MONOCYTE INTEGRIN EXPRESSION AND MONOCYTE–PLATELET COMPLEX FORMATION IN HUMANS WITH CORONARY RESTENOSIS

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SUMMARY

1. In the present study, we sought to determine whether patients with restenosis after coronary stenting possess increased monocyte reactivity, as manifested by a higher level of adhesion molecule expression and an enhanced propensity to form monocyte–platelet aggregates after activation in vitro.

2. Anti-coagulated peripheral venous blood from 24 patients, 10 with and 14 without angiographically verified restenosis, was obtained. Leucocyte antigen expression and the number of leucocyte–platelet complexes were measured by flow cytometry after activation in whole blood.

3. Surface integrin Mac-1 (CD11b/CD18) and VLA-4 (CD49d/CD29) expression on monocytes and the relative number of monocyte–platelet complexes after in vitro activation were significantly elevated in patients with restenosis compared with patients without restenosis (fluorescence intensities of 1425 ± 76 vs 1195 ± 71, 87 ± 7 vs 65 ± 6 and 47 ± 4 vs 29 ± 3% for for Mac-1, VLA-4 and monocyte–platelet complexes, respectively; \( P < 0.05 \) for each parameter).

4. The results suggest that restenosis is associated with increased monocyte VLA-4 and Mac-1 integrin expression and monocyte–platelet complex formation, which can be revealed after activation in vitro.

Key words: angioplasty, leucocytes, platelets, restenosis, stents.

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTCA) has found widespread application in the management of coronary artery disease (CAD). With improvements in techniques and tools, more complex lesions are being treated. Unfortunately, restenosis continues to limit the long-term success of the procedure, resulting in the need for repeat revascularization in approximately 30% of patients.\(^1\)

The pathogenesis of restenosis is not completely understood. Two principal mechanisms, namely excessive neointimal growth and negative remodelling, have been proposed to contribute towards restenosis.\(^2\) The failure of stenting to considerably improve the prognosis and the high incidence of late in-stent restenosis suggests that neointimal thickening plays a leading role.\(^3,4\) Emerging experimental and clinical data indicate that inflammation triggers neointimal formation and, thus, restenosis. Immunohistochemical analysis has revealed that inflammatory cells (monocytes/macrophages) are prevalent in human restenotic lesions and comprise substantial lesion volume.\(^3,6\) Moreover, PTCA-induced appearance of activated leucocytes (monocytes and granulocytes) and leucocyte–platelet aggregates in blood,\(^7,9\) as well as elevated plasma concentrations of monocyte-related cytokine interleukin (IL)-6,\(^10\) have been observed to correlate with later clinical outcome.

In animal models of balloon angioplasty and stenting, it was shown that monocytes are recruited early and abundantly to the denuded vessels and, by producing bioactive mediators, stimulate smooth muscle cell migration and proliferation leading to neointimal hyperplasia.\(^11\) A strong correlation between the extent of inflammatory reaction, characterized by mononuclear cell infiltration, and late neointimal thickening was found.\(^12\) In addition, blockade of MCP-1 (monocyte chemoattractant protein-1)\(^13\) and integrins VLA-4\(^14\) (CD49d/CD29) and Mac-1\(^15\) (CD11b/CD18), molecules responsible for leucocyte recruitment to the damaged area, or infusion of IL-10, a potent monocyte deactivator,\(^16\) were effective in the attenuation of experimental restenosis.

The above evidence that monocytes may be central promoters of neointimal hyperplasia after vascular injury prompted us to question whether patients with restenosis may exhibit elevated leucocyte reactivity after activation in vitro. We have analysed the expression of several monocyte antigens that can participate in cell adhesion and inflammatory reactions in patients with and without restenosis after stenting. Our results demonstrate that monocyte expression of the integrins Mac-1 and VLA-4 and the number of monocyte–platelet complexes after activation in whole blood are significantly higher in patients with angiographically verified restenosis.

METHODS

Patients

For this pilot study, we enrolled 24 patients who were to undergo coronary angioplasty due to stable coronary artery disease and follow-up angiography. The study was approved by the Hospital Ethics Board of the Cardiology Research Center of Ministry of Health (Moscow, Russia) and subjects gave informed consent to participate in the study. The inclusion criteria were
successful PTCA with stenting of a single lesion in a native coronary artery. Exclusion criteria included recent (< 2 weeks before the procedure) myocardial infarction, bypass graft lesions and concurrent illnesses (cancer or chronic inflammatory diseases). Coronary angioplasty with insertion of metallic stents of the same design was performed according to standard techniques by two operators (ANS and SIP). The procedure was considered successful when the residual diameter stenosis immediately after the procedure was ≤ 10% and if no major complications occurred (in-hospital death, urgent bypass surgery, redo-PTCA, myocardial infarction). All patients received the same medications (aspirin 125 mg/day, beta-blockers and hypolipidaemic agents) before PTCA and during the follow-up period. Blood samples were obtained 6 months after stenting. Using sodium citrate (Becton Dickinson Immunocytometry Systems) and at least 13 000 events were measured. The instrument settings were the same for all measurements and setting stability was regularly checked by fluorescent beads (Calibrite; Becton Dickinson Immunocytometry Systems). Data were transferred to IBM format using Consort 30 to IBM-PC Data TRANSFER (Becton Dickinson Immunocytometry Systems) and analysed using WinMDI software (Scripps Research Institute; http://www.facs.scripps.edu). Granulocytes were distinguished from other leucocytes by the combination of forward- and side-scattered laser light, monocytes were distinguished by CD14 expression and leucocyte–platelet complexes were distinguished by labelling with platelet-specific CD42a antibody. The relative cell surface antigen level was expressed as mean fluorescence intensity units, calculated by subtracting non-specific fluorescence, using a linear scale.

Statistical analysis

Results are presented as the mean ± SEM (mean ± SD for kinetic studies). The variables from each group (patients with and without restenosis) were analysed separately using Student’s t-test for independent samples. Linear regression analysis was used to compare measurements of monocyte and granulocyte surface CD11b fluorescence intensity and the number of monocyte– and granulocyte–platelet complexes.

RESULTS

Clinical and angiographic results

The study population consisted of 24 patients, all male. The angioplasty procedure was successful in all patients and no significant complications occurred. None of the patients had early clinical events. Angiographic follow up was obtained and restenosis (diameter stenosis > 50%) was revealed in 10 patients. Table 1 summarizes the main clinical and angiographic characteristics of the patients, obtained before PTCA (subsequently, patients were divided into two groups according to the results of the control angiography). By the time of expiry of the follow-up period, 90% of patients with restenosis had effort angina. None of the patients had de novo-formed coronary lesions (with diameter stenosis > 50%) and symptoms of cardiac failure.

Dynamics of leucocyte surface antigen expression and leucocyte–platelet complex formation during activation in whole blood

The dynamics of monocyte surface expression of integrins Mac-1, VLA-4 (evaluated with specific antibodies against their respective α-subunits CD11b and CD49d) and the LPS-binding molecule CD14, as well as the time-course of monocyte–platelet interaction in whole blood were analysed using blood samples from three

### Table 1 Baseline patient characteristics

<table>
<thead>
<tr>
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<th>Patients with restenosis</th>
<th>Patients without restenosis</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53 ± 2</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>No. with diabetes</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>% Vessels involved (LAD/RCA/LCx)</td>
<td>10/50/40</td>
<td>29/43/28</td>
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<tr>
<td>Reference diameter (mm)</td>
<td>2.8 ± 0.2</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Diameter stenosis (%)</td>
<td>75 ± 5</td>
<td>77 ± 7</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM.

LAD, left anterior descending coronary artery; RCA, right coronary artery; LCx, left circumflex artery.
patients. As shown in Fig. 1, monocyte surface exposure of Mac-1 integrin molecules significantly increased during incubation of blood with LPS and reached maximal values within 2 h. The effect of LPS on monocyte CD14 expression (Fig. 1) was analogous to that of Mac-1 and is in agreement with previous data indicating a two-fold upregulation of CD14 that peaked between 1 and 3 h after LPS addition to blood. Although we observed some monocyte–platelet association after blood storage on ice, incubation of blood with LPS elicited a significant increase in the number of platelet-bearing monocytes (Fig. 1). Surface VLA-4 integrin expression by monocytes was not upregulated and remained at the same level at all time points.

In our model of whole blood stimulation increases in granulocyte Mac-1 expression and the proportion of granulocytes associated with platelets were similar to observations made for monocytes (data not shown). Accordingly, all further measurements were performed under standard experimental conditions of storage of blood on ice for 1 h followed by a 2 h incubation with LPS (1 μg/mL) at 37°C.

Surface Mac-1, VLA-4 and CD14 expression by activated monocytes

We determined that, after LPS activation of whole blood, monocyte expression of Mac-1 and VLA-4 was significantly higher for patients with restenosis than patients without restenosis (fluorescence intensities 1425 ± 76 vs 1195 ± 71 and 87 ± 7 vs 65 ± 6 for Mac-1 and VLA-4, respectively; all P < 0.05). Values for the expression of the LPS-binding molecule CD14 did not differ significantly between study populations. The results obtained for Mac-1, VLA-4 and CD14 expression in each individual are shown in Fig. 2.

Monocyte–platelet interactions

The number of monocyte–platelet complexes formed after LPS activation in whole blood was calculated as the percentage of CD42a-positive cells among CD14-expressing cells. As shown in Fig. 3, the quantity of monocyte–platelet complexes was significantly higher for patients with restenosis than patients without restenosis (47 ± 4 vs 29 ± 3%, respectively; P < 0.05).

Granulocyte Mac-1 expression and granulocyte–platelet interactions

Surface expression of Mac-1 by granulocytes and the number of granulocyte–platelet complexes after activation in whole blood were also estimated. We found correlations between granulocyte and monocyte Mac-1 expression (r = 0.77) and between the quantities of granulocyte–platelet and monocyte–platelet complexes (r = 0.75). However, the measured parameters for granulocytes did not differ significantly between patients with and without restenosis (fluorescence intensities of 1126 ± 106 vs 1050 ± 72 and 76 ± 3 vs 65 ± 6 for Mac-1 and VLA-4, respectively; all P < 0.05).
Muscle cells. Thus, VLA-4 can be used by monocytes for fibronectin and vascular cell adhesion molecule (VCAM)-1 adhesion. Here, we demonstrate, for the first time, that an increased number of intimal monocytes/macrophages and neointimal thickening in baboons, anti-VLA-4 antibody has been shown to reduce the further invasion within an injured arterial wall. In endarterectomized patients with restenosis, Mac-1 and VLA-4 molecules participating in the total number of Mac-1 and VLA-4 molecules participating in restenosis in humans. This observation provides the first evidence that restenosis may, at least in part, be associated with increased monocyte–platelet association, with the alteration in platelet function being evident both before PTCA and 1–4 years after the procedure.

**DISCUSSION**

The present study has demonstrated that both monocyte Mac-1 and VLA-4 integrin expression and the propensity to form monocyte–platelet aggregates are altered in patients who develop postangioplasty restenosis. Monocyte accumulation in the injured vessel is a key event in the initiation of inflammatory reactions that can lead to neointimal hyperplasia. Monocytic integrins Mac-1 and VLA-4 seem to be principal participants in monocyte recruitment to inflamed areas. Mac-1, a member of leucocyte-restricted β2-integrins, directly facilitates cell adhesion and transmigration at sites of fibrinogen and platelet deposition. Blockade of Mac-1 with antibodies has been shown to reduce neointimal thickening in animal models of angioplasty or stent implantation. In Mac-1-deficient mice, an impaired transplatelet leucocyte migration, together with diminished neointimal formation after mechanical artery injury, have been observed. Clinical studies have shown that Mac-1 expression on monocytes and granulocytes is increased locally and systemically after PTCA and that the magnitude of Mac-1 upregulation was higher in patients with subsequent clinical events. Leucocyte adhesion to platelets has important consequences. It can trigger leucocyte migration at the sites of vessel injury through the deposited platelet layer. Moreover, the interaction between monocytes and platelets may result in modification of monocyte function, such as induction of tissue factor in monocytes.

Known ligands of β2-integrin VLA-4 include matrix protein fibronectin and vascular cell adhesion molecule (VCAM)-1 adhesion molecules expressed on activated endothelial and smooth muscle cells. Thus, VLA-4 can be used by monocytes for further invasion within an injured arterial wall. In endarterectomized baboons, anti-VLA-4 antibody has been shown to reduce the number of intimal monocytes/macrophages and neointimal thickening. Here, we demonstrate, for the first time, that an increased monocyte surface VLA-4 expression may be characteristic of patients with restenosis. This observation provides the first evidence that, like Mac-1, VLA-4-mediated monocyte interactions with other cell types and matrix may also be important in the pathogenesis of restenosis in humans.

Our ‘whole blood’ model has allowed us to simultaneously evaluate the total number of Mac-1 and VLA-4 molecules participating in cell adhesion at sites of acute inflammation or vessel wall injury. It is well established that the majority of Mac-1 integrin molecules in resting monocytes are stored within intracellular compartments. Upon activation, monocytes rapidly transport Mac-1 receptors to the cell surface membrane, thus ensuring firm adhesion to specific ligands. This explains the significant increase of surface Mac-1 exposure after LPS stimulation of monocytes in vitro. In contrast, the number of VLA-4 molecules was not upregulated by LPS. This finding is in accordance with previous observations indicating that VLA-4-dependent adhesion does not require cell activation and is principally mediated by affinity changes in the pre-existing molecules.

The appearance of heterophilic leucocyte–platelet aggregates in peripheral blood is indicative of a pro-inflammatory and prothrombotic status and is characteristic of patients with acute coronary syndromes, cardiopulmonary bypass and peripheral vascular disease. It has been reported that PTCA-induced leucocyte–platelet complex formation was more pronounced in those patients who experienced late clinical events. Leucocyte adhesion to platelets has important consequences. It can trigger leucocyte migration at the sites of vessel injury through the deposited platelet layer. Moreover, the interaction between monocytes and platelets may result in modification of monocyte function, such as induction of tissue factor in monocytes.

The formation of monocyte–platelet aggregates is a complex process that involves several types of monocyte- and platelet-adhesion receptors. Although in the present study we did not investigate the mechanism of monocyte–platelet association, we consider that it may result from gradual platelet and monocyte activation. Blood cooling has been shown to activate platelets and promote platelet aggregation and, indeed, we observed some platelet-bearing monocytes after blood storage on ice. Stimulation of monocytes by subsequent incubation of blood with LPS at 37°C leads to further monocyte–platelet association. Thus, our data demonstrating an increased propensity for the formation of monocyte–platelet complexes in patients with restenosis compared with patients without restenosis may reflect monocyte and platelet hyperreactivity in the former group. Platelet hyperreactivity has already been observed in other models of platelet activation, with the alteration in platelet function being evident both before PTCA and 1–4 years after the procedure.

Using the in vitro activation model described herein, we have found that for any given individual, both the expression of surface antigens on monocytes and the number of monocyte-platelet complexes are preserved at a constant level (CV < 10%) over a period of at least several (2–3) months (TI Arefieva et al., unpubl. obs., 2000), possibly reflecting an intrinsic reactivity level for each individual. For the present study, we have enrolled patients with analogous baseline characteristics. None of the patients presented with established clinical factors that could increase the likelihood of developing restenosis (acute coronary syndromes or a small diameter of the damaged vessel). Therefore, we speculate that restenosis may, at least in part, be associated with increased monocyte and platelet ‘reactivity’, as revealed for the restenotic group in the present study. Future investigations are aimed at establishing whether the measurement of these parameters may be of assistance in identifying patients prone to restenosis after PTCA or stenting.
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REFERENCES