c7E3Fab Reduces Postischemic Leukocyte-Thrombocyte Interaction Mediated by Fibrinogen
Implications for Myocardial Reperfusion Injury

Christian Kupatt, Helmut Habazettl, Peter Hanusch, Reinhard Wichels, Daniela Hahnel, Bernhard F. Becker, Peter Boekstegers

Abstract—Reperfusion injury after coronary occlusion is in part mediated by leukocyte activation and adhesion. Platelets may interact with polymorphonuclear granulocytes (PMNs), causing aggravated reperfusion injury. We studied whether c7E3Fab, a chimeric Fab fragment blocking platelet glycoprotein (GP) IIb/IIIa, decreases PMN-platelet–dependent myocardial dysfunction after ischemia. Isolated guinea pig hearts (n=5 per group) perfused at a constant flow of 5 mL/min were subjected to ischemia (15 minutes, 37°C) and reperfusion. Human PMNs (10×10⁶ cells, 3 mL), platelets (400×10⁶, 3 mL), and fibrinogen (1 mg/mL) were infused for 3 minutes after 2 minutes of reperfusion, with or without c7E3Fab. Flow cytometry detected GPIIb/IIIa (platelets) and MAC-1 (aMβ2, PMNs) as well as coaggregates of both in the effluent, whereas double-fluorescence microscopy visualized intracoronary PMN-platelet coaggregates. Postischemic recovery of pressure-volume work (12–cm H₂O preload and 60–mm Hg afterload) was defined as the ratio of postischemic to preischemic external heart work (mean±SEM). c7E3Fab reduced platelet GPIIb/IIIa detection to 10% of controls, blocked a transcoronary MAC-1 increase (+25% without versus −23% with c7E3Fab), and inhibited PMN-platelet coaggregation in the effluent (49±12% without versus 17±2% with c7E3Fab) as well as in the hearts themselves (5.0±0.7/cm² without versus 1.2±0.3/cm² surface area with c7E3Fab). Postischemic recovery of external heart work (83±5% in cell-free hearts) declined to 46±4% after postischemic PMN-platelet infusion, but not in the presence of c7E3Fab (74±11%) or LPM19c (71±6%). We conclude that c7E3Fab inhibits formation of PMN-platelet aggregates during myocardial reperfusion, an effect that protects against PMN-platelet–dependent stunning. (Arterioscler Thromb Vasc Biol. 2000;20:2226-2232.)

Key Words: abciximab ■ polymorphonuclear neutrophils ■ platelets ■ myocardial stunning ■ microcirculation ■ fibrinogen

Pharmacological and interventional strategies aimed at reperfusion of an occluded coronary artery are standard therapeutic regimens, although a certain percentage of treatments remain unsuccessful. Aimed at reducing this margin, inhibitors of the platelet fibrinogen receptor protein, glycoprotein (GP) IIb/IIIa (α2b/β3, CD41/CD61), have been introduced, eg, a Fab fragment of the chimeric monoclonal antibody 7E3 (c7E3Fab, Abciximab [Centocor]) against human GPIIb/IIIa.1–3 Besides improving coronary flow reserve, c7E3Fab protects from myocardial stunning in humans, which is expressed as regional wall shortening of the reperfused myocardium.4 Factors beyond inhibition of platelet aggregation might contribute to the improvement in flow and myocardial function, eg, inhibition of platelet-endothelium or platelet-leukocyte interactions. Fibrinogen competition studies with monoclonal antibodies suggest that MAC-1 (aMβ2, CD11b/CD18) is a major, if not unique, fibrinogen receptor on the polymorphonuclear neutrophil (PMN) surface.5–6 Interestingly, MAC-1 expression decreases after application of c7E3Fab in patients undergoing percutaneous transluminal coronary angioplasty.7 These observations suggest that attenuation of fibrinogen-mediated PMN-platelet interactions might contribute to the beneficial effects of c7E3Fab on myocardial infarction, myocardial stunning,4 and clinical restenosis.1

Experimentally, coinfusion of platelets, PMNs, and plasma in isolated hearts exacerbated myocardial dysfunction after ischemia and reperfusion, an effect involving adhesion molecules of the selectin class.8 Generally, however, selectins mediate a first contact between cell membranes, inducing deceleration of cell movement (rolling).9 For firm adhesion, subsequent interaction of adhesion molecules, eg, GPIIb/IIIa on platelets and MAC-1 on PMNs with their common ligand, fibrinogen, has to prevail.6

Although the clinical and experimental evidence is compelling, PMN-platelet interaction inside the heart has not yet been studied. Therefore, in the present study, we investigated whether platelets interact with PMNs via fibrinogen binding...
to its receptors during posts ischemic reperfusion. We used ex vivo microscopy to analyze whether c7E3Fab and LPM19c, a blocking antibody against the fibrinogen-binding site of MAC-1, could reduce PMN-platelet coaggregate formation in the coronary effluent and in epicardial microvessels. Moreover, the influence of both fibrinogen receptor blockade strategies on external heart work before and after an ischemic period was studied. The study was conducted to reveal whether the therapeutic benefit of GPIIb/IIIa blockade implicates, beyond inhibition of platelet aggregation, a functionally relevant reduction of heterotypic PMN-platelet coaggregate formation.

Methods

Materials
CD15 magnetic bead antibody from Miltenyi Biotec, FITC-labeled CD41 antibody (MCA467), phycoerythrin (PE)-labeled CD11b antibody (MCA551), and FITC and PE isotype controls (MCA928F and 928PE, respectively) were purchased from Serotec; blocking CD11b antibody LPM19c and FITC-labeled fibrinogen antibody F0111 were from Dako; c7E3Fab was from Eli Lilly; calcium-free PBS and Tyrode’s solutions were from c冷静; human fibrinogen, F2883 were from Sigma.

Preparation of Human PMNs and Platelets
Because c7E3Fab was raised against the activated human platelet epitope GPIIIa, human PMNs and platelets were used throughout the study. Human PMNs were isolated from peripheral venous blood of healthy volunteers by magnetic bead separation, as described elsewhere. In brief, purification of neutrophils from 10 mL of human blood (0.1% EDTA) was achieved by magnetic separation of cells previously labeled with a CD15 antibody carrying iron microbeads (Miltenyi Biotec). Cells were analyzed by flow cytometry (FACScan, Becton Dickinson) and proved to be 99% PMNs.

Preparation of Isolated Hearts
The care of the animals and all experimental procedures conformed with the Guide for the Care and Use of Laboratory Animals. Hearts were isolated and perfused as previously described. In brief, male guinea pigs (250 to 350 g) were anesthetized (20 mg ketamine) and decapitated. The aorta was quickly cannulated, and hearts were retrogradely perfused with a modified Krebs-Henseleit buffer gassed with 94.5% O₂ and 5.5% CO₂ (37°C, pH 7.40 ± 0.05).

The working heart preparation was established as previously described. The caval and aygos veins were ligated. Pulmonary venous entry was used for a canula that, in the working mode of the heart, provided orthograde access for the perfusate. In the perfusion apparatus, it was possible to switch between nonworking (Langendorff) mode and working heart mode. External heart work was calculated as the sum of pressure-volume work (developed aortic pressure multiplied by cardiac output) and acceleration work. For microscopic evaluation (Langendorff preparation), caval, aygos, and pulmonary veins were ligated. Retrograde perfusion was maintained throughout the experiment and during the in situ microscopic period.

Flow Cytometry
FITC- or PE-labeled isotype controls of either fluorescence with a nonbinding, fluorescence-labeled antibody (anti-mouse IgG1, MCA928F, or MCA928PE) served to determine nonspecific binding and background fluorescence in a flow cytometer (FACScan, Becton Dickinson). For isolated PMNs and platelets, flow cytometric analysis was performed before and after posts ischemic coronary passage. CD11b and GPIIb/IIIa (CD41) were detected by Serotec antibodies MAC551 and MCA467, respectively, except that after LPM19c incubation, CD11b was detected by the PE-labeled LPM19c antibody (Dako), because MCA551 did not recognize the LPM19c binding.

In fluorescence microscope experiments, detection of the interaction of rhodamine 6G–labeled PMNs and BCECF-AM–labeled platelets in the coronary effluent (see below) was performed by flow cytometry. Data analysis of 10,000 events was performed with LYSIS II software on a Hewlett-Packard system.

Fluorescence Microscopy
For the purpose of in situ microscopy, hearts were arrested in cold cardioplegic solution (28 mmol/L potassium) after ischemia, 5 minutes of reperfusion, and infusion of PMNs and platelets for 3 minutes (Figure 1). Thereafter hearts were placed on a specially designed microscopic stage holder, with the surface of the left ventricle exposed for microscopic observation (Ploemopak, Leitz) with a 10× objective (L10, 0.22 numerical aperture; Leitz). Images were generated by a charge-coupled-device camera (COHU 4400, Prospective Measurements). The distribution of platelets that were labeled with BCECF-AM was analyzed by epi-illumination with an H130 mercury light source and a I2,3 filter block (Leitz). The distribution of PMNs was studied under a rhodamine 6G–selective N₂ filter block (Leitz).

Figure 1. External heart work in phase 1 (EHW1): Langendorff phase 1, L1 (5 mL/min); Langendorff phase 2, L2 (5 mL/min); external heart work phase 2, EHW2; Langendorff phase 2, L2. Thereafter, hearts were placed on a microscopic stage, and epicardial tissue was analyzed for reperfusion.

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This method allowed for quantitative analysis of coaggregate retention of PMNs and platelets in epicardial regions of the myocardium (0.5- to 1.5-mm depth). Moreover, PMN-platelet interaction was detected in cell clusters containing both rhodamine 6G (PMN-specific) and BCECF-AM (platelet-specific) fluorescence. The resolution would not allow for detection of single platelets on PMNs, thus revealing heterotypic coaggregates of 2 or more platelets on 1 or more PMNs.

External Heart Work
Saline-perfused, isolated hearts performing pressure-volume work at 12 cm H₂O preload and 60 mm Hg afterload (20 minutes) were subjected to ischemia (37°C, 15 minutes) and reperfused in Langendorff mode (20 minutes). After 2 minutes of reperfusion, which allowed for fibrinogen saturation of the perfusion system, PMNs and platelets were simultaneously infused with fibrinogen for 3 minutes.
(with or without previously incubated antibody). After 20 minutes of reperfusion, external heart work was initiated for a second time (20-minute duration). The ratio of external heart work achieved postischemically divided by the preischemic level was defined as recovery of external heart work, given as a percentage of the preischemic value.

**Statistical Methods**

The results are given as mean±SEM. Statistical analysis was performed with 1-way ANOVA. Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests between the groups by using the Student Newman Keuls procedure (SPSS statistical software package). Differences between groups were considered significant for \( P<0.05 \).

**Results**

In the present study, the efficacy of fibrinogen receptor blockade as a strategy to attenuate platelet interaction with PMNs and the subsequent functional detriment during postischemic myocardial reperfusion was assessed. Flow cytometric studies were performed to evaluate the surface expression of the adhesion molecules GPIIb/IIIa on platelets and MAC-1 on PMNs.

**Fibrinogen Receptor Expression on Platelets and PMNs**

Pharmacological studies revealed that c7E3Fab dose-dependently competed with MCA467, a GPIIb/IIIa-recognizing antibody, and with fibrinogen (Figure 2A). On the other hand, LPM19c dose-dependently bound to its epitope, MAC-1. Both c7E3Fab and LPM19c competed with PMN fibrinogen binding (Figure 2B).

GPIIb/IIIa expression on platelets before and after coronary passage did not change significantly (238±54 FU before versus 262±52 FU after coronary passage). Incubation of platelets with c7E3Fab reduced GPIIb/IIIa detection with the MCA 467 to 12±5FU before and 10±4 FU after coronary passage (Figure 3A). Concomitantly, P-selectin detection on platelets (145±16 FU before and 117±13 FU after coronary passage) decreased after c7E3Fab incubation (54±11 FU before and 45±11 FU after coronary passage; data not shown). Concerning neutrophils, although no significant difference was found in MAC-1 detection by MCA551 with or without c7E3Fab treatment, an increase of PMN MAC-1 detection during postischemic coronary passage was reversed by c7E3Fab (Figure 3B). Application of LPM19c reduced available MAC-1, as detected by a PE-labeled LPM19c antibody, from 36.5±3.1 to 3.2±1.7 FU (Figure 3b), whereas platelets, which were not incubated with LPM19c before coronary passage, were left unchanged (Figure 3a).

**PMN-Platelet Interaction in Coronary Effluent After Ischemia**

In addition to its inhibitory effects on adhesion molecule expression, the potential of c7E3Fab to reduce coaggregate formation was more directly assessed by labeling whole PMNs and platelets with 2 different fluorescent dyes, rhodamine 6G and BCECF-AM, respectively. Thereafter, PMNs and platelets were infused with or without fibrinogen in a postischemic guinea pig heart.

Compared with nonischemic control hearts, which released 16±2% of the PMNs occupied with platelets, ischemia (15 minutes, 37°C) enhanced PMN-platelet interaction, the extent of which depended on the presence of fibrinogen (49±13% versus 30±15% with or without fibrinogen, respectively; Figure 4). Inhibition of GPIIb/IIIa (c7E3Fab) or MAC-1 (LPM19c) reduced PMN-platelet interaction to control levels (17±2% and 14±3%, respectively), whereas control antibodies had no effect (data not shown). Moreover, the number of platelets interacting with each PMN was analyzed, expressed as the mean of platelet-specific fluorescence on PMNs. The results paralleled the percentage of coaggregates found in the PMN population (Figure 4).

**Retention of PMN-Platelet Aggregates in the Reperfused Heart**

Using double-fluorescence videomicroscopy ex vivo, we studied the retention of PMNs, platelet aggregates, and heterotypic PMN-platelet aggregates in the epicardial microcirculation. Examples of this method are given in Figure 5, showing PMN retention (rhodamine fluorescence) (Figure 5A) and platelet aggregation (BCECF-AM fluorescence) (Figure 5B) at the same site, whereas c7E3Fab inhibited the interaction (Figure 5C and D). Quantitative analysis revealed
that postischemic retention of PMN-platelet aggregates in the presence of fibrinogen increased from $1.7 \pm 0.2/cm^2$ (nons ischemic or postischemic controls) to $5.0 \pm 0.7/cm^2$ (Figure 6). This PMN-platelet coaggregation was blocked by the addition of c7E3Fab and LPM19c (Figure 6).

**Figure 3.** Flow cytometric analysis of platelet GPIIb/IIa (A) and PMN MAC-1 expression (B) before (pre) and after (post) passage through a postischemic, isolated guinea pig heart, given as fu (fluorescence units). *P < 0.05 vs ischemic/reperfused (I/R) + fibrinogen (Fib) and I/R+Fib+LPM19c. #P < 0.05 vs I/R+Fib and I/R+Fib+c7E3Fab.

Analysis of the homotypic platelet aggregates revealed a similar impact of ischemia and reperfusion, fibrinogen, or c7E3Fab treatment on the distribution in the epicardial microcirculation (Figure 6). The latter reduced the platelet

**Figure 4.** PMNs ($10^5$) were evaluated per experiment, and 5 experiments per group were conducted. PMN-platelet coaggregates are given as a percentage of total PMN number. Platelet adhesion on PMNs, given as platelet BCECF-AM fluorescence units (fu; mean of 10 000 events). *P < 0.05 vs control (normoxia), ischemia (15 minutes) and reperfusion (I/R) + fibrinogen (Fib, 1 mg/mL) + c7E3Fab, and I/R+Fib+LPM19c.

**Figure 5.** Representative example of postischemic myocardium subjected to PMN-platelet and fibrinogen coinfusion. A, rhodamine-specific fluorescence of PMNs. B, BCECF-AM–specific fluorescence of platelets. Large arrows indicate PMNs; small arrows, platelet aggregates. C and D, Representative example of postischemic myocardium subjected to PMN, platelet, and fibrinogen coinfusion after pretreatment of cells with c7E3Fab.

**Figure 6.** Quantitative analysis of PMN-platelet coaggregates (left column) and platelet aggregates (right column) (5 fields per heart were examined; 5 hearts in all groups): Control (normoxia) + fibrinogen (Fib, 1 mg/mL), and ischemia (15 minutes) and reperfusion (I/R). *P < 0.05 vs all other groups, #P < 0.05 vs control, I/R, and I/R+Fib+c7E3Fab. Quantitative evaluation of platelet aggregates (5 fields per heart). *P < 0.05 vs all other groups, #P < 0.05 vs control, I/R, and I/R+Fib+c7E3Fab.
aggregate retention from $21\pm3/cm^2$ to $7\pm1/cm^2$. LPM19c had no effect exceeding the use of an unspecific antibody ($13\pm2/cm^2$).

**Myocardial Function**

At a constant preload of 12 cm H$_2$O and an afterload of 60 mm Hg, isolated guinea pig hearts performed an external heart work of $377\pm37\text{ mJ} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$. After ischemia and reperfusion without further intervention (Figure 7), heart work decreased to $313\pm34\text{ mJ} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, indicating a postischemic recovery of $83\%$. Simultaneous addition of washed PMNs, platelets, and fibrinogen during early reperfusion (2 to 5 minutes) further decreased the postischemic external heart work to $46\%$ of the preischemic level, an effect reversed by addition of c7E3Fab (74\% of the preischemic level). These changes in recovery were not related to changes in heart rate. Thus, postischemic recovery of external heart work was sensitive to the application of a PMN-platelet bolus in the presence of fibrinogen, a phenomenon inhibited by the coapplication of c7E3Fab or LPM19c.

**Discussion**

In the present study, we have demonstrated that reperfusion of an ischemic myocardium activates fibrinogen-dependent interactions of PMNs with platelets, leading to aggregate formation. Antibody studies of the fibrinogen receptors revealed that GPIb/IIa on platelets and MAC-1 on PMNs were essential for the fibrinogen-dependent PMN-platelet interaction during postischemic reperfusion (Figures 3 and 4). Microscopic analysis of epicardial microvessels demonstrated that heterotypic aggregates of PMNs and platelets were retained in the capillary system (Figures 5 and 6). The functional detriment caused by PMN-platelet interaction was reversed in the presence of the fibrinogen receptor antibodies c7E3Fab (GPIb/IIa, platelets) and LPM19c (MAC-1, PMNs; Figure 7).

Although the leukocyte contribution to myocardial reperfusion injury has been demonstrated and discussed before, only have recent studies revealed an interaction between the leukocyte and platelet compartments as effectors of reperfusion injury. In particular, the study of Lefer and coworkers suggested that blockade of P-selectin and its ligands, eg, PSGL-1, reduced PMN-platelet–dependent myocardial reperfusion injury. PMN interaction with activated platelets resembles PMN–endothelial cell interaction, wherein a first, reversible contact of both cell types is mediated preferentially by selectins. This process is followed by the highly selective, firm adhesion only if integrin activation is achieved by other substances, eg, platelet-activating factor or chemokines, among others. Firm adhesion of PMNs on platelets preferentially involves the integrins MAC-1 (aM\text{\beta}2, PMNs) and GPIb/IIa (aI\text{b}3, platelets) and their mutual ligand fibrinogen. Accordingly, in our study, the blockade of its receptors by c7E3Fab or LPM19c specifically reduced fibrinogen-dependent PMN-platelet coaggregate formation (Figure 4) and retention (Figure 6) in reperfused hearts. However, other PMN-platelet interactions may have contributed to the observed effects. Interestingly, platelet inhibition through c7E3Fab extends to P-selectin reduction, since P-selectin expression was reduced by two thirds after c7E3Fab incubation. Therefore, the initial contact between platelets and PMNs is also reduced by this intervention. However, P-selectin inhibition is exceeded by the reduction of PMN-platelet coaggregation achieved by c7E3Fab and particularly LPM19c, which was not added to the platelet compartment, suggesting an essential role of the fibrinogen receptor interaction. On the other hand, coaggregate formation was still observed without fibrinogen, possibly due to additional firm adhesion-molecule interactions, eg, CD11a/CD18 (PMNs) and intercellular adhesion molecule-2 (platelets).

By virtue of the chosen model, interaction of human PMN MAC-1 with endothelial intercellular adhesion molecule-1 was excluded (data not shown). Therefore, the protective effect of c7E3Fab and LPM19c on myocardial function (Figure 7) depends on a reduction of coaggregate formation (Figure 6) and nonspecific PMN plugging in capillaries. Although the latter effect was more pronounced in the presence of LPM19c (data not shown), functional improvement with both interventions was similar (Figure 7). Taken together, formation of a fibrinogen bridge between PMNs and platelets appears to be a crucial step for postischemic functional detriment in our model. Moreover, because LPM19c was incubated only with PMNs and not with the platelet compartment, inhibition of fibrinogen binding on PMNs appears sufficient for cardioprotection in this model. Fibrinogen-dependent interaction of PMNs and platelets itself signals cell activation through tyrosine kinases. In addition, coaggregation decreases the distance for platelet-derived activation factors, eg, platelet-activating factor or interleukin-1, to their receptors on PMNs and may subsequently induce PMN activation.

Beyond platelet GPIb/IIa inhibition, did c7E3Fab interfere with MAC-1 on PMNs? Although c7E3Fab was not directly competing with the MAC-1 detection antibody used (Figure 3B), it interfered with MAC-1 upregulation (Figure 3B, cf Reference 7) and PMN-fibrinogen binding (Figure 2B). A functional interaction of the original clone 7E3 or c7E3Fab with purified leukocytes has been found for a review, see Reference 35), although no direct binding of FITC-labeled c7E3Fab to purified PMNs could be detected. Moreover, in a homologous model of postischemic PMN-platelet infusion in rat hearts, an \&lnot;4-fold decrease of PMN

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![Figure 7. Recovery of external heart work (percentage of postischemic/preischemic value) was examined in 5 hearts per group. I/R indicates ischemia and reperfusion; Fib, fibrinogen (1 mg/mL). *P<0.05 vs all other groups.](image-url)
retention was described,36 which in such a model implies an MAC-1/intercellular adhesion molecule-1 interaction.37 Therefore, the functional blockade of MAC-1 appears to be an effect of c7E3Fab application, in addition to platelet GPIIb/IIIa blockade.

Clinically, the effectiveness of GPIIb/IIIa blockade is confirmed by a reduction in the incidence of death, myocardial infarction, or revascularization procedures after acute coronary syndromes.1–3,38 An association between restenosis and PMN-platelet interactions has been suggested before.39 Interestingly, the occurrence of restenosis, 1 clinical end point reduced by c7E3Fab treatment, is experimentally also attenuated by an MAC-1 antibody.40 In addition, c7E3Fab application improves regional myocardial function after acute myocardial infarction and primary percutaneous transluminal coronary angioplasty with stent implantation.4 The functional impact of c7E3Fab might involve the reduced formation of platelet-PMN coaggregates.7,41 Therefore, it is tempting to speculate that the beneficial effect of adjuvant c7E3Fab therapy on myocardial reperfusion injury in patients has an anti-PMN component.

In conclusion, we have shown that c7E3Fab reduces the formation of PMN-platelet coaggregates concomitantly with the retention of such aggregates in the postischemic myocardium, the latter phenomenon inducing a distinct impairment of postischemic myocardial function. Further studies are needed to characterize the contribution of PMNs to postischemic microthrombosis. A strict separation of inflammatory and thrombotic events may give way to a more interactive paradigm of both processes, since c7E3Fab appears as adjuvant cardiac therapy aimed at both events.

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