Reduction of monocyte–platelet interaction and monocyte activation in patients receiving antiplatelet therapy after coronary stent implantation

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Background: Monocyte activation induces different procoagulant and proadhesive inflammatory responses and thus may play a role in thrombotic complications after coronary interventions. Monocyte–platelet interaction may trigger these effects inducing monocyte activation.

Aims: To characterize the effect of antiplatelet vs anticoagulation therapy on monocyte–platelet interaction and monocyte function after intracoronary stenting.

Methods and Results: Immediately before, and during the first 12 days after successful coronary stenting, monocyte–platelet conjugates and monocyte function were assessed by flow cytometric detection of GPIIb/IIIa (CD41) on monocytes and by monocyte surface exposure of Mac-1 (CD11b/CD18) and L-selectin (CD62L). Twenty patients receiving combined antiplatelet therapy (ticlopidine, aspirin) were compared to 20 patients with standard anticoagulation (phenprocoumon, overlapping heparin, aspirin). Before stenting, monocyte–platelet conjugates and Mac-1 surface expression in both groups were significantly increased, while L-selectin was significantly diminished. Anticoagulation did not change these variables significantly during the subsequent 12 days. In contrast, antiplatelet therapy reduced platelet–monocyte conjugates by 46 ± 9.3% (mean ± SEM, P=0.0019) within 4 days, which was associated with a decrease in Mac-1 expression (28 ± 6.7%, P=0.0013) and an increase in L-selectin (56 ± 15.0%, P=0.0061).

Conclusion: After intracoronary stenting, combined antiplatelet therapy, but not anticoagulation, causes reduction of monocyte–platelet interaction, which is associated with monocyte deactivation. This may contribute to a decreased risk for thrombotic events.

Key Words: Stent, monocytes, ticlopidine, anticoagulation.

Introduction:
Coronary stenting is an established treatment for suboptimal immediate results after angioplasty. Moreover, recent data suggest that in selected patients elective coronary stenting may improve long-term outcome. However, despite strict anticoagulation therapy, subacute stent thrombosis remains a major early complication. The activated platelet appears to play a central role in this event. Apart from their direct haemostatic effect, activated platelets may stimulate procoagulant inflammatory responses. Activated platelets are known to attach to monocytes and thereby to initiate monocyte activation. Activated monocytes may play a role in the pathogenesis of thrombosis via several pathways: they initiate the extrinsic pathway of coagulation via surface expression of tissue factor. Moreover, activated monocytes rapidly show enhanced surface expression of the β2-integrin Mac-1 (CD11b/CD18). Mac-1 binds and converts factor X to Xa, leading to rapid fibrin formation. Mac-1 also binds to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, facilitating tight adhesion as a prerequisite of monocyte transendothelial migration. In addition, activated monocytes release a variety of promoters of acute inflammatory response, such as metabolites of arachidonic acid, leukotrienes and interleukines. In patients with coronary heart disease, increased monocyte surface expression of both the adhesion molecule Mac-1 and tissue factor were found.

We have previously shown that after coronary stenting combined antiplatelet therapy with ticlopidine and aspirin reduces platelet activation, as compared with standard anticoagulation regimen using a vitamin K antagonist, overlapping heparin and aspirin. The
The purpose of this work was to characterize the influence of antiplatelet vs standard anticoagulation therapy on platelet–monocyte interaction and monocyte activation during the first 12 days after coronary stent implantation.

**Methods**

**Patients**

Patients with successful intracoronary stent placement after PTCA, considered to be at low risk for subacute stent thrombosis, were eligible for the study. Indications for stenting were extensive coronary dissections after PTCA, complete vessel closure, residual stenosis of 30% or more, and lesions in venous bypass grafts. The risk of subacute stent thrombosis was stratified by a panel of angiographic and clinical risk factors and was considered low if the patients fulfilled less than two of the criteria shown in Table 1. We included 20 consecutive patients of a pilot study for the ISAR trial, treated by combined antiplatelet therapy, and 20 consecutive patients treated with anticoagulation who were recruited during the 3 months preceding this pilot study. The baseline clinical and angiographic characteristics of the study population are shown in Tables 2 and 3. The study was approved by our institutional ethics committee, and all patients gave written informed consent. In addition, 50 healthy volunteers (mean age 27 years; range 24–40 years) were recruited from the hospital staff to establish reference range values for cytometry.

**Stent placement**

Stent implantation was performed as previously described. Before PTCA, heparin 15 000 units and aspirin 500 mg were given intravenously. Conventional monorail balloon catheters were used for angioplasty (Express; Scimed, Verviers, Belgium). The 7 mm or 15 mm standard Palmaz-Schatz stent (Johnson & Johnson, Warren, NJ, U.S.A.) were hand-crimped on the angioplasty balloon. Balloon catheters

<table>
<thead>
<tr>
<th>Table 1 Criteria for increased risk for subacute stent thrombosis</th>
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<tr>
<td>Stent as bailout for acute occlusion after coronary balloon angioplasty</td>
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<tr>
<td>Stent in left anterior descending coronary artery</td>
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<tr>
<td>Major side branches within stented segment</td>
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<tr>
<td>Dissection not fully covered by stent</td>
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<tr>
<td>Thrombolysis in Myocardial Infarction grade 1 or 2 distal runoff after stenting</td>
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<tr>
<td>Acute myocardial infarction within the last two weeks</td>
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<tr>
<td>Intracoronary thrombus within stented segment</td>
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<tr>
<td>Recanalized chronic occlusion</td>
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<tr>
<td>Minimal lumen diameter within stent &lt;3 mm</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2 Main patient characteristics</th>
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<tbody>
<tr>
<td>Antiplatelet (n=20)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>Age, years, mean ± SD (range)</td>
</tr>
<tr>
<td>Active smokers, n (%)</td>
</tr>
<tr>
<td>Hypercholesterinaemia, n (%)</td>
</tr>
<tr>
<td>Systemic hypertension, n (%)</td>
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<tr>
<td>Diabetes mellitus, n (%)</td>
</tr>
<tr>
<td>Prior myocardial infarction, n (%)</td>
</tr>
<tr>
<td>Reduced left ventricular function (ejection fraction &lt;50%), n (%)</td>
</tr>
<tr>
<td>Unstable angina, n (%)</td>
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<tr>
<td>Multivessel disease, n (%)</td>
</tr>
<tr>
<td>Prior balloon angioplasty, n (%)</td>
</tr>
</tbody>
</table>

None of the differences between the two groups were statistically significant (P>0.15).

**Antiplatelet and anticoagulation regimen**

Immediately after pressure bandage application, a continuous heparin infusion, titrated to a partial thromboplastin time of 80 to 100 s, was started in all patients. In patients receiving anticoagulation, the vitamin K antagonist phenprocoumon (Marcumar; Hoffmann-La Roche, Grenzach-Wyhlen, Germany) was initiated immediately after the intervention. Heparin infusion was continued for 5 to 10 days until a stable level of oral anticoagulation was achieved. The target International Normalized Ratio (INR) was between 3.5 and 4.5. Partial thromboplastin time and INR were monitored twice daily. In patients assigned to antiplatelet therapy, heparin was discontinued 12 h after the intervention. Ticlopidine 250 mg twice daily (Tiklyd, Sanofi-Winthrop, Munich, Germany) was started immediately after the procedure. Ticlopidine and phenprocoumon

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Flow cytometry

Blood samples were handled as described earlier[23]. In brief, peripheral venous blood, anticoagulated with 1:5 (vol/vol) CPDA (sodium citrate, phosphate buffer, dextrose, adenine; Greiner, Germany), was obtained immediately before the intervention and thereafter daily in the morning for 12 days. Staining was performed immediately after blood sampling in whole blood. Whole blood (25 μl) and an equal volume of phosphate buffered saline (pH 7.4) were incubated with saturating concentrations of phycoerythrin(PE)-conjugated anti-CD14 and with one of the fluoresceinisothiocyanate (FITC)-labelled monoclonal antibodies (mAbs) for 30 min in the dark at room temperature. Erythrocytes were lysed and leukocytes were fixed (Lysing solution and fixing reagent, Coulter Electronics, Krefeld, Germany). Finally, the cells were washed three times and stored in paraformaldehyde (1%) at 4 °C in the dark. Flow cytometric analysis was performed within 24 h on a FACScan flow cytometer (Beckton Dickinson). The day-to-day reproducibility was controlled by fluorescent beads of defined various fluorescence (CaliBRITE, Becton Dickinson). Monocytes were identified by CD14-positive PE-fluorescence. In each sample, FITC-fluorescence intensity of 2000 CD14-positive cells was analysed over a logarithmic scale of 1 to 1026 channels. Results are expressed as mean channel of fluorescence intensity (mean fl.).

Monoclonal antibodies

All mAbs were obtained from Immunotech. MAbs anti-CD11b (clone, Bear1), anti-CD62L: (clone, DREG56) and anti-CD41a (clone, P2) were obtained FITC-labelled. MAbs anti-CD11b and anti-CD62L both served as markers of monocyte activation[24,25] showing no cross-reaction with resting or activated platelets in control experiments. Activation of monocytes is typically accompanied by upregulation of the Mac-1-complex (CD11b/CD18) and shedding of α2-selectin from the cellular surface. MAb anti-CD41 is directed against the glycoprotein IIb/IIa, which is the inducible fibrinogen receptor, expressed on platelets[26]. GPIIb/IIa was analysed on the monocyte surface and served as a marker of platelet or platelet particle binding to monocytes[11,27,28]. PE-conjugated mAb anti-CD14 (clone, TUK4) was used to identify monocytes. CD14 is a myeloid differentiation protein typically expressed on the surface of mature monocytes.

Statistical analysis

The Student’s t-test was used to test quantitative coronary angiography data. The Kolmogorov Smirnov test showed that the flow cytometry data were not normally distributed. The results are reported as median (interquartile range), unless otherwise indicated. The study primarily sought to test the hypothesis that immunofluorescence variables vary between the two treatment groups. Second, we analysed the time course of the immunofluorescence variables within each group.
Mean channel fluorescence intensity is expressed as median [interquartile range]. P values refer to the differences between the two treatment groups. All markers in the control group showed a significant difference ($P<0.01$) to both patient groups.

Accordingly, we first tested differences between the two groups by comparing the sum of each measurement in a patient using the Mann–Whitney U-test. If this revealed significant differences, we compared the variable at the individual time points. To analyse time courses, differences were tested by Friedman’s test followed by the Wilcoxon rank sum test. A $P$ value less than 0.05 was regarded as significant.

**Results**

**Baseline characteristics of the study group**

Twenty patients treated with anticoagulation and 20 patients receiving antiplatelet therapy were studied. As shown in Tables 2 and 3, the two patient groups were comparable with regard to their clinical and angiographical characteristics.

Before stent implantation, both patient groups showed significantly ($P<0.01$) increased monocyte immunofluorescence of Mac-1 and GPIIb/IIIa and significantly ($P<0.01$) decreased surface density of L-selectin compared to the reference range reflecting increased monocyte–platelet interaction and monocyte activation. Before stent implantation the three surface markers did not significantly differ between the two patient groups (Table 4).

**Platelet–monocyte conjugates**

In the anticoagulation group, GPIIb/IIIa on monocytes was constantly elevated for 12 days, whereas antiplatelet therapy caused a significant ($P<0.01$) persistent decrease in anti-GPIIb/IIIa immunofluorescence on monocytes after the intervention (day 4, 80[48–172] vs pre-stent, 209 [142–352], $P<0.002$) (Fig. 1). Significant differences between the two patient collectives were found on days 1, 2, 4, 5 and 8 after the intervention.

Table 4  Monocyte surface markers before stent implantation

<table>
<thead>
<tr>
<th>Markers</th>
<th>Anticoagulation (n=20)</th>
<th>Antiplatelet (n=20)</th>
<th>$P$</th>
<th>Control (n=50)</th>
</tr>
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<tbody>
<tr>
<td>Mac-1</td>
<td>107 [84;155]</td>
<td>128 [78;162]</td>
<td>0.68</td>
<td>71 [52;103]</td>
</tr>
<tr>
<td>L-selectin</td>
<td>30 [24;39]</td>
<td>38 [34;44]</td>
<td>0.11</td>
<td>62 [56;72]</td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>167 [74;260]</td>
<td>209 [142,352]</td>
<td>0.03</td>
<td>39 [34;85]</td>
</tr>
</tbody>
</table>

Figure 1  (a) Monocyte surface exposure to GPIIb/IIIa (CD41) before stent implantation and during the first 12 days after intervention. Standard anticoagulation (n=20) (○) was compared to combined antiplatelet therapy (n = 20) (●). *indicates significant ($P<0.05$) differences between both patient groups. □ indicates reference range. (b) Plot of the individual changes in the surface exposure of GPIIb/IIIa (CD41) on monocytes before stent implantation and at day 4 after the procedure.
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Figure 2 (a) Monocyte surface expression of Mac-1 (CD11b/CD18) before stent implantation and during the first 12 days after intervention. Standard anticoagulation (n=20) (♦) was compared to combined antiplatelet therapy (n=20) (●). *indicates significant (P<0.05) differences between both patient groups. □ indicates reference range. (b) Plot of the individual changes in the surface expression of Mac-1 (CD11b/CD18) on monocytes before stent implantation and at day 4 after the procedure.

Monocyte surface markers

In patients receiving anticoagulation, Mac-1 surface expression remained elevated at the pre-stent level for the entire study period (Fig. 2(a)). In contrast, in the antiplatelet group Mac-1 decreased after stenting, reaching statistical significance vs pre-stent values at day 4 (mean channel fluorescence intensity, median interquartile range; day 4, 77 [60–106] vs pre-stent, 128 [78–162], P<0.002) (Fig. 2(b)). Thereafter, Mac-1 essentially remained at the same level during the whole study period. Significant differences between the two patient groups were found on days 4, 5, 9, 10 and 11.

In patients with anticoagulation, L-selectin surface expression essentially remained constant at the low peri-interventional level. In contrast, in the antiplatelet group, L-selectin on monocytes increased within 4 days up to the level of the healthy control group (mean channel fluorescence intensity, median interquartile range; day 4, 66 [43–81] vs pre-stent, 36 [31–48], P<0.01) and remained constant thereafter (Fig. 3). Significant differences between the two patient groups were found on days 1–4 after the intervention.

Discussion

This study compares antiplatelet with anticoagulation therapy after coronary stenting and their effect on platelet–monocyte interaction and monocyte function during the first 12 days after intervention. In both groups, platelet–monocyte conjugates were substantially above the reference range at pre-stent, and the surface expression of Mac-1 and L-selectin indicated monocyte activation. In patients receiving combined antiplatelet therapy, we found normalization of platelet–monocyte conjugates after stenting, which was associated with monocyte deactivation. Anticoagulation therapy, however, did not affect platelet–monocyte conjugates and concomitantly the level of monocyte activation assessed by surface markers remained elevated throughout the observation period.

Platelet–monocyte conjugates

Platelets adhere to leukocytes in an activation-dependent manner[28] and modulate the activation state of leukocytes[11,12]. Specifically, we showed that adherence of monocytes to platelets induces the expression of interleukin-1β, interleukin-8 and MCP-1[12]. Previous studies showed platelet activation in patients with advanced coronary artery disease[10,11] and demonstrated that platelet function was predictive for the risk of acute ischaemic events after coronary interventions[8,29]. In accordance with our previous study[12], we found elevated levels of platelet–monocyte interaction before coronary stenting. This finding may be explained as a consequence of platelet activation due to high shear stresses at narrow coronary artery stenoses. After coronary stenting, combined antiplatelet therapy decreases platelet activation[9]. Consistent with these findings, the present study shows decreased platelet–monocyte interaction in patients treated with antiplatelet therapy.
Monocyte activation

Platelet–leukocyte interaction is associated with leukocyte activation. It induces upregulation of Mac-1, proteolytic shedding of L-selectin [30], superoxide anion release [31] and increased tissue factor expression [32, 33]. In our patients, platelet–monocyte interaction paralleled the increased surface expression of Mac-1 and shedding of L-selectin. Both platelet–monocyte conjugates and monocyte activation markers were lowered by combined antiplatelet therapy after the intervention. The surface markers of monocyte activation investigated in this study are of major physiological importance: they promote local inflammation and thrombogenesis by mediating intercellular adhesion and induction of procoagulant activity; whereas L-selectin initiates the primary contact ('rolling') of monocytes onto endothelium [34], Mac-1 mediates tight monocyte-endothelial adhesion ('arrest') [35]. Moreover, occupancy of the Mac-1 receptor sets the signal for increased surface expression of tissue factor [36]. In addition, Mac-1 can bind and convert factor X to factor Xa [37, 14]. Therefore, activation-dependent modulation of these two surface molecules may directly influence inflammatory and procoagulant processes such as subacute stent thrombosis.

Limitations of the study

Assignment to anticoagulation or antiplatelet therapy was not randomized in our study. Hence, there is concern that the results of this study might have been affected by differences in clinical angiographic characteristics or changes in the stenting procedure. However, the inclusion of patients with low risk of stent thrombosis only, ensured that the study population was quite homogeneous and our procedural approach was not changed during the study period. Accordingly, the two study groups did not differ with respect to any of the relevant procedural, angiographic or clinical characteristics. Therefore, we do not have sound reason to assume that the results of our study would have been substantially different, if assignment to treatment had been randomized.

We found persistently increased levels of platelet–monocyte conjugates and monocyte activation markers in patients treated with anticoagulation. In patients with antiplatelet therapy a decrease in platelet–monocyte conjugates and monocyte deactivation were found. The present study does not allow to attribute these deactivating effects of antiplatelet therapy to the ticlopidine therapy alone. Heparin has been shown to induce platelet and leukocyte activation. In addition, with respect to monocyte function the two treatment groups separate at day one after intervention. At this time ticlopidine medication cannot be assumed to have a measurable effect [37]. Thus, the differential effects of the two treatment regimens may be, at least in part, due to a direct action of heparin.

Clinical implications

Our study demonstrates that, compared with conventional anticoagulation, combined antiplatelet therapy after coronary stenting may exert anti-inflammatory
effects by decreased surface expression of Mac-1. Put together with the results of previous in vitro studies\[22,23,24\] our findings suggest that this effect is mediated through reduction of monocyte activation by adherent activated platelets. It is known that inhibition of monocyte activation not only affects the surface expression of adhesion molecules and procoagulant activity, but also the secretion of cytokines\[25\]. Monocyte-derived cytokines such as interleukin-1β and tumour necrosis factor-α, play an important role in the initiation of the systemic inflammatory response syndrome\[26\] commonly found in acute coronary syndromes\[22\]. Among the sequelae of these inflammatory responses, the increase in plasma fibrinogen is a notorious cardiovascular risk factor\[24\], so far difficult to treat. Ticlopidine administration has already been shown to reduce plasma fibrinogen levels\[27\]. The potential indirect anti-inflammatory effects of antiplatelet therapy that we describe may be one of the mechanisms for this effect. These beneficial effects of antiplatelet vs anticoagulation therapy on monocyte function may contribute to a decreased risk for subacute stent thrombosis and increased survival in patients receiving antiplatelet therapy after intracoronary stenting, as found in the ISAR trial\[22\]. If the findings of the present study can be extrapolated to other manifestations of ischaemic heart disease, potent inhibition of platelet function may be considered as a potential means of inhibiting detrimental systemic inflammatory responses in acute coronary syndromes.

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References


