Novel Role of Platelets in Mediating Inflammatory Responses and Ventricular Rupture or Remodeling Following Myocardial Infarction


**Objective**—The goal of this study was to investigate the role of platelets in systemic and cardiac inflammatory responses and the development of postinfarct ventricular complications, as well as the efficacy of antiplatelet interventions.

**Methods and Results**—Using a mouse myocardial infarction (MI) model, we determined platelet accumulation and severity of inflammation within the infarcted myocardium by immunohistochemistry and biochemical assays, analyzed peripheral blood platelet-leukocyte conjugation using flow cytometry, and tested antiplatelet interventions, including thienopyridines and platelet depletion. Platelets accumulated within the infarcted region early post-MI and colocalized with inflammatory cells. MI evoked early increase in circulating platelet-leukocyte conjugation mediated by P-selectin/P-selectin glycoprotein ligand-1. Antiplatelet interventions inhibited platelet-leukocyte conjugation in peripheral blood, inflammatory infiltration, content of matrix metalloproteinases or plasminogen activation, and expression of inflammatory mediators in the infarcted myocardium (all P<0.05) and lowered rupture incidence (P<0.01). Clopidogrel therapy alleviated the extent of chronic ventricular dilatation by serial echocardiography.

**Conclusion**—Platelets play a pivotal role in promoting systemic and cardiac inflammatory responses post-MI. Platelets accumulate within the infarcted myocardium, contributing to regional inflammation, ventricular remodeling, and rupture. Antiplatelet therapy reduces the severity of inflammation and risk of post-MI complications, demonstrating a previously unrecognized protective action. (Arterioscler Thromb Vasc Biol. 2011;31:834-841.)

Key Words: ischemic heart disease ■ leukocytes ■ platelets ■ thienopyridines ■ inflammation ■ myocardial infarction ■ ventricular rupture

The role of platelets in atherosclerotic lesions and acute coronary syndrome has been well documented. The proinflammatory actions of platelets have received increasing attention.1,2 Platelets contribute to inflammatory responses through release of inflammatory mediators and platelet-leukocyte interactions by which platelets mediate leukocyte activation and infiltration into inflamed tissues.1,2 There are several reports of an elevated proportion of platelet-leukocyte aggregates tested ex vivo in blood samples from patients with acute coronary syndromes.3-5 The current rationale for routine use of the platelet P2Y12 receptor inhibitors thienopyridines (clopidogrel and prasugrel) is to prevent arterial thrombosis following coronary intervention.6 Thienopyridine treatment is known to inhibit platelet-leukocyte interactions in the peripheral blood of patients with peripheral atherosclerotic vascular disease, coronary artery disease, or renal transplantation.5,6

Myocardial infarction (MI) evokes intense inflammatory responses both systemically and within the infarcted myocardium, with adverse consequences.7 The potential contribution of platelets to postinfarct cardiac inflammation remains unexplored. Relevant to this is the question of whether thienopyridines exert cardiac protection through inhibition of platelet’s inflammatory action in the infarcted myocardium, independent of vascular thrombosis.

Ventricular wall rupture is a fatal complication of acute MI, with a death rate of 70% to 90%.8,9 Recent experimental studies, including ours, have provided strong evidence that wall rupture is the consequence of severe regional inflammation and elevation of proteinases, particularly matrix metalloproteinases (MMPs) and plasmin, with subsequent damage to extracellular matrix proteins and reduction in tissue tensile strength.10-14 Thus, interventions such as MMP inhibitors and gene disruption of MMP-9, MMP-2, or urokinase plasminogen activator inhibitors may offer protection against or reduce the incidence of post-MI ventricular wall rupture.
gen activator (uPA) reduced rupture incidence. More severe inflammation, with a higher level of MMP-9 or C-reactive protein, was also reported in infarcted patients who had rupture compared with those without rupture. The mouse is the only laboratory species that develops rupture after acute MI. The mouse model of rupture simulates many features seen in human ventricular rupture and also displays overt infarct expansion in the acute phase and ventricular remodeling chronically.

Here, we report findings suggesting a pivotal role for platelets in systemic and myocardial inflammation and wall rupture pathogenesis following acute MI that were effectively inhibited by treatment with thienopyridines. These findings reveal additional efficacy of antiplatelet interventions in the setting of acute MI.

Methods
A detailed description is presented in the Supplemental Materials, available online at http://atvb.ahajournals.org.

Animals and Surgery
All experimental procedures were approved by a local animal ethics committee. Male C57BL/6 mice were operated on for permanent occlusion of the left coronary artery or sham operation. To generate tissues for assays, animals were killed at day 2 or 3 after MI, and infarct and noninfarct portions of the left ventricle (LV) were separated. Animals allocated to the rupture incidence were closely monitored for 7 days; autopsy was performed once animals were found dead to determine the reason of death, and infarct size was determined.

Treatment With Test Agents
Animals were randomly assigned to different groups after surgery, and all treatment started 2 hours after MI and lasted for 3 days. Clopidogrel was given by daily gavage at 2 different doses (50/15/5 mg/kg). Prasugrel was tested at 3, 6, 12, 24, and 72 hours after MI. CD41 antibody (BD Biosciences) or control IgG antibody was used at 0.5 mg/kg IP for 3 days to induce thrombocytopenia. Inhibition of platelet activity by the interventions was assessed by tail bleeding test.

To investigate the role of P-selectin glycoprotein ligand-1 (PSGL-1) in platelet-leukocyte conjugation, 2 hours after MI, mice were treated with PSGL-1 blocking antibody (BD Pharmingen, 2 mg/kg IP). Blood samples were collected 24 hours after MI.

Immunohistochemistry and Carstair Stain
Mouse hearts were harvested at different time points after MI, and paraffin or cryostat sections prepared for Carstair stain or immunohistochemistry, respectively. For platelet detection, immunohistochemistry was performed using anti-mouse glycoprotein IIb (CD41) monoclonal antibody, and images were analyzed digitally using ImagePro software. The average of percentages per fields was used. For quantitation of inflammatory cell density, frozen sections were stained with rat anti-mouse CD45 antibody, and nuclei were stained using 4',6-diamidino-2-phenylindole. The number of inflammatory cells was counted.

Flow Cytometry for Platelet-Leukocyte Conjugation and P-Selectin* Platelets
Blood samples were collected and processed within 30 minutes at room temperature. After lysing red blood cells, leukocytes were labeled with PerCP-conjugated anti-mouse CD45 and platelets with fluorescein isothiocyanate–conjugated anti-mouse CD62P (P-selectin). Leukocytes (20 000/sample) were analyzed with a Becton-Dickinson FACSCalibur flow cytometer and FlowJo software.

Washed platelets (10^6 cells) were labeled with phycoerythrin-conjugated anti-mouse CD41, and activated platelets were labeled with fluorescein isothiocyanate–conjugated anti-mouse CD62P; 10^5 platelets per sample were analyzed with the flow cytometer.

Western Blotting
Content of CD41 or plasminogen activator inhibitor-1 (PAI-1) in sham-operated and infarct cardiac tissue (day 3) was determined by Western blot.

Quantitative Real-Time Polymerase Chain Reaction, Gelatin Zymography, and ELISA
Infarct and noninfarct portions of the LV were collected from infarct mice for gene expression of inflammatory mediators by real-time polymerase chain reaction or for gelatin zymography and ELISA.

Plasminogen Activation Assay
Amidolytic assays with modifications were performed in lysates of heart tissues from sham-operated and infarct mice for detection of plasminogen activation parameters. Activities of urokinase plasminogen activator (uPA) or tissue-type PA (tPA) were determined using their respective inhibitors.

Echocardiography
Echocardiography was performed as described previously. Short-axis view of the LV was obtained. Images were analyzed for LV dimensions and cross-sectional areas at end-diastole.

Statistics
Results are expressed as either mean±SEM or percentages. Using GraphPad Instat software, results were analyzed by ANOVA followed by Newman-Keuls multiple comparison test or Fisher’s exact test, respectively. Echocardiographic data were analyzed by 2-way ANOVA for repeated measures. P<0.05 was considered statistically significant.

Results
Platelet Accumulation Within the Infarcted Myocardium
Time-dependent accumulation of platelets within the infarcted myocardium was estimated by immunohistochemistry using CD41 antibody at 3, 6, 12, 24, and 72 hours after MI or by Carstair’s stain at 24 and 72 hours (n=4 to 5 at each time point per staining). We observed that platelets accumulated in the interstitium of the infarcted region as early as 6 hours after MI and becoming increasingly prominent thereafter (Figure 1A to 1D). At 72 hours after MI, platelet accumulation was widespread within the infarcted myocardium. Colocalization of platelets with dense inflammatory cells was also common within the infarcted region after 6 hours (Figure 1B and 1C), with platelet particles visible under high magnification (Figure 1B). Although aggregates of platelets and leukocytes were visible in microvessels, positive platelet stain was largely extravascular in location. Both quantitative histology and Western blot showed substantial platelet accumulation in the infarcted myocardium (Figure 1D and 1E).

Antiplatelet Therapy Suppressed Inflammatory Responses in the Infarcted Myocardium
We then examined effects of thienopyridines or platelet depletion in the MI model. These therapies significantly prolonged tail bleeding time (Figure 2A). Whereas the bleeding time appeared similarly prolonged in mice receiving...
clopidogrel at high or low dose, the amount of bleeding was greater in the high-dose group (0.8 to 1.1 mL/20 minutes) than in the low-dose group (0.2 to 0.4 mL/20 minutes). The severity of myocardial inflammation was estimated by quantification of (1) density of CD45\(^+\) cells by immunohistochemistry (n=7 to 8/group), (2) content and activity of MMP-2 and MMP-9 by gelatin zymography (n=4 to 5/group), (3) gene expression of inflammatory mediators by quantitative real-time polymerase chain reaction (n=7 to 8/group), and (4) assay of selected cytokines by ELISA. Measurements were performed using infarcted and noninfarcted LV tissues collected at day 2 (for gene expression) or day 3 (for zymography, immunohistochemistry, or ELISA).

Immunohistochemistry revealed that treatment with clopidogrel, prasugrel, or CD41 antibody significantly and similarly reduced the density of leukocytes within the infarcted area by 20% to 30% (Figure 2B and 2C). Furthermore, quantitative real-time polymerase chain reaction showed a significant suppression by clopidogrel therapy of elevated expression of MMP-9, MMP-13, interleukin-1\(\beta\), and tumor necrosis factor-\(\alpha\), although other selected genes were not altered by this treatment (Supplemental Figure I). Previous studies have shown that increased levels of MMP-2 and MMP-9 are critical for rupture development. Gelatin zymography revealed lower levels of both MMP-9 and MMP-2 in the infarcted myocardium of mice treated with clopidogrel (Supplemental Figure IIA). Clopidogrel therapy also significantly reduced protein content of tumor necrosis factor-\(\alpha\) and interleukin-1\(\beta\) by ELISA (Supplemental Figure IIB) or Carstair stain (C, light purple-blue) showing platelet accumulation within the infarcted myocardium from 6 hours after MI and becoming prominent by 72 hours. IZ indicates infarct zone; NIZ, noninfarct zone. Arrows indicate colocalization of platelet particles and leukocytes in the infarcted region. D, Histological quantitation of platelet stain (n=4 to 5/group) as averaged percentages of the entire infarcted region. E, Western blot shows marked increases in CD41 abundance in the infarcted myocardium. The positive control was purified platelets from 5 \(\mu\)L of blood.

**Figure 1.** Platelet accumulation within the infarcted myocardium in relation to leukocyte infiltration. A to C, Representative images of immunohistochemistry (A and B, red for CD41\(^+\)) or Carstair stain (C, light purple-blue) showing platelet accumulation within the infarcted myocardium from 6 hours after MI and becoming prominent by 72 hours. IZ indicates infarct zone; NIZ, noninfarct zone. Arrows indicate colocalization of platelet particles and leukocytes in the infarcted region. D, Histological quantitation of platelet stain (n=4 to 5/group) as averaged percentages of the entire infarcted region. E, Western blot shows marked increases in CD41 abundance in the infarcted myocardium. The positive control was purified platelets from 5 \(\mu\)L of blood.

**Plasminogen Activators and Effect of Antiplatelet Interventions**

The plasminogen/PA system is known to contribute to ventricular rupture and remodeling postinfarction.\(^{12,14}\) Using the amidolytic assay on mouse heart protein lysates, we found that uPA was the predominant PA in the mouse heart and that MI was associated with increased uPA but decreased tPA activity relative to sham values (Figure 3A and 3B). Clopidogrel significantly prevented the MI-induced increase in uPA activity, and platelet depletion tended to lower it. uPA activity is endogenously suppressed by PAI-1. Western blot revealed that the tissue content of PAI-1 was not significantly altered either by MI or by antiplatelet interventions (Figure 3C).

**Acute MI Evoked Platelet-Leukocyte Conjugation and the Mechanism Involved**

Using flow cytometry, we measured platelet-leukocyte conjugation in peripheral blood from mice with sham surgery or MI (n=5 to 12/group). Acute MI led to a clear increase over the baseline value in platelet-monocyte conjugation as early as 6 hours, which progressively increased up to 72 hours after MI (Figure 4A and 4B). Platelet conjugation with granulocytes and lymphocytes was also significantly elevated by 72 hours (Supplemental Figure III, Figure 4B). Platelet aggregation was also detected microscopically in peripheral blood mononuclear cells from mice following MI (Figure 4C).

We then explored the mechanism underlying the postinfarct platelet-leukocyte interaction, focusing on P-selectin/PSGL-1. Following MI, the proportion of P-selectin expressing platelets increased significantly at 24 and 72 hours, indicating platelet activation (Figure 5A). PSGL-1 blocking
against antibody given 2 hours after surgery abolished MI-stimulated platelet-monocyte conjugation (Figure 5B).

**Effects of Antiplatelet Therapy in the MI Model**

Effect of clopidogrel (50/15/15 mg/kg) therapy on flow cytometry-derived parameters were analyzed in blood samples collected at 24 and 72 hours after MI. Clopidogrel therapy inhibited MI-induced increase in proportion of P-selectin expressing platelets (Figure 5A) and abolished platelet-monocyte conjugation without influencing the basal value (Figure 6). Platelet depletion prevented not only MI-induced but also the baseline conjugation measured at 72 hours post-MI (Figure 6).

Postinfarct ventricular rupture and remodeling are important clinical events and closely related to cardiac inflammation. We further evaluated the efficacy of antiplatelet interventions on rupture incidence and degree of ventricular remodeling. A total of 117 mice with MI were monitored up to 7 days. In an untreated group, 46% of animals (24 of 52) died of rupture during 3 to 6 days. Treatment with clopidogrel at 2 dosages or with CD41 antibody for 3 days after MI similarly reduced rupture incidence (P<0.01 versus untreated group, Figure 7A). Infarct size was comparable among the groups (Figure 7A). Surviving mice (n=15) in the clopidogrel-treated group were further monitored up to 14 days, but no rupture occurred during this extended week, confirming that rupture was prevented, rather than delayed.

We further examined the potential long-term effect of clopidogrel therapy in infarct mice without and with 3-day clopidogrel treatment (50/15/15 mg/kg). Serial echocardiography was performed before and during the 1 to 4 weeks after MI. Both groups had comparable infarct size (treated: 38.2±1.2%; untreated: 39.9±1.0%). Echocardiography showed significant LV enlargement post-MI; measured independently by LV dimension or cross-sectional area, LV dilatation was significantly less in the clopidogrel-treated group (Figure 7B). This beneficial effect was already detectable at week 1 post-MI and was maintained throughout the 4-week period. There was no significant difference in LV contractile function between the 2 groups (data not shown).

**Discussion**

We have made several novel findings in this study. (1) Platelets are among the first wave of inflammatory cells accumulating within the infarcted myocardium colocalizing with infiltrated leukocytes. (2) MI promotes conjugation of circulating platelets and leukocytes, which is largely medi-
ated by P-selectin/PSGL-1. (3) Antiplatelet therapy significantly suppressed the extent of regional cardiac inflammation, evidenced by reduced inflammatory cell density, MMP abundance/activity, and expression of key inflammatory markers, together with platelet-leukocyte conjugation in peripheral blood, effects mostly likely due to inhibition of platelet-mediated leukocyte activation occurring at peripheral blood and tissue levels and subsequent infiltration into infarct tissues. (4) Clopidogrel therapy prevented MI-induced increase in uPA. (5) Clopidogrel therapy suppressed the incidence of ventricular rupture in the acute phase and mitigated ventricular remodeling in chronic phase of MI, complications closely related to regional inflammation.10–14 These findings provide proof-of-concept evidence for cardiac protection of antiplatelet therapy in addition to its antithrombotic action in acute MI.

Intravascular thrombosis has been the focus of interest in acute coronary syndromes and forms the rationale for routine thienopyridine treatment. Clinical trials have documented broad benefits, including survival, when administered at the time of presentation of symptoms.19–21 Experimental studies have shown that ischemia-reperfusion triggers platelet aggregation within coronary vessels that can be inhibited by interventions such as deletion of P-selectin or the intracellular adhesion molecule-1 gene.22–24 In our study, platelet accumulation in the mouse infarcted myocardium is unique in its largely extravascular location, early presence, and close association with infiltrated inflammatory cells. How do circulating platelets enter into the interstitial space of the infarcted myocardium? One mechanism indicated by our findings involves platelets aggregating with circulating leukocytes and then piggy-backing into the infarcted myocardium. Platelet-leukocyte conjugation in peripheral blood and colocalization of platelets and inflammatory cells within the tissue occur as early as 6 hours after MI. A higher level of platelet-monocyte conjugation detected in peripheral blood soon after MI is in...
keeping with the fact that majority of infiltrates in the infarcted myocardium are monocytes or macrophages during the acute phase. Considering the dynamic nature of platelet-leukocyte aggregation and leukocytes infiltrating into the infarcted region, our quantitation of P-selectin-expressing platelets and platelet-leukocyte aggregates likely underestimates the degree of systemic inflammatory responses. Platelets may also enter into the infarcted myocardium, with intramural bleeding occurring in the mouse or human patients with MI likely because of vessel damage or increased vascular permeability. Thus, platelets might leave the blood stream through injured microvessels and, once in the infarcted myocardium, be tethered to exposed matrix materials or injured cells through specific ligands or receptors. We recently observed that clopidogrel therapy had no effect on the extent of intramyocardial hemorrhage.

Higher levels of ex vivo platelet-leukocyte aggregates have been reported in patients with acute coronary syndrome, and clopidogrel treatment is known to inhibit platelet-leukocyte conjugation stimulated ex vivo in patients with peripheral atherosclerotic vascular disease or coronary artery disease. There has been no report on such efficacy in vivo or its relation to inflammation in the infarcted myocardium.

Here, we observed simultaneous suppression by clopidogrel therapy of platelet-leukocyte conjugation in peripheral blood and the severity of inflammation in the infarcted myocardium, in which platelets contribute significantly to both systemic and regional inflammatory responses. P2Y12 expression is largely limited to platelets. The similarity of antiinflammatory efficacy achieved by clopidogrel, prasugrel, or platelet depletion strongly suggests that platelet inhibition by thienopyridines is via blocking P2Y12.

Platelets function as inflammatory cells through numerous membrane molecules and granular release of inflammatory mediators. Platelets promote inflammatory responses by 2 fundamental mechanisms: granular release of inflammatory mediators, such as serotonin, histamine, chemokines, cytokines, and MMPs; and platelet-leukocyte interactions contributing to leukocyte activation and recruitment to inflamed tissues. On platelet activation and degranulation, P-selectin rapidly translocates from α-granule to platelet surface. P-selectin can then bind to PSGL-1 on the surface of leukocytes, forming aggregates. We showed that blockade of PSGL-1 prevented MI-stimulated platelet-monocyte aggregation in circulating blood. Interestingly, treatment with clopi-
the inhibition of rupture events by gene disruption of MMP-9 or MMP-2 observations. Contributing to cardiac inflammation. Considering that inflammatory cell-derived MMPs and subsequent breakdown of collagen fibers are the central mechanism of postinfarct ventricular rupture, the proinflammatory nature of activated platelets is expected to contribute to the development of ventricular rupture. Similar to the inhibition of rupture events by gene disruption of MMP-9 or MMP-2 or by treatment with MMP inhibitors, here we showed, for the first time, inhibition by antiplatelet interventions on the severity of inflammation and wall rupture following MI. Our findings indicate that platelet-leukocyte aggregates, particularly mononuclear cells, play a critical role in initiating early inflammatory responses post-MI.

In addition to activation of MMPs, plasminogen activation also promotes post-MI ventricular rupture and healing. Whereas MI-induced wall rupture is abolished in uPA (but not tPA)--deficient mice, PAI-1 disruption exacerbates post-MI wall rupture. We observed for the first time that antiplatelet therapy suppressed uPA activity in the infarcted myocardium without change in PAI-1 abundance. Thus, antiplatelet interventions profoundly inhibit an array of inflammatory responses following MI, including the plasmin/MMP proteinase system.

Although the current incidence is low (1% to 3%), ventricular rupture post-MI remains a lethal complication. Clinical information is limited regarding thienopyridine therapy and risk of postinfarct rupture. Of several large-scale clinical trials on clopidogrel, COMMIT and CLARITY are the only ones on patients with acute MI. Although both trials showed significant benefits by thienopyridine treatment, including several primary end points, there was no significant change in the incidence of rupture in COMMIT trial (0.8% versus 0.9%) and no report of rupture incidence in CLARITY trial, although the former was limited by the lack of a loading dose of clopidogrel. In a large registry (GRACE), it was found that the use of thienopyridine was significantly lower in patients who developed rupture post-MI compared with rupture-free patients. Using the mouse model, we demonstrated a potent inhibition by antiplatelet interventions on ventricular rupture, with its onset closely related to infarct expansion. We also observed alleviated chronic ventricular dilatation by clopidogrel therapy, an efficacy detectable at day 7 post-MI, suggesting inhibition of acute infarct expansion as the explanation for this long-term benefit. Importantly, these benefits were achieved by administering antiplatelet agents within the first 3 days after MI. Although these findings were made on the murine MI model, their clinical relevance is indicated by studies showing severe inflammatory cell infiltration and intramyocardial bleeding in human hearts, detected by histopathologic or clinical imaging means, and an increased platelet-leukocyte aggregates in patients with acute coronary events. Additional clinical trials are warranted to test whether these findings might pertain to patients with acute MI.

Earlier studies using cardiac ischemia/reperfusion models have shown that because of formation of microthrombi and release of vasoconstrictors, such as thromboxanes, platelets exacerbate cardiac injury and dysfunction with reduced coronary blood flow. Recent studies revealed increased infarct size following ischemia/reperfusion due to an activated platelet activity. To limit the influence of vascular thrombosis, we adopted a permanent coronary artery occlusion model, although microthrombi were still visible within the infarct myocardium. Furthermore, the efficacy of thienopyridine was observed, with comparable infarct size between treated and untreated groups. The dosages of clopidogrel tested in the current study would be expected to inhibit platelet activity by 50% to 90%. Clinically, a steady-state effect of clopidogrel is 50% to 60% inhibition of ADP-induced platelet activation. However, 20% to 40% of patients display clopidogrel resistance, for a variety of reasons.

It would be important to examine whether patients who respond poorly to antiplatelet drugs might have more severe inflammation and higher risk of ventricular rupture post-MI. Furthermore, our findings of increased platelet-leukocyte conjugation in the peripheral blood of infarcted mice and the effect of clopidogrel therapy strongly indicate that this measure is a useful clinical biomarker.

In conclusion, we have shown that increased platelet-leukocyte aggregation and regional accumulation and activation of platelets contribute to myocardial inflammation and wall rupture in the mouse MI model. These changes are inhibited by antiplatelet interventions, indicating direct cardiac protection via attenuation of inflammatory responses. Although our findings were made in the murine MI model, they are in keeping with the current consensus of platelets being a class of inflammatory cells and highlight the previously unrecognized efficacy of antiplatelet therapy in the setting of acute MI.

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Disclosures
None.

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Supplemental Materials

A. Supplemental methods

Animals and surgery

Male C57Bl/6 mice (12-14-week-old) were used. All experimental procedures were approved by a local animal ethics committee and conformed to the guide for the Care and Use of Laboratory Animals published by the Australian Government National Health and Medical Research Council (7th Edition, 2004). Animals were anesthetised with the mixture of ketamine/xylazine/atropine (100/20/1.2 mg/kg, i.p.). After tracheal intubation, artificial ventilation and left thoractomy, MI was induced by permanent occlusion of the left coronary artery, as previously described. Animals were treated pain-reliever carprofen (5 mg/kg, s.c.). Surgery-related loss that occurred within the 24 h post-operation was 5%. To generate tissues for a variety of assays, some animals were killed at days 2 or 3 after MI and infarct and non-infarct portions of the left ventricle (LV) were separated using fine scissors under a surgical microscope as the demarcation of an interacted region was clear when infarct was over 24 h. Animals allocated to the rupture incidence were closely monitored for 7 days and autopsy was performed once animals were found dead to determine the reason of death. Infarct size was determined using the method of fractional LV surface area, as described previously. To keep for the extent of disease insult, animals with an infarct size less than 25% or over 60% were excluded.

Treatment with anti-platelet agents

We tested several anti-platelet interventions including clopidogrel (Sanofi Aventis), prasugrel (Lilly) and CD41 antibody (BD Biosciences) to induce thrombocytopenia. Animals were randomly assigned to different groups after surgery and all treatment started 2 h after induction of MI. Tablets containing clopidogrel (Plavix) or prasugrel (Effient) were ground into fine powder and freshly made in an emulsion in 0.5% methylcellulose solution and administered by gavage once daily for 3 days. Clopidogrel was tested at a high (50/15/15
mg/kg) and a low dosage (15/5/5 mg/kg). Previous reports showed a 90% inhibition of platelet activity by clopidogrel at the higher dosage used.\(^2\)\(^3\) Prasugrel was tested at 5 mg/kg daily according to a recent report.\(^3\) CD41 or control IgG antibody was used at 0.5 mg/kg, i.p. for 3 days.

**Determination of bleeding time**

Inhibition of platelet activity by interventions was assessed by measuring tail bleeding time as previously reported.\(^4\) Mice were anesthetized with the mixture of ketamine/xylazine/atropine (100/20/1.2 mg/kg, i.p.) and a 10-mm segment of the tail tip was cut off and the tip of the tail immersed in saline (37°C). Bleeding was monitored for up to 20 min. In some experiments, the volume of blood loss over the 20 min period was determined by measuring reduction in the body weight.

**Immunohistochemistry (IHC) and Carstair stain**

Mice were killed and LVs were harvested at 3, 6, 12, 24 and 72 h after MI, respectively. Hearts were either frozen or fixed in 4% paraformaldehyde (pH 7.4). Paraffin-embedded or cryostat tissues were serially cut into 5 \(\mu\)m sections and stained with Carstair (paraffin sections)\(^5\) or IHC (frozen sections) to detect platelets or inflammatory cells.

For platelet detection, IHC was performed using anti-mouse glycoprotein IIb (CD41) monoclonal antibody (0.67 \(\mu\)g/ml, Beckman Coulter) following blocking with MOM kit (Vector). Mouse IgG isotype control (Jackson ImmunoResearch) was used at the same concentration. Primary antibody was detected by using the EnVision™ G|2 System/AP (Dako) containing the mouse link, enzyme enhancer and permanent Red Chromogon. Images were acquired and analysed using the Olympus BX50 microscopy and Qcapture (Qimaging) software. To quantify platelet-stained (red) areas within the infarcted region, images were acquired with 10\(\times\) objective magnification and 5-6 fields covering the entire infarct area of the section were analysed digitally using ImagePro software. The average of percentages per fields was used.
For quantitation of inflammatory cell density, frozen sections were stained with rat anti-mouse CD45 primary antibody (BD Biosciences) with second Alexa Fluor 555 goat anti-rat antibody (Invitrogen). Rat IgG2b isotype control (BD Pharmingen) was used as recommended by BD Pharmingen. Nuclei were stained using Prolong Gold antifade reagent with DAPI (Invitrogen). Multiple images (8-11 at 20× magnification) covering the entire infarct region of the LV section were acquired digitally using Olympus BX61 fluorescence microscopy and AnalySIS FIVE software (Olympus). The number of inflammatory cells was counted by a single investigator blind of the group information.

**Preparation and staining of peripheral blood mononuclear cells (PBMC)**

Blood samples were collected from animals at day-3 after MI. As described previously, PBMCs were prepared by gradient centrifugation, suspended and slides prepared. Monocytes were stained using DAPI (Invitrogen) for nuclei and using CD68 antibody (BD Biosciences) for monocyte membrane. Platelets were identified by IHC using CD41 antibody (Beckman Coulter). Platelet binding to PBMC was identified by a fluorescence microscopy.

**Whole-blood flow cytometry for platelet-leukocyte conjugation**

Blood was collected by cardiac puncture using a 25G needle and heparin (40 U/ml) was used as anticoagulant. Caution was taken to minimize agitation during withdrawal and the initial portion of blood was discarded. Samples (100 μl) were processed within 30 min at room temperature. After lysing red blood cells using a lysing buffer (0.8% NH₄Cl, 0.1% KHCO₃ in distilled water, centrifugation (500g for 5 min) and washing twice with 1% fetal bovine serum in PBS (FBS/PBS, pH 7.6), pellets were resuspended in 100 μl FBS/PBS. Leukocytes and platelets were labelled with PerCP-conjugated anti-mouse CD45 (BD Pharmingen) or with FITC-conjugated anti-mouse CD62P (P-selectin, BD Pharmingen), respectively, for 30 min in darkness. After washing with 1% FBS/PBS, pellets were resuspended in 600 μl 1% FBS/PBS and analyzed with a FACSCalibur (Beckton-Dicinson) flow cytometer. FITC-conjugated anti-CD62P and PerCP-conjugated anti-CD45 were designated as FL1 and FL3, respectively, and
20,000 leukocytes per sample were analysed by using FlowJo software. Subgroups of leukocytes (monocytes, lymphocytes, and granular cells) were distinguished based on their distinct laser scatter properties.

To investigate the role of P-selectin glycoprotein ligand-1 (PSGL-1) in platelet-leukocyte conjugation, mice were treated 2 h after MI with PSGL-1 blocking antibody (BD Pharmingen, 2 mg/kg, i.p.). Blood samples were collected 24 h after MI for analysis.

**Platelet preparation and detection of P-selectin positive platelets**

Washed platelets were prepared from 1 ml blood according to a previously described method. Platelets (10^6 cells) were labelled with PE-conjugated anti-mouse CD41 (BD Pharmingen, 5 ul) and activated platelets labelled with FITC-conjugated anti-mouse CD62P (5 μl) for 30 min in darkness. After washing twice using 1% FBS/PBS, pellets were resuspended in 600 μl 1% FBS/PBS and analyzed with the FACSCalibur flow cytometer. PE or FITC conjugated rat isotype control was used. FITC-conjugated anti-CD62P and PE-conjugated anti-CD41 were designated as FL1 and FL2, respectively, and 10^5 platelets per sample were collected and analysed by using FlowJo software.

**Western blotting**

A portion of sham-operated and the entire piece of infarcted LV tissues or purified blood platelets (positive control) were lysed in lysis buffer containing: Tris 0.125 M (pH 6.8), SDS 4%, Glycerol 20%, PMSF 1 mM and protease inhibitor cocktails (Sigma Aldrich) but. Equal amount of protein (10 μg) was separated on 10% SDS-PAGE under non-reducing conditions for CD41 blotting but under reducing conditions for PAI-1 blotting. Membranes were incubated with antibodies against mouse CD41 (BD Pharmingen, 1:2,000), PAI-1 (a gift from Prof. Daniel A. Lawrence; Ann Arbor, MI, USA) or α-tubulin (Sigma Aldrich, 1:20,000), and exposed using enhanced chemiluminescence reagent (Millipore).

**Quantitative real-time PCR, gelatine zymography and ELISA**
Infarct and non-infarct portions of the LV were collected from mice at day-2 (for gene expression) or day-3 (for gelatin zymography) after MI. The entire piece of infarct tissue was used for assays. Gelatine zymography was performed to determine levels of MMP-2 and MMP-9, as previously reported.\(^5\) Total RNA was isolated and reverse transcribed and qRT-PCR was performed using a SYBR green mix (Invitrogen) and on the ABI Prism 7500 system (Applied Biosystems). Expression levels of the following genes were determined: tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, monocyte chemoattractant protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), L-selectin, P-selectin, macrophage migration-inhibitory factor (MIF), MMP-8, MMP-9 and MMP-13. Expression levels were calculated using the method of \(2^{-\Delta\Delta Ct}\) and normalized to 18S.

Content of IL-1β and TNFα were also measured in LV tissues from infarcted and sham-operated mice using commercial ELISA kits (R&D System) following the manufacturer’s instructions. All samples and standards were measured in duplicates.

**Plasminogen activation (PA) assay**

Heart tissues were homogenized to 50 mg (wet weight)/ml PBS with 1% (v/v) Triton-X100 and sonicated. Amidolytic assays were performed in 96-well plates, with modification for detection of plasminogen activation parameters.\(^8,9\) Each reaction had 2.86 mg (wet weight)/ml lysate, 0.05 mg/ml cyanogen bromide-digested fibrinogen and 2 mM S-2251. Where appropriate, reactions were supplemented with 500 nM plasminogen, 200 µM amiloride and 2 µM tPA-STOP. Reactions were stopped with mineral oil and absorbance \(\lambda=405\)nm was measured at 37°C every 6 minutes for 25 h. Second-order polynomial equations were best-fitted to “absorbance versus time” curve using GraphPad Prism v5.03 software, as previously described.\(^9\) The second-order coefficient of each equation was taken as half the initial rate of plasminogen activation. To measure urokinase uPA activity, the second-order coefficient in the presence of amiloride was subtracted from that in the absence of amiloride. To measure tissue-
type tPA activity, the second-order coefficient in the presence of tPA-STOP was subtracted from that in the absence of tPA-STOP. Heart tissues were homogenized to 50 mg (wet weight)/ml PBS with 1% (v/v) Triton-X100 and sonicated. Amidolytic assays were performed in 96-well plates, with modification for detection of PA parameters. Each reaction had 2.86 mg (wet weight)/ml lysate, 0.05 mg/ml cyanogen bromide-digested fibrinogen and 2 mM S-2251. Where appropriate, reactions were supplemented with 500 nM plasminogen, 200 µM amiloride and 2 µM tPA-STOP.

**Whole-blood flow cytometry for platelet-leukocyte conjugation**

Blood was collected by cardiac puncture using a 25G needle and heparin (40 U/ml) was used as anticoagulant. Caution was taken to minimize agitation during withdrawal and the initial portion of blood was discarded. Samples (100 μl) were processed within 30 min at room temperature. After lysing red blood cells using a lysing buffer (0.8% NH₄Cl, 0.1% KHCO₃ in distilled water, centrifugation (500g for 5 min) and washing twice with 1% fetal bovine serum in PBS (FBS/PBS, pH 7.6), pellets were resuspended in 100 μl FBS/PBS. Leukocytes and platelets were labelled with PerCP-conjugated anti-mouse CD45 (BD Pharmingen) or with FITC-conjugated anti-mouse CD62P (P-selectin, BD Pharmingen), respectively, for 30 min in darkness. After washing with 1% FBS/PBS, pellets were resuspended in 600 μl 1% FBS/PBS and analyzed with a FACSCalibur (Beckton-Dicinson) flow cytometer. FITC-conjugated anti-CD62P and PerCP-conjugated anti-CD45 were designated as FL1 and FL3, respectively, and 20,000 leukocytes per sample were analysed by using FlowJo software. Subgroups of leukocytes (monocytes, lymphocytes, and granular cells) were distinguished based on their distinct laser scatter properties.

To investigate the role of P-selectin glycoprotein ligand-1 (PSGL-1) in platelet-leukocyte conjugation, mice were treated 2 h after MI with PSGL-1 blocking antibody (BD Pharmingen, 2 mg/kg, i.p.). Blood samples were collected 24 h after MI for analysis.

**Platelet preparation and detection of P-selectin positive platelets**
Washed platelets were prepared from 1 ml blood according to a previously described method. Platelets (10^6 cells) were labelled with PE-conjugated anti-mouse CD41 (BD Pharmingen, 5 ul) and activated platelets labelled with FITC-conjugated anti-mouse CD62P (5 μl) for 30 min in darkness. After washing twice using 1% FBS/PBS, pellets were resuspended in 600 μl 1% FBS/PBS and analyzed with the FACSCalibur flow cytometer. PE or FITC conjugated rat isotype control was used. FITC-conjugated anti-CD62P and PE-conjugated anti-CD41 were designated as FL1 and FL2, respectively, and 10^5 platelets per sample were collected and analysed by using FlowJo software.

**Echocardiography**

Echocardiography was performed and images analysed as described previously. In brief, mice were anesthetised with isoflurane and short-axis view of the left ventricle (LV) was obtained using an iE33 ultrasound system and a 15MHz liner probe (Philip). Images were analysed by a single investigator blind of animal information. LV dimensions at end-diastole and end-systole (LVDd, LVDs), LV cross-sectional areas at end-diastolic and end-systolic (LVAd, LVAs) were measured from echocardiographic images and fractional shortening (FS) and fractional area change (FAC) were calculated.

**B. Supplemental Results**

**Supplemental Fig. I.** Effect of clopidogrel therapy on gene expression of inflammatory mediators by the infarcted myocardium at 48 h after myocardial infarction (MI). MI is associated with significant elevation in the expression of all genes studied (all P<0.01 vs. non-infarct tissues, NMI), with exception for VCAM-1 as revealed by qRT-PCR. Clopidogrel therapy significantly suppressed elevated expression of MMP-9, MMP-13, IL-1β and TNF-α, albeit that other selected genes were not altered by this treatment. N=6-7 per group. *P<0.01 vs. untreated MI control.
Supplemental Fig. II. Effect of clopidogrel therapy on tissue content of MMPs and cytokines at 72 h after MI. A, Gelatine zymography revealed lower levels of content/activity of MMP-9 and MMP-2 in the infarcted myocardium of mice treated with clopidogrel. *P<0.05 vs. non-infarcted myocardium (NIM) and †P<0.05 vs. untreated MI control. N=4-5 per group. B, clopidogrel treatment significantly reduced protein content of TNFα and IL-1β in infarcted tissues as determined by ELISA. *P<0.05 vs. untreated MI control. N=6 per group.

Supplemental Fig. III. Clopidogrel therapy abolished MI-evoked platelet-monocyte conjugation. Representative forward and side scatter properties by gated flow cytometry of monocytes from mice 72 h after surgery for sham-operation (A), MI (B) or MI treated with clopidogrel at 50/15/15 mg/kg (C).

C. References


Supplemental Fig. 1

- MMP-8
- L-selectin
- ICAM-1
- MMP-9
- MMP-13
- IL-1β
- IL-6
- TNF-α
- MCP-1
- MIF
- MMP-8
- iNOS
- L-selectin
- VCAM-1
- ICAM-1

Fold change

Control    Clop

NMI

MI
Supplemental Fig. II

A

<table>
<thead>
<tr>
<th></th>
<th>Non-infarct</th>
<th>Infarct</th>
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<tr>
<td></td>
<td>control</td>
<td>clopidogrel</td>
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<td></td>
<td>control</td>
<td>clopidogrel</td>
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- Latent MMP-9 (OD)
- Active MMP-9 (OD)
- Latent MMP-2 (OD)
- Active MMP-2 (OD)

B

- TNFα (pg/mg)
- IL-1β (pg/mg)
Supplemental Fig. III

A

MI (72 h)
Conjugated (11.2%)

Sham (72 h)
Conjugated (4.4%)

B

MI (72 h)
Conjugated (11.2%)

C

MI (72 h)
+Clopidogrel
Conjugated (5.0%)
심근경색증 후 염증반응과 심실파열 혹은 리모델링 등의 심실합병증에 영향을 미치는 혈소판의 역할

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Summary

목적
본 연구의 목적이 먼저 심장의 염증반응을 그리고 심근경색증 후 심실합병증의 발병에 있어서 혈소판의 역할을 조사하는 것이다.

방법 및 결과
마우스 심근경색증 모델을 이용하여 심근경색증이 유발될 심근에서 면역학적 및 생화학적 방법을 동반하여 혈소판 습적과 염증의 중증도를 연구하였다. 유세포분석기를 이용하여 말초혈액 내 혈소판-백혈구 접합을 분석하였고, thienopyridine과 혈소판 결합을 포함한 항혈소판 치료에 대하여 연구를 진행하였다.

혈소판은 조기 심근경색증 후 총 농도 내에 누적되었고, 염증세포 내에도 존재하였다. 심근경색증은 P-select/P-select glycoprotein ligand-1에 의해 유발되는 혈소판-백혈구 접합의 조기 증가를 유발하였다. Clopidogrel 치료는 심근경색증 내에서 말초혈액의 염증세포, matrix metalloproteinases에서 혈소판-백혈구 접합, plasminogen 활성화 및 염증 매개 물질의 표현을 억제하였다(all P<0.05). 또한 심실판막 가능성을 감소시켰으며(P<0.01), 고통적인 심장조음검사로 인하여 확인한 결과 만성 심실 확장의 정도를 완화시켰다.

결론
혈소판은 심근경색증 후 전신 및 심장 염증반응을 증가시키는데 있어서 중요한 역할을 한다. 심근경색증 후 심근 내의 혈소판 총량은 유사적인 염증과 심근 리모델링 및 살해에 있어서 중요한 역할을 한다. 항혈소판 치료는 염증의 중증도와 심근경색증 후 혈관증의 위험도를 감소시키고 심장 보호가능이 있음을 증명할 수 있었다.
Commentary

이 연구는 급성 심근경색증에 있어서 clopidogrel 등의 항혈소판 치료가 기존에 알려진 항혈관 효과에 부가하여 심근 내 염증 및 섬진과일이나 재 {! templates/loki/extracted_text_document.png !} 

급성 심장동맥 주요은 파손된 경화된 벽 접촉 및 읍고인자가 의한 혈관성과 혈관경화가 동반되어 발생한다. 혈소판은 두 가지 기본적인 기전을 통하여 염증반응을 촉진한다. 첫 번째는 세로토닌, 히스타민, chemokines, cytokines, MMPs와 같은 염증 중개물질의 과일 분비이고, 두 번째는 혈소판-백혈구의 상호작용으로 인한 염증세포의 활성화이다.

급성 관상동맥 주요군의 파열된 경화된 주변에서 는 혈소판 응집과 함께 혈소판 표면에 P-selectin의 발현이 증가한다. P-selectin은 혈소판 α-granule에 존재하는 단백질로서 혈소판이 활성화될 경우, 혈소판막 표면에 발현되어 증상수와 단백구의 수용체로 작용하며 혈관내벽으로 백혈구를 유인한다. 혈소판막 단백의 변화는 혈소판-백혈구의 응집을 증가시키고, 염증세포를 활성화시킴으로써 염증과 혈관성화를 촉진한다. 동맥 경화판의 파열 후 6시간이 경과할 때 발로 혈관내벽 또한 나타나, 손상된 심근조직 내에서도 마세혈관의 손상 및 투과성의 증가가 관찰에 혈소판-백혈구 접합이 존재하게 되며, 특정 레간드나 수용체들을 통해 손상된 세포에 부착하여 심근 내 속열을 유발 할 수 있다.

본 연구에서 clopidogrel 치료는 혈소판-백혈구 접합체의 형성과 P-selectin을 표현하는 혈소판의 바이 온젝 및 심근경색증에서의 국소적인 염증 반응을 동시에 억제하였다. 이러한 결과는 P2Y12가 심근경색증 후 혈소판 활성화와 혈소판-백혈

구 상호작용으로 인한 염증에 중요한 역할을 담당하며, clopidogrel, prasugrel 등의 thienopyridines계열 약물들의 항혈소판 효과 및 항혈증 효과가 P2Y12의 억제를 통해 이루어질 수 있게 사항한다. 심근경색증 후 심실파열의 발생률은 1-3% 정도로 낮지만, 일반 발생하면 생명을 위협하는 지명적인 합병증이다. 심근경색증 후 좌심실의 파열은 활성화된 혈소판에 의한 염증반응에서 비롯된 MMPs와 결제조직 성유의 고장이 중요한 기전으로 작용한다. 또한 MMPs 활성화와 더불어 플라스마노겐 활성화는 심근경색증 후 심실파열을 촉진하는 역할을 한다. 본 연구에서는 항혈소판 치료가 plasmin/MMP 단백 분해효소 시스템을 통해 심근경색증 후 염증반응 및 심실파열을 한정한 억제하는 기전을 밝혔으며, 이는 MMP-9 또는 MMP-2의 유전자 억제 또는 MMP 억제 치료로 인한 심실파열의 예방기전과 유사하다. 또한 clopidogrel 치료의 통해 단핵 심근화장의 활화를 관찰할 수 있었다.

결론적으로 이 연구에서는 마우스 심근경색증 모델에서 혈소판의 활성화 및 혈소판-백혈구 접합체의 형성의 심근경색 중증에서의 역할이 심근의 염증과 파열에 관련되어 있으며, 이러한 변화가 항혈소판 치료의 항혈증 효과 및 염증 억제효과에 의하여 심장을 보호함을 밝혔다. 비록 이 연구가 심근경색증의 동물모델에서 나온 결과이지만, 혈소판과 염증세포에 대한 현대의 연구 흐름과 일치하며, 급성 심근경색에서 이전에 밝혀진 것과 동일한 현상소한 치료의 부가적인 효과의 기전을 밝힐 수 있을 것이다.
REFERENCES


