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FIGURE CAPTIONS

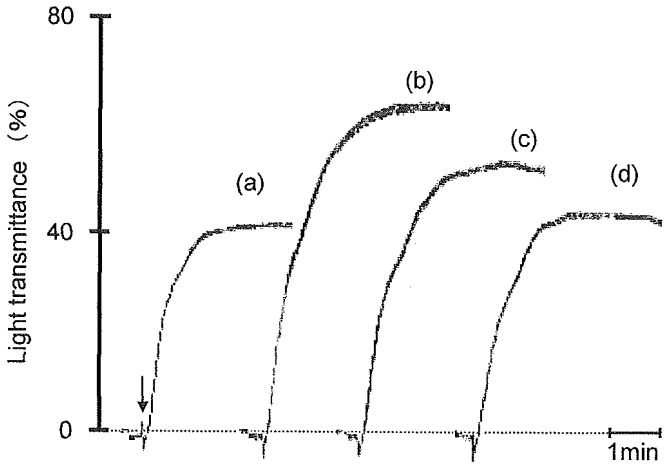
Fig. 1 ADP (3 μM)-induced platelets (final count; 2.0 x 10^5 /μL) aggregation in the presence of latex beads samples (a) non-coated latex beads, (b) H12-latex beads, (c) and (d) H12/rGPIbα-latex beads). The final concentration of beads was 2.0 x 10^3 /μL. Number of H12 molecules conjugated to the one latex bead was (a) 0, and (b)-(d) 2.0 x 10^5. Number of rGPIbα molecules conjugated to one latex bead was (a) 0, (b) 0, (c) 1.7 x 10^3, and (d) 5.0 x 10^3. Arrow shows addition of ADP.

Fig. 2 Average surface coverage of (a) latex beads samples or (b) thrombocytopenia-imitation blood (platelet count; 2.0 x 10^4 /μL) mixed with sample latex beads and passed over the

surface; control latex beads (●; 1.0 x 10^5 /μL), H12-latex beads (◊; 1.0 x 10^5 /μL), rGPIbα-latex beads (△; 1.0 x 10^5 /μL), a mixture of H12-latex beads (5.0 x 10^4 /μL) and rGPIbα-latex beads (5.0 x 10^4 /μL) (○), and calculation value of ((◊+△)/2) at various shear rates (□). The Average surface coverage was calculated after blood was circulated for 180 s. All measurements were performed in triplicate.

Fig. 3 Average surface coverage of H12-latex beads in thrombocytopenia-imitation blood containing (○) H12-latex beads (5.0 x 10^4 /μL) and rGPIbα-latex beads (5.0 x 10^4 /μL), (△) H12-latex beads (1.0 x 10^5 /μL), and (□) control latex beads (1.0 x 10^5 /μL) at a shear rate of 1600 s^{-1}.

Fig. 4 (a) SEM images of platelet thrombus formed on the collagen surface in the presence of H12-latex beads and rGPIbα-latex beads at a shear rate of 1600 s^{-1}. **(b)** Number of H12-latex beads (opened bar) or rGPIbα-latex beads (filled bar) on the thrombus was estimated using scanning electron microscope after blood was passed over the collagen surface (the number of thrombus: N=120). Error bars represented standard deviation.



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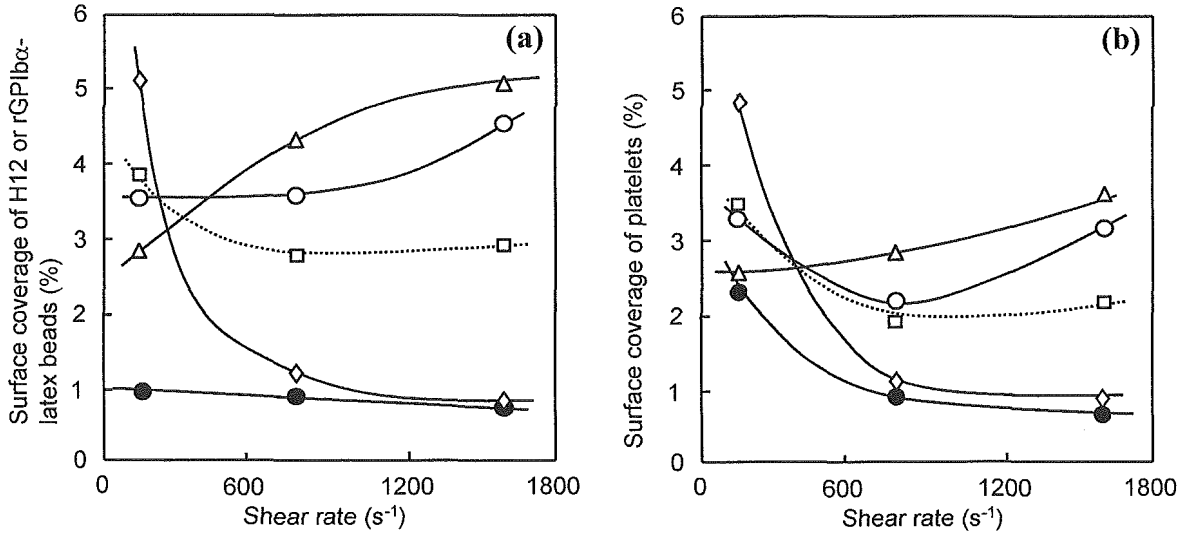

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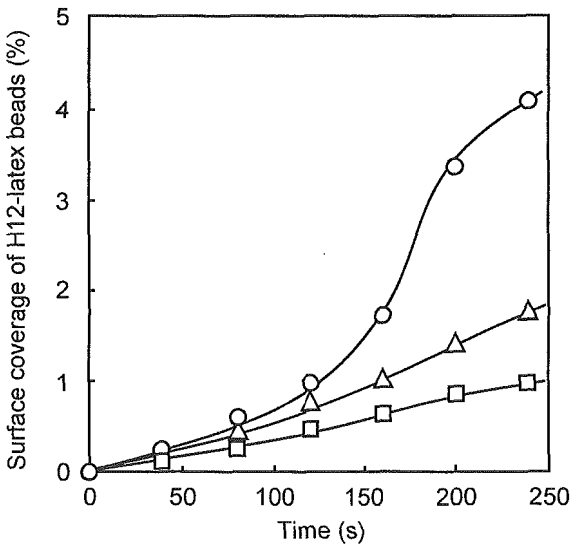

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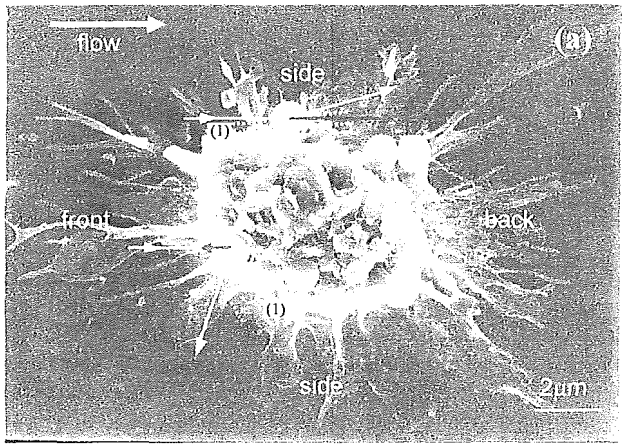

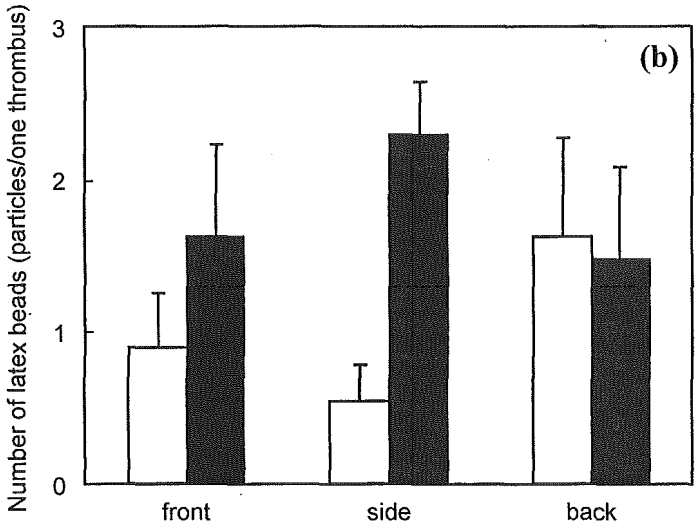

Fig. 4 Okamura et al.

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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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Kanagawa, Japan; and La Jolla, California

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

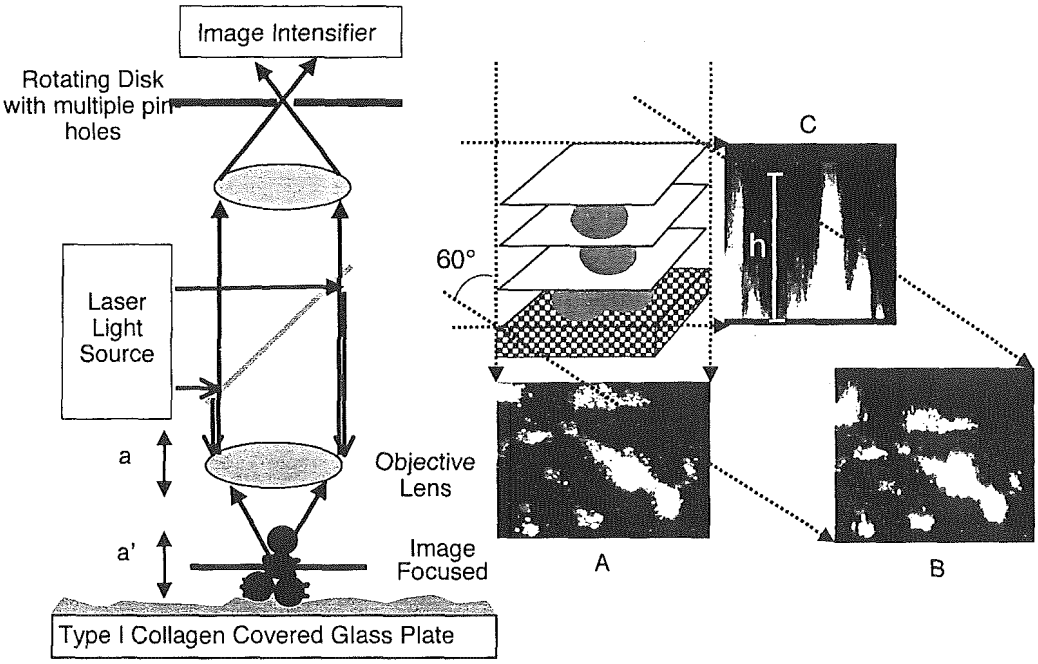

Figure 1. Three-dimensional projection imaging of platelet thrombi. A piezo-electric motor **(a)** moved the objective lens at a constant speed of 0.4 μmol/l/s to provide scanning images of the platelet thrombi **(a')**. The sum of the confocal images in a bottom to top stack (z-axis) was projected on 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

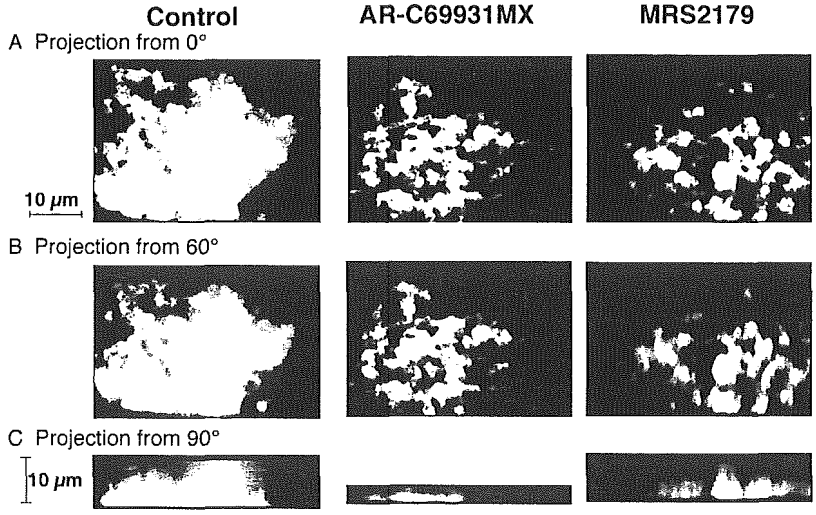

Figure 2. Three-dimensional projection images of thrombi formed in the presence or absence of adenosine diphosphate receptor antagonists. Blood with fluoresceinated platelets was perfused over immobilized collagen type I fibrils for 6 min at the wall shear rate of 1,500 s^{-1} in the absence (Control) 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.

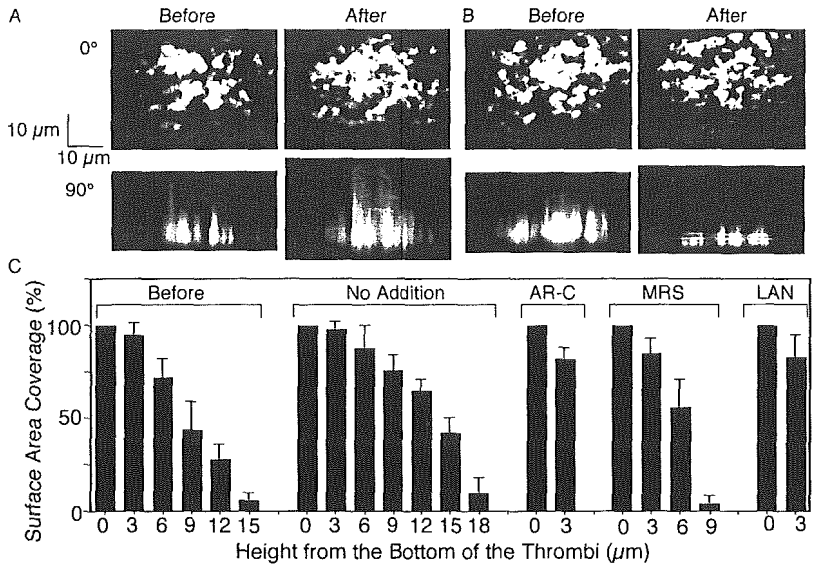

Figure 3. Reduction in the size of platelet thrombi exposed to blood containing different antagonists of platelet function. These experiments were performed as described in the caption of Figure 2, with the difference that the surface was exposed to two subsequent aliquots of blood each perfused for 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

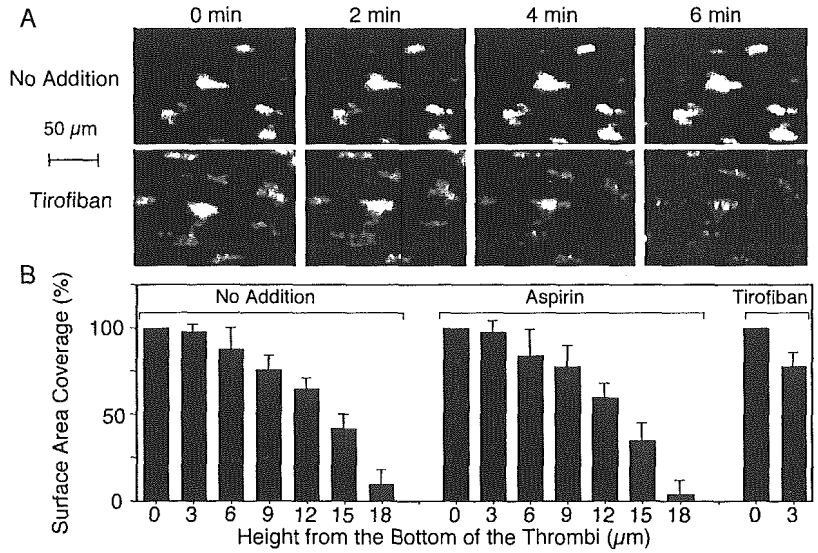

Figure 4. Changes in the two-dimensional and three-dimensional structure of platelet thrombi exposed to blood containing antiplatelet agents. These experiments were performed essentially as described in the caption of Figure 3. **(A)** Representative two-dimensional fluorescence microscopy images of 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.

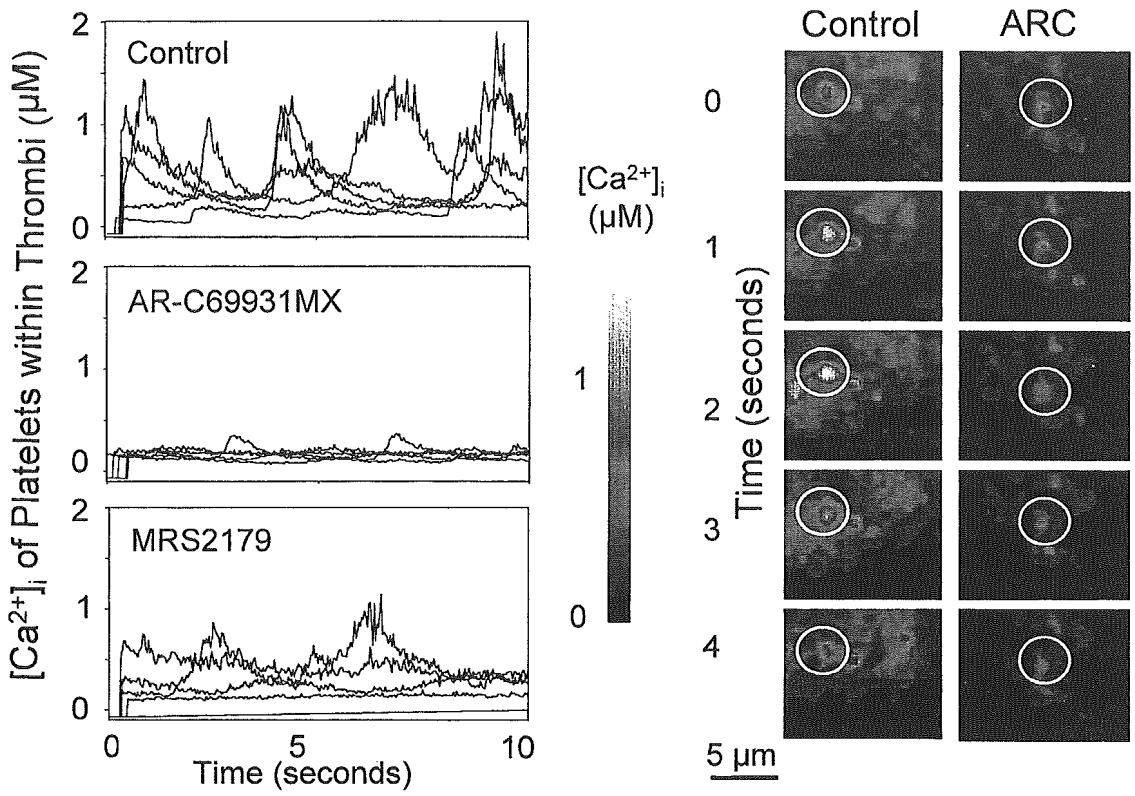

Figure 5. Changes in the intracytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) of platelet thrombi caused by adenosine diphosphate receptor antagonists. These experiments were conducted as described in the caption of Figure 3, except that blood was replaced with a cell suspension containing fluo-3AM–loaded 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).

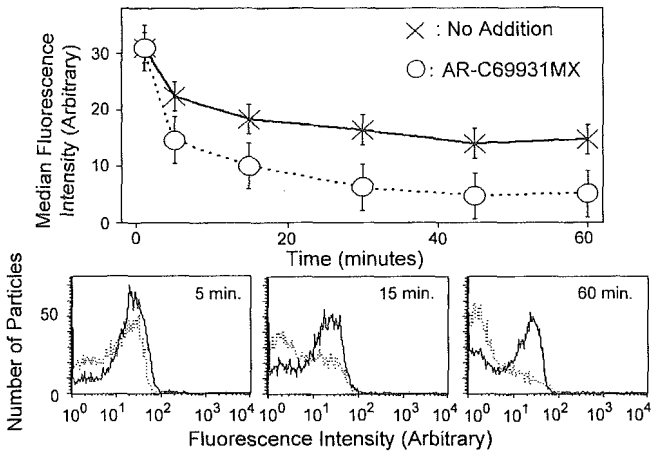

Figure 6. Changes in PAC-1 binding to activated platelets caused by adenosine diphosphate (ADP) receptor antagonists. To activate platelets, 25 μl of HEPES buffer containing ADP and epinephrine (400 μmol/l each) was mixed with 375 μl of platelet-rich plasma and incubated for 20 min. Fifty μl of fluorescein 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

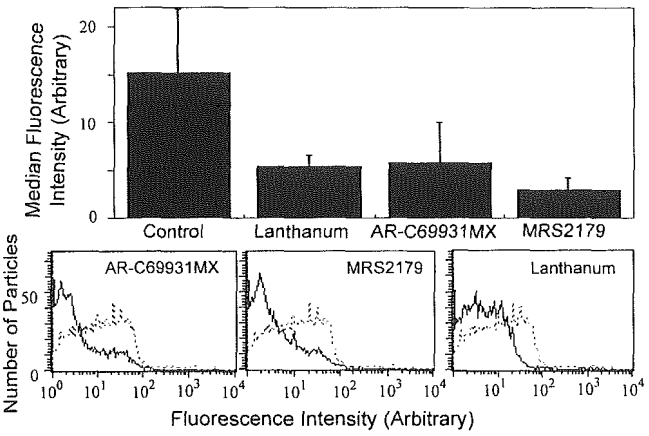

Figure 7. Effect of different platelet inhibitors on PAC-1 binding to activated platelets. These experiments were performed as described in the caption for Figure 6, with the only difference that the $P2Y_1$ inhibitor, MRS2179, or the putative Ca^{2+} channel blocker, lanthanum chloride, was also added 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.

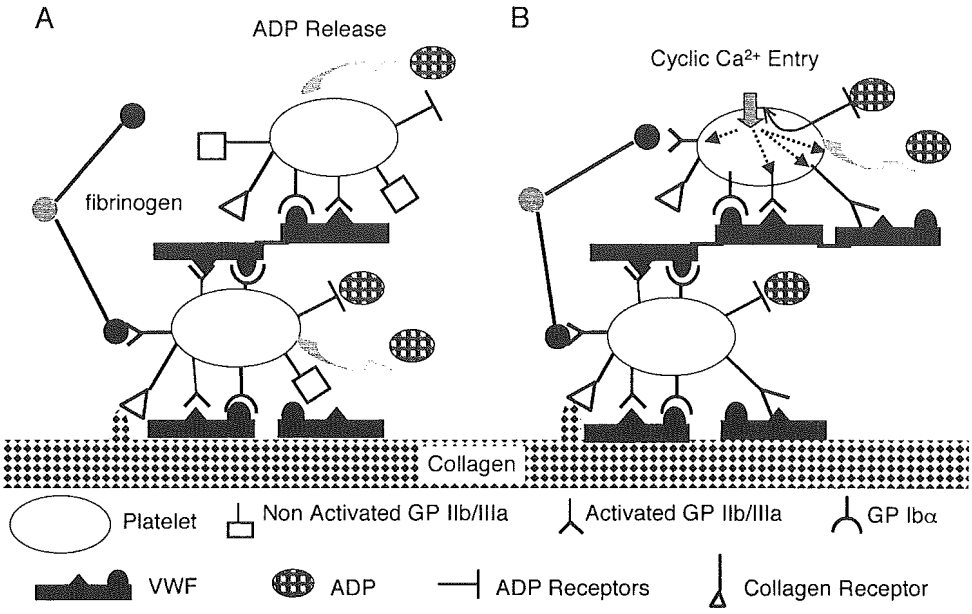

Figure 8. Schematic representation of the mechanism that stabilizes platelets at the edge of a growing thrombus exposed to elevated shear rates. **(A)** Circulating platelets adhere and become activated onto collagen through multiple adhesive interactions, initiated by glycoprotein (GP) Ibα binding to 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.

Acknowledgments

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APPENDIX

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

人工赤血球・人工血小板の開発の現状

武岡真司
早稲田大学理工学術院

はじめに

筆者の所属する研究グループ（早稲田大学理工学総合研究センター）は，慶應義塾大学医学部と共同して厚生労働省科学研究，医薬品・医療機器などレギュラトリーサイエンス総合研究事業，H 16-医薬-067, 069, 071により人工赤血球と人工血小板の研究を推進している．人工血液全体の現状に関しては，厚生労働省科学研究の研究代表者小林紘一教授による本誌「講座」に詳しい[1]．本「講座」では，これらの厚生労働科学研究の成果の一部も含めて報告する．人工赤血球は，リン脂質の二分子膜小胞体（リポソーム）に酸素を酸素分圧に応じて吸・脱着する分子（ヘモグロビン）を内包させた酸素運搬体であり，これが血中に長く留まって安全，安定に酸素運搬機能を発現し続ける．それに対して人工血小板は，血管損傷部位や活性化した血小板のみを認識する分子をリポソームやアルブミン重合体に担持した微粒子であり，これが血中に長く留まって血管損傷部位に特異的に粘着して止血機能を発現する．筆者らは理工学の立場から人工赤血球や血小板の材料となる担体の設計，製造，物性評価を行ってきた．担体には，適当な血液適合性や血中滞留性が求められるが，分解性や代謝物の低毒性も重要な検討項目である．現在担体としてリン脂質分子の集合体（リポソーム）や遺伝子組み換えヒトタンパク質の複合体や重合体を選択して用いている．

キーワード：人工赤血球，人工血小板，ヘモグロビン

Seminar
Current Development of Artificial Red Blood Cells and Artificial Platelets
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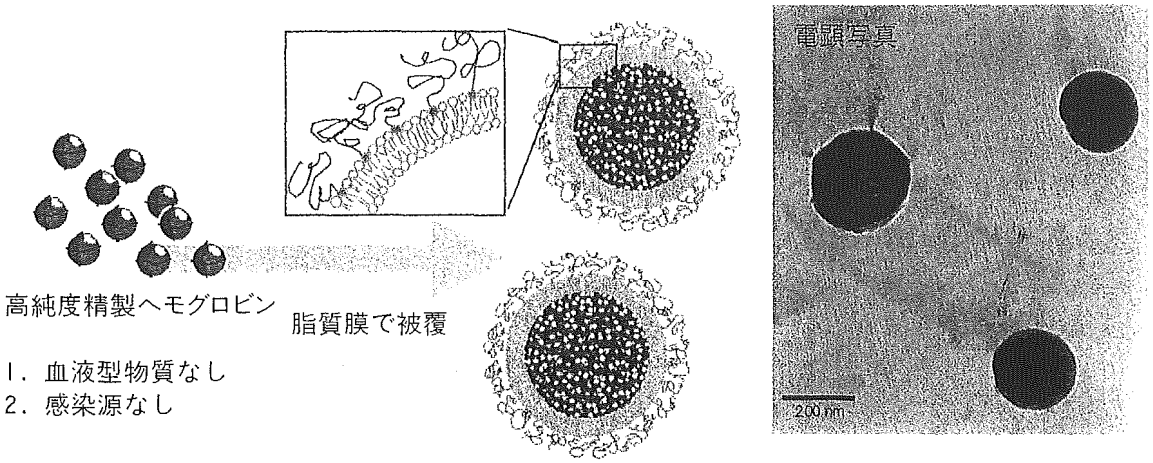
早稲田大学理工学術院；教授

1. ヘモグロビン小胞体の構造

人工赤血球としてパーフルオロカーボン乳剤や修飾ヘモグロビンなどが検討され臨床使用されてきたが，機能や安全性の観点から満足できるものではなかった．筆者の所属するグループで開発を進めている，高濃度ヘモグロビンをリポソームの内水相に内包させた，赤血球と類似構造のヘモグロビン小胞体(図❶)は，最も安全度と機能が高いため早期の臨床試験着手が期待されている[2]．現段階では期限の切れた献血血液由来のヘモグロビンの有効利用が進められているが，将来的には遺伝子組み換えヒトヘモグロビンが利用されるであろう．赤血球からヘモグロビンを精製する際に，血液型を決める型物質やヘモグロビン以外のタンパク質，ウイルスや菌（もし含まれたとしても）を加熱やフィルター処理で除去されている．生理活性なヘモグロビンを安定なリン脂質膜で包むことによって，ヘモグロビンに由来する副作用（血管収縮や腎毒性，神経毒など）を回避できる．ヘモグロビン小胞体は生理食塩液に分散され，脱酸素状態で容器に密封されているため，室温で2年間の液状保存（赤血球製剤では採血後3週間の冷蔵保存）が保証されており，乾燥粉末ではさらに長期間の保存が可能である．製剤のヘモグロビン濃度



図❶　ヘモグロビン小胞体の構造と模式図ならびに透過型電子顕微鏡写真．

は10 g/dlであり，ヒト血液の値（11～15 g/dl）と比較して遜色ない．また，ヘモグロビン分子が封入されているため製剤の膠質浸透圧はほとんどゼロである．したがって，膠質浸透圧の調節が必要となる場合にはアルブミン（リコンビナント）や多糖類などのコロイド製剤と併用となる．図❶の電子顕微鏡写真では，ヘモグロビンの鉄が染色されており，数多くのヘモグロビンが脂質分子膜で包まれた小胞体構造であることと，粒子径が約250 nmに厳密に調節されていることがわかる．これは，赤血球の約1/30程度の粒子径であるので，梗塞部位の透過など赤血球にはない機能が期待できる．酸素親和度はアロステリック因子，ピリドキサール5′-リン酸の共封入により適当値に調節されている．脂質類の成分組成には，ヘモグロビンのカプセル化効率，常温で2年間液状保存できる安定性，血流中での溶血の回避と適度な血中滞留時間（人では3日程度の半減期予測），血小板や補体の活性化の回避など，に対する工夫が施されている．製造面でも分子集合技術を利用した粒子径の厳密な制御と高濃度ヘモグロビンの内包など，従来の小胞体における課題が解決できている[3]．

2. 動物試験による機能と安全性の評価

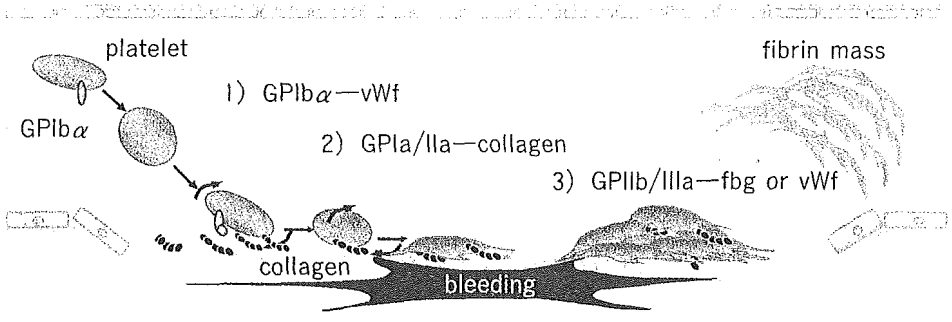
現在までに結果が得られているヘモグロビン小胞体に関する評価試験成績を簡単に紹介する．主にラットやハムスターを用いた試験であるが，基本的な安全性と酸素輸送効果は十分確認できている．また，現在霊長類を用いた安全性試験が進行している．酸素運搬効果を確認する試験として，ラット全血液量の90%をアルブミン単独で交換した場合には，70%交換あたりから血圧と腎皮質酸素分圧の低下が顕著となって死亡したが，ヘモグロビン小胞体をアルブミン溶液に分散させた系で90%交換した場合には，血圧，腎皮質酸素分圧ともに維持された[4]．ヘモグロビン小胞体のアルブミン分散液によるハムスター80%交換輸血試験では，非侵襲に測定した皮下微小循環系の組織酸素分圧は交換前の60～70%に低下するものの，対照アルブミン投与群よりも5倍以上の値が維持されていた[5]．さらにNZW兎を用いた検討では，人工呼吸下，脱血し平均血圧を30～35 mmHgに低下させた後，ヘモグロビン小胞体分散液を投与し，組織酸素分圧の多点測定を実施，とくに脳と腎臓でヘモグロビン小胞体が有意な回復効果を発揮することを明らかにしている[6]．中型動物を用いた実験としてビーグル犬（約7 kg）を用い人工呼吸下，脾臓摘出後アルブミンで75%血液希釈後さらに30%脱血し，30分経過後に人工赤血球を投与し，循環動態，血液ガス組成，組織酸素分圧，組織酸素化度，心拍出量，血中酸素濃度の回復が確認されている[7]．

安全面では，血管弛緩因子である一酸化窒素や一酸化炭素が関与してヘモグロビンに認められる抵抗血管の収縮と血圧亢進は，ヘモグロビン小胞体では認められなかった[8,9]．これは，ヘモグロビン小胞体の大きさが寄与しているものと思われる．ヘモグロビン小胞体の血中滞留時間は，ラット，ラビット，カニクイザルからヒトへ類推すると，3日間程度の半減期として見積もられ，緊急時の単回投与では十分とされる．ラットでは，血中半減期が1～1.5日間であるので，脾臓や肝臓の病理組織学的所見では，投与1日後には脾臓や肝臓に貪食されていたヘモグロビン小胞体は3日後には激減し，投与7日以内にはほとんど消失していた[10]．また，ラットでの単回交換投与（循環血液量の40%交換），反復負荷投与（10 m*l*/kg/day，14日間）による血液生化学試験（30項目）や病理試験での詳細から，ヘモグロビン小胞体成分である，脂質分解に関わるリパーゼの亢進，コレステロール値の上昇，鉄の沈着，細網内皮系の肥大が一過性に認められた以外に変動を認めていない[11]．その他免疫系，凝固系への影響も認められておらず，大量出血時の緊急対応では十分なる機能を発現するものと期待されている．

上述のような効果と安全性の高い人工酸素運搬体では，輸血の代替以外にさまざまな適応が検討されている．たとえば体外循環回路補充液としての利用の検討では，ラット体外循環モデルの作成のため小型人工心肺を試作し，ヘモグロビン小胞体分散液を充塡液として使用，血液交換率が50%以上になる条件で灌流させた後，灌流回路中のヘモグロビン小胞体を分離除去して赤血球を回収して投与し，長期生存できることを確認している[12]．虚血性疾患の治療への利用においても，虚血再灌流実験などで小粒径のヘモグロビン小胞体の効果を実証する *in vivo* 実験が進められている．*in vitro* では，微小血管モデル内を流動するヘモグロビン小胞体の酸素放出挙動の解析から，虚血領域酸素化の機序解明を進められている[13,14]．人工酸素運搬体は，腫瘍組織酸素化にも有効であることを実証し，新しい適応の可能性を提示された[15]．

3. 人工血小板の開発の考え方

血小板は出血部位に対し特異的粘着，伸展，凝集，放出，血液凝固系の活性化などの複雑な機能をもち，これらのすべてを兼備した血小板代替物の構築は事実上不可能であろう．しかし，血小板の粘着と凝集に着目して，これらの機能を付与させた担体の投与によっても，少数残存する血小板の機能補助ができるものと考えられる．筆者らは慶應義塾大学医学部内科 池田康夫教授のグループ



図❷　血小板の止血機構．
一次止血（血小板血栓）
1）接着（tethering → rolling）GPIbα—vWf
2）粘着（adhesion）GPIa/IIa—collagen
3）凝集（aggregation）GPIIb/IIIa—fbg or vWf
二次止血（フィブリン血栓）

とともに，血小板膜タンパク質の一部の遺伝子組換え体や合成オリゴペプチドを担持させた微粒子を作成し，これらが血小板を巻き込んで出血部位へ集積することによって止血能が発現されることを期待して，研究を進めている[16]．

血小板による止血は，高ずり速度の血流と低ずり速度の血流では機構が異なる．図❷に示したように，高ずり速度の出血に対する血小板の止血は，出血部位に露出する血管内皮下組織であるコラーゲンに結合したフォンビルブランド因子(vWf) に対して，血小板が認識して接着して転がることから始まる．*in vitro* 観測で抗 GPIIb/IIIa 抗体を添加して GPIIb/IIIa の機能を阻害した血小板では，vWf 固定化基板上を流動方向に沿って転がる現象がみられ，この認識能は血小板表面の GPIb/V/IX 複合体の GPIbα 部が担っている[17]．次に血小板表面の GPIaIIa ($\alpha_2\beta_1$ インテグリン) や GPVI が直接コラーゲンと相互作用して血小板は粘着し，そこで活性化されると血小板は伸展して顆粒を放出するが，最も重要な過程は GPIIb/IIIa ($\alpha_{IIb}\beta_3$ インテグリン) が活性体となる現象である．フィブリノーゲンは，この活性体を認識して血小板間を架橋し凝集体を形成して一次止血を担う．引き続く凝固系の誘導によるフィブリン塊の形成 (二次止血) によって止血が完成する．そこで，高ずり速度の血流下で vWf を介してコラーゲンを認識する GPIbα，低ずり速度でコラーゲンを直接認識する GPIaIIa，活性化血小板上の GPIIb/IIIa を認識するフィブリノーゲン (Fbg) やその認識部位であるペプチドを候補とした．

4. 人工血小板の研究動向

採血液に抗 GPIIb/IIIa 抗体を添加して GPIIb/IIIa を阻害すると，血小板表面の GPIbα との相互作用によって vWf 固定化基板上を流動方向に沿って転がる現象がみられる．そして，rGPIbα を担持させたリン脂質小胞体でも血小板と同様に vWf 基板上を転がることが確認された[18]．転がる小胞体の数はずり速度が高くなるほど多くなり，rGPIbα の特性が確認できた．また，その転がり速度は小胞体を構成する膜の柔軟性と相関した[19]．すなわち“柔らかい”小胞体では転がり速度は低くなり，“硬い”小胞体では転がり速度は高くなった．他方，アルブミン重合体は，内部が充塡された無定形な綿雪のような形態をとっており，出血部位での充塡効果が期待できる．表面に rGPIbα を結合させたところ，小胞体のような vWf 基板を転がる挙動は全く認められず，高ずり速度下でも粘着する挙動が認められた．ラテックスビーズに rGPIbα を結合させた系でも粘着することから，担体が重合体である場合と膜構造をもつ場合では rGPIbα 機能の発現の仕方が異なることが示唆された[20]．

他方，主に低ずり速度の血流下でコラーゲンに直接結合する血小板膜タンパク質の遺伝子組換え体 (rGPIaIIa) を結合させた小胞体は，コラーゲン基板を特異的に認識して粘着 (停止) することが西谷ら[21]によって確認された．また，ずり速度が高くなるにつれ粘着数は減少するが，rGPIbα と rGPIaIIa ともに担持させた小胞体では，低ずり速度から高ずり速度までコラーゲン基板を粘着できる系が構築されている[22]．

さらに減少した残存血小板の凝集を補助するために，粘着して活性化した血小板同士を架橋するフィブリノーゲンを結合させたアルブミン重合体も検討した[23]．活性化血小板の固定化基板を作成し，フィブリノーゲン結合アルブミン重合体を流動させたところ基板上に一様に粘着し，抗 GPIIb/IIIa 抗体を添加した系やアルブミン重合体のみの系では粘着が抑制された．血小板数が正常値の 1/5 程度に調節された血小板減少モデル血液にフィブリノーゲン結合アルブミン重合体を添加したところ，濃度増大とともに流動血小板の粘着数が増大したことから，フィブリノーゲン結合アルブミン重合体は血小板粘着増強効果を有する微粒子であることが示唆された．しかし，フィブリノーゲンは不安定であり，しかも現状ではヒト血液由来となるため，Fbg の γ 鎖 C 末端アミノ酸序列 (H 12：HHLGGAKQAGDV) を結合さ

せた系を用いた研究を重点的に進めている[24]．H 12結合アルブミン重合体を血小板減少血液（[血小板]＝2.0×10⁴/μl）に添加しコラーゲン基板上に流動させたところ，粘着血小板の占有率が増加し，そこにH 12結合アルブミン重合体が巻き込まれていたのでH 12-polyAlbは血小板凝集を補強する効果があると考えられた．

筆者ら[25]は抗がん剤であるブスルファン投与の副作用によって血小板が減少したラットを用いて *in vivo* 効果試験を行っている．血小板数が正常値の1/5程度まで減少した状態のラットに対して，セボフルラン麻酔後試料を尾静脈投与した．試料投与5分経過後，尾先端から1 cmの部位にクイックヒール（ベクトン・ディッキンソン社製）を用いて傷（長さ2.5 mm，深さ1 mm）をつけ，尾先端を生理食塩水液に浸して止血時間を計測した．また，試料投与5分前，投与30分後に採血し，各血球変動を観察した．コントロールとして生理食塩液を投与した血小板減少症モデルラット群（[血小板]＝19.8±2.8/μl）の出血時間は609±153秒であり，正常ラット群（[血小板]＝80.9±8.6/μl）の出血時間（178±56秒）と比較して約3.4倍延長した．H 12結合していないアルブミン重合体を40 mg/kg投与したところ，出血時間は短縮し(184±69秒)，投与量の減少に伴いその効果は減少した．したがって，アルブミン重合体自体でも止血効果を有することが示唆された．そこで，出血時間に影響しないアルブミン重合体の投与量（4 mg/kg）でH 12-アルブミン重合体の止血能を検討した．H 12-polyAlbの投与では，出血時間352±73秒となり出血時間は半分に短縮したが，逆配列H 12を結合させたアルブミン重合体では出血時間を短縮させないので，H 12の効果が確認された．さらに検体投与5分前，投与30分後に採血し，各血球変動を観察したところ，各検体投与前後における血球変動は生じていないことから，H 12-アルブミン重合体は血液適合性の高い微粒子系と思われた．さらにポリエチレングリコールでアルブミン重合体を表面修飾し，一部のポリエチレングリコール鎖末端にH 12を結合させた系では，投与後3時間後に同様の試験を行っても止血効果が持続していることが確認された．

他方，rGPIaIIaを担持させたアルブミン重合体ではX線照射で血小板数を正常値の1/5程度に減少させたマウスに投与したところ，コントロール群の出血時間（730±198秒）と比較して，投与量依存的に出血時間の短縮が認められた（たとえば2.4×10¹¹ particles/kgでは出血時間は337±46秒）[26]．

現在，GPIbαを結合させたアルブミン重合体やリポソームの系で *in vivo* 試験が進行中であるが，予想どおりの結果が得られつつあるので，今後はこれらの混合系における最適化を目指している．

おわりに

人工赤血球は臨床試験を目指して，企業がGLP製造を行う段階に入っている．また，人工血小板の研究は，動物試験での効果と安全性を多角的に確認している段階にある．これらの製剤はいずれもわが国が最先端にあるため，有効性や安全性の試験項目や方法の設定やガイドラインの作成に対して迅速で慎重なる検討が必要である．そのためには，産官学の共同体制での研究や協議の場として，学会（たとえば日本血液代替物学会や関連学会）の果たす役割と責任も大きいと思われる．産業においては，ナノバイオロジクス領域における具体的な成果として，わが国の近未来医療への貢献はもとより，安全な血液が不足している多くの国に対しても大きな国際貢献と成り得る．まずは長期的そして全人類的な視野に立った開発を期待したい．

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