診断基準が確立できていないことが指摘されている。また，アスピリンに限らず，どんな薬剤でもすべての患者に有効であるわけではない。したがって，アスピリン抵抗性と定義せずに“treatment failure”（治療不成功）とすべきで，特別な疾患として分類する必要はないと指摘している。さらに，アスピリン服薬のコンプライアンスを確認する必要があること，また，血栓塞栓症は血小板機能のみに依存するのではなく，さまざまな要因に依存すること，アスピリンは血小板凝集に関わる刺激伝達系のたった一つの経路を抑えるにすぎないことなどの理由で，アスピリンに過大な期待をもつこと自体が誤りであるとしている。したがって，現時点で血小板機能を測定して，その結果をもってアスピリン治療に介入することを臨床現場で行うべきではないと強調している。

さいごに

われわれは，これらアスピリン抵抗性に関する問題，実態を明確にするために，多施設共同前向きコホート研究「アスピリン抵抗性の実態ならびにその遺伝子背景に関する研究」(The Study on Profile and Genetic factors of Aspirin Resistance：ProGEAR Study) を，全国 20 施設の協力のもとに開始した[17]。この試験は，脳梗塞/TIA および急性冠症候群の二次予防としてアスピリンの投与を受けている長期服薬患者 1000 症例の登録を目標に，3 種類の血小板機能検査，2 種類の COX-1 機能測定を測定項目とし，登録後 2 年間の血栓塞栓症の発症を追跡する試験である。同時に，その遺伝子背景を COX-1，COX-2 遺伝子を中心として解析する予定である。

本邦でのアスピリン抵抗性の実態ならびにその背景因子について明らかにできるものと期待している。

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PRECLINICAL STUDY

Dependence of Platelet Thrombus Stability on Sustained Glycoprotein IIb/IIIa Activation Through Adenosine 5′-Diphosphate Receptor Stimulation and Cyclic Calcium Signaling

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OBJECTIVES We sought to evaluate the mechanisms that support the stability of platelet aggregates on a thrombogenic surface exposed to flowing blood.

BACKGROUND Activation of the membrane glycoprotein (GP) IIb/IIIa—mediated in part through the $P2Y_1$ and $P2Y_{12}$ adenosine 5′-diphosphate (ADP) receptors—is necessary for platelet aggregation. Platelets in growing thrombi exhibit cyclic calcium signal, suggesting that sustained activation may be required for thrombus stability.

METHODS Blood was perfused over type I collagen fibrils at the wall shear rate of 1,500 s^{-1}. Three-dimensional visualization of platelet thrombi was obtained in real time with confocal microscopy. The intracytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in fluo-3AM–loaded platelets.

RESULTS The height of platelet thrombi in control blood was 13.5 ± 3.3 μm after 6 min, and increased to 16.3 ± 4.5 μm (n = 8) after an additional 6 min. In contrast, the height was reduced to 5.4 ± 2.2 and 3.3 ± 1.3 μm, respectively ($p < 0.01$, n = 8), when the blood used in the second 6-min perfusion contained a $P2Y_1$ (MRS2179) or $P2Y_{12}$ (AR-C69931MX) inhibitor. The $[Ca^{2+}]_i$ of platelets within forming thrombi oscillated between 212 ± 38 nmol/l and 924 ± 458 nmol/l, with cycles lasting 4.2 ± 2.8 s that were inhibited completely by AR-C69931MX and partially by MRS2179. Accordingly, thrombi became unstable upon perfusion of blood containing the Ca^{2+} channel blocker, lanthanum chloride. Flow cytometric studies demonstrated that AR-C69931MX, MRS2179, and lanthanum chloride reduced monoclonal antibody PAC-1 binding to platelets, indicating a decrease of membrane-expressed activated GP IIb/IIIa.

CONCLUSIONS Continuous $P2Y_1$ and $P2Y_{12}$ stimulation resulting in cyclic $[Ca^{2+}]_i$ oscillations is required for maintaining the activation of GP IIb/IIIa needed for thrombus stability in flowing blood. (J Am Coll Cardiol 2006;47:155–62) © 2006 by the American College of Cardiology Foundation

Arterial thrombosis may initiate after the rupture of an unstable atherosclerotic plaque, and it involves multiple platelet adhesion and agonist receptors (1) as well as activation of clotting with fibrin deposition (2,3). Two adenosine 5′-diphosphate (ADP) receptors, $P2Y_1$ and $P2Y_{12}$, mediate platelet stimulation induced not only by exogenous ADP (4–6), but also by shear forces or interactions with extracellular matrixes that cause ADP release from storage granules (7–9). In particular, $P2Y_{12}$ concurs to the stability of platelet aggregates (10) and may exert a similar effect in developing arterial thrombi (11). In experimental studies, platelets that adhere and aggregate onto immobilized von Willebrand factor (VWF) exhibit cyclic oscillations in intracytoplasmic Ca^{++} concentration ($[Ca^{2+}]_i$) (12). These have been shown to depend on the concurrent function of $P2Y_{12}$ and glycoprotein (GP) IIb/IIIa (integrin $\alpha_{IIb}\beta_3$), and are linked to the recruitment and activation of flowing platelets into growing thrombi (12). Such findings, and the results obtained with a thrombosis model in $P2Y_{12}$-deficient mice (13), suggest that continuing stimulation of ADP receptors may be required to initiate as well as propagate thrombus growth on damaged vascular surfaces. Experiments with selective inhibitors have confirmed the importance of $P2Y_{12}$ in this regard (12), while $P2Y_1$ may be key for the initial activation of ADP-stimulated platelets (14) but have no role in thrombus propagation. In the present study, we have used a fibrillar type I collagen surface exposed to flowing blood and specific antagonists of the ADP receptors and GP IIb/IIIa to evaluate how aggregating platelets are incorporated irreversibly into a thrombus. Our findings may contribute to

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Abbreviations and Acronyms

ADP	= adenosine diphosphate
$[Ca^{2+}]_i$	= intracytoplasmic Ca^{2+} concentration
FITC	= fluorescein isothiocyanate
GP	= glycoprotein
PPP	= platelet-poor plasma
PRP	= platelet-rich plasma
VWF	= von Willebrand factor

clarifying the mechanism of action of antithrombotic agents.

METHODS

Blood samples. Venous blood was obtained from medication-free volunteers (6 men, 2 women; age 28 to 43 years) with their informed consent, and transferred into plastic tubes containing 1/10 volume of the thrombin inhibitor Argatroban (Mitsubishi Kagaku, Tokyo, Japan) to yield a final concentration of 100 μmol/l (15,16), which does not decrease the plasma divalent cation concentration. Platelet-rich plasma (PRP) was separated by centrifugation at 100 *g* for 15 min and platelet-poor plasma (PPP) by further centrifugation at 800 *g* for 10 min. The platelet count in PRP was adjusted to $3 \times 10^5/\mu l$.

Reagents. AR-C69931MX (17) was from AstraZeneca (Loughborough, Leicestershire, United Kingdom). MRS2179 (18) was obtained from Dr. Savi (Sanofi-Synthelabo Recherche, Toulouse, France). Lanthanum chloride (19), acid insoluble fibrillar collagen type I from bovine Achilles tendon, mepacrine (quinacrine hydrochloride), acetyl salicylic acid, ADP, and epinephrine were from Sigma Chemical Co. (St. Louis, Missouri). Tirofiban (Aggrastate) was from Merck & Co. (Allentown, Pennsylvania). Fluo-3 acetoxymethyl ester (Fluo-3AM) was from Molecular Probe (Eugene, Oregon).

Measurement of thrombus volume. Platelets were rendered fluorescent by adding 10 μmol/l mepacrine (16,20) or 1 μg/ml of the fluorescein isothiocyanate (FITC)-labeled Fab fragment of the anti-GP IIb/IIIa monoclonal antibody, YM337 (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan). Thrombi of similar size were obtained in either case (not shown). A rectangular flow chamber with type I collagen fibrils coated on the glass bottom (15,16,20) was assembled onto the stage of an inverted epifluorescence microscope (Leica, Germany). Blood was aspirated through the chamber with a syringe pump (Harvard Apparatus, Holliston, Massachusetts) at a constant flow rate to yield a wall shear rate of 1,500 s^{-1}. Images were digitized on-line with a color CCD video camera (L-600, Leica, Germany). Thrombus growth was evaluated in two dimensions by measuring the surface area covered by platelets (16) and in three dimensions by confocal microscopy (Fig. 1) as previously reported (15). The effect of inhibitors of platelet function on the stability of platelet thrombi was tested in two-stage experiments, consisting in the perfusion of untreated blood for 6 min followed by blood containing or not a test substance for an additional 6 min.

Measurement of $[Ca^{2+}]_i$. Platelets in PRP were incubated for 30 min at 37°C with fluo-3AM (8 μmol/l), then mixed with erythrocytes separated from the same blood and

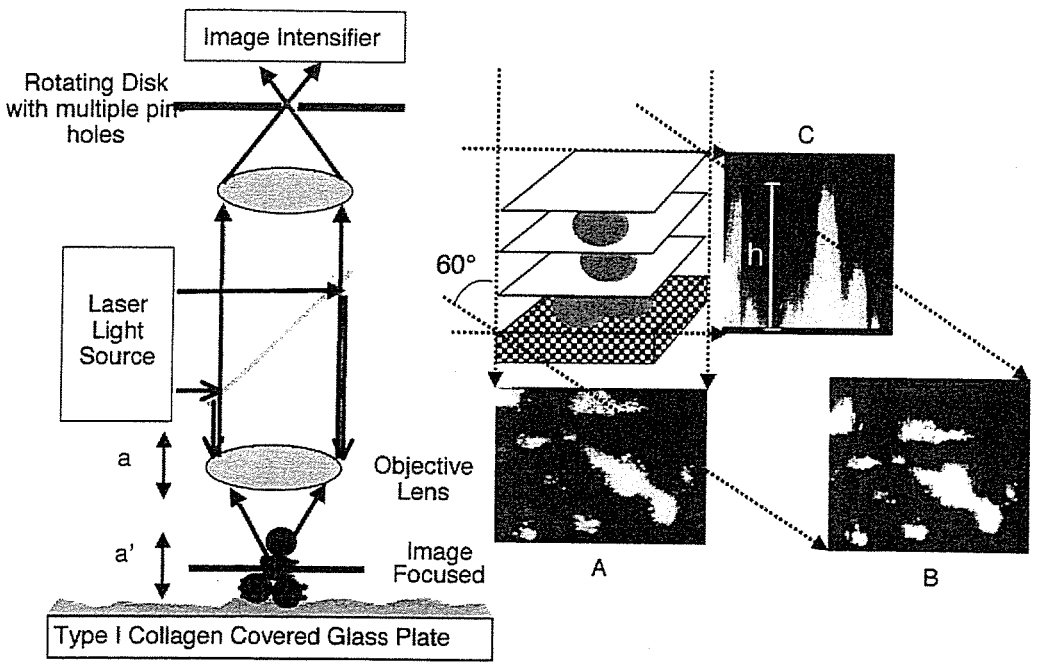

Figure 1. Three-dimensional projection imaging of platelet thrombi. A piezo-electric motor (**a**) moved the objective lens at a constant speed of 0.4 μmol/l/s to provide scanning images of the platelet thrombi (**a'**). The sum of the confocal images in a bottom to top stack (z-axis) was projected on planes at 10° intervals relative to the x axis to obtain the three-dimensional projection images shown on the **right**, including projections at 0 degrees (top view; **A**), 60° (**B**), and 90° (front view; **C**). The maximum height (**h**) of the platelet thrombi was calculated from the front view projection, as shown in **C**.

washed three times by centrifugation and resuspension in a buffer composed of 10 mmol/l HEPES, 140 mmol/l NaCl, pH 7.4 (HEPES buffer). The washed cells were resuspended in homologous PPP containing Argatroban (100 μmol/l) at a 40% hematocrit. Unlike previously suggested (21) but in agreement with recent reports (22), we did not use probenecid to prevent the leakage of fluo-3 because of its effects on platelet function (23). The $[Ca^{2+}]_i$ of 10 randomly selected platelets incorporated at different positions within a thrombus was measured using confocal microscopy. Variations in the fluorescence intensity of fluo-3AM were converted into $[Ca^{2+}]_i$ using the equation:

$$[Ca^{2+}]_i = Kd(F - Fmin)/(Fmax - F)$$

where Kd (495 nmol/l) is the dissociation constant of fluo-3AM for the interaction with Ca^{2+} (21); F is the measured fluorescent intensity of single platelets; Fmax is the fluorescence intensity of single platelets treated with the Ca^{2+} ionophore A23187 (10 μmol/l; Sigma) in the presence of 2 mmol/l Ca^{++}; and Fmin is the fluorescent intensity of unstimulated single platelets.

Flow cytometry. The platelet binding of FITC-conjugated PAC-1, a monoclonal antibody that selectively interacts with activated GP IIb/IIIa, was measured by flow cytometry (FACScan, Becton-Dickinson, San Jose, California). Platelets in PRP were activated with the combination of ADP and epinephrine (25 μmol/l each) or with the thrombin receptor activation peptide (1 mmol/l). Then, FITC-conjugated PAC-1 was added at a final concentration of 2.77 μg/ml, followed by HEPES buffer containing or not AR-C69931MX (100 nmol/l), MRS2179 (100 μmol/l), or lanthanum chloride (1 mmol/l). PAC-1 binding was measured 5, 10, 30, 45, and 60 min after addition of the last solution. All these experiments were performed under static conditions. The median fluorescence of 10,000 single platelets was calculated using the CellQuest software (Becton Dickinson Biosciences).

Statistical analysis. All numerical data are expressed as mean values ± SD unless otherwise specified. The effect of various concentrations of AR-C69931MX and MRS2179 on the surface coverage by platelets was tested by one-way analysis of variance. Differences between two groups of data were compared by Newman-Keuls test. A p value of <0.05 was considered to denote statistical significance.

RESULTS

$P2Y_1$ and $P2Y_{12}$ antagonists inhibit platelet thrombus growth. In agreement with previous results (7,8), ADP receptor antagonists inhibited thrombus growth on type I collagen fibrils exposed to blood flowing with a wall shear rate of 1,500 s^{-1}. Platelet surface coverage decreased from 38.0 ± 6.6% to 13.6 ± 3.9% after blocking $P2Y_{12}$ with 100 nmol/l AR-C69931MX, and to 19.4 ± 5.4% after blocking $P2Y_1$ with 100 μmol/l MRS2179 ($p < 0.01$). Both antagonists also inhibited thrombus volume (Fig. 2). Untreated blood perfused for 6 min formed multilayered thrombi with a height of 13.2 ± 2.3 μm (n = 8), which was reduced to a single layer of platelets with a height of 3.2 ± 1.1 μm by 100 nmol/l AR-C69931MX (n = 8). With blood containing 100 μmol/l MRS2974, the thrombus height was 6.1 ± 3.5 μm (n = 8), less than with untreated blood ($p < 0.01$) but more than with the $P2Y_{12}$ antagonist ($p < 0.01$).

Inhibitors of $P2Y_1$, $P2Y_{12}$, and Ca^{2+} channels reduce platelet thrombus size. Blood perfused over collagen type I fibrils for 6 min at the wall shear rate of 1,500 s^{-1} formed

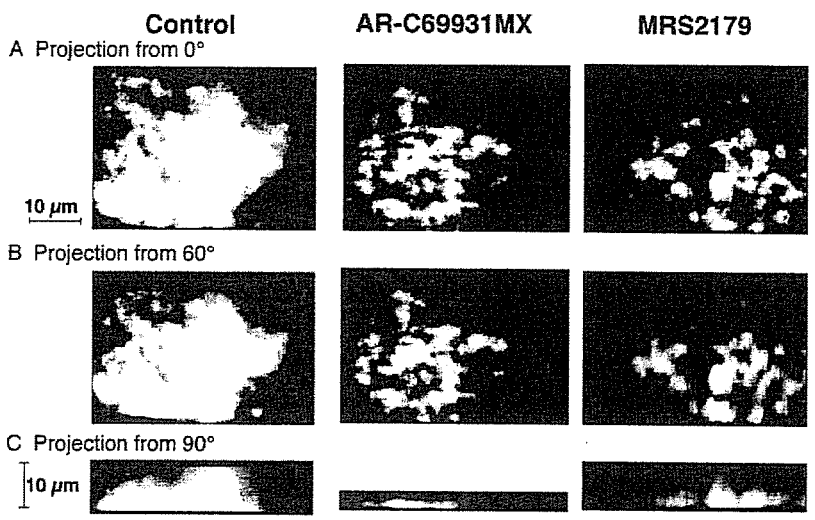

Figure 2. Three-dimensional projection images of thrombi formed in the presence or absence of adenosine diphosphate receptor antagonists. Blood with fluoresceinated platelets was perfused over immobilized collagen type I fibrils for 6 min at the wall shear rate of 1,500 s^{-1} in the absence (Control) or presence of MRS2179 (100 μmol/l) or AR-C69931MX (100 nmol/l), as indicated. The platelet thrombi were scanned in the z-axis by confocal microscopy, and the resulting images were projected on planes rotated around the x-axis at 10° intervals (please see the online version of this article for supplemental videos). The figure shows projection images from the top **(A)**, 60° **(B)**, and the front **(C)**. These images are representative of the results obtained in eight separate experiments.

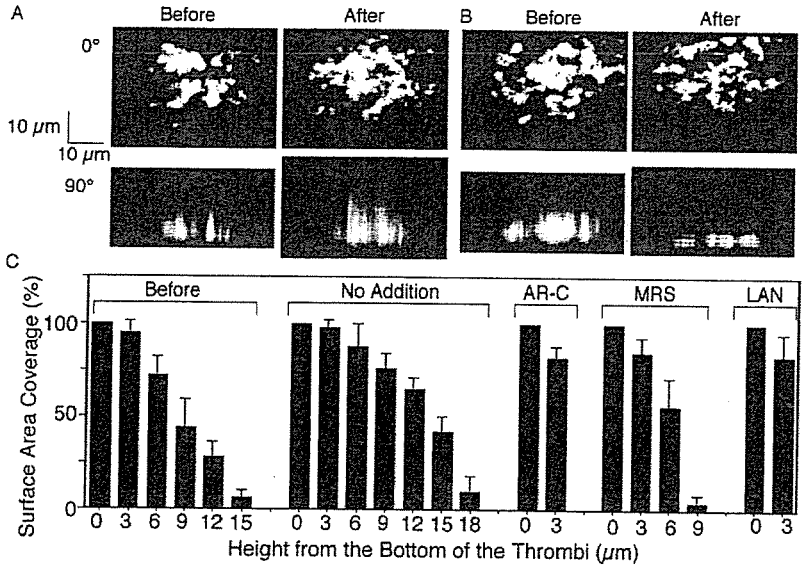

Figure 3. Reduction in the size of platelet thrombi exposed to blood containing different antagonists of platelet function. These experiments were performed as described in the caption of Figure 2, with the difference that the surface was exposed to two subsequent aliquots of blood each perfused for 6 min. **(A)** Representative images (0-degree and 90° projections) of platelet thrombi after perfusion of the first (before) or second (after) control blood aliquot (please see the online version of this article for supplemental videos). **(B)** Representative images of platelet thrombi after perfusion of the first control blood aliquot (before) or a second blood aliquot containing the $P2Y_{12}$ inhibitor AR-C69931MX (100 nmol/l) (please see the online version of this article for supplemental videos). **(C)** Cross-sectional area occupied by fluorescent platelets in horizontal planes passing through the thrombi at the indicated distance from the collagen surface, calculated as percentage of the area in the plane closest to collagen surface. The **bars labeled "Before"** show the area of thrombi formed after perfusion of untreated blood for 6 min. The **bars labeled "No Addition," AR-C, MRS, and LAN** show the area of thrombi remaining on the surface after an additional 6 min perfusion of untreated blood, or blood treated with the $P2Y_{12}$ inhibitor AR-C69931MX (100 nmol/l), or the $P2Y_1$ inhibitor MRS2179 (100 μmol/l), or the putative Ca^{2+} channel inhibitor lanthanum chloride (LAN) (1 mmol/l), respectively. Mean and SEM of eight experiments are shown.

platelet thrombi with a height of 13.5 ± 3.3 μm (n = 8; Fig. 3), increasing to 16.3 ± 4.5 μm after an additional 6 min. In contrast, when blood perfused in the second 6-min period contained 100 nmol/l AR-C69931MX, thrombus size progressively decreased until a single layer of adherent platelets with a height of 3.3 ± 1.3 μm remained. A similar effect, resulting in a 3.4 ± 1.2 μm high single layer of adherent platelets, was observed with the Ca^{2+} channel blocker, lanthanum chloride (1 mmol/l), whereas 100 μmol/l MRS2974 reduced thrombus height to 5.4 ± 2.2

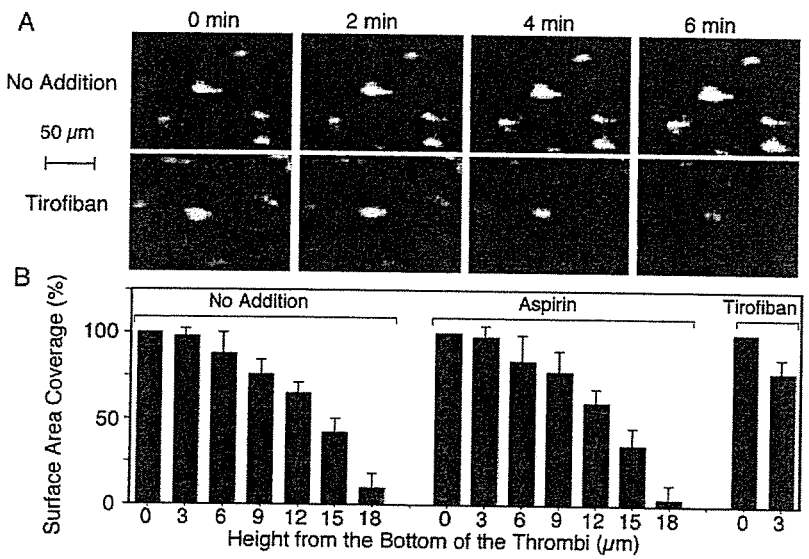

Figure 4. Changes in the two-dimensional and three-dimensional structure of platelet thrombi exposed to blood containing antiplatelet agents. These experiments were performed essentially as described in the caption of Figure 3. **(A)** Representative two-dimensional fluorescence microscopy images of platelet thrombi immediately after perfusion of the first control blood aliquot (0 min) or at different times after beginning the second perfusion with either untreated blood (No Addition) or blood containing tirofiban (0.5 μmol/l), as indicated (please see the online version of this article for supplemental videos). **(B)** Cross-sectional area of thrombi at the indicated distances from the collagen surface after perfusion of untreated blood for 6 min (No Addition), or after perfusion for an additional 6 min of blood containing aspirin (100 μmol/l) or tirofiban (0.5 μmol/l), as indicated. See Figure 3C for additional details.

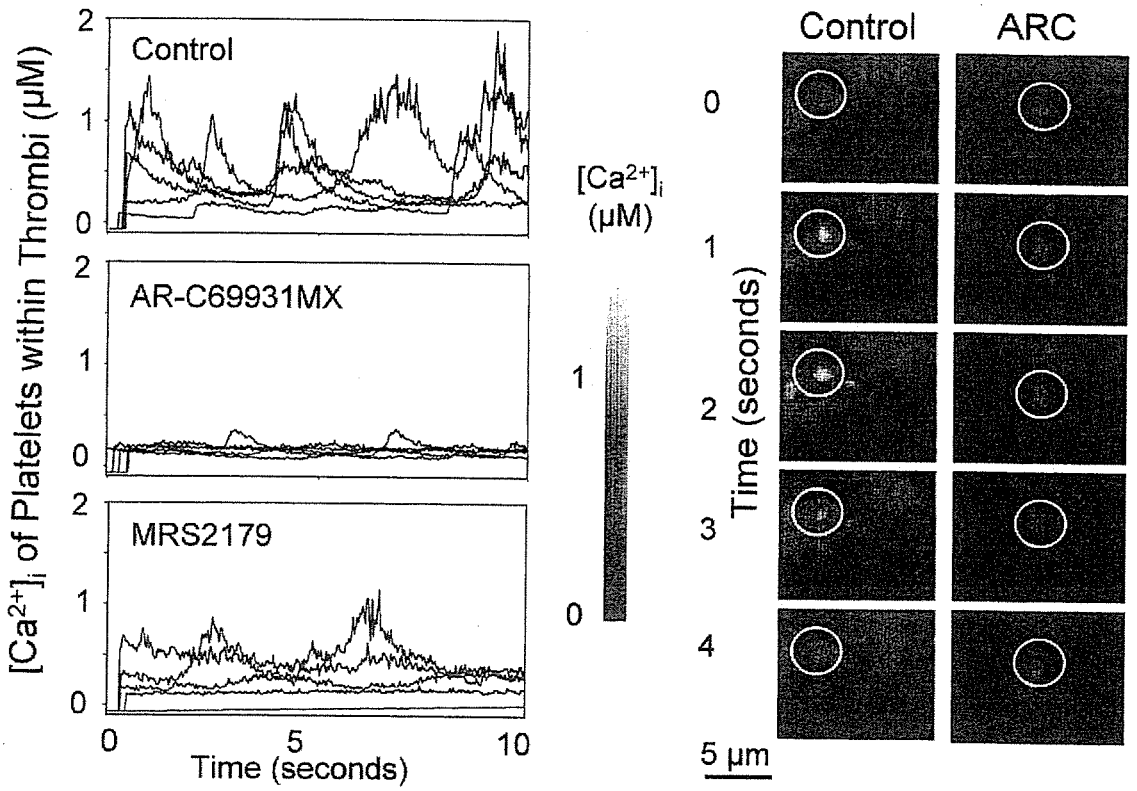

Figure 5. Changes in the intracytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) of platelet thrombi caused by adenosine diphosphate receptor antagonists. These experiments were conducted as described in the caption of Figure 3, except that blood was replaced with a cell suspension containing fluo-3AM–loaded platelets, washed erythrocytes, and homologous platelet-poor plasma with the specific thrombin inhibitor argatroban (100 μmol/l) as the anticoagulant. The cell suspension was perfused over type I collagen fibrils at the shear rate of 1,500 s^{-1} for 4 min to form platelet thrombi. Then, the same cell suspension without or with the addition of the $P2Y_{12}$ antagonist AR-C69931MX (100 nmol/l), or the $P2Y_1$ antagonist MRS2179 (100 μmol/l), was perfused for an additional 4 min. The $[Ca^{2+}]_i$ of platelets incorporated into thrombi was measured during the second perfusion. **(Left panels)** Intracytoplasmic Ca^{2+} concentration of five randomly selected platelets recorded for 10 s beginning 2 min after the start of the second perfusion. **(Right panels)** Images reflecting the concentration of Ca^{2+} ions in platelets within thrombi formed during perfusion of untreated blood (Control) or blood containing 100 nmol/l AR-C69931MX (ARC).

μm. The latter value was less than with untreated blood but more than with the anti-$P2Y_{12}$ antagonist ($n = 8$; $p < 0.01$ for both comparisons; Fig. 3). Addition of 0.5 μmol/l tirofiban, a GP II/IIIa antagonist (15), to the blood used in the second perfusion also reduced thrombi to a single layer of adherent platelets with a height of 3.1 ± 1.1 μm ($n = 8$; Fig. 4), whereas 100 μmol/l aspirin allowed continued growth to a height of 15.8 ± 5.5 μm ($n = 8$; Fig. 4).

Platelets within thrombi exhibited cyclic $[Ca^{2+}]_i$ oscillations (Fig. 5). The lowest average $[Ca^{2+}]_i$ was 212 ± 38 (SEM) nmol/l and the highest 924 ± 458 nmol/l, with a cycle length from peak to peak of 4.2 ± 2.8 s (Fig. 5). These $[Ca^{2+}]_i$ variations appeared to depend on specific ion channels because they were blocked by the Ca^{2+} channel antagonist lanthanum chloride (not shown). Addition of the $P2Y_{12}$ antagonist to the perfused blood rapidly decreased the platelet $[Ca^{2+}]_i$ within formed thrombi; after 2 min the value ranged between 182 ± 22 nmol/l and 244 ± 96 nmol/l without detectable cycles in most platelets (Fig. 5). After addition of the $P2Y_1$ antagonist, the low and high $[Ca^{2+}]_i$ values ranged between 192 ± 34 nmol/l and 558 ± 211 nmol/l, respectively, and some but not all platelets showed measurable cycles of 5.8 ± 2.5 s (Fig. 5). Neither tirofiban, in spite of the pronounced effect on platelet thrombus size, nor aspirin had any influence on the cyclic $[Ca^{2+}]_i$ of aggregated platelets (not shown).

Inhibition of $P2Y_1$ and $P2Y_{12}$, and blockade of Ca^{2+} entry reduce activated GP IIb/IIIa on platelets. Fluorescein isothiocyanate-conjugated PAC-1 binding to platelets, measured as the median fluorescence intensity of 10,000 platelets, increased when platelets were activated by the combination of ADP and epinephrine or the thrombin receptor activating peptide (the results were similar and only the former are shown). Bound PAC-1 slowly but significantly decreased in time when no additional exogenous agonist was added after the initial activation (Fig. 6). The reduction was more marked after adding the $P2Y_{12}$ inhibitor (Fig. 6), suggesting that continuous stimulation is necessary to maintain the active state of GP IIb/IIIa. A similar effect was observed with the $P2Y_1$ inhibitor or the Ca^{2+} channel blocker (Fig. 7), but not with aspirin (data not shown).

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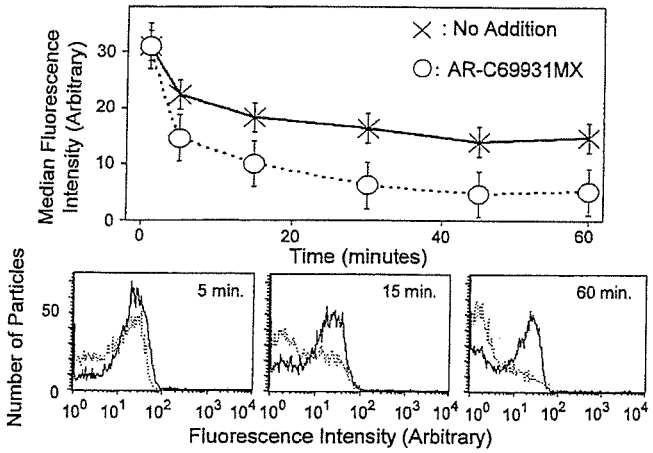

Figure 6. Changes in PAC-1 binding to activated platelets caused by adenosine diphosphate (ADP) receptor antagonists. To activate platelets, 25 μl of HEPES buffer containing ADP and epinephrine (400 μmol/l each) was mixed with 375 μl of platelet-rich plasma and incubated for 20 min. Fifty μl of fluorescein isothiocyanate-conjugated PAC-1 (25 μg/ml) was then added, and the fluorescence of individual platelets was measured 5, 15, 30, 45, and 60 min after the addition of 50 μl of HEPES buffer containing or not the $P2Y_{12}$ antagonist AR-C69931MX (100 nmol/l). **(Upper panel)** Mean and SEM of the median fluorescence of 10,000 platelets in eight experiments. **(Lower panels)** Representative flow cytometric results at selected time points in one experiment without **(solid lines)** or with **(dotted lines)** the addition of AR-C69931MX.

DISCUSSION

Our findings show that platelet thrombi growing on collagen may disperse several minutes after the initial aggregation when exposed to blood containing an ADP receptor antagonist or a putative Ca^{2+} channel blocker or a GP IIb/IIIa inhibitor. Thrombus stability, therefore, may depend on sustained ligand binding to activated GP IIb/IIIa, which in turn may be mediated by $[Ca^{2+}]_i$ elevations induced by the continuous stimulation of specific signaling pathways (Fig. 8). Our findings add to the concept of intercellular calcium communication, suggesting that it operates bidirectionally from platelets at the growing edge of a thrombus not only to activate newly recruited platelets (12) but also to ensure stability throughout the entire aggregate. Such a conclusion is based on the observation that perfusion of blood containing ADP receptor antagonists reduced the cyclic $[Ca^{2+}]_i$ increases within platelet thrombi even several minutes after the initial adhesive contacts had been established. Our experiments, however, could not define whether platelets at the center of thrombi differed in their Ca^{2+} responses from those at the outer edges. It has been proposed that cooperation between $P2Y_{12}$-mediated activation and GP IIb/IIIa engagement by ligand promotes the activation of newly recruited platelets in a thrombus (12). We observed, however, that blocking GP IIb/IIIa on the perfused platelets had no effect on the $[Ca^{2+}]_i$ oscillations within the thrombus even though it caused progressive disaggregation. Thus, the effect of GP IIb/IIIa inhibition on Ca^{2+} signals may reflect the need of maintaining newly recruited platelets in contact with adherent ones until activation is induced. In the case of platelets already aggregated into a thrombus, the replacement of an adhesive ligand by a GP IIb/IIIa antagonist may result in the detachment of platelets at the edge without effects on the activation of those still in the thrombus.

We propose that cyclic $[Ca^{2+}]_i$ increases within platelet thrombi reflect a mechanism that maintains GP IIb/IIIa activation and, thus, thrombus stability. The experiments with lanthanum, a putative Ca^{2+} channel blocker, support such a concept, but in this study we could not identify the ion channel involved or the signaling pathway regulating its function. Lanthanum, therefore, could have other effects, such as altering the ion binding sites of adhesive receptors and/or ligands (24) resulting in thrombus dissolution independently of Ca^{2+} channel blocking. Moreover, the cyclic $[Ca^{2+}]_i$ increases of aggregated platelets within thrombi could be a consequence, not the cause of, maintaining GP IIb/IIIa activation. In the alternative, the observation that $[Ca^{2+}]_i$ oscillations are linked to ADP receptor function may suggest an involvement of store-dependent Ca^{2+} entry (25–27) mediated by $P2Y_{12}$ simulation. From a technical viewpoint, changes in platelet volume could have affected the results obtained with fluo-3AM, a single-wave Ca^{2+} indicator, thus influencing our conclusions. It should be noted that we have attempted to minimize such possible effects by using ultra-fast confocal microscopy, and no cyclic platelet volume changes have been reported during thrombus growth. In the end, sustained platelet activation necessary for thrombus stability may involve various signaling molecules, including ephrin/eph kinases (28), regulated by

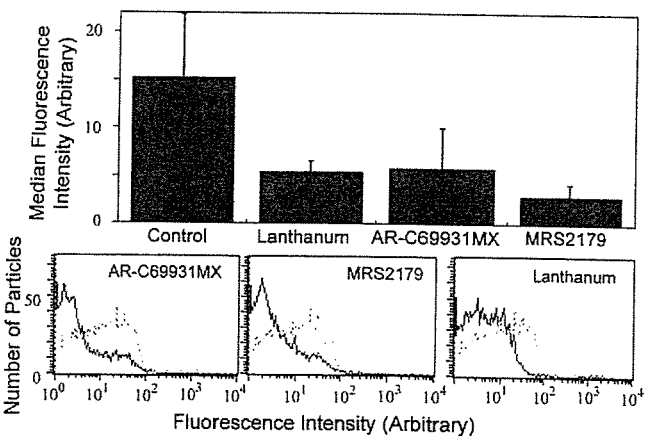

Figure 7. Effect of different platelet inhibitors on PAC-1 binding to activated platelets. These experiments were performed as described in the caption for Figure 6, with the only difference that the $P2Y_1$ inhibitor, MRS2179, or the putative Ca^{2+} channel blocker, lanthanum chloride, was also added to platelets after activation by adenosine diphosphate and epinephrine. The **upper panel** shows the mean and SEM of the median fluorescence of 10,000 platelets measured 30 min after addition of AR-C69931MX (final concentration: 100 nmol/l), MRS2179 (final concentration: 100 μmol/l), or lanthanum chloride (final concentration: 1 mmol/l) as compared to control in which only buffer was added (n = 8). The **lower panels** show the actual flow cytometric results of one representative experiment. **Solid lines** represent the results in the presence of the inhibitor shown in each panel, while **dotted lines** represent the results in the absence of inhibition.

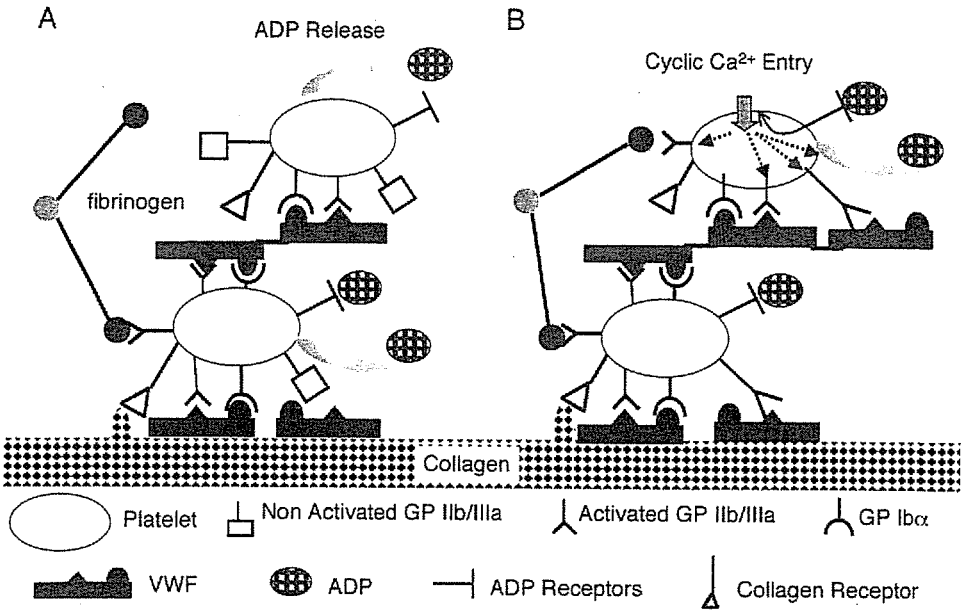

Figure 8. Schematic representation of the mechanism that stabilizes platelets at the edge of a growing thrombus exposed to elevated shear rates. (A) Circulating platelets adhere and become activated onto collagen through multiple adhesive interactions, initiated by glycoprotein (GP) Ibα binding to von Willebrand factor (VWF) under high shear rate conditions. Full activation depends on released adenosine diphosphate (ADP) and leads to the binding of soluble adhesive ligands such as VWF and fibrinogen. These form the new substrate for the recruitment of circulating platelets, again initiated under high shear rate conditions by GP Ibα-VWF binding. Adhesive interactions and soluble agonists present in the environment of the growing thrombus lead to activation of the newly recruited platelets and further ADP release. (B) Cyclic Ca^{2+} signaling induced by released ADP and mediated by $P2Y_1$ and $P2Y_{12}$ maintains GP IIb/IIIa activation necessary for the sustained binding of adhesive molecules and stability of the aggregate. The first layer of platelets interacting with the collagen surface may not require sustained ADP stimulation for stable adhesion.

platelet-platelet contacts at the upper edge of the growing thrombus.

Although there is agreement that $P2Y_{12}$ is involved in the calcium signaling that sustains activation, conclusions on the role of $P2Y_1$ are not univocal, perhaps because previous studies were focused on the activation of newly recruited platelets (12) while we analyzed the activation of platelets already incorporated within thrombi. The two processes may be distinct, or the discrepant results may be caused by methodological differences. For example, continuous rather than periodic monitoring of $[Ca^{2+}]_i$ cycles may better reveal the relatively weak effect of blocking $P2Y_1$. Moreover, others have reported that only the combined blockade of $P2Y_1$ and $P2Y_{12}$ can inhibit platelet thrombus formation on the surface of collagen (7), whereas we have shown previously (8) and confirm here that inhibition of either receptor is almost equally effective in doing so. This discrepancy may result from differences in experimental observation times, because we measured thrombus volume after several minutes when the stability may be more influenced by interplatelet communication and less by the effects of the platelet-collagen interactions at the base of the thrombus.

Perhaps the main limitation of an ex vivo blood perfusion model is the need to use an anticoagulant to preserve blood fluidity, which in turn prevents the generation of thrombin and, consequently, of fibrin, both likely to have a role in providing thrombus stability (3,29). Nonetheless, ADP-induced signaling pathways may contribute to the overall effects of other agonists, including thrombin, that lead to nucleotide release from storage granules after primary platelet stimulation. Moreover, the mechanism highlighted here may provide initial stability to aggregated platelets exposed to flowing blood and, through the procoagulant function of activated platelets (30), contribute to fibrin formation. Our results, therefore, provide new insights into the mechanism of action of antiplatelet agents used in clinical practice and currently targeted against the $P2Y_{12}$ receptor. In situations such as acute coronary syndromes or coronary interventions with elevated thrombotic risk, $P2Y_{12}$ inhibitors may be used in combination with anticoagulants such as heparin, suggesting that an experimental model in which thrombin activity and fibrin formation are inhibited may be representative of at least some clinical conditions.

Conclusions. Our studies, although directly relevant only for thrombus formation on collagen fibrils under artificial blood flow conditions, suggest a possible role for distinct ADP receptors and their operating calcium signals in sustaining the long-term GP IIb/IIIa activation required to maintain platelet aggregate stability before the occurrence of fibrin generation. Such a mechanism may provide a more comprehensive understanding of the pharmacological effects of anti-$P2Y_{12}$ drugs, and suggest novel strategies to achieve the dispersion of platelet thrombi after they are formed.

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APPENDIX

For the supplemental videos, please see the online version of this article.

Journal of Thrombosis and Haemostasis, 3: 114–120

ORIGINAL ARTICLE

Co-localization of von Willebrand factor with platelet thrombi, tissue factor and platelets with fibrin, and consistent presence of inflammatory cells in coronary thrombi obtained by an aspiration device from patients with acute myocardial infarction

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Summary. *Background:* Detailed histochemical analysis of coronary thrombi obtained freshly from acute phase of myocardial infarction patients may provide information necessary to understand the mechanism of coronary occlusive thrombus formation. *Methods and Results:* Coronary thrombi causing myocardial infarction were obtained from 10 consecutive patients of myocardial infarction in the acute phase, using a newly developed aspiration catheter. All the fixed specimens of coronary thrombi, by hematoxylin and eosin staining, were found to contain three major constituents, namely, platelets, densely packed fibrin and inflammatory cells, including polymorphonuclear and mononuclear cells, although their distribution in each specimen is totally heterogeneous. Immunohistochemical staining revealed the prominent presence of von Willebrand factor (VWF) at the sites of platelet accumulation, presence of tissue factor and platelets at the sites of deposition of fibrin fibrils. It also revealed the presence of CD16-, CD45- and CD34-positive cells, yet the functional roles of these cells have still to be elucidated. There are weak positive correlation between the number of inflammatory cells involved in the unit area of coronary thrombi specimen and the time of collection of the specimens after the onset of chest pain. *Conclusions:* In spite of various limitations, our results contain information suggesting the possible role of VWF in platelet-thrombus formation, possible important role played by tissue factor and activated platelets in the formation of fibrin fibrils, and the positive relationship between inflammatory cells migration and the formation of occlusive thrombi in human coronary arteries.

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Introduction

Clinical experience with the use of antiplatelet [1,2] and anticoagulant agents [3] in the prevention of acute myocardial infarction along with the findings demonstrating the role of inflammatory maker as a predictor for its onset [4–6] suggests the important roles played by platelets, coagulation cascade and inflammation, in the formation of coronary thrombi; however, the exact mechanism of coronary thrombus formation, especially the mechanism of platelet and inflammatory cell accumulation and fibrin deposition under conditions of arterial blood flow still remains to be elucidated [7–9]. Indeed, several *ex vivo* studies have demonstrated the possible roles of von Willebrand factor (VWF), platelets, leukocytes, tissue factor, and fibrin deposition in experimental occlusive thrombus formation [10–13]. However, their roles in the formation of human coronary thrombi causing myocardial infarction are still to be elucidated.

Recently, aspiration of coronary thrombi via an ultra-thin aspiration catheter, which can selectively be introduced into the coronary arterial tree, has been developed as an alternative choice of reperfusion treatment [14–16]. By the use of this technique, specimens of occlusive thrombi causing acute myocardial infarction can be obtained freshly from acute phase of myocardial infarction patients [17]. Although the thrombus specimens obtained by this method are usually not as big as those obtained in autopsy studies, and the fragmented thrombus specimens do not always represent the whole and global distribution of thrombus components, this technique has possible methodological advantages although not proven so far [18] and not clarified even in our present study; e.g. thrombus specimen can be obtained without any postmortem histological changes taking place.

In the present study, we have attempted histochemical clarification of the content of coronary thrombi with the specimens obtained freshly from acute phase patients with myocardial infarction by the use of an aspiration device. Although it is known that the mechanism of thrombus formation is influenced by the accompanying risk factors of patients such as gender, smoking or diabetes mellitus [19], we focus in our study the common findings, appearing in all the thrombus specimens because sample size of our study is not large enough to address the contributing role of the accompanying risk factors. We have shown here the presence of VWF at the site where platelets were densely accumulated, the presence of tissue factor at the site of deposition of fibrin fibrils, and the presence of various inflammatory cells in coronary thrombi.

Methods

Patient selection and sample preparation

From March 2003 to August 2003, 10 consecutive patients admitted to the Tokai University Hospital with acute myocardial infarction were enrolled in this study. All the patients were brought to the Emergency Room because of prolonged (lasting > 30 min) and nitroglycerin-resistant chest pain having the typical characteristics of ischemic cardiac pain. The diagnosis of acute myocardial infarction and acute coronary syndrome was based on the clinical guidelines published previously [20,21]. The electrocardiogram obtained in the Emergency Room revealed that all of the patients, except one (case 7 in Table 1) who showed ST-segment depression in leads V_4 to V_6, had ST-segment elevation in two or more leads. The standard treatment protocol, including aspirin administration [21] was instituted promptly after the diagnosis of acute myocardial infarction was made. The study was approved by the Internal Review Board of the Tokai University Hospital, and written informed consent was obtained from all of the enrolled patients. The clinical characteristics of the patients are summarized in Table 1. Accompanying risk factors, which may influence the content of thrombi, such as diabetes mellitus, hyperlipidemia, smoking and hypertension, was also shown in the Table 1. Definition of diabetes mellitus, hyperlipidemia and hypertension were based on the clinical guideline published in Japan (not cited because they are written in Japanese). Current and previous smokers are considered to have risk factor of smoking.

Emergency coronary angiography, thrombus aspiration, and preparation of the histological samples

After visualizing the occlusions of the coronary arteries by emergency coronary angiography (Table 1), the occlusive thrombi were aspirated using the newly developed ultra-thin aspiration catheter, which is designed exclusively for aspiration of thrombi from coronary arteries (Thrombuster, Kaneka, Co. Tokyo, Japan). In detail, we have settled the aspiration device at site of coronary occlusion. Then, we produced negative pressure by drawing a 20 mL syringe to aspirate the occlusive thrombi before any other intervention was applied. All the patients were then treated by balloon angioplasty and subsequent stent implantation to protect against re-occlusion. Standardized anticoagulation with unfractionated heparin was instituted during the cardiac catheterization [21]. Ticlopidine, 200 mg day^{-1}, was started immediately after the stent implantation, and continued for at least 1 month.

The thrombi obtained by aspiration were immediately fixed in a fixative solution of 0.1 M phosphate buffer saline (pH 7.3) containing 4% paraformaldehyde (EM Science, Fort Washington, PA, USA) and 0.2% glutaraldehyde (EM Science) for 2 h, and embedded in paraffin in accordance with previously published procedures [22]. Then, serial sections were stained by hematoxylin and eosin to identify the basic constituents of the thrombi. Mallory–Azan and phosphotungstic acid hematoxylin (PTAH) stainings were performed to clarify the distribution of platelets and fibrin. For immunohistochemical identification of platelet, VWF, tissue factor, P-selectin, and inflammatory cell specific antigens, the sections were immunohistochemically stained using monoclonal antibodies against VWF (DAKO Japan, Kyoto, Japan), fibrin (Takeda Chemical Industries, Ltd, Osaka, Japan), tissue factor (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), P-selectin (DAKO Japan), CD16 (DAKO Japan) for neutrophils, CD45 (DAKO Japan) for white blood cells

Table 1 Patient characteristics

Case	Age	Sex	Diagnosis	Culprit lesion	Hours after the onset	Risk factors
1	59	M	AMI	LCX (no. 11)	18	Smoking
2	74	M	AMI	LAD (no. 6)	2	
3	81	M	AMI	RCA (no. 1)	2	HT, HL
4	63	M	AMI	LAD (no. 7)	7	Smoking
5	74	M	AMI	SVG	2	HT, HL
6	54	M	AMI	LAD (no. 6)	2	Smoking, HL
7	74	F	ACS	LAD (no. 7)	1	HT, HL
8	75	M	AMI	LAD (no. 7)	9	HT, HL
9	76	F	AMI	RCA (no. 1)	14	HT, DM
10	28	M	AMI	RCA (no. 1)	14	

AMI, acute myocardial infarction [20]; ACS, acute coronary syndrome [21]; LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery; RCA, right coronary artery; SVG, Saphenous vein graft; HT, hypertension; HL, hyperlipidemial; DM, diabetes mellitus.

(mainly mononuclear cells), and CD34 for premature endothelial and blood progenitor cells (DAKO Japan). Sheep polyclonal antibody against platelet-specific protein GPIIb/IIIa (Affinity Biologicals, South Bend, Canada) was also used. In general, specificity of staining by the above antibodies was tested in comparison with the staining of negative control using non-immune mouse IgG or normal sheep serum. To confirm the specificity of the staining with anti-GPIIb/IIIa antibody in our study, the distribution of positive staining with this antibody was compared with the distribution of the positive staining with a mouse-derived monoclonal antibody against human GPIIb (DAKO Japan). The antibody against fibrin is originally developed by using fibrin like β peptide as immunogen. Thus, this antibody theoretically cross react with fibrinogen [23]. Specificity of the immunohistochemical identification of fibrin was confirmed by other staining such as Mallory–Azan and PTAH stainings. In the quantitative analysis, the numbers of CD16-, CD45-, and CD34-positive cells were calculated in the samples in which the cell counts could be conducted over an area of at least 1 mm^2 and the number of cell in the unit area of 1 mm^2 was calculated. Indeed, all the samples obtained from right coronary arteries and four from left coronary arteries were suitable for these criteria. It is noteworthy that the researcher working at pathological laboratory did not aware clinical characteristic of the patient when they conducted pathological investigations including the counting of the number of leukocytes involved in thrombus specimens.

Statistical analysis

All the numerical data were shown as mean ± SD, unless otherwise stated. Comparison of the data between two groups was conducted by Students' paired and the unpaired *t*-test. A *P*-value of <0.05 was considered to indicate statistical significance.

Results

Activated platelets, tissue factor and fibrin are involved in the formation of coronary thrombi

As shown in Fig. 1, the coronary thrombi were composed mainly of platelets and densely packed fibrin. Inflammatory cells, including neutrophils and mononuclear cells, are also involved in thrombus specimen (Fig. 1A). These three components were included in all the thrombus specimens we tested. Immunohistochemical staining revealed the presence of tissue factor at sites of dense fibrin deposition (Figs 1C,D and 2C,D). Co-localization of tissue factor and fibrin could be shown in all the thrombi specimens. A small number of fibrin fibers were also identified around activated platelets by Mallory–Azan staining (Fig. 1B).

As shown in Fig. 2B, the presence of platelets was also confirmed immunohistochemically by demonstrating the presence of the platelet-specific protein, GPIIb/IIIa. Not

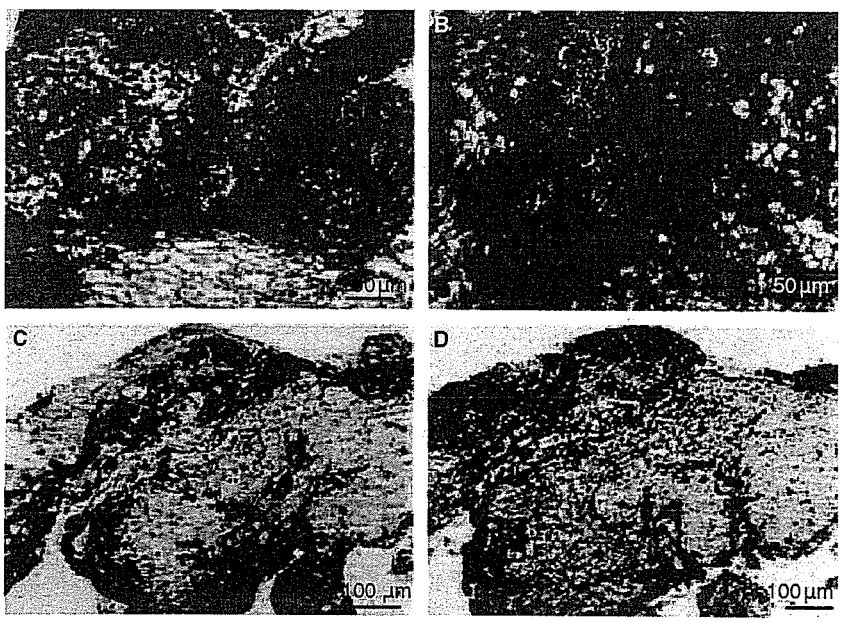

Fig. 1. Histological features of coronary thrombi obtained during the acute phase from patients with acute myocardial infarction. (A) Hematoxylin & eosin staining. The coronary thrombus specimen obtained from patient 10 in Table 1 is composed of inhomogeneous distribution of platelets, fibrin and inflammatory cells. (B) Mallory–Azan staining. Coronary thrombus specimen obtained from the same patient reveals platelets, stained blue, packed at the center of the specimen. A dense fibrin network can be seen around the packed platelets. It is important to note that a certain amount of fibrin fibrils is stained on the surface of activated platelets. C and D. Immunohistochemical staining of fibrin (C) and tissue factor (D). Two consecutive specimens of coronary thrombi obtained from patient 10 in Table 1 were immunohistochemically stained with anti-fibrin (C) and anti-tissue factor antibodies (D). A large amount of fibrin fibers co-localized with tissue factor was demonstrated. Although all the panels of Fig. 1 demonstrate representative results obtained from a single case, similar results, demonstrating the co-localization of tissue factor and activated platelets with fibrin, could be seen in the thrombi obtained from all the examined cases.

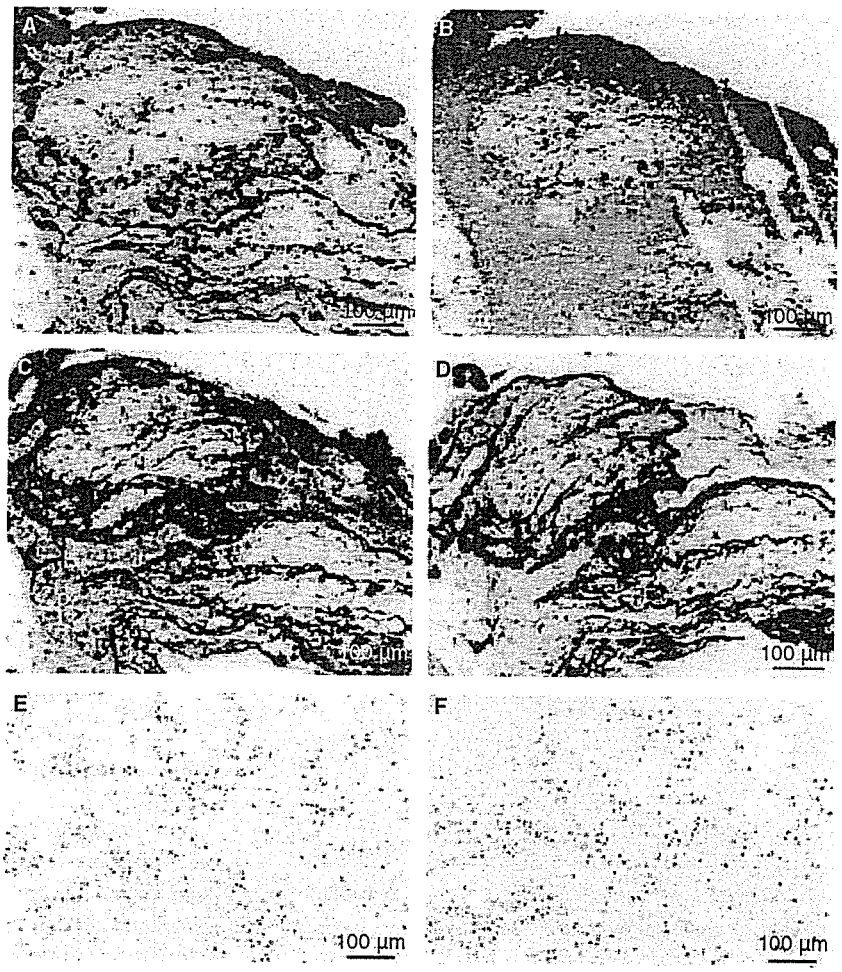

Fig. 2. Histochemical staining of coronary thrombi obtained freshly from patients with acute myocardial infarction. (A) Immunohistochemical staining of von Willebrand factor (VWF). A coronary thrombus specimen obtained from patient 3 in Table 1 demonstrated the prominent presence of VWF, especially at sites of accumulation of platelets. (B) Immunohistochemical staining for GP IIb/IIIa. A consecutive slice of the coronary thrombus specimen obtained from the same patient as above demonstrated the co-localization of platelets stained by platelet-specific anti-GP IIb/IIIa antibody with the VWF shown in panel A. (C and D) Immunohistochemical staining of tissue factor (C) and fibrin (D). Two consecutive specimens of coronary thrombi obtained from patient 3 in Table 1 were immunohistochemically stained with anti-tissue factor (C) and anti-fibrin antibodies (D). Similar to the results shown in the panels C and D in Fig. 1, a large amount of fibrin fibers co-localized with tissue factor was demonstrated. (E and F) Negative control using non-immune mouse IgG and normal sheep serum, respectively.

only fibrin/fibrinogen, but also VWF, was present at the sites of platelet accumulation (Fig. 2A). The presence of VWF at site of platelet accumulation could be demonstrated in all the thrombi specimens. The specificity of the antibodies used in this study was confirmed in comparison with the results shown by the negative control antibodies (Fig. 2E,F).

Characterization of the inflammatory cells identified in the coronary thrombi

As shown in Fig. 3, immunohistochemical staining revealed the heterogeneous but constant presence of CD16- and CD45-positive cells in all of the specimens obtained from the 10 cases of acute myocardial infarction. All of the specimens, except those obtained from one patient (case 3, the oldest patient), contained more CD16-positive cells (251.4 ± 198.7 mm^{-2}, $n = 7$) than CD45-positive cells (148.7 ± 78.0 mm^{-2}, $n = 7$). Thrombi obtained from the right coronary arteries (CD16: 312.3 ± 266.3 mm^{-2}, CD45: 123.7 ± 105.2 mm^{-2}, $n = 3$) tended to contain larger numbers of CD16-positive cells than those obtained from the left coronary arteries (CD16: 242.2 ± 173.5 mm^{-2}, CD45: 149.3 ± 117.6 mm^{-2}, $n = 4$). As shown in Fig. 4, there was weak but significant positive correlation between the number of CD16- and CD45-positive cells in the thrombi and the time of collection of the specimens after the onset of chest pain, with an r-value of 0.73 and 0.86, respectively ($P < 0.05$ in both). No significant relationship was found between the distribution of P-selectin and the number of inflammatory cells involved in the thrombus specimens. It was noteworthy that CD34-positive cells were present in abundance in some samples, while they were sparse in others. These cells were either incorporated deep within the thrombi or were present on their surface.

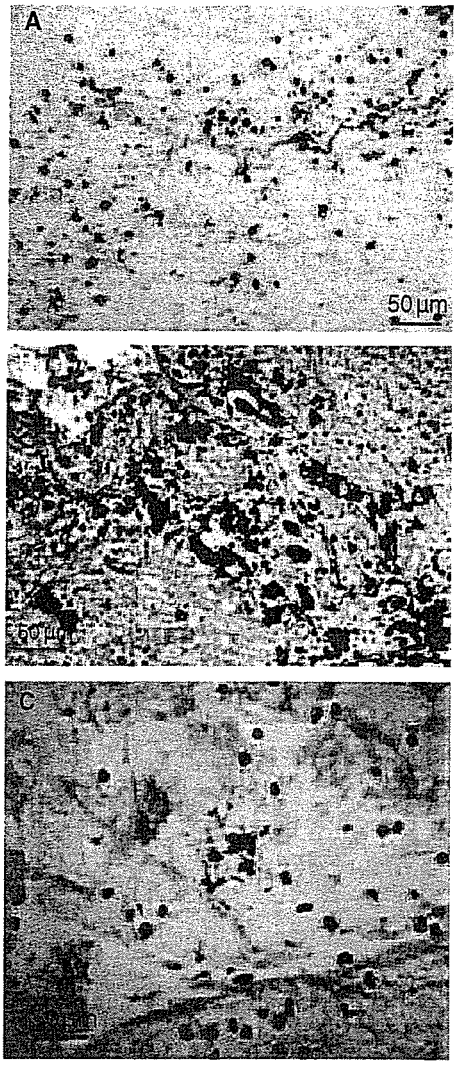

Fig. 3. Characterization of the inflammatory cells involved in the formation of coronary thrombi. All the specimens of coronary thrombi obtained from the 10 cases of acute myocardial infarction shown in Table 1 contained inflammatory cells stained positively by anti-CD16 (A), anti-CD45 (B), and anti-CD34 monoclonal antibody (C). CD16- and CD45 positive cells were present in abundance in all of the specimens; while a large number of CD34-positive cells was noted in some specimens (C: case 10 in Table 1), only a few were noted in others (case 8 in Table 1, results not shown in the Figure).

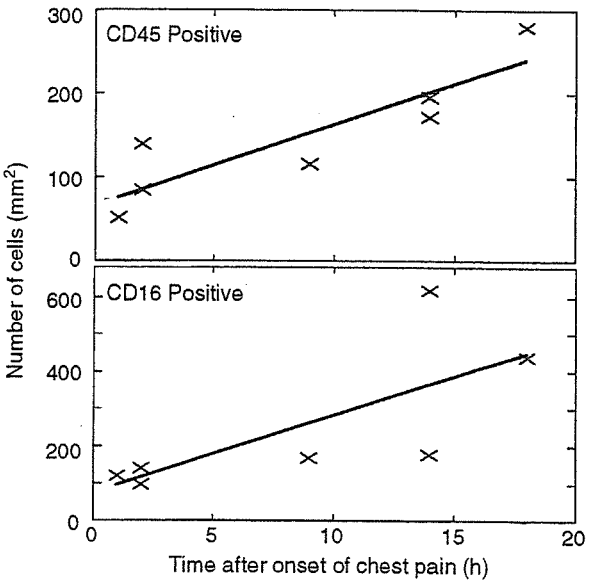

Fig. 4. Relationship between the number of leukocytes in the thrombus specimens and the time of specimen collection after the onset of chest pain. The upper and lower panels demonstrate the relationship between the time of specimen collection after the onset of chest pain and the number of CD16- and CD45-positive cells, respectively, in the thrombus specimens in which cell count could be conducted over an area of at least 1 mm^2 (n = 7). A significant positive relationship was noted for both ($P < 0.05$).

Discussion

Our results represent the first clear demonstration of the co-localization of VWF with platelets, and of tissue factor and platelets with fibrin fibrils, in human coronary thrombi causing acute myocardial infarction, confirming their possible contribution suggested by experimental thrombosis [10–13]. Characterization of inflammatory cells involved in coronary thrombi by cell surface markers revealed the constant presence of CD45- and CD16-positive cells. The revealed constituents of the coronary thrombi in our study, namely, platelets [24], fibrin [24], tissue factor [25] and inflammatory cells [26], are in complete agreement with previously published autopsy finding. As compared to the autopsy study, our method has both possible advantages as well as obvious disadvantages; the confounding effect of histological changes taking place after the formation of the coronary thrombi, in particular, post-mortem changes, could be avoided, where fresh thrombi causing acute myocardial infarction were obtained without delay; on the other hand, the spatial distribution of the constituents of thrombi is broken by catheter aspiration, which makes it difficult to exclude the possible contamination of the thrombi by cells and substances from an atheroma disrupted by the catheter aspiration technique. Nevertheless, our main findings, that is, co-localization of VWF with platelets and of tissue factor with fibrin, as well as the involvement of inflammatory cells in coronary thrombi, are unlikely to have been influenced by our methodological limitations.

One of our findings, co-localization of VWF with platelet, may suggest the causative role of VWF in platelet thrombus formation in human coronary arteries, the concept of which have been suggested by many *in vitro* and *ex vivo* investigations [9,10,27] and observational clinical studies [28–31]. One of the other finding, the presence of tissue factor at sites of fibrin deposition, also provide important information to gain further insight into the role of tissue factor in the formation. Although our finding do not exclude possible contributory role of other coagulation related proteins such as factor Xa [32], and factor XIa [33], our results confirm previously published findings demonstrating the crucial role of tissue factor in the formation of arterial-occlusive thrombus formation with the experimental thrombosis [11–13,34,35]. Co-localization of platelet and fibrin shown in our finding also suggested the possible contributory role of activated platelet in the formation of fibrin fibrils previously shown in the experimental conditions [36,37]. As a

whole, our present findings are confirmation of the experimental results in real pathological thrombus formation.

Our study has obvious methodological limitations, particularly in regard to applying results to the understanding of the functional role of these specific cells and proteins in the formation of coronary thrombi. For example, only thing we have shown here is the co-localization of cells and proteins such as VWF and platelet without demonstrating any causative relation. We cannot exclude the possibility of VWF accumulating later at sites of platelet thrombi, without playing any roles in their formation. The same argument is valid for tissue factor and fibrin deposition. In spite of these limitations, we would still like to emphasize the findings, i.e. the presence of VWF around platelets and the co-localization of tissue factor with fibrin in human coronary thrombi presumably causing the myocardial infarction, represent direct finding in human, which could never have been obtained from animal experiments or *ex vivo* and *in vitro* perfusion studies even using human blood. Another important issue we could not address in our manuscript is the contributing role of gender and the accompanying risk factors in the histological contents of coronary thrombi. Indeed, previous study suggested the mechanisms of the onset of coronary thrombi might be influenced by the accompanying risk factors; e.g. coronary thrombus formation in smoking women is often initiated by atheromatous erosion rather than its rupture [19]. We could not address this issue in our present study because the sample size of the study was not large enough to clarify the role of accompanying risk factors.

The presence of inflammatory cells, including CD16-, CD45- and CD34-positive cells, might represent contamination from an underlying atheroma, as the specimens were obtained by aspiration from sites of coronary occlusion demonstrated by angiography. Obviously, because of the nature of the aspiration, we cannot exclude the possible contamination of the thrombi by components of the atheroma. Indeed, previous studies have demonstrated the presence of inflammatory cells in atheromatous plaque, and suggested a possible role of these cells in the formation and disruption of an atheroma [38,39]. While we can speculate that the inflammatory cells found at sites packed densely with platelet thrombi migrated from the peripheral circulation, mediated by adhesion molecules appearing on the surface of activated platelets, as suggested experimentally [40,41], we could not demonstrate the relationship between the distribution of P-selectin and the migration of leukocyte in thrombi. Moreover, the pathological roles of these inflammatory cells, including CD16-, CD45- and CD34-positive cells, especially CD34-positive cells, which can be differentiated into endothelial progenitors [42], possibly functionally modified by contact with the thrombi containing the activated platelets, remain to be elucidated. Further investigations focusing on the relationship between thrombosis and inflammation must, therefore, be conducted.

In conclusion, we examined the coronary thrombi specimen obtained by the use of aspiration device from acute phase of myocardial infarction by detailed histochemical staining. Our results, demonstrating the co-localization of VWF with platelet, tissue factor with fibrin, and the presence of various inflammatory cells, suggest causative role of VWF in platelet thrombus formation, the role played by tissue factor and activated platelets in the formation of fibrin fibrils, and the close relationship between inflammatory cells migration and the formation of occlusive thrombi in human coronary arteries.

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