Platelets activated by transient coronary occlusion exacerbate ischemia-reperfusion injury in rat hearts

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THERE IS INCREASING EVIDENCE that platelet function is altered in patients with acute coronary syndromes. Increased proportions of activated platelets expressing P-selectin have been observed in blood from patients with unstable angina or acute myocardial infarction (1, 26). The plasma concentration of the soluble form of P-selectin, a protein derived from activated platelets and endothelial cells (39), is increased in these patients (22, 23). Moreover, treatments that are essential in the management of patients with acute coronary syndromes, such as heparins (40), glycoprotein IIb/IIIa blockers (8, 33), or thrombolytics (5, 17), activate platelets. Although indexes of platelet activation have been found to be useful markers in the prognostic assessment of patients with acute coronary syndromes (16, 24), the consequences of platelet activation on myocardial injury secondary to ischemia-reperfusion are not known.

However, it is well known that ischemia-reperfusion induces the accumulation of platelets in the myocardium (3, 27). Although myocardial platelet accumulation can have deleterious effects on myocardial cell injury through microvessel obstruction (9) and the release of toxic substances such as thromboxane A2, platelet-activating factor, and serotonin (14, 34), the consequences of the accumulation of platelets in reperfused myocardium have not been clarified. It has been described that the addition of platelets to the perfusate impairs coronary blood flow (28, 35), reduces posts ischemic contractile recovery (21, 35, 37) and increases arrhythmias (12) and ischemic injury (35) in isolated rat, guinea pig, or rabbit hearts subjected to ischemia-reperfusion. However, other studies (21, 37, 41) have found no effects or even beneficial effects of platelets. Although it has been reported (37) that the deleterious effects of platelets on isolated guinea pig hearts depend on platelet adhesion to coronary endothelium, the correlation between platelet activation, myocardial platelet accumulation, and platelet-induced myocardial injury after transient occlusion has not been established.

Therefore, this study investigated the influence of platelet activation induced by ischemia-reperfusion on myocardial platelet accumulation and myocyte cell injury. Platelets from pigs with or without transient coronary occlusion (CO) were assessed for P-selectin expression and added to the perfusate of isolated rat hearts subjected to transient global ischemia and reperfusion.
Platelets From Pigs Submitted to Transient CO

Platelets were isolated from systemic venous blood samples from 30 pigs included in control groups of studies that involved transient CO and that underwent identical instrumentation. A schematic presentation of the experimental protocol that was followed to obtain blood samples is shown in Fig. 1A. Animals were premedicated with 10 mg/kg azaperone, anesthetized with 10 mg/kg iv thiopental sodium, intubated, and mechanically ventilated with room air. A continuous infusion of thiopental sodium was used to maintain anesthesia. The right femoral vein was catheterized, a midline sternotomy was performed, and the pericardium was opened. The left anterior descending (LAD) coronary artery was dissected free at its midpoint and surrounded by an elastic snare. After being stabilized, the LAD coronary artery was ligated for 48 min and then reperfused. Blood was obtained from the femoral vein before CO (pre-CO) and 10 min after reperfusion (R). An additional group of pigs was submitted to identical instrumentation but did not undergo transient CO. Platelets were obtained in these animals 1 h after stabilization (sham). After the first 5 ml were discarded, 20 ml of blood were collected in a syringe containing acid citrate dextrose A (ACD-A) anticoagulant composed of whole blood (1:4:10 vol/vol; Laboratorios Grifols) and 2 μg/ml prostacyclin (Flolan; Glaxo Wellcome) and centrifuged immediately at 200 × g for 15 min at room temperature. The top two-thirds of the supernatant (platelet-rich plasma) were collected and recentrifuged at 900 × g for 10 min. The supernatant was removed and the platelet pellet was washed at 700 × g for 10 min with modified Tyrode buffer composed of (in mM) 129 NaCl, 2.8 KCl, 0.8 KH₂PO₄, 5 glucose, and 10 HEPES and 0.02% bovine serum albumin (pH 7.4) and 0.3 μg/ml prostacyclin. Platelets were finally resuspended in modified Tyrode buffer and counted with a Coulter STKS cell counter. Platelet concentration was adjusted to 4 × 10¹¹ platelets/l for perfusion of isolated hearts.

For in vitro activation, the final platelet suspension was preincubated for 5 min at 37°C in the presence or absence of 1 μM tirofiban (Aggrastat; Merck Sharp & Dome). Human thrombin (0.1 U/ml final concentration; Sigma) was then added, followed by a 10-min incubation at 37°C.

P-Selectin Expression

The KO 2.9 anti-P-selectin monoclonal antibody was used to determine P-selectin surface expression in platelets by flow cytometry. This antibody was obtained by immunization of P-selectin-deficient mice with a pre-B-cell line transfected with human P-selectin cDNA and was demonstrated to recognize P-selectin on activated pig platelets (30). Aliquots of platelet suspensions were incubated for 25 min at room temperature with the KO 2.9 antibody, washed with phosphate-buffered saline (PBS)-0.5% fetal bovine serum (FBS), and further incubated for 25 min with F(ab')₂ fragments of a goat anti-mouse fluorescein isothiocyanate-conjugated antibody (3.5 μg/ml; Caltag Laboratories). Platelets were washed again and resuspended in PBS-0.5% FBS for flow cytometry. Negative controls were performed using an equivalent volume of PBS-2% FBS instead of the anti-P-selectin antibody. The platelet count was analyzed with a flow cytometer (FACScalibur, Becton Dickinson; San Jose, CA) calibrated weekly for fluorescence and light scatter using fluorescent beads. Platelets were identified on the basis of forward and sideward scatter parameters in the logarithmic mode. For each sample, 20,000 platelets were collected. Data were analyzed with CELLQuest software (Becton Dickinson) and results were expressed as a percentage of specific antibody-positive platelets, defined as those with a fluorescence intensity >99% of negative control platelets.

Platelet Aggregation

The aggregation of platelets was analyzed by flow cytometry. Just before being infused into isolated rat hearts, aliquots of platelet suspensions were fixed with 2% paraformaldehyde and kept on ice until analysis. The fixed samples were then further diluted 1:10 with modified Tyrode buffer and 20,000 events were collected per sample. Platelet aggregates were identified by means of forward and sidescatter parameters.
Thrombin-induced aggregability of modified Tyrode-washed platelets was analyzed with a four-channel aggregometer (model 570 VS, Chrono-log, Havertown, PA). Platelet suspensions were prepared as described above but platelet count was adjusted to $35 \times 10^{10}$ platelets/L. Samples were distributed in siliconized cuvettes and warmed to 37°C. Aggregation was induced by addition of thrombin and measured as the maximal increase in light transmittance as a percentage of that observed with modified Tyrode buffer. When tirofiban was used, a 5-min preincubation with this drug was performed before the addition of thrombin.

Ischemia-Reperfusion in Isolated Rat Hearts

Male Sprague-Dawley rats weighing 300–350 g ($n = 128$) were anesthetized with intraperitoneal injection of thiopental sodium (150 mg/kg). The hearts were transferred to a nonrecirculating Langendorff apparatus and perfused at 10 ml/min as described earlier (13) with a modified Krebs-Henseleit bicarbonate buffer composed of (in mM) 140 NaCl, 24 NaHCO$_3$, 2.7 KCl, 0.4 KH$_2$PO$_4$, 1 MgSO$_4$, 1.8 CaCl$_2$, and 11 glucose at 37°C and continuously oxygenated with 95% O$_2$-5% CO$_2$. Two sidearms of the apparatus just proximal to the heart inflow cannula allowed the infusion of platelets and the recording of coronary perfusion pressure (CPP). Contractile function was assessed by means of a water-filled latex balloon inserted into the left ventricle.

Experimental Protocol and Groups of Treatment

The experimental protocol used with rat hearts is outlined in Fig. 1B. Isolated hearts were allowed to equilibrate for 30 min and were then subjected to 60 min of no-flow ischemia, followed by 60 min of reperfusion. Platelets or platelet suspension buffer were infused directly into the coronary flow through a side port situated in the perfusion line just proximal to the inflow cannula allowed the infusion of platelets and the recording of coronary perfusion pressure (CPP). Contractile function was assessed by means of a water-filled latex balloon inserted into the left ventricle.

The percentage of positive P-selectin platelets in pre-CO samples averaged 16 ± 2%. This percentage was higher in R platelets (21 ± 2%; 33 ± 13% of increase respect to pre-CO, $P = 0.02$). The P-selectin expression in sham platelets was not different from that observed in pre-CO platelets (Fig. 2A).

Effect of Platelets on Ischemic-Reperfused Isolated Hearts

Left ventricular pressure and CPP. Values corresponding to left ventricular (LV) end-diastolic pressure (LVEDP), LV developed pressure, and CPP are shown in Table 1. In control hearts, no-flow ischemia resulted in cessation of LV contractile activity and in a steep increase in LVEDP with a peak 18.1 ± 2.3 min after the onset of the ischemic period. Reperfusion induced a further increase in LVEDP with a peak 2.8 ± 0.2 min after its onset and increased CPP. LV developed pressure recovered only to 8 ± 2% of its initial value after 60 min of reperfusion. Addition of 10$^8$ sham or pre-CO platelets to the perfusion buffer just before the onset of ischemia did not induce changes in either LVEDP or CPP compared with hearts infused with platelet suspension buffer. However, the addition of R platelets before ischemia induced a significant further increase in peak LVEDP observed during early reperfusion ($P = 0.02$) and increased CPP at reperfusion ($P = 0.009$).

LDH release. In hearts receiving platelet-diluting buffer, no measurable LDH release was detected during equilibration and an abrupt LDH release was induced by reperfusion after 60 min of ischemia (32 ± 3 U/g dry wt for first 15 min). Addition of pre-CO or sham platelets had no effect on LDH release compared with the control group. However, hearts receiving R platelets before ischemia showed a significant increase in LDH release (60% increase respect to controls, $P = 0.004$) (Fig. 2B).

Effect of in vitro activation with thrombin. Activation of pre-CO platelets with 0.1 U/ml thrombin induced a marked increase in P-selectin expression ($P < 0.001$) (Fig. 3A). This thrombin concentration did not induce...
aggregation in modified Tyrode-washed platelets as detected by conventional aggregometry. The absence of microaggregates in the infused samples was assessed by flow cytometry. No differences in forward scatter means were observed between activated and nonactivated samples, whereas particles with higher size were detected when platelets were resuspended at 35\(\times\)10\(^8\)/l and stimulated with 0.3 U/ml thrombin (data not shown). Preincubation of platelets with 1 \(\mu\)M tirofiban, a concentration that abolished platelet aggregation in platelet-rich plasma in response to 10 \(\mu\)M ADP and inhibited aggregation of washed platelets in response to 0.3 U/ml thrombin to a 41 \(\div\)5% by conventional aggregometry, did not affect thrombin-induced increase in P-selectin expression (Fig. 3A).

Table 1. Hemodynamic parameters from control, sham, precoronary occlusion, or reperfused animals

<table>
<thead>
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<th>Preischma</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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<tr>
<td></td>
<td>LVEDP</td>
<td>LVdevP</td>
<td>CPP</td>
</tr>
<tr>
<td>Control</td>
<td>4.3 \pm 1.2</td>
<td>104.4 \pm 11.3</td>
<td>57.6 \pm 3.4</td>
</tr>
<tr>
<td>Sham</td>
<td>5.6 \pm 0.8</td>
<td>110.3 \pm 8.8</td>
<td>61.3 \pm 4.8</td>
</tr>
<tr>
<td>Pre-CO</td>
<td>4.5 \pm 0.6</td>
<td>109.5 \pm 9.7</td>
<td>58.9 \pm 5.7</td>
</tr>
<tr>
<td>R</td>
<td>5.2 \pm 1.1</td>
<td>101.3 \pm 8.5</td>
<td>60.3 \pm 4.5</td>
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</tbody>
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Values are means \pm SE and are expressed in mmHg. LVEDP, left ventricular end-diastolic pressure; LVdevP, LV developed pressure; CPP, coronary perfusion pressure; LVEDP\(_{\text{max}}\), maximal value for LVEDP during the corresponding period. Control, isolated rat hearts perfused with platelet suspension buffer; Sham, platelets obtained from pigs without coronary occlusion; Pre-CO, before coronary occlusion; R, 10 min after reperfusion. *P < 0.05 vs. control group.
Thrombin-activated platelets added during the last 5 min of the equilibration period markedly increased LDH release from isolated rat hearts submitted to ischemia and reperfusion compared with controls ($P < 0.001$). This effect was independent of the presence of tirofiban (Fig. 3B). Perfusion pressure at reperfusion was also increased in hearts that received thrombin-activated platelets both in the absence and in the presence of tirofiban (121 ± 14 and 116 ± 12 mmHg, respectively, vs. 84 ± 13 mmHg in controls, $P < 0.001$). Tirofiban and thrombin infused alone did not modify LDH release (37 ± 6 U/g dry wt after 15 min for thrombin infusion and 32 ± 6 U/g dry wt after 15 min for thrombin + tirofiban infusion) or perfusion pressure (79 ± 6 and 83 ± 6 mmHg, respectively) compared with control hearts at the dosage used in these experiments.

When thrombin-activated platelets were infused into isolated hearts that were not subsequently submitted to ischemia-reperfusion, no hemodynamic changes were observed with respect to hearts perfused with control buffer and no LDH release occurred (data not shown).

**Correlation between platelet activation and ischemia-reperfusion injury.** When considering the whole series of experiments, there was a close correlation between P-selectin expression in platelets added before ischemia and LDH release induced by ischemia-reperfusion in isolated rat hearts ($r = 0.84$, $P < 0.001$) (Fig. 4).

**Accumulation of platelets in isolated rat hearts.** $^{99m}$Tc-HMPAO-labeled platelets isolated from porcine pre-CO blood scarcely accumulated in isolated rat hearts that were subjected to ischemia and reperfusion. The percentage of accumulated platelets was significantly increased when R platelets were used. Thrombin-activated platelets were retained in the isolated hearts in a higher percentage than R platelets independently of the presence of 1 μM tirofiban (Fig. 5A). There was a clear correlation between the degree of P-selectin expression and myocardial platelet accumulation ($r = 0.85$, $P < 0.001$) (Fig. 5B). A significant correlation was also found between the percentage of platelets retained in the isolated hearts and LDH release detected at reperfusion ($r = 0.97$, $P < 0.001$) (Fig. 6).

**DISCUSSION**

This study investigates the consequences of platelet activation induced by transient CO on myocardial injury secondary to ischemia-reperfusion. The main findings are the following: 1) transient CO induces platelet activation as assessed by membrane expression of P-selectin; and 2) the effects of platelets on myocardium subjected to ischemia-reperfusion depend on the presence and extent of platelet activation. Increased platelet activation results in larger myocardial platelet accumulation and additional myocardial damage. These results may help to clarify the conflicting results on the mechanisms and consequences of platelet accumulation in reperfused myocardium.
There is solid proof that unstable angina and acute myocardial infarction are associated with increased platelet aggregability (7) and increased proportions of activated platelets expressing P-selectin in blood (1, 26). The magnitude of these changes has been consistently found to correlate with the severity of the clinical manifestation of the acute coronary syndrome (1, 16, 24).

Preinfarction angina has been found to be associated to a reduced extent of necrosis in patients with acute myocardial infarction (25). The apparent discrepancy between these findings and the adverse effect of platelet activation on infarct size described in the present study can be explained by the potent protective effect of brief episodes of ischemia against cell death secondary to subsequent prolonged CO or ischemic preconditioning (IPC). It has been described that IPC reduces the thrombotic potential of platelets and improves vessel patency in animal experimental models due to inhibiting platelet aggregation via stimulation of platelet adenosine receptors (18, 19). Because adenosine released by cardiomyocytes during IPC is an important mediator of protection in most animal species (36), and has a potent inhibitory effect on platelet activation-aggregation, it is expected that IPC inhibits platelet activation as it does adenosine (31).

The mechanisms responsible for platelet activation have not been clarified. Exposure of prothrombotic elements in active culprit coronary lesions has been proposed to play a role (29). It has also been shown that treatment with heparin (40), anti-glycoprotein IIb/IIIa agents (8, 33), and thrombolytic drugs (5, 17), enhance platelet activation in acute coronary syndromes. It has been suggested that myocardial ischemia may directly activate platelets independently of changes in the culprit coronary lesion or the effect of treatment. The mechanism by which myocardial ischemia activates platelets is not known. It has been suggested that it could be mediated by activation of microvascular coronary endothelium (15) or by thromboxane release and the sympathetic response (12) elicited by myocardial ischemia. In this study, the fact that platelet activation was not observed in pigs submitted to sham operation without LAD coronary artery occlusion rules out that it could be only a consequence of surgery and instrumentation associated to the experimental procedure.

Although it has been consistently shown that platelets accumulate in myocardium reperfused after transient ischemia (3, 27), the mechanism of this accumulation is not well known. It does not seem to depend mainly on microembolization from upstream culprit coronary lesions, but on local adhesion of platelets to activated endothelium (4, 21), and is not reduced by glycoprotein IIb/IIIa blockers (2, 21). Consistent with this interpretation, the present results show that myocardial accumulation of platelets is enhanced by their activation and does not depend on platelet aggregation.

The available information on the effects of platelets on myocardial injury secondary to ischemia is more controversial. The consequences of addition of platelets to the perfusion media in isolated hearts subjected to transient ischemia have been analyzed in several studies. Some studies have found that platelets have a negative effect on posts ischemic coronary flow (28, 35), functional recovery (21, 35, 37), and arrhythmias (12). However, other studies (41) have found that the presence of platelets in the perfusate preserves posts ischemic cell energy, function, and viability. Platelet depletion by treatment with antiplatelet antiserum failed to reduce infarct size in dogs (32). In the present study, the addition of platelets to the perfusate had an adverse effect on posts ischemic myocardium only when the proportion of activated platelets was high. According to this study, the discrepancies observed in previous studies could be explained, at least in part, by differences in the magnitude of platelet activation induced by platelet manipulation or the presence of thrombin (37).

The mechanism by which platelets may be harmful to ischemic-reperfused myocardium has not been elucidated. One possibility is that platelet activation results in the formation of microaggregates that occlude small vessels (9). However, in the present study, washed platelets activated by either in situ ischemia-reperfusion or ex vivo thrombin-stimulation were not aggregated, as demonstrated by flow cytometry. Moreover, the addition of tirofiban failed to prevent myocardial accumulation of platelets or their adverse effects. Some studies (28, 35, present study) but not others (21, 37) have found an increase in coronary resistance in reperfused hearts in the presence of platelets. In the present study, the increase in coronary resistance was proportional to the magnitude of platelet accumulation. However, because platelet accumulation was closely correlated with cell injury, as assessed by LDH release, the increase in resistance could be the consequence instead of the cause of increased injury. This possibility seems more likely, because the important increase in LDH release associated with platelet activation was out of proportion relative to the slight increase observed in coronary vascular resistance.
H1140  PLATELET ACTIVATION IN ISCHEMIA-REPERFUSION INJURY

Another possibility is that products of the platelet release reaction cause the adverse effects on ischemic-reperfused cardiomyocytes. The contribution of serotonin, thromboxane A₂, thrombin, and platelet-activating factor to arrhythmogenesis and vasoconstriction has been described and recently reviewed (10). It has also been demonstrated that substances released by platelets are able to mimic the electrophysiological effects produced by the presence of platelets during ischemia in isolated hearts (11). However, in a previous study (21), infusion of the supernatant of activated platelets failed to reproduce the effect of the presence of platelets in the perfusate, although this could be due to ex vivo inactivation of some platelet-derived products.

Several studies have reported a protective effect of treatments against platelet-induced damage in isolated hearts. Abciximab has been described to reverse the negative effects of platelets on coronary flow and contractility on rat-isolated hearts (6). The blocking effect of the antibody on endothelial cell-platelet adhesion has been proposed as the main explanation for these results, although no data on platelet accumulation were reported. On the other hand, administration of superoxide dismutase significantly improved the contractile recovery of platelet-perfused hearts without influencing platelet retention (38). Antagonization of isoprostane-thromboxane receptors also reversed the cardiodepressive effect associated with the presence of platelets in the perfusate without reducing platelet adhesion (20). In the present study, there was a close correlation between the amount of platelets retained in myocardium and the severity of myocardial damage, indicating that platelet adhesion to coronary microvasculature is at least a prerequisite for expression of the adverse effects of activated platelets.

The observation that platelet activation may have adverse myocardial effects on ischemic-reperfused myocardium may be relevant in patients with certain forms of acute coronary syndrome. Platelet activation appears as a potential pharmacological target in the protection against myocardial injury secondary to ischemia-reperfusion. The ability of different drugs interfering with platelet activation, adhesion, and aggregation to limit myocardial injury needs to be further investigated.

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