TABLE 2. Associations of Serum IL-18 Levels With Atherosclerotic Risk Factors (n=366)

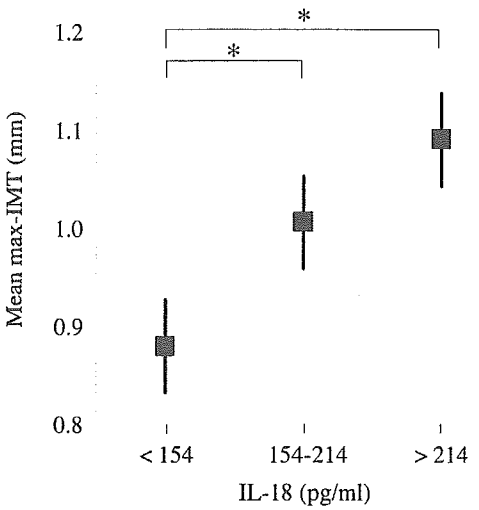
	r or Mean±SD	P
Age, y	0.17	0.001
Sex, men/women	208±85/180±74	<0.001
Body mass index, kg/m^2	0.11	0.041
Hypertension, yes/no	202±82/173±73	<0.001
Systolic blood pressure	0.16	0.002
Diastolic blood pressure	0.01	0.81
Diabetes mellitus, yes/no	205±92/191±78	0.37
Fasting blood glucose	−0.01	0.88
Dyslipidemia, yes/no	195±81/191±81	0.58
Total cholesterol	−0.10	0.06
Triglyceride	0.10	0.056
HDL-cholesterol	−0.17	0.001
Smoking, yes/no	206±86/183±74	0.008
IL-6, pg/mL	0.23	<0.001
hs-CRP, mg/dL	0.29	<0.001

Levels of inflammatory markers were analyzed as log-transformed values. HDL indicates high-density lipoprotein.

significant correlations with carotid IMT (r=0.25 and r=0.23, both P<0.001), neither of such associations was significant when IL-18 and traditional atherosclerotic risk factors were simultaneously included in the model (model 3).

Additionally, given different IL-18 levels between men and women, we have performed separate analyses. Similar to the results obtained for all 366 patients, IL-18 levels were correlated with IMT both in men (r=0.36, P<0.001) and in women (r=0.31, P<0.001), and the associations remained significant when controlling for age, body mass index, and traditional atherosclerotic risk factors (in men, β=0.18, P=0.01; and in women, β=0.25, P<0.001).

Given the association between IL-18 and carotid IMT, the mean max-IMT was compared across the tertiles of IL-18 levels (Figure). IMT was greater in the highest and the middle



Mean max-IMT according to tertiles of IL-18. Error bars are 95% CI. *P<0.001

tertile of IL-18 than in the lowest tertile (Table 4). Moreover, the differences persisted when adjusting traditional atherosclerotic risk factors, log-transformed IL-6 and hs-CRP, and medication usages.

Discussion

In the present study, we have found that elevated serum IL-18 levels are associated with increased carotid IMT as evaluated by B-mode ultrasound. Also, the association was independent of traditional atherosclerotic risk factors, IL-6, and hs-CRP levels. To our knowledge, this is the first study that demonstrates the associations between IL-18 levels and carotid atherosclerosis, with IL-6 and hs-CRP taken into account.

In the current study, IL-18 levels were higher in hypertensive patients and in smokers than in those who were not, and had significant correlations with traditional atherosclerotic risk factors (Table 2). These findings are approximately in line with those of Ferrucci et al,[19] who showed associations of higher IL-18 levels with such risk factors. Also, in accor-

TABLE 3. Multivariate Analyses of Mean Max-IMT

	Model 1		Model 2		Model 3	
Variables	β	P	β	P	β	P
IL-18*, pg/mL	0.26	<0.001	0.22	<0.001	0.20	<0.001
Age, y	0.34	<0.001	0.36	<0.001	0.34	<0.001
Sex, men/women	0.21	<0.001	0.11	0.08	0.10	0.09
Body mass index, kg/m^2			−0.08	0.10	−0.10	0.05
Hypertension, yes/no			0.17	<0.001	0.17	<0.001
Diabetes mellitus, yes/no			0.03	0.55	0.02	0.65
Total cholesterol, mg/dL			0.01	0.93	0.01	0.84
Triglycerides, mg/dL			0.01	0.83	0.01	0.84
HDL cholesterol, mg/dL			0.07	0.18	−0.05	0.33
Smoking, yes/no			0.14	0.021	0.13	0.026
IL-6,* pg/mL					0.05	0.34
hs-CRP,* mg/dL					0.08	0.16

*Levels of inflammatory markers were analyzed as log-transformed values.

TABLE 4. Mean Max-IMT Stratified by IL-18 Tertiles

	IL-18 tertile		
	Lowest (<154 pg/mL)	Middle (154–214 pg/mL)	Highest (>214 pg/mL)
Observed IMT, mm	0.88	1.00*	1.09*
95% CI	0.83–0.92	0.96–1.05	1.04–1.13
Adjusted IMT,‡ mm	0.92	1.01†	1.04*
95% CI	0.87–0.96	0.96–1.05	1.00–1.09
Adjusted IMT,§ mm	0.92	1.01†	1.04*
95% CI	0.88–0.97	0.97–1.05	1.00–1.08

*$P<0.005$ vs lowest tertile.

†$P<0.05$ vs lowest tertile.

‡When controlling for age, sex, body mass index, hypertension, diabetes mellitus, smoking status, total cholesterol, triglyceride and HDL cholesterol.

§When additionally controlling for use of stains, aspirin, and ACEI/ARB, and log transformed hs-CRP and IL-6.

dance with previous studies,[5,6,20] IL-18 levels had modest correlations with other inflammatory markers. Nevertheless, studies that examined the associations of IL-18 levels with atherosclerotic risk factors and other inflammatory markers are limited, requiring further studies to clarify their linkages.

Although elevated IL-18 levels can predict the development of CVD,[5,6] their association with carotid IMT remains to be examined. In the present study, we have found that higher IL-18 levels are associated with greater IMT, suggesting their link with carotid atherosclerosis. However, because of the impact of atherosclerotic risk factors,[21–23] the association between IL-18 and IMT may need to be examined with such factors taken into accounts. When controlling for age and sex, IL-18 was significantly associated with IMT (Table 3, model 1), and the association was independent of traditional atherosclerotic risk factors (Table 3, model 2). Furthermore, similar results were obtained when separate analyses were performed for men and women. These findings support the association between IL-18 and carotid atherosclerosis. Additionally, such association was little modified when levels of IL-6 and hs-CRP were further controlled for (Table 3, model 3), suggesting that the association is independent of such inflammatory markers. Of note, despite the associations of IL-6 or hs-CRP with IMT,[24–26] neither had significant association with IMT in the multiple regression model, which could be because of the lack of our statistical power.

To further demonstrate the associations between carotid atherosclerosis and IL-18, mean max-IMT was compared across the tertiles of IL-18 levels. IMT was greater in patients belonging to the highest and the middle tertiles than in those belonging to the lowest tertile (Figure, Table 4), and the differences persisted when adjusting traditional atherosclerotic risk factors. The greater IMT in patients with higher IL-18 appears to be congruent with Aso et al[11] Moreover, although inflammatory markers levels can be modified by aspirin, statins, angiotensin-converting enzyme inhibitors, or angiotensin II type 1 receptor blocker,[27–30] the differences between IL-18 and IMT were not virtually modified when such medication usages were considered (Table 4), further supporting the link between IL-18 and carotid IMT.

IL-18 is highly expressed in human carotid atherosclerotic plaques, predominantly colocalized with macrophages.[9] Thus, increased IL-18 production from severe atherosclerotic lesions could contribute to the higher IL-18 found in this study. Also, experimental studies have shown that IL-18 enhances atherosclerosis through release of interferon-γ[10] and induces expression of IL-6 in vascular endothelial and smooth muscle cells.[31] Inversely, IL-18 deficiency reduces the extent of atherosclerosis in apolipoprotein E–knockout mice.[32] These findings are in accordance with the hypothesis that IL-18 plays a key role in atherogenesis, supporting the link between IL-18 and carotid atherosclerosis.

There are some limitations for the current study. First, because this study is cross-sectionally designed, we cannot determine the causal relationships between higher IL-18 levels and greater IMT. Second, we used single blood sampling for the measurements of IL-18 levels, which does not guarantee the average levels in our patients. However, our IL-18 measurements were relatively stable over 1 year, supporting the link between IL-18 and chronic atherosclerosis. Third, this study included substantial number of patients on medications, requiring larger studies to separate the effects of such medications.

In conclusion, we have demonstrated an association between higher serum IL-18 level and greater carotid IMT, suggesting the link between IL-18 and atherosclerosis. This finding can offer a clue to understand the role of IL-18 in the development of atherosclerotic diseases.

Acknowledgments

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References

1. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation.* 2002;105:1135–1143.
2. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med.* 2000;342:836–843.
3. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation.* 2000;101:1767–1772.
4. Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger WH Jr, Heimovitz H, Cohen HJ, Wallace R. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am J Med.* 1999;106:506–512.
5. Blankenberg S, Tiret L, Bickel C, Peetz D, Cambien F, Meyer J, Rupprecht HJ. Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina. *Circulation.* 2002;106:24–30.
6. Blankenberg S, Luc G, Ducimetiere P, Arveiler D, Ferrieres J, Amouyel P, Evans A, Cambien F, Tiret L. Interleukin-18 and the risk of coronary heart disease in European men: the Prospective Epidemiological Study of Myocardial Infarction (PRIME). *Circulation.* 2003;108:2453–2459. Epub 2003 Oct 2427.
7. Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K, Kenji Akita, Motoshi Namba, Fujimi Tanabe, Kaori Konishi, Shigeharu Fukuda, Masashi Kurimoto. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature.* 1995;378:88–91.
8. Szmitko PE, Wang CH, Weisel RD, de Almeida JR, Anderson TJ, Verma S. New markers of inflammation and endothelial cell activation: Part I. *Circulation.* 2003;108:1917–1923.

9. Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A. Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation.* 2001;104:1598–1603.
10. Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein E(−/−) mice through release of interferon-gamma. *Circ Res.* 2002;90:E34–E38.
11. Aso Y, Okumura K, Takebayashi K, Wakabayashi S, Inukai T. Relationships of plasma interleukin-18 concentrations to hyperhomocysteinemia and carotid intimal-media wall thickness in patients with type 2 diabetes. *Diabetes Care.* 2003;26:2622–2627.
12. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, Clegg LX. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987–1993. *Am J Epidemiol.* 1997; 146:483–494.
13. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation.* 1997;96:1432–1437.
14. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group [see comments]. *N Engl J Med.* 1999;340:14–22.
15. Chambless LE, Folsom AR, Clegg LX, Sharrett AR, Shahar E, Nieto FJ, Rosamond WD, Evans G. Carotid wall thickness is predictive of incident clinical stroke: the Atherosclerosis Risk in Communities (ARIC) study. *Am J Epidemiol.* 2000;151:478–487.
16. Mallat Z, Henry P, Fressonnet R, Alouani S, Scoazec A, Beaufils P, Chvatchko Y, Tedgui A. Increased plasma concentrations of interleukin-18 in acute coronary syndromes. *Heart.* 2002;88:467–469.
17. Yamashita H, Shimada K, Seki E, Mokuno H, Daida H. Concentrations of interleukins, interferon, and C-reactive protein in stable and unstable angina pectoris. *Am J Cardiol.* 2003;91:133–136.
18. Zaremba J, Losy J. Interleukin-18 in acute ischaemic stroke patients. *Neurol Sci.* 2003;24:117–124.
19. Ferrucci L, Corsi A, Lauretani F, Bandinelli S, Bartali B, Taub DD, Guralnik JM, Longo DL. The Origins of Age-Related Pro-Inflammatory State. *Blood.* 2005;105:2294–2299.
20. Esposito K, Pontillo A, Di Palo C, Giugliano G, Masella M, Marfella R, Giugliano D. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. *JAMA.* 2003; 289:1799–1804.
21. Howard G, Sharrett AR, Heiss G, Evans GW, Chambless LE, Riley WA, Burke GL. Carotid artery intimal-medial thickness distribution in general populations as evaluated by B-mode ultrasound. ARIC Investigators. *Stroke.* 1993;24:1297–1304.
22. Heiss G, Sharrett AR, Barnes R, Chambless LE, Szklo M, Alzola C. Carotid atherosclerosis measured by B-mode ultrasound in populations: associations with cardiovascular risk factors in the ARIC study. *Am J Epidemiol.* 1991;134:250–256.
23. Crouse JR, Goldbourt U, Evans G, Pinsky J, Sharrett AR, Sorlie P, Riley W, Heiss G. Risk factors and segment-specific carotid arterial enlargement in the Atherosclerosis Risk in Communities (ARIC) cohort. *Stroke.* 1996;27:69–75.
24. Wang TJ, Nam BH, Wilson PW, Wolf PA, Levy D, Polak JF, D'Agostino RB, O'Donnell CJ. Association of C-reactive protein with carotid atherosclerosis in men and women: the Framingham Heart Study. *Arterioscler Thromb Vasc Biol.* 2002;22:1662–1667.
25. Magyar MT, Szikszai Z, Balla J, Valikovics A, Kappelmayer J, Imre S, Balla G, Jeney V, Csiba L, Bereczki D. Early-onset carotid atherosclerosis is associated with increased intima-media thickness and elevated serum levels of inflammatory markers. *Stroke.* 2003;34:58–63.
26. Kato A, Odamaki M, Takita T, Maruyama Y, Kumagai H, Hishida A. Association between interleukin-6 and carotid atherosclerosis in hemodialysis patients. *Kidney Int.* 2002;61:1143–1152.
27. Ikonomidis I, Andreotti F, Economou E, Stefanadis C, Toutouzas P, Nihoyannopoulos P. Increased proinflammatory cytokines in patients with chronic stable angina and their reduction by aspirin. *Circulation.* 1999;100:793–798.
28. Albert MA, Danielson E, Rifai N, Ridker PM. Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study. *JAMA.* 2001;286:64–70.
29. Rezaie-Majd A, Maca T, Bucek RA, Valent P, Muller MR, Husslein P, Kashanipour A, Minar E, Baghestanian M. Simvastatin reduces expression of cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol.* 2002;22:1194–1199.
30. Lauten WB, Khan QA, Rajagopalan S, Lerakis S, Rahman ST, Parthasarathy S, Khan BV. Usefulness of quinapril and irbesartan to improve the anti-inflammatory response of atorvastatin and aspirin in patients with coronary heart disease. *Am J Cardiol.* 2003;91:1116–1119.
31. Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U. Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J Exp Med.* 2002;195:245–257.
32. Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, Bayard F, Hansson GK. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res.* 2003;59: 234–240.

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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

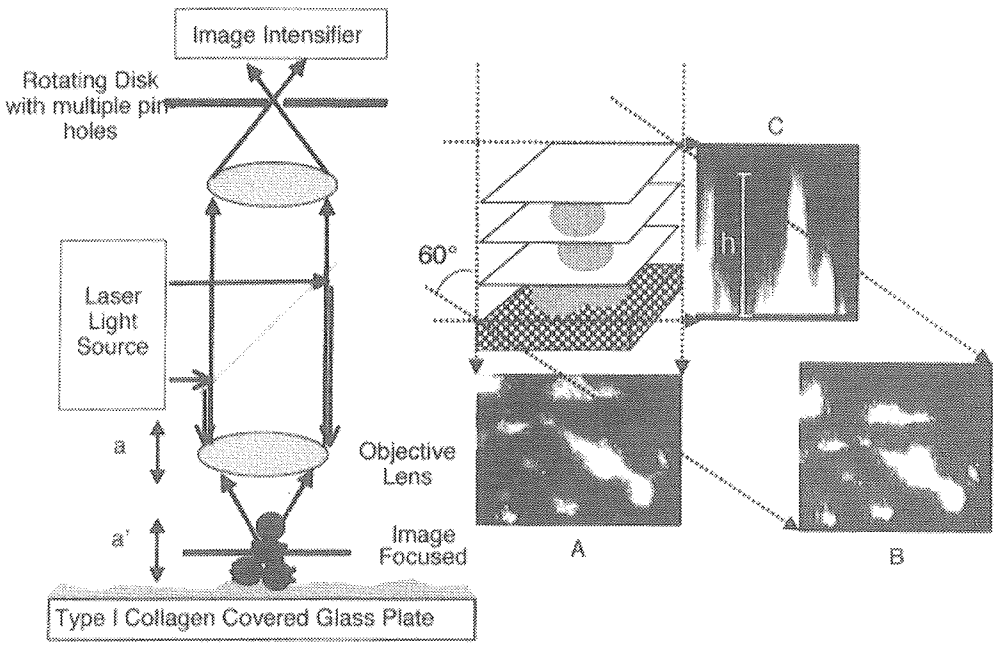

Figure 1. Three-dimensional projection imaging of platelet thrombi. A piezo-electric motor **(a)** moved the objective lens at a constant speed of 0.4 μmol/l/s to provide scanning images of the platelet thrombi **(a')**. The sum of the confocal images in a bottom to top stack (z-axis) was projected on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

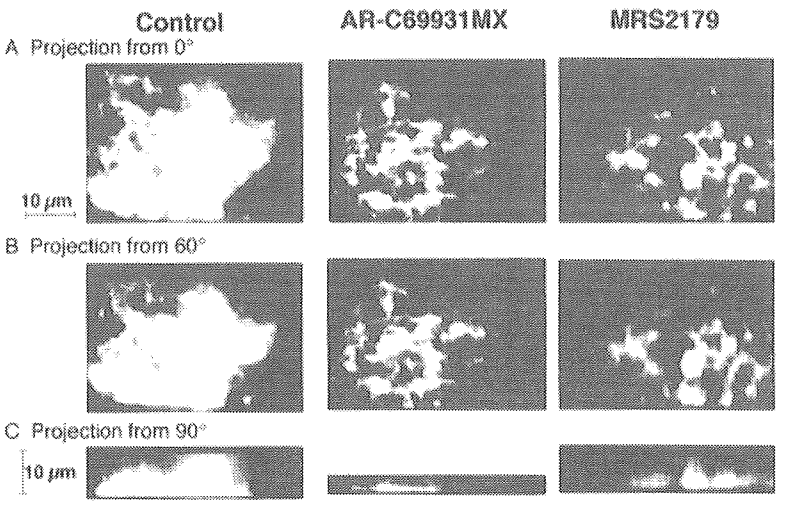

Figure 2. Three-dimensional projection images of thrombi formed in the presence or absence of adenosine diphosphate receptor antagonists. Blood with fluoresceinated platelets was perfused over immobilized collagen type I fibrils for 6 min at the wall shear rate of 1,500 s^{-1} in the absence (Control)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

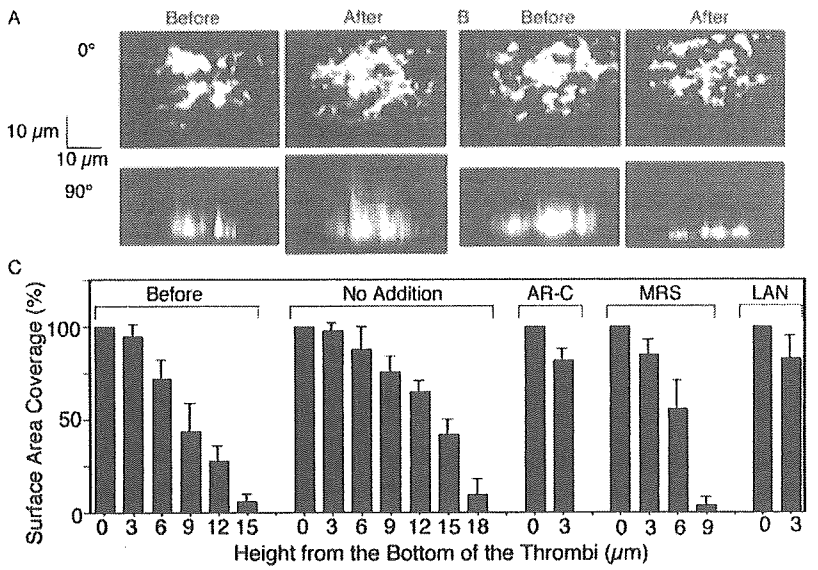

Figure 3. Reduction in the size of platelet thrombi exposed to blood containing different antagonists of platelet function. These experiments were performed as described in the caption of Figure 2, with the difference that the surface was exposed to two subsequent aliquots of blood each perfused for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

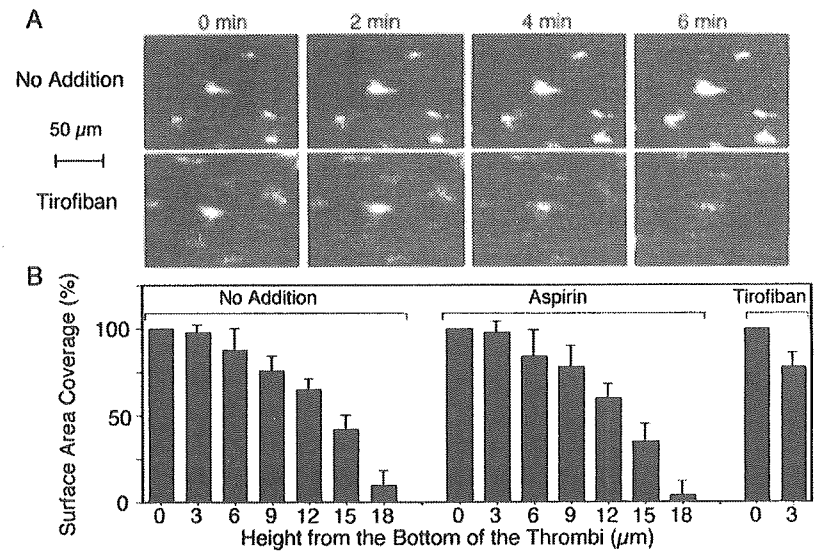

Figure 4. Changes in the two-dimensional and three-dimensional structure of platelet thrombi exposed to blood containing antiplatelet agents. These experiments were performed essentially as described in the caption of Figure 3. **(A)** Representative two-dimensional fluorescence microscopy images of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

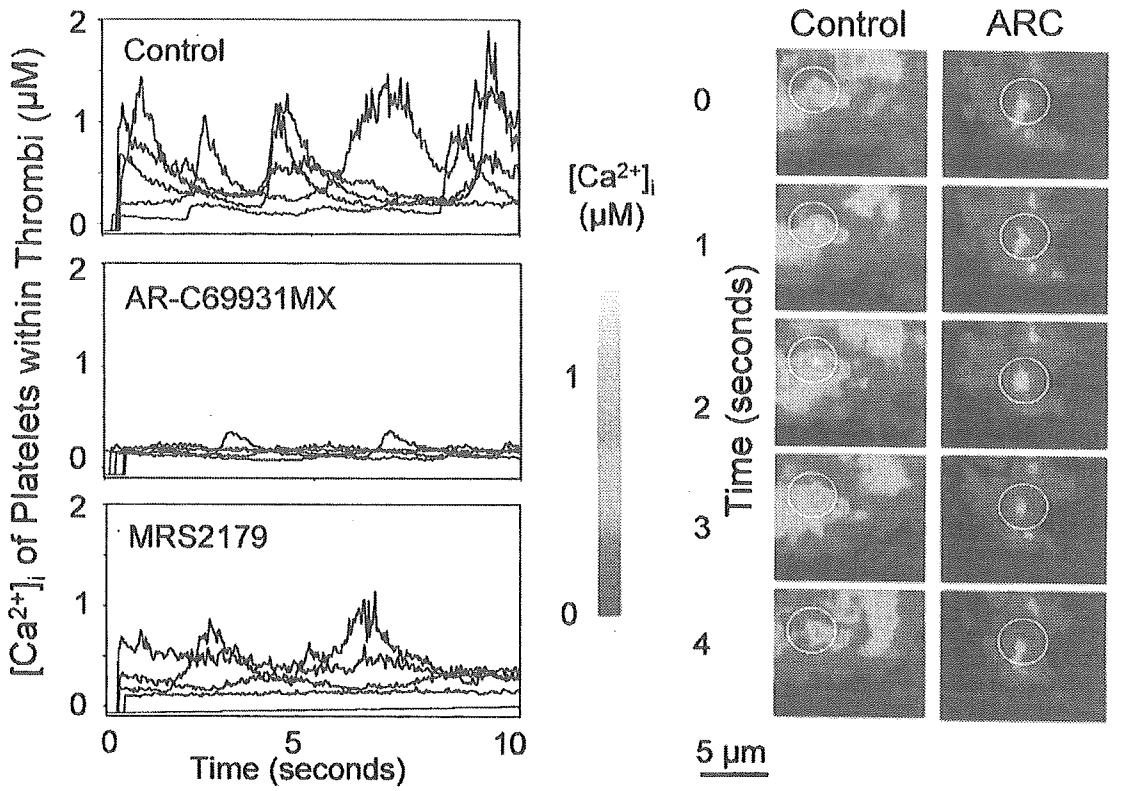

Figure 5. Changes in the intracytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) of platelet thrombi caused by adenosine diphosphate receptor antagonists. These experiments were conducted as described in the caption of Figure 3, except that blood was replaced with a cell suspension containing fluo-3AM–loaded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

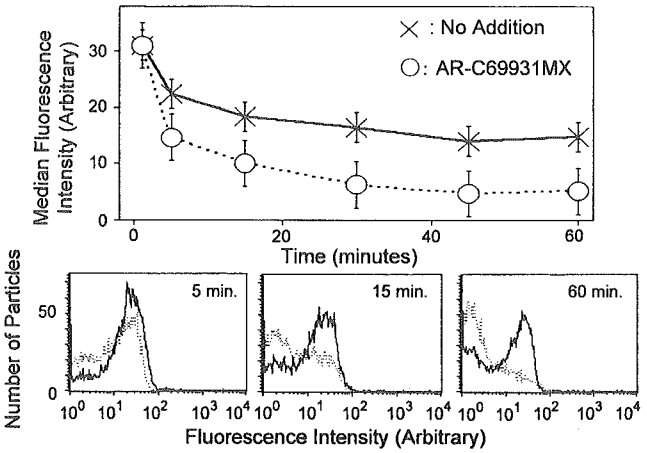

Figure 6. Changes in PAC-1 binding to activated platelets caused by adenosine diphosphate (ADP) receptor antagonists. To activate platelets, 25 μl of HEPES buffer containing ADP and epinephrine (400 μmol/l each) was mixed with 375 μl of platelet-rich plasma and incubated for 20 min. Fifty μl of fluorescein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

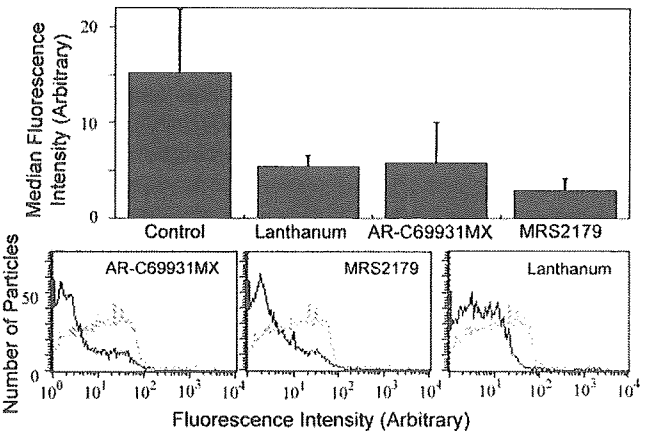

Figure 7. Effect of different platelet inhibitors on PAC-1 binding to activated platelets. These experiments were performed as described in the caption for Figure 6, with the only difference that the $P2Y_1$ inhibitor, MRS2179, or the putative Ca^{2+} channel blocker, lanthanum chloride, was also added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

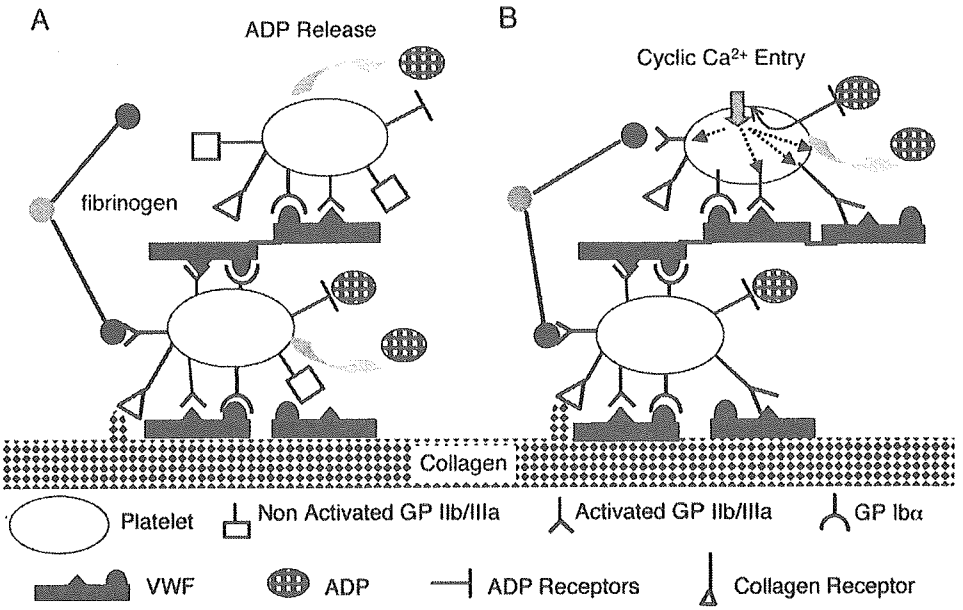

Figure 8. Schematic representation of the mechanism that stabilizes platelets at the edge of a growing thrombus exposed to elevated shear rates. **(A)** Circulating platelets adhere and become activated onto collagen through multiple adhesive interactions, initiated by glycoprotein (GP) Ibα binding to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

1. Ruggeri ZM. Platelets in atherothrombosis. Nat Med 2002;8:1227–34.
2. Goto S. Propagation of arterial thrombi: local and remote contributory factors. Arterioscler Thromb Vasc Biol 2004;24:2207–8.
3. Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. Nat Med 2002;8:1175–81.
4. Hollopeter G, Jantzen HM, Vincent D, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001;409:202–7.
5. Savi P, Combalbert J, Gaich C, et al. The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P450-1A. Thromb Haemost 1994;72:313–7.
6. Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. Blood 2002;99:193–8.
7. Turner NA, Moake JL, McIntire LV. Blockade of adenosine diphosphate receptors P2Y(12) and P2Y(1) is required to inhibit platelet aggregation in whole blood under flow. Blood 2001;98:3340–5.
8. Goto S, Tamura N, Handa S. Effects of adenosine 5′-diphosphate (ADP) receptor blockade on platelet aggregation under flow. Blood 2002;99:4644–6.
9. Remijn JA, Wu YP, Jeninga EH, et al. Role of ADP receptor P2Y(12) in platelet adhesion and thrombus formation in flowing blood. Arterioscler Thromb Vasc Biol 2002;22:686–91.
10. Trumel C, Payrastre B, Plantavid M, et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. Blood 1999;94:4156–65.
11. Bhatt DL, Topol EJ. Scientific and therapeutic advances in antiplatelet therapy. Nat Rev Drug Discov 2003;2:15–28.
12. Nesbitt WS, Giuliano S, Kulkarni S, Dopheide SM, Harper IS, Jackson SP. Intercellular calcium communication regulates platelet aggregation and thrombus growth. J Cell Biol 2003;160:1151–61.
13. Andre P, Delaney SM, LaRocca T, et al. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest 2003;112:398–406.
14. Gachet C. ADP receptors of platelets and their inhibition. Thromb Haemost 2001;86:222–32.
15. Goto S, Tamura N, Ishida H. Ability of anti-glycoprotein IIb/IIIa agents to dissolve platelet thrombi formed on a collagen surface under blood flow conditions. J Am Coll Cardiol 2004;44:316–23.
16. Goto S, Tamura N, Handa S, Arai M, Kodama K, Takayama H. Involvement of glycoprotein VI in platelet thrombus formation on both collagen and von Willebrand factor surfaces under flow conditions. Circulation 2002;106:266–72.
17. Ingall AH, Dixon J, Bailey A, et al. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 1999;42:213–20.
18. Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK. Competitive and selective antagonism of P2Y1 receptors by N6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate. Br J Pharmacol 1998; 124:1–3.
19. Friedman H, Meir S, Rosenberger I, Halevy AH, Kaufman PB, Philosoph-Hadas S. Inhibition of the gravitropic response of snapdragon spikes by the calcium-channel blocker lanthanum chloride. Plant Physiol 1998;118:483–92.
20. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996;84:289–97.
21. Merritt JE, McCarthy SA, Davies MP, Moores KE. Use of fluo-3 to measure cytosolic Ca2+ in platelets and neutrophils. Loading cells with the dye, calibration of traces, measurements in the presence of plasma, and buffering of cytosolic Ca2+. Biochem J 1990;269:513–9.
22. Heemskerk JW, Willems GM, Rook MB, Sage SO. Ragged spiking of free calcium in ADP-stimulated human platelets: regulation of puff-like calcium signals in vitro and ex vivo. J Physiol 2001;535: 625–35.
23. Packham MA, Rand ML, Perry DW, Ruben DH, Kinlough-Rathbone RL. Probenecid inhibits platelet responses to aggregating agents in vitro and has a synergistic inhibitory effect with penicillin G. Thromb Haemost 1996;76:239–44.
24. Achyuthan KE, Mary A, Greenberg CS. Tb(III)-ion-binding-induced conformational changes in platelet factor XIII. Biochem J 1989;257: 331–8.
25. Redondo PC, Harper AG, Salido GM, Pariente JA, Sage SO, Rosado JA. A role for SNAP-25 but not VAMPs in store-mediated Ca2+ entry in human platelets. J Physiol 2004;558:99–109.
26. Rosado JA, Lopez JJ, Harper AG, et al. Two pathways for store-mediated calcium entry differentially dependent on the actin cytoskeleton in human platelets. J Biol Chem 2004;279:29231–5.
27. Rosado JA, Sage SO. The ERK cascade, a new pathway involved in the activation of store-mediated calcium entry in human platelets. Trends Cardiovasc Med 2002;12:229–34.
28. Prevost N, Woulfe DS, Jiang H, et al. Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. Proc Natl Acad Sci U S A 2005;102: 9820–5.
29. Gast A, Tschopp TB, Baumgartner HR. Thrombin plays a key role in late platelet thrombus growth and/or stability. Effect of a specific thrombin inhibitor on thrombogenesis induced by aortic subendothelium exposed to flowing rabbit blood. Arterioscler Thromb 1994;14: 1466–74.
30. Goto S, Tamura N, Li M, et al. Different effects of various anti-GP IIb-IIIa agents on shear-induced platelet activation and expression of procoagulant activity. J Thromb Haemost 2003;1:2022–30.

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

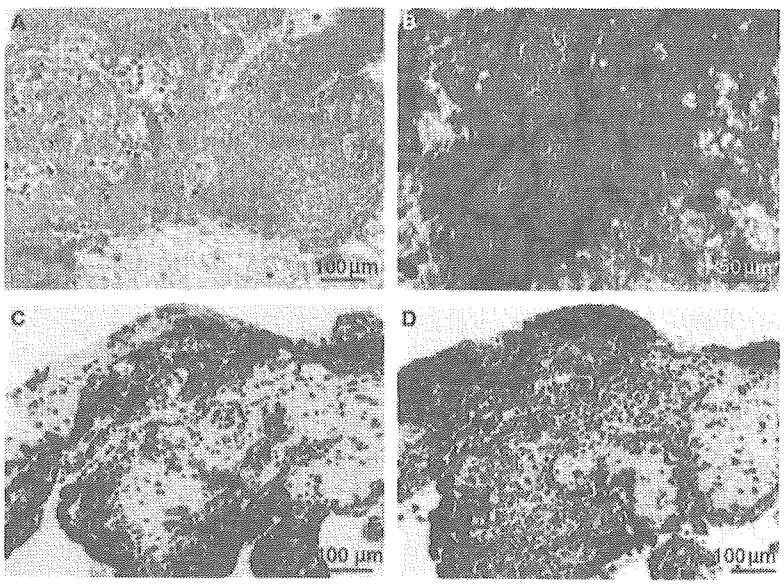

Fig. 1. Histological features of coronary thrombi obtained during the acute phase from patients with acute myocardial infarction. (A) Hematoxylin & eosin staining. The coronary thrombus specimen obtained from patient 10 in Table 1 is composed of inhomogeneous distribution of platelets, fibrin and inflammatory 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.

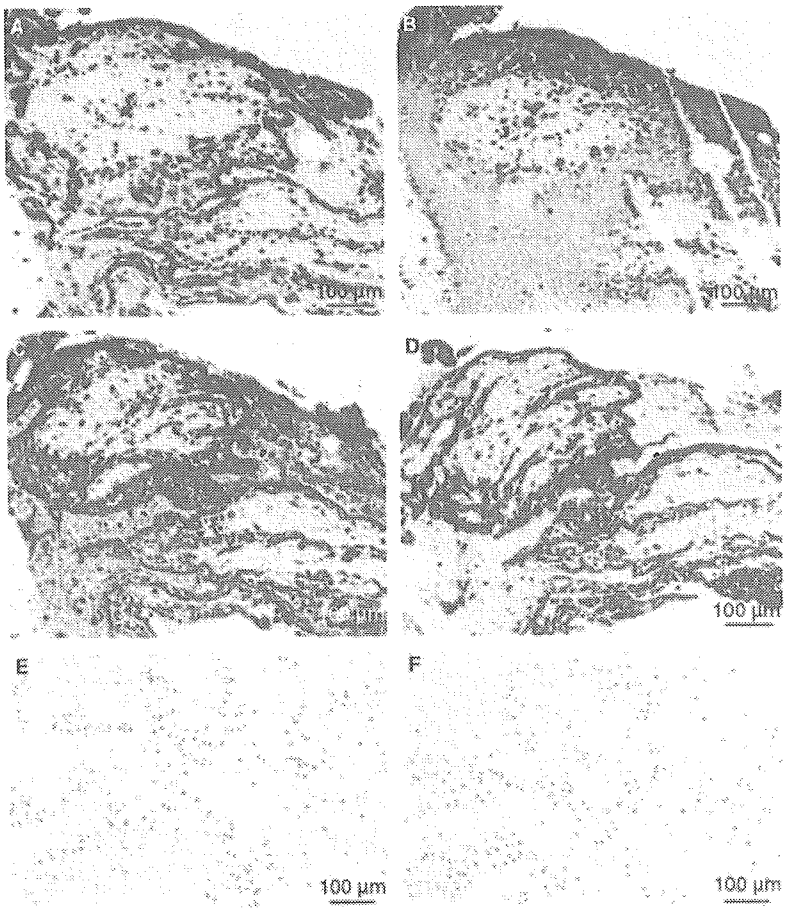

Fig. 2. Histochemical staining of coronary thrombi obtained freshly from patients with acute myocardial infarction. (A) Immunohistochemical staining of von Willebrand factor (VWF). A coronary thrombus specimen obtained from patient 3 in Table 1 demonstrated the prominent presence of VWF, especially at 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.

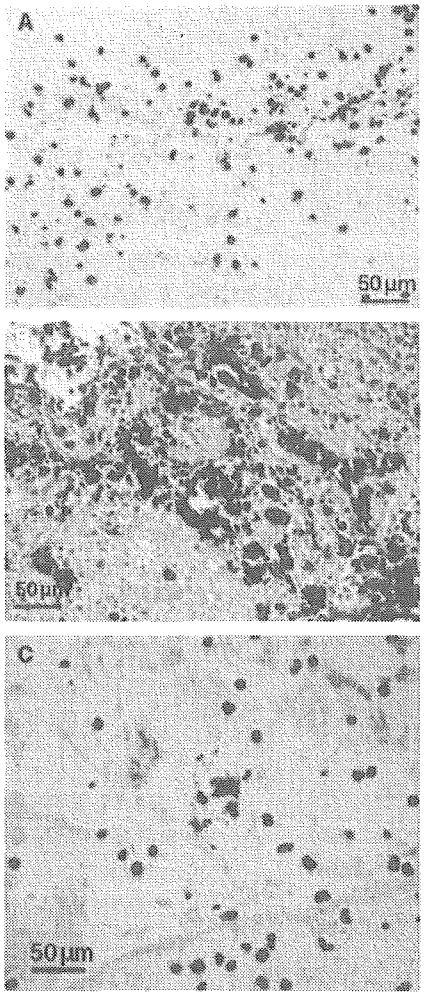

Fig. 3. Characterization of the inflammatory cells involved in the formation of coronary thrombi. All the specimens of coronary thrombi obtained from the 10 cases of acute myocardial infarction shown in Table 1 contained inflammatory cells stained positively by anti-CD16 (A), anti-CD45 (B), and anti-CD34 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).

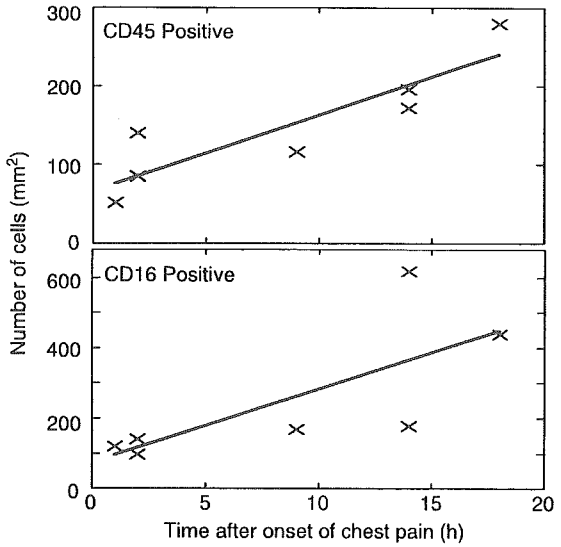

Fig. 4. Relationship between the number of leukocytes in the thrombus specimens and the time of specimen collection after the onset of chest pain. The upper and lower panels demonstrate the relationship between the time of specimen collection after the onset of chest pain and the number of CD16- and 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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References

1 Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ* 2002; **324**: 71–86.

2 Gluckman TJ, Baranowski B, Ashen MD, Henrikson CA, McAllister M, Braunstein JB, Blumenthal RS. A practical and evidence-based approach to cardiovascular disease risk reduction. *Arch Intern Med* 2004; **164**: 1490–500.

3 Hurlen M, Abdelnoor M, Smith P, Erikssen J, Arnesen H. Warfarin, aspirin, or both after myocardial infarction. *N Engl J Med* 2002; **347**: 969–74.

4 Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med* 2000; **342**: 836–43.

5 Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med* 2004; **350**: 1387–97.

6 Brennan ML, Penn MS, Van Lente F, Nambi V, Shishehbor MH, Aviles RJ, Goormastic M, Pepoy ML, McErlean ES, Topol EJ, Nissen SE, Hazen SL. Prognostic value of mycloperoxidase in patients with chest pain. *N Engl J Med* 2003; **349**: 1595–604.

7 Goto S. Propagation of arterial thrombi: local and remote contributory factors. *Arterioscler Thromb Vasc Biol* 2004; **24**: 2207–8.

8 Goto S. Understanding the mechanism and prevention of arterial occlusive thrombus formation by anti-platelet agents. *Curr Med Chem* 2004; **2**: 149–56.

9 Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002; **8**: 1227–34.
10 Goto S, Ikeda Y, Saldivar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. *J Clin Invest* 1998; **101**: 479–86.
11 Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med* 2002; **8**: 1175–81.
12 Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furie BC, Furie B. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J Exp Med* 2003; **197**: 1585–98.
13 Palmerini T, Coller BS, Cervi V, Tomasi L, Marzocchi A, Marrozzini C, Leone O, Piccioli M, Branzi A. Monocyte-derived tissue factor contributes to stent thrombosis in an in vitro system. *J Am Coll Cardiol* 2004; **44**: 1570–7.
14 Lablanche JM, Fourrier JL, Gommeaux A, Becquart J, Bertrand ME. Percutaneous aspiration of a coronary thrombus. *Cathet Cardiovasc Diagn* 1989; **17**: 97–8.
15 Wang HJ, Kao HL, Liau CS, Lee YT. Export aspiration catheter thrombosuction before actual angioplasty in primary coronary intervention for acute myocardial infarction. *Catheter Cardiovasc Interv* 2002; **57**: 332–9.
16 Yamauchi T, Furui S, Isshiki T, Toyoizumi H, Kohtake H, Takeshita K, Suzuki S, Harasawa A, Sasaki Y. Emergent right coronary artery thrombectomy with a jet aspiration thrombectomy catheter. *Cardiovasc Intervent Radiol* 1999; **22**: 340–2.
17 Murakami T, Mizuno S, Takahashi Y, Ohsato K, Moriuchi I, Arai Y, Mifune J, Shimizu M, Ohnaka M. Intracoronary aspiration thrombectomy for acute myocardial infarction. *Am J Cardiol* 1998; **82**: 839–44.
18 Naruko T, Ueda M, Haze K, van der Wal AC, van der Loos CM, Itoh A, Komatsu R, Ikura Y, Ogami M, Shimada Y, Ehara S, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker AE. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 2002; **106**: 2894–900.
19 Burke AP, Farb A, Malcom GT, Liang Y, Smialek J, Virmani R. Effect of risk factors on the mechanism of acute thrombosis and sudden coronary death in women. *Circulation* 1998; **97**: 2110–6.
20 Antman EM, Anbe DT, Armstrong PW, Bates ER, Green LA, Hand M, Hochman JS, Krumholz HM, Kushner FG, Lamas GA, Mullany CJ, Ornato JP, Pearle DL, Sloan MA, Smith SC Jr, Alpert JS, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Gregoratos G, Halperin JL, Hiratzka LF, Hunt SA, Jacobs AK. ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Revise the 1999 Guidelines for the Management of Patients with Acute Myocardial Infarction). *Circulation* 2004; **110**: e82–292.
21 Braunwald E, Antman EM, Beasley JW, Califf RM, Cheitlin MD, Hochman JS, Jones RH, Kereiakes D, Kupersmith J, Levin TN, Pepine CJ, Schaeffer JW, Smith EE III, Steward DE, Theroux P, Gibbons RJ, Alpert JS, Faxon DP, Fuster V, Gregoratos G, Hiratzka LF, Jacobs AK, Smith SC Jr. ACC/AHA guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction – 2002: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on the Management of Patients With Unstable Angina). *Circulation* 2002; **106**: 1893–900.
22 Yamashita A, Asada Y, Sugimura H, Yamamoto H, Marutsuka K, Hatakeyama K, Tamura S, Ikeda Y, Sumiyoshi A. Contribution of von Willebrand factor to thrombus formation on neointima of rabbit stenotic iliac artery under high blood-flow velocity. *Arterioscler Thromb Vasc Biol* 2003; **23**: 1105–10.
23 Kurokawa T, Iwasa S, Kakinuma A, Stassen JM, Lijnen HR, Collen D. Enhancement of clot lysis in vitro and in vivo with a bispecific monoclonal antibody directed against human fibrin and against urokinase-type plasminogen activator. *Thromb Haemost* 1991; **66**: 684–93.
24 Velican C, Velican D. Are routine autopsy protocols adequate for accurate appraisal of coronary atherosclerosis? *Zentralbl Allg Pathol* 1989; **135**: 619–29.
25 Badimon L, Badimon JJ, Cohen M, Chesebro JH, Fuster V. Vessel wall-related risk factors in acute vascular events. *Drugs* 1991; **42**(Suppl. 5): 1–9.
26 Roberts WC, Buja LM. The frequency and significance of coronary arterial thrombi and other observations in fatal acute myocardial infarction: a study of 107 necropsy patients. *Am J Med* 1972; **52**: 425–43.
27 Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell* 1998; **94**: 657–66.
28 Jansson JH, Nilsson TK, Johnson O. Willebrand factor in plasma: a novel risk factor for recurrent myocardial infarction and death. *Br Heart J* 1991; **66**: 351–5.
29 Folsom AR, Wu KK, Rosamond WD, Sharrett AR, Chambless LE. Prospective study of hemostatic factors and incidence of coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. *Circulation* 1997; **96**: 1102–8.
30 Goto S, Sakai H, Goto M, Ono M, Ikeda Y, Handa S, Ruggeri ZM. Enhanced shear-induced platelet aggregation in acute myocardial infarction. *Circulation* 1999; **99**: 608–13.
31 Sakai H, Goto S, Kim JY, Aoki N, Abe S, Ichikawa N, Yoshida M, Nagaoka Y, Handa S. Plasma concentration of von Willebrand factor in acute myocardial infarction. *Thromb Haemost* 2000; **84**: 204–9.
32 Weitz JI, Hirsh J, Samama MM. New anticoagulant drugs: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest* 2004; **126**: 265S–86S.
33 Gruber A, Hanson SR. Factor XI-dependence of surface- and tissue factor-initiated thrombus propagation in primates. *Blood* 2003; **102**: 953–5.
34 Yamashita A, Furukoji E, Marutsuka K, Hatakeyama K, Yamamoto H, Tamura S, Ikeda Y, Sumiyoshi A, Asada Y. Increased vascular wall thrombogenicity combined with reduced blood flow promotes occlusive thrombus formation in rabbit femoral artery. *Arterioscler Thromb Vasc Biol* 2004; **24**: 2420–4.
35 Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982; **122**: 429–36.
36 Goto S, Tamura N, Li M, Handa M, Ikeda Y, Handa S, Ruggeri ZM. Different effects of various anti-GPIIb-IIIa agents on shear-induced platelet activation and expression of procoagulant activity. *J Thromb Haemost* 2003; **1**: 2022–30.
37 Ramstrom S, Ranby M, Lindahl TL. Platelet phosphatidylserine exposure and procoagulant activity in clotting whole blood – different effects of collagen, TRAP and calcium ionophore A23187. *Thromb Haemost* 2003; **89**: 132–41.
38 Zeiher AM, Goebel H, Schachinger V, Ihling C. Tissue endothelin-1 immunoreactivity in the active coronary atherosclerotic plaque. A clue to the mechanism of increased vasoreactivity of the culprit lesion in unstable angina. *Circulation* 1995; **91**: 941–7.
39 Kolodgie FD, Gold HK, Burke AP, Fowler DR, Kruth HS, Weber DK, Farb A, Guerrero LJ, Hayase M, Kutys R, Narula J, Finn AV, Virmani R. Intraplaque hemorrhage and progression of coronary atheroma. *N Engl J Med* 2003; **349**: 2316–25.
40 Goto S, Eto K, Ikeda Y, Handa S. Abciximab not RGD peptide inhibits von Willebrand factor-dependent platelet activation under shear. *Lancet* 1999; **353**: 809.
41 Evangelista V, Manarini S, Coller BS, Smyth SS. Role of P-selectin, beta2-integrins, and Src tyrosine kinases in mouse neutrophil-platelet adhesion. *J Thromb Haemost* 2003; **1**: 1048–54.
42 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964–7.

Detection of von Willebrand Factor and Tissue Factor in Platelets-Fibrin Rich Coronary Thrombi in Acute Myocardial Infarction

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The rapid closure of coronary arteries due to occlusive thrombi is the major cause of acute myocardial infarction. However, the mechanisms of coronary thrombus formation have not been elucidated. We immunohistochemically assessed the localizations and their changes over time of glycoprotein IIb/IIIa, fibrin, von Willebrand factor (vWF), and tissue factor (TF), after the onset of chest pain (<4, 4 to 6, or 6 to 12 hours), in fresh coronary thrombi causing acute myocardial infarction. The occlusive thrombi were consistently composed of platelets, fibrin, vWF, and TF from the early phase of onset, and glycoprotein IIb/IIIa and fibrin were closely associated with vWF and TF, respectively. vWF and/or TF may contribute to occlusive thrombus formation and be novel therapeutic candidates for treating patients with coronary thrombosis. © 2006 Elsevier Inc. All rights reserved. **(Am J Cardiol 2006;97:26–28)**

Many thrombotic factors are involved in acute thrombus formation. In particular, von Willebrand factor (vWF) binding to glycoprotein (GP) Ibα and GP IIb/IIIa plays an important role in initial platelet aggregation under rapid flow conditions.[1] Tissue factor (TF) expressed in disrupted atheromatous plaques potently activates the coagulation cascade, leading to thrombin generation. Thrombin is a powerful platelet agonist, in addition to fibrin formation, and may amplify thrombus growth.[2] Although vWF and TF play significant roles in thrombus formation, their localization and how they change over time have not been examined in fresh thrombi that cause acute myocardial infarction (AMI). We therefore examined platelet, vWF, TF, and fibrin localization immunohistochemically in fresh occlusive thrombi from patients with AMI. We also examined the proportion of these constituents <4, 4 to 6, and 6 to 12 hours after the onset of chest pain. Thrombi were obtained with a novel ultra-thin aspiration catheter, which allows the pathologic evaluation of occlusive thrombi in vivo without postmortem changes.[3,4]

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We examined 21 patients with AMI who had undergone thrombus removal using an aspiration catheter (Thrombuster, Kaneka, Japan). The diagnosis of AMI was based on published clinical guidelines.[5] All patients provided written informed consent to participate in the study, and the institutional ethics committees approved the study protocol. We examined thrombi obtained from 17 patients within 12 hours of chest pain onset. Thrombi that appeared organized (2 patients) were excluded from this study. Thus, 15 patients were separated into 3 groups according to the time after onset (<4, 4 to 6, and 6 to 12 hours). The risk factors for coronary artery disease consisted of hypertension (systolic pressure >140 mm Hg and/or diastolic pressure >90 mm Hg), hypercholesterolemia (total cholesterol >220 mg/dl), diabetes mellitus, smoking, and obesity (body mass index >30 kg/m^2). Aspirated thrombi were immediately fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. Sections were stained immunohistochemically using antibodies against platelet GP IIb/IIIa (Affinity Biologicals, Hamilton, California), vWF (Santa Cruz Biotechnology, Santa Cruz, California), TF (Kaketsuken, Kumamoto, Japan)[6] and fibrin (CHEMICON International, Temecula, California) with the peroxidase-labeled secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania), or were double-stained with fluorescein isothiocyanate- or Cy3-labeled secondary antibodies (Jackson ImmunoResearch). Immunofluorescent and immunohistochemical positive areas were analyzed using a spectral confocal scanning system (TCS SP2, Leica, Tokyo, Japan) and a color imaging morphometric analysis system (Mac SCOPE, Mitani, Fukui, Japan), respectively.[6] One-way analysis of variance was used to

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Table 1
Clinical characteristics of study patients

	Hours After Onset		
	0–4 (n = 5)	4–6 (n = 5)	6–12 (n = 5)
Age (yrs)	65.2 ± 7.6	60.6 ± 2.3	67.4 ± 3.7
Men	5	4	5
Hypercholesterolemia*	3	3	3
Hypertension†	3	3	3
Diabetes mellitus	2	1	1
Obesity	2	3	4
Smoker	3	4	4

* Hypercholesterolemia (total cholesterol >220 mg/dl).
† Hypertension (systolic pressure >140 mm Hg and/or diastolic pressure >90 mm Hg).

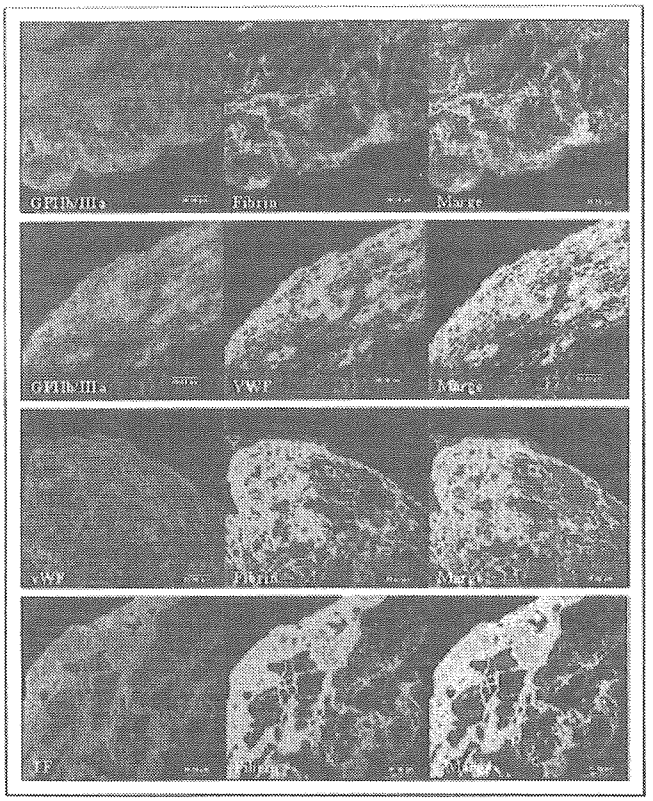

Figure 1. Representative immunofluorescent micrographs of fresh coronary thrombi from patients with AMI. *Left*, staining with fluorescein isothiocyanate-labeled GP IIb/IIIa, vWF, and TF *(green). Center*, staining with Cy3-labeled with fibrin and vWF *(red). Right*, merged immunofluorescent images. Co-localized areas of each factor are stained *yellow*.

compare values among the groups. The data are expressed as means ± SE. A p value <0.05 was considered statistically significant.

Table 1 shows the clinical characteristics of the patients. Risk factors for coronary artery disease among the 3 groups did not differ significantly. All coronary thrombi specimens were composed mostly of aggregated platelets and densely packed fibrin (Figure 1), along with inflammatory-related polymorphonuclear and mononuclear cells (data not shown). Immunohistochemical staining revealed the constitutive presence

Table 2
Immunopositive area for antibodies in thrombi

	Hours After Onset		
	0–4 (n = 5)	4–6 (n = 5)	6–12 (n = 5)
GP IIb/IIIa (%)	24.1 ± 3.1	30.7 ± 5.4	34.9 ± 8.0
Fibrin (%)	28.4 ± 4.3	29.3 ± 7.0	30.5 ± 5.9
vWF (%)	28.3 ± 4.2	30.5 ± 8.9	34.3 ± 6.2
TF (%)	19.9 ± 4.6	26.2 ± 4.8	18.9 ± 3.5

of GP IIb/IIIa, fibrin, vWF, and TF in all the thrombi. The immunopositive area (percentage) of GP IIb/IIIa and vWF tended to increase in older thrombi, but did not significantly differ with time after onset (Table 2). Immunofluorescent staining showed that GP IIb/IIIa intermingled with fibrin, and that the surface of the thrombi was mainly covered with GP IIb/IIIa and vWF. GP IIb/IIIa co-localized with vWF, TF was closely associated with fibrin, and vWF somewhat co-localized with fibrin (Figure 1).

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The occlusive thrombi causing AMI were rich in fibrin, as well as platelets, from the early phase of onset. In contrast to our findings, Nagata et al[7] recently reported a higher platelet component of many aspirated thrombi using thrombectomy catheters during the early phase of AMI. This discrepancy may have been because they stained samples with hematoxylin and eosin or azan, and we used immunohistochemical means with a specific antibody to detect fibrin. Therefore, the results of the present study seem to be more objective and specific. We found that the proportion of platelets and fibrin in thrombi did not significantly differ within 12 hours after onset. This suggests that the coagulation system, as well as platelets, plays a crucial role in thrombus formation in AMI. In contrast, thrombin generation on activated platelets involves complex interactions between platelets and coagulation proteins. Activated platelets provide a procoagulant lipid surface to assemble coagulation factors and promote thrombin generation, resulting in additional platelet activation and fibrin formation. Immunofluorescent staining showed that GP IIb/IIIa and vWF intermingled with fibrin, which closely co-localized TF. The findings suggest that accumulation of these molecules on activated platelets amplifies thrombus growth. Because fibrin is essential for stabilizing initial and loosely packed platelets aggregates during high blood flow, our results support the relevance of fibrinolysis therapy for AMI.[8]

Many in vitro studies have revealed that vWF plays a pivotal role during the initial step of platelet adhesion and aggregation under rapid flow conditions.[9,10] We found that vWF was closely localized with platelets and partly with fibrin, which suggests that vWF plays an important role in platelet–platelet[9,10] and platelet–fibrin interactions[11] during thrombus growth in AMI. Because vWF is present in plasma and in platelet α-granules, a positive immunoreaction for vWF in thrombi does not necessarily reflect functional vWF. How-