Effect of Glycoprotein IIb/IIIa Receptor Blockade on Platelet-Leukocyte Interaction and Surface Expression of the Leukocyte Integrin Mac-1 in Acute Myocardial Infarction

Franz-Josef Neumann, MD, Dietlind Zohlnhöfer, MD, Leila Fakhoury, MD, Ilka Ott, MD, Meinrad Gawaz, MD, Albert Schömig, MD

Munich, Germany

OBJECTIVES
This prospective randomized study investigated platelet-induced upregulation of Mac-1 on monocytes and its inhibition by glycoprotein (GP) IIb/IIIa blockage in patients with acute myocardial infarction (AMI).

BACKGROUND
In experimental AMI, Mac-1 on leukocytes is the pivotal adhesion molecule for detrimental inflammatory responses. In vitro, platelet adhesion to monocytes upregulates Mac-1.

METHODS
Patients undergoing stenting in AMI within 48 h after onset of symptoms were randomly assigned to receive either standard-dose heparin (n = 50) or abciximab plus low-dose heparin (n = 50). In serial blood samples, we assessed platelet-monocyte interaction and Mac-1 surface expression by triple color immunofluorescence flow cytometry.

RESULTS
Compared with platelet-negative monocytes, Mac-1 surface expression on monocytes with attached platelets was upregulated (median fluorescence intensity [interquartile range]: 259 [179 to 367] vs. 135 [78 to 195] arbitrary units, p < 0.001). As an indicator of platelet-monocyte interaction, mean fluorescence of the platelet marker GP Ibα in the monocytes population decreased after abciximab, although it remained unaffected by heparin alone. Abciximab achieved this effect by a reduction in platelet mass attached to monocytes (GP Ibα fluorescence intensity of heterotypic aggregates at 24 h [arbitrary units]: 187 [143 to 236] after abciximab vs. 228 [156 to 332] after heparin, p = 0.02), whereas it did not affect the percentage of monocytes with adherent platelets. Reduction of platelet-monocyte interaction resulted in decreased Mac-1 surface expression (fluorescence intensity at 24 h [arbitrary units]: 116 [68 to 153] after abciximab vs. 162 [117 to 239] after heparin, p = 0.001).

CONCLUSIONS
In patients with AMI, platelet-leukocyte interactions modulate Mac-1 expression on monocytes. Glycoprotein IIb/IIIa blockade is a therapeutic option to interfere with this mechanism. (J Am Coll Cardiol 1999;34:1420–6) © 1999 by the American College of Cardiology

A large body of evidence from animal experiments indicates that the interaction of neutrophils and monocytes with the microvasculature limits reperfusion in acute myocardial infarction (AMI) (1–5). In this interaction, the β₂ integrin Mac-1 plays a pivotal role (1–4). Inhibition of Mac-1 dependent leukocyte adhesion markedly improves microvascular reflow and myocardial salvage in animal models of AMI (1–3). This study as well as other recent studies have found upregulation of Mac-1 on leukocytes of patients with AMI (6,7). The underlying mechanism for this phenomenon remained obscure.

In this study, we hypothesized that platelet-induced leukocyte activation may play a role in this respect. We previously described increased platelet leukocyte interaction in patients with AMI (8). In this interaction, primary attachment of platelets to myeloid leukocytes occurs by tethering of the platelet’s P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes (9–14). The heterotypic adhesion is stabilized by binding Mac-1 to an unknown counter-receptor on the platelet (12–15). This is enabled by Mac-1 activation as a consequence of tyrosine...
phosphorylation and mitogen-activated protein kinase activation due to PSGL-1 ligation (13,16). As part of this cell activation, platelet binding to myeloid leukocytes also upregulated the magnitude of Mac-1 surface expression on leukocytes (17,18). The increased platelet leukocyte interaction in patients with AMI may thus contribute to the upregulation of Mac-1 on leukocytes in this condition.

This prospective randomized study investigated platelet-induced upregulation of Mac-1 and its inhibition by glycoprotein (GP) IIb/IIIa receptor blockade in patients with AMI.

METHODS

Study design. We investigated patients in a prospective randomized trial comparing GP IIb/IIIa receptor blockade with usual care for stenting in AMI (19). The study included patients with AMI undergoing revascularization by stent placement within 48 h after onset of pain. Inclusion criteria were: 1) typical anginal pain lasting >30 min, 2) ST-segment elevation of at least 1 mm in two or more contiguous leads, 3) elevation in creatine kinase to at least three times the upper limit of normal with a concomitant rise in MB-isoenzyme, and 4) coronary artery occlusion with angiographic appearance of fresh thrombus. We recruited patients who met the first criterion and at least one of the other criteria. Exclusion criteria were inability to give informed consent and contraindications to one of the study drugs. By means of sealed envelopes, patients were randomized to one of two open label treatment regimens: patients assigned to treatment with GP IIb/IIIa receptor blockade received a bolus of abciximab, 0.25 mg per kilogram of body weight before the intervention, followed by continuous infusion, 10 \( \mu \)g per min for 12 h, plus heparin, 7,500 U. In patients assigned to usual care, we administered 15,000 units of heparin before the intervention followed by intravenous heparin infusion, 1,000 units/h, for the first 12 h after sheath removal. All patients received aspirin, 500 mg intravenously, before catheterization, and postinterventional antithrombotic therapy consisted of ticlopidine (250 mg bds) and aspirin (100 mg bds) throughout the study. Stent placement was performed, as described previously (19). In all patients the procedure was successful and achieved Thrombolysis in Myocardial Infarction (TIMI) grade 3 flow.

The study consisted of 50 patients assigned to usual care and 50 patients assigned to abciximab. The study groups were homogeneous with respect to baseline demographic, clinical and angiographic characteristics (Table 1). All patients gave written informed consent. The study was carried out according to the Declaration of Helsinki and was approved by our institutional ethics committee.

Immunostaining and flow cytometry. Blood samples were obtained before the initiation of in-hospital therapy and subsequently at 12 h and at days 1, 3, 5 and 10 after revascularization of the occluded vessel. Samples were collected into a fixative solution (1:3 vol/vol) containing methacroleine (Cyfix II, gift of Dr. Andreas Ruf, Karlsruhe, Germany) (20,21). After 10 min samples were diluted in 1:10 (vol/vol) ice-cold phosphate buffered saline (PBS) and stored at 4°C until staining was performed within 12 h.

### Table 1. Baseline Demographic, Clinical and Angiographic Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>Abciximab (n = 50)</th>
<th>Usual Care (n = 50)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>61.3 ± 10.5</td>
<td>62.6 ± 10.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Women</td>
<td>8</td>
<td>13</td>
<td>0.3</td>
</tr>
<tr>
<td>Smoker</td>
<td>30</td>
<td>20</td>
<td>0.1</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>36</td>
<td>32</td>
<td>0.2</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>29</td>
<td>36</td>
<td>0.2</td>
</tr>
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<td>Diabetes mellitus</td>
<td>4</td>
<td>8</td>
<td>0.4</td>
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<tr>
<td>Multivessel disease</td>
<td>34</td>
<td>34</td>
<td>1.0</td>
</tr>
<tr>
<td>Previous CABG</td>
<td>1</td>
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<td>1.0</td>
</tr>
<tr>
<td>Previous PTCA</td>
<td>2</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>Time from onset of pain</td>
<td>17</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>to intervention</td>
<td>6–12 h</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>12–24 h</td>
<td>11</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>24–48 h</td>
<td>12</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Prior thrombolytic</td>
<td>10</td>
<td>5</td>
<td>0.3</td>
</tr>
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<td>therapy</td>
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<td>I</td>
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</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TIMI grade 0/1 flow</td>
<td>39</td>
<td>44</td>
<td>0.3</td>
</tr>
<tr>
<td>Q-wave MI</td>
<td>38</td>
<td>33</td>
<td>0.3</td>
</tr>
<tr>
<td>Target vessel</td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>LAD</td>
<td>25</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>LCX</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>RCA</td>
<td>18</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Venous bypass graft</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Peak creatine kinase</td>
<td>1074 ± 1005</td>
<td>1009 ± 1115</td>
<td>0.8</td>
</tr>
</tbody>
</table>

(U/liter)

Data are expressed as mean value ± SD or number of patients.

CABG = coronary artery bypass graft surgery; LAD = left anterior descending coronary artery; LCx = left circumflex coronary artery; RCA = right coronary artery.
Immunostaining and flow cytometry were performed as previously described (4,8,20). We stained leukocytes in whole-blood by triple-color immunofluorescence with the use of fluoresceine isothiocyanat (FITC)-labeled monoclonal antibodies (mAbs) for the \( \alpha_{M} \)-chain of Mac-1 (CD11b), P-selectin (CD 62P), fragment E epitope of fibrinogen (all from Immunotech, Hamburg, Germany), and receptor induced binding site (RIBS neoepitope) on fibrinogen (gift of Dr. M. Ginsberg), phycoerythrine (PE)-labeled mAbs for GP Ibα of von Willebrand-factor receptor complex (CD 42b, Immunotech), PE-Cy5-labeled mAbs for the monocyte marker CD 14 (endotoxin receptor, Immunotech), quantum red-labeled monoclonal antibodies (mAbs) for the \( \alpha_{M} \)-chain of Mac-1 (CD11b, Sigma, München, Germany). Preliminary experiments revealed that binding of the anti-CD11b mAbs used in our study was not affected by abciximab at doses 10 times the therapeutic range (50 mg/L). We incubated aliquots of 40 \( \mu \)L with saturating concentrations of mAbs for 30 min at room temperature. Thereafter, erythrocytes were lysed with Immunolyse (Coulter Electronics, Krefeld, Germany).

For flow cytometry, we used a FACS-Calibur (Becton-Dickinson, Heidelberg, Germany) equipped with a 488-nm argon laser at 500 mW. We identified myeloid leukocytes as anti-CD11b-positive events in the histogram generated by the FITC- or PE-fluorescence and monocytes as anti-CD11b-positive events in the histogram generated by the PE-Cy5 fluorescence. In the GPIbα histogram, GPIbα-positive leukocytes with a fluorescence intensity above that of an isotype-matched irrelevant control antibody always could be clearly differentiated from GPIbα-negative leukocytes. The mean channel fluorescence intensity was a measure for antibody binding and, thus, antigen surface exposure.

**In vitro experiments.** Blood samples from healthy volunteers were anticoagulated in 1:10 (vol/vol) CPDA (citrate phosphate dextrose adenine) (Greiner) and were incubated for 15 min with or without 50 \( \mu \)mol/L ADP. In some experiments, we added blocking anti-P-selectin mAbs (clone G1, Centocor, Malvern) at a concentration of 10 mg/L or abciximab at a concentration of 50 mg/L before stimulation with ADP. After incubation, samples were washed three times with PBS and stained with FITC-conjugated anti-CD42 and PE-conjugated anti-CD14 mAbs for 30 min at room temperature. We then performed flow cytometry as described above. In some samples we confirmed the flow cytometry findings by confocal laser scanning microscopy (Axiovert35, Zeiss, Oberkochen, Germany), as previously described (17).

**Statistical analysis.** Discrete variables, expressed as counts, were tested by Fisher’s exact test. Results of normally distributed continuous variables were reported as mean \( \pm \) standard deviation and were tested by the \( t \) test for paired and unpaired samples, as appropriate. The Kolmogorov-Smirnov test showed that the flow cytometry data were not normally distributed. Results are reported as median (interquartile range). Differences between treatment groups were tested by the Mann-Whitney \( U \) test. We first analyzed differences in the entire time course by testing the sum of each measurement in a given patient. If this evaluation revealed significant differences, we compared the variable at individual time points. Time courses were tested with Friedman’s test followed by Wilcoxon’s rank sum test. To corroborate these analyses, we also performed analysis of variance (ANOVA) for repeated measure which always confirmed the results of the nonparametric tests. We used SPSS 8.01 for Macintosh software package for all statistical analyses. A \( p \) value less than 0.05 in the two-tailed test was regarded as significant.

**RESULTS**

**Effect of abciximab on platelet-leukocyte interaction in vitro.** After stimulation of whole blood with ADP, almost 80% of myeloid leukocytes had platelets attached (Fig. 1). Confocal laser microscopy revealed adhesion of platelet aggregates as the predominant pattern of platelet-leukocyte interaction (Fig. 2). Abciximab did not affect the percentage of myeloid leukocytes with adherent platelets but reduced the intensity of GP Ibα immunofluorescence of platelet-leukocyte aggregates (Fig. 1). On microscopy, attachment of single platelets was the predominant pattern of platelet-leukocyte interaction in the presence of abciximab (Fig. 2). With abciximab, we found a conspicuous reduction in the amount of platelets attached to myeloid leukocytes as compared with stimulation in absence of abciximab (Fig. 2). Blocking P-selectin mAbs completely prevented ADP-induced platelet-leukocyte interaction (Fig. 1). Stimulation of whole blood samples with LPS (lipopolysaccharide) at 1 \( \mu \)g/L had no effect on leukocyte-platelet interaction.
Platelet-leukocyte interaction before intervention. In patients, analysis of GP Ibα immunofluorescence revealed that 7% (5.1% to 9.1%) of the monocytes and 3.9% (2.9% to 5.2%) of all myeloid leukocytes had platelets attached before the intervention. Analysis of P-selectin immunofluorescence in myeloid leukocytes yielded similar percentages of coaggregates (4.7% [3.3% to 8.7%]). Mac-1 surface expression on monocytes with adherent platelets was increased substantially as compared with that on platelet-negative monocytes (Fig. 3), whereas CD14 did not show significant differences. The pattern of platelet-dependent Mac-1 surface expression in the entire population of myeloid leukocytes was similar to that in monocytes (Fig. 3). In platelet-monocyte aggregates, immunofluorescence for bound fibrinogen (fragment E epitope) was increased compared with single monocytes (Table 2). Analyzing both fragment E epitopes and receptor-induced neoepitopes on fibrinogen, we obtained similar results in the entire myeloid leukocyte population (Table 2).

Effect of abciximab on platelet-leukocyte interaction in reperfused AMI. At 24 h and 72 h after the intervention, the level of platelet-monocyte interaction was significantly lower in the abciximab group than in the control group (Fig. 4). In the abciximab group, platelet-monocyte interaction decreased during the first 24 h of reperfusion whereas it did not change in the control group (Fig. 4). Compared with usual care, abciximab did not affect the percentage of monocytes with adherent platelets (7.2% [5.2% to 8.9%] vs. 6.7% [5% to 9.7%], p = 0.73) but reduced the amount of platelets incorporated into platelet-monocyte aggregates. This was shown by a reduction in the GP Ibα immunofluorescence intensity of platelet-monocyte aggregates (187 [143 to 236] vs. 228 [156 to 332] arbitrary units, p = 0.02). Similar to the time course of platelet-monocyte interaction, we found a decrease in Mac-1 on monocytes within 24 h of reperfusion in the abciximab group, but an increase in the control group (Fig. 5). At 24 h and at 72 h after the intervention, Mac-1 surface expression on monocytes in the abciximab group was significantly lower than in the control group (Fig. 5). Findings in the entire population of myeloid leukocytes were similar to those in monocytes (Table 3). Without affecting the percentage of myeloid leukocytes with adherent platelets, abciximab reduced the platelet load in platelet-monocyte aggregates. This was shown by a reduction in the GP Ibα immunofluorescence intensity of platelet-monocyte aggregates compared with usual care. At 24 h after the intervention, surface exposure of the platelet receptors GP Ibα and P-selectin was lower in the myeloid leukocyte population of the abciximab group than in that of the usual care group (Table 3). The same was found for fibrinogen bound to myeloid leukocytes (Table 3). The lower platelet load in the myeloid leukocyte population of the abciximab group was associated with a lower surface expression of Mac-1 at 24 h (Table 3) and at 72 h after the intervention (85 [56 to 164] vs. 131 [94 to 204], p = 0.03), compared with usual care.

DISCUSSION

Our study demonstrates the modulation of Mac-1 expression on myeloid leukocytes by platelet-leukocyte interactions in patients with AMI. It also indicates that GP IIb/IIIa blockade is a therapeutic option to interfere with this mechanism.

Platelet-induced upregulation of Mac-1. Experimental studies indicate that heterotypic adhesion of platelets and leukocytes is not just a mere marker of ongoing platelet activation but also has important consequences for leukocyte function (8,17,18,22). In vitro, binding of activated platelets to myeloid leukocytes induces expression of proinflammatory cytokines, oxidative burst and increased surface expression of Mac-1 (8,17,18,22). Confirming the relevance of platelet-induced leukocyte activation in vivo, we found that Mac-1 expression was higher on circulating monocytes and neutrophils than platelets attached to preactivated leukocytes. As a potential explanation for this finding, we can exclude preferential binding of platelets to preactivated leukocytes. Due to sequestration
of surface exposed PSGL-1 (23), leukocyte preactivation either does not enhance or even decreases platelet-leukocyte interaction as shown in this study and in previous studies (23,24).

**Increased adhesiveness of platelet-leukocyte aggregates.** Compared with platelet-negative cells, the adhesive properties of platelet-leukocyte aggregates are markedly enhanced. Apart from increased levels of Mac-1, they are endowed with the entire arsenal of platelet adhesion. As shown by our study, this includes von Willebrand-factor receptor, mediating adhesion to subendothelial structures under high shear (25), P-selectin, involved in interactions with endothelial cells (5,26) and receptor bound fibrinogen. Receptor bound fibrinogen has the ability to bind nonactivated GP IIb/IIIa and to induce activation of resting platelets by outside-in integrin signaling (27). This is an important mechanism for the recruitment of circulating platelets to the site of vascular injury (27). In addition, monocytes promote thrombin formation by surface exposure of tissue factor that is also increased in AMI (6). Platelet-monocyte aggregates, therefore, constitute an ideal nidus for intravascular thrombus formation (28).

**Effect of abciximab.** Several independent studies suggested that GP IIb/IIIa is not involved in the interaction of activated platelets to myeloid leukocytes (11,13–15,29). Nevertheless, abciximab may interfere with the formation of platelet-monocyte aggregates in at least two ways: 1) due to its cross-reactivity with Mac-1 (30), abciximab may inhibit the stabilization of platelet-leukocyte aggregates by binding Mac-1 to an unknown counter-receptor on the platelet, 2) abciximab prevents the formation and, hence, attachment of platelet aggregates, thereby reducing the platelet mass in the heterotypic aggregates. Our study suggests that only the latter mechanism is relevant. In both our in vitro experiments as well as in blood samples taken from patients with AMI, abciximab reduced platelet-leukocyte interaction by reducing the platelet mass in platelet-monocyte aggregates while the percentage of monocytes with platelets attached remained unchanged. These findings by flow cytometry were also confirmed by laser scanning microscopy.

Abciximab substantially reduced Mac-1 surface expression on myeloid leukocytes compared with conventional heparin treatment. Our preliminary experiments ensured that abciximab did not affect the recognition of Mac-1 by

**Table 2.** Fibrinogen Binding on Single Myeloid Leukocytes (GP Ibα −) and on Platelet-Leukocyte Aggregates (GP Ibα +)

<table>
<thead>
<tr>
<th></th>
<th>GP Ibα −</th>
<th>GP Ibα +</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen fluorescence intensity</td>
<td>31 (25,43)</td>
<td>240 (147,459)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>RIBS fluorescence intensity</td>
<td>21 (16,26)</td>
<td>185 (132,332)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen fluorescence intensity</td>
<td>20 (15,29)</td>
<td>532 (248,958)</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

Data are expressed as median (quartiles). Fluorescence intensity is expressed in arbitrary units.

RIBS = receptor induced binding sites on fibrinogen.

**Figure 4.** Overall GP Ibα fluorescence intensity (arbitrary units) of monocytes in patients assigned to heparin treatment (closed circles) and in patients assigned to abciximab (open circles). Circles represent median and error bars interquartile range. *p < 0.002 for the comparison between groups. p Values for the comparison with the initial value in each treatment group are given at the bottom and at the top of the graphs. GP = glycoprotein.

**Figure 5.** Mac-1 fluorescence intensity (arbitrary units) of monocytes in patients assigned to heparin treatment (closed circles) and in patients assigned to abciximab (open circles). Circles represent median and error bars interquartile range. *p < 0.004 for the comparison between groups. p Values for the comparison with the initial value in each treatment group are given at the bottom and at the top of the graph.
the mAbs used in our study. We, thus, attribute the reduced Mac-1 surface expression after treatment with abciximab to the reduction in the mass of platelets attached to myeloid leukocytes. This could have led to less paracrine or direct receptor stimulation of leukocytes (16,29). The reduction in Mac-1 surface expression by abciximab supports the concept that platelet-leukocyte interactions contribute to the up-regulation of Mac-1 expression in patients with AMI.

Reduction by abciximab in platelet-leukocyte interaction and leukocyte Mac-1 surface exposure became apparent at the first sampling after initiation of therapy and persisted during the first three days. This is consistent with the durable receptor occupancy and inhibition of platelet aggregation by abciximab, even after discontinuation of the infusion (20). In addition, the observed long-lasting effect of abciximab may be related to plaque passivation due to adhesion of platelets with blocked GP IIb/IIIa receptors. This could have reduced ongoing platelet activation at the treated plaque (31).

Methodologic considerations and study limitations. To avoid ex vivo platelet-leukocyte interaction, we drew blood samples directly into Cyfix II, a fixative solution developed for flow cytometry. Previous studies verified that, after fixation with Cyfix II, mAb binding remains adequate and allows detection of subtle changes in the expression of activation-dependent cell surface markers (20,21).

Our study did not address consequences of platelet-induced leukocyte activation apart from Mac-1 surface expression. Nevertheless, it is tempting to speculate that the reduction in platelet-leukocyte interaction by abciximab could have reduced oxidative burst and cytokine elaboration, which are also stimulated by platelet adhesion (8,17,18,22).

Implications. As indicated by the surface exposure of von Willebrand-factor receptor, P-selectin and receptor-bound fibrinogen, the platelet-leukocyte aggregates formed under treatment with abciximab were less bulky and less adhesive than those under standard heparin (5,25–27). Moreover, the decreased platelet-leukocyte interaction under abciximab led to downregulation of the surface expression of Mac-1. Our findings, thus, elucidate a novel mechanism by which abciximab can reduce the interaction of platelet-leukocyte aggregates with the microvasculature and limit inflammatory responses during reperfusion in AMI. Therefore, abciximab may help to optimize reperfusion at the microvascular level.

Consistent with this inference, we recently found that, compared with standard heparin treatment, abciximab substantially improved microvascular flow after stent placement in AMI (19). This benefit was functionally relevant as it conferred increased recovery of contractile function in the area at risk (19). This study suggests that reduction of bulky, adhesive platelet-leukocyte aggregates may have contributed to the beneficial effect of abciximab during reperfusion in AMI.

Acknowledgments
We appreciate the invaluable assistance of Tanja Nordte, Rainer Huppmann, Sergej Kammerzell and Michael Schleef. We thank Dr. Mark Ginsberg, Scripps Clinic, for providing the anti-RIBS monoclonal antibody and Dr. Andreas Ruf, Klinikum Karlsruhe, for providing Cyfix II.

Reprint requests and correspondence: Dr. Franz-Josef Neumann, Deutsches Herzzentrum an der Technischen Universität, Lazarettstr. 36, 81636 München, Germany. E-mail: neumann@dhm.mhn.de.

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