Stent-Induced Expression and Activation of the Leukocyte Integrin Mac-1 Is Associated With Neointimal Thickening and Restenosis

Teruo Inoue, MD; Toshihiko Uchida, MD; Isao Yaguchi, MD; Yoshihiko Sakai, MD; Kan Takayanagi, MD; Shigenori Morooka, MD

Background—Increased expression of the β2 integrin Mac-1 (CD11b/CD18, αMβ2), which is responsible for firm leukocyte adhesion to platelets and fibrinogen at injured vessels, is found in association with neointimal hyperplasia after coronary interventions. The role of Mac-1 in the pathophysiology of restenosis is incompletely defined. To clarify further the role of Mac-1, we determined whether coronary stenting induced activation of Mac-1, which is required for high-affinity receptor-ligand interactions.

Methods and Results—Expression of CD11b (α-subunit of Mac-1) and binding of 8B2 (monoclonal antibody against an activation-dependent neoepitope of Mac-1) on the surface of polymorphonuclear leukocytes were analyzed in 62 patients undergoing coronary stenting using flow cytometric analysis of whole blood obtained from the coronary sinus and femoral vein. Transcardiac CD11b expression increased significantly at 24 hours and maximally at 48 hours after stenting; 8B2 began to increase at 10 minutes and was maximally increased at 48 hours after stenting. These changes were more prominent in patients with subsequent restenosis. Multiple regression analysis showed that the late lumen loss by quantitative coronary angiographic analysis was independently correlated with the CD11b increase (R²=0.42, P<0.01) and the 8B2 increase (R²=0.55, P<0.001) 48 hours after the procedure. Mac-1 activation, as assessed by 8B2 binding, was the most powerful predictor of late lumen loss.

Conclusion—Coronary stenting produced upregulation and early activation of the leukocyte integrin Mac-1, which is associated with late lumen loss and restenosis. These data support a role for inflammation in neointimal thickening and suggest the validity of targeting leukocyte recruitment for preventing clinical restenosis. (Circulation. 2003;107:1757-1763.)

Key Words: stents ■ restenosis ■ hyperplasia ■ leukocytes ■ cell adhesion molecules

Coronary stents have been developed to improve the short- and long-term results of percutaneous coronary intervention (PCI). Compared with balloon coronary angioplasty, coronary stents have demonstrated the ability to prevent abrupt vessel closure1,2 and to reduce restenosis.3-6 However, in-stent restenosis, which occurs in 20% to 30% of patients,3-6 remains a significant limitation of percutaneous revascularization.

Increasing experimental and clinical evidence suggests that leukocytes may play an important pathophysiological role in the development of restenosis after PCI.7,8 Stent deployment is associated with significant platelet activation, which promotes leukocyte recruitment to the injured vessel wall.9,10 Leukocyte firm adhesion and transplatelet migration are mediated by the β2 integrin Mac-1 (CD11b/CD18, αMβ2), which is expressed on activated leukocytes and binds to a broad repertoire of ligands, including fibrinogen,11-13 platelet glycoprotein Ibα,14 and possibly intercellular adhesion molecule-2.11 Clinically, we observed serial changes in the leukocyte surface expression of Mac-1 during the course after PCI using flow cytometric analysis, and we demonstrated its upregulation on the surface of polymorphonuclear leukocytes (PMNs) 24 to 48 hours after interventions.15-18 Specific receptor-ligand interactions require the transition of Mac-1 from a latent, relaxed state to a high-affinity, activated state. This transition is presumed to require a qualitative remodeling of the receptor through conformational changes associated with exposure or reorganization of new protein surface structures, ie, neoepitopes.19 The high affinity monoclonal antibody, 8B2, recognizes an activation-dependent neoepitope of Mac-1 associated with fibrinogen binding and cellular adhesion.20

In the present study, we observed serial increased expression of Mac-1 and its activation neoepitope on the surface of PMNs after coronary stenting by flow cytometric analysis. We also investigated the relationship between the expression...
of the Mac-1 activation neoepitope and angiographic late lumen loss to determine whether Mac-1 is a determinant of neointimal growth and restenosis.

**Methods**

**Subjects**

The subjects included 62 patients with isolated atherosclerotic coronary artery disease of the proximal left anterior descending artery who underwent initial elective coronary stent implantation (from April 1999 to March 2001) and follow-up coronary angiography. All patients exhibited clinically stable class I or II effort angina without previous myocardial infarction, according to the Canadian Cardiovascular Society (CCS). The target lesions were all type A or type B lesions, as described by the American College of Cardiology (ACC)/American Heart Association (AHA) Task Force. All of the patients had received the standard daily oral medications for angina, including 81 mg of aspirin, and none of these medications was discontinued or exchanged during the PCI or the post-PCI follow-up term. The patients started receiving 200 mg of oral ticlopidine 2 days before the intervention procedure as a specific post-stent antplatelet medication, which is the standard regimen in Japan, and it was continued until 1 month after PCI. When ticlopidine produced any side effects, including headache, skin rash, liver dysfunction, leukocytopenia, or bleeding tendency, the drug was discontinued immediately, and the patients were excluded from the study. The other exclusion criteria included receipt of other cardioactive or antplatelet drugs and the presence of other cardiac or noncardiac complications that could affect the analysis. The study protocol was approved by the Dokkyo University Institutional Review Board, and written informed consent was obtained from each patient.

**Procedure of Coronary Stent Implantation**

Coronary stent implantation was performed using the standard Judkins technique with the femoral approach. All patients were premedicated intravenously with 10,000 U of heparin to maintain an activated clotted time (ACT) of 200 to 250 s before the procedure. A Multi-Link stent (American Cardiovascular System), NIR stent (Boston Scientific) or S670 stent (Medtronic) was placed after an optimal or suboptimal lesion dilation was obtained by the initial balloon dilation(s). Patients who underwent implantation with other types of stents were excluded from this study. Post-stent balloon dilation was then performed at a pressure of 12 or 14 atm for 15 s. The contrast medium used during the procedure was a nonionic dextrose.

**Quantitative Coronary Angiographic Analysis**

Coronary lesions were assessed by the quantitative coronary angiographic (QCA) measurements using a computer-based QUANTCOR system (Siemens). The quantitative measurements were performed on end-diastolic frames from the angiograms by one investigator who was unaware of the study design. The reference diameter, lesion length, and minimal lumen diameter were measured before and after PCI and at the time of the follow-up coronary angiography. On the basis of these measurements, we obtained the value of acute gain (minimal lumen diameter after PCI minus minimal lumen diameter before PCI) and late lumen loss (minimal lumen diameter after PCI minus minimal lumen diameter at follow-up angiography) for the lesions. Restenosis was defined as >50% diameter stenosis at follow-up angiography.

**Blood Sampling**

Before the PCI procedure, a coronary sinus catheter (Goodman Corp) was inserted through the right femoral venous sheath and was positioned in the coronary sinus. The catheter was left in the coronary sinus for 48 hours after the procedure. Coronary sinus blood and peripheral blood were taken via the coronary sinus catheter and via the femoral venous sheath at the following 4 time points: (1) before PCI (ie, after heparin was administrated and the ACT was maintained); (2) 10 minutes after coronary stenting (ie, after PCI was finished and the system was retrieved); and (3) 24 hours after and (4) 48 hours after coronary stenting. Blood sampling was performed carefully and gently to avoid cell activation by the sampling procedure itself. A 3.5 mL sample of whole blood was immediately collected in a tube containing 1 mL of acid citrate dextrose.

**Reagents**

The monoclonal antibodies used in this experiment were purified Leu15 (Becton Dickinson Immunocytometry Systems) as anti-CD11b and 8B2 (provided by Dr Thomas Edgington, Department of Immunology, The Scripps Research Institute, La Jolla, Calif). Purified mouse immunoglobulin G1 was also used as an isotype negative control. The fluorescein-conjugated second step reagents for indirect immunofluorescence used were fluorescein isothiocyanate–conjugated F(ab')2 fragment of anti-mouse immunoglobulin G goat immunoglobulins (Dako).

**Indirect Immunofluorescence Assay**

Indirect immunofluorescence labeling was performed on 100 μL of well-mixed whole blood incubated with 20 μL each of Leu15 (50 μg/mL) and 8B2 (100 μg/mL) under pretreated saturating conditions for 30 minutes at 4°C. After the incubation, the samples were washed twice in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), and supernatant was removed. Then, 100 μL of second-step reagent was added at a dilution of 1/50 in PBS containing BSA for 45 minutes at 4°C. Lysing solution (8.26 g NH4Cl, 1.00 g KHCO3, and 0.04 g EDTA-4Na in 1 L of distilled water) was added to the specimen for hemolysis and incubated for 5 minutes at room temperature. After centrifugation, the supernatant was removed. Then, the sample solution was fixed in 1% paraformaldehyde solution. After the final washing, 0.7 mL of PBS was added to the samples, and the sample solution was stored at 4°C until analysis.

**Flow Cytometric Analysis**

The flow cytometric analysis was performed within 2 hours using an EPICS XL flow cytometer (Coulterronics). The flow cytometer was calibrated each day with standard fluorescent microbeads (CalibRITE: Becton Dickinson Immunocytometry Systems). This analysis focused on the PMN leukocytes identified by their forward (cell size) and right angle (inner structure of cells) scatter features. A PMN cluster of small size and complex inner structure could be distinguished from other leukocyte clusters of large size and simpler inner structure. For each sample, 10,000 events were collected and analyzed. Data acquisition was performed with CELLQuest software (Becton Dickinson Immunocytometry Systems). Mean channel fluorescence intensity was calculated and then converted into antigen density.

**Quantification Assay**

In this study, quantification of cell surface antigen expression was performed using the quantitative indirect immunofluorescence (QIFI) technique.21,22 This technique is based on the linear relationship observed between fluorescence intensity and antigen density when tested on a flow cytometer. When added in saturating concentrations, the binding of monoclonal antibodies to most available antigen sites is dominantly monovalent. Under these conditions, the number of antigen sites per cell, the frequency referred to as antigen density, corresponded to the number of cell-bound monoclonal antibody molecules, which in turn is often referred to as antibody-binding capacity (ABC). This analysis was performed using the QIFKIT (Biocytex). The kit has a series of 5 beads with well-defined quantities of a primary CDS monoclonal antibody in ABC units ranging from 2500 to 500 000. A second vial has a blank bead
Patients with restenosis presented for follow-up angiography ≈2 months earlier than patients without restenosis (4.2±0.3 versus 6.2±0.1 months). Late lumen loss (1.09±0.09 versus 0.42±0.08 mm; P<0.001) and percent diameter stenosis (60.0±0.9% versus 36.3±1.1%; P<0.001) were significantly greater in patients with restenosis compared with those without restenosis (Table 2).

**Expression of Mac-1 and Mac-1 Neopeptide After Coronary Stenting**

Expression of CD11b and the 8B2 activation neopeptide on the surface of PMNs were analyzed using flow cytometric analysis of whole blood obtained from the coronary sinus and femoral vein. The expression of CD11b and 8B2 on coronary sinus leukocytes was evaluated in patients with and without restenosis (Figure 1). The ABC for CD11b did not change 10 minutes after coronary stenting compared with the baseline values before the procedure. However, CD11b expression increased significantly at 24 hours and maximally at 48 hours after stenting. These changes were more prominent in patients with restenosis than in patients without restenosis. The relative increase in the ABC for CD11b at 48 hours from the baseline value (48 h/baseline) was 1.55±0.09 versus 1.21±0.04×10³ sites/cell (P<0.01), respectively, in patients with and without restenosis.

The ABC for 8B2 increased 10 minutes after coronary stenting and continued to rise 48 hours after stenting. These changes were also more prominent in patients with restenosis than in patients without restenosis. The relative increase in the ABC for 8B2 at 48 hours was 1.55±0.11 versus 1.23±0.06×10³ sites/cell (P<0.01), respectively, in patients with and without restenosis. The transcardiac gradients (coronary sinus blood minus peripheral blood) of the ABC for CD11b and 8B2 were increased at 48 hours after stenting compared with the baseline values, and these increases were more prominent in patients with restenosis than in patients without restenosis (Figure 2).

**Association Between Mac-1 Kinetics and Neointimal Hyperplasia After Coronary Stenting**

The relative increase in the ABC for CD11b at 48 hours correlated positively with the late lumen loss (R=0.38, P<0.01). Late lumen loss showed a stronger correlation for the increase in the ABC for 8B2 (R=0.50, P<0.001; Figure 3).

Multiple regression analysis of the patients showed that the late lumen loss was not correlated with age, coronary risk factors, any procedural variables, or other quantitative coronary angiographic variables but rather with the increase in CD11b (R=0.42, P<0.01) or its neopeptide, 8B2, (R=0.55, P<0.001) expression. The change in expression of 8B2 activation neopeptide was the most powerful predictor of the late lumen loss (Table 3).

**Effect of Heparin on In Vivo CD11b Expression and 8B2 Binding**

Because heparin binds to the I-domain of CD11b, it is conceivable that heparin might affect the binding of 8B2 to CD11b. To eliminate this possibility, we examined the effect of heparin on total CD11b (Leu15-positive cells) and active
CD11b (8B2-positive cells) in vitro and in vivo. Both Leu15 and 8B2 binding were unaffected by the addition of 1 U/mL heparin to whole blood, indicating that the presence of heparin does not interfere with the assessment of CD11b expression by flow cytometry (data not shown). We also collected whole blood from patients who received intravenous heparin to maintain an ACT of 200 to 250 s. Again, Leu15 and 8B2 binding were indistinguishable before and after heparin administration. These results have been incorporated into Figure 4.

**Discussion**

We demonstrated that expression and activation of the leukocyte integrin Mac-1 increased after coronary stenting and that they were associated with late lumen loss and restenosis. Transcardiac measurements determined that the changes in Mac-1 expression and activation occurred across the stented vascular bed, indicating that local leukocyte activation is temporally and spatially associated with mechanical vascular injury.

**Mac-1 Activation: Indicator of Vascular Injury and Inflammation After PCI**

PCI produces local mechanical vascular injury, which promotes leukocyte recruitment and vascular inflammation. Accumulating PMNs and monocytes release a variety of inflammatory mediators, including cytokines, chemokines, growth factors, and reactive oxygen intermediates, which potentiate injury and promote smooth muscle cell proliferation, migration, and extracellular matrix deposition. Mac-1 orchestrates the recruitment of leukocytes by promoting firm adhesion to and transmigration across fibrinogen and platelet ligands such as glycoprotein Ibα and possibly intercellular adhesion molecule-2, which is present at sites of injured vessel wall. Prior experimental and clinical studies have implicated Mac-1 in restenosis. Monoclonal antibody blockade and absence of Mac-1 reduce neointimal thickening after experimental angioplasty and stenting. We demonstrated that balloon angioplasty and stenting upregulated Mac-1 expression on the surface of PMNs and was associated with subsequent development of restenosis. Maximum up-regulation of Mac-1 was observed 48 hours after PCI in our serial sample analysis up to 144 hours after PCI.
Ligand interactions require transition of integrins from inactive to active, high-affinity states. This transition likely requires remodeling of the receptor through conformational changes associated with exposure or reorganization of new protein surface structures, ie, neoepitopes. 19 8B2 is a monoclonal antibody that only recognizes a Mac-1 neoepitope associated with fibrinogen binding and cellular adhesion. 20 In the present study, we observed that 8B2 neoepitope expression increased 10 minutes after coronary stenting, whereas CD11b expression did so only 24 hours later, suggesting that stenting promoted activation-dependent conformational change of existing Mac-1 on the surface of PMNs before upregulation of Mac-1 expression. Maximal increases in 8B2 neoepitope expression were observed 48 hours after stenting and was similar to total Mac-1 (ie, CD11b) expression. On the basis of our prior observations, we expect that Mac-1 expression would then decrease over time.15 In addition, multiple regression analysis suggested that 8B2 neoepitope expression was more closely correlated with the late lumen loss than total CD11b expression.

There is limited information on the molecular mechanisms that regulate the adhesion and subsequent trafficking of leukocytes to the vessel wall in the absence of the arterial endothelium such as occurs after angioplasty. On the basis of in vitro observations using monoclonal antibodies, Diacovo and coworkers11 have proposed a model of leukocyte recruitment at sites of platelet and fibrin deposition in which Mac-1 is required for PMN diapedesis. Additional in vitro observations indicated that platelets are capable of upregulating and activating Mac-1 as a consequence of P-selectin/PSGL-1 signaling.27 The first in vivo data supporting Diacovo et al’s 11 model was reported by Simon et al. 26 They used Mac-1-deficient mice subjected to carotid artery injury and showed that Mac-1 is required for transplatelet leukocyte migration and vessel wall accumulation. Although such studies have

**TABLE 3. Multiple Regression Analysis for Predicting Late Lumen Loss**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Standard Regression Coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>0.124</td>
<td>0.308</td>
</tr>
<tr>
<td>Hypertension (yes/no)</td>
<td>0.158</td>
<td>0.161</td>
</tr>
<tr>
<td>Diabetes mellitus (yes/no)</td>
<td>0.212</td>
<td>0.095</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>0.256</td>
<td>0.052</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>−0.235</td>
<td>0.072</td>
</tr>
<tr>
<td>No. of balloon inflations</td>
<td>0.156</td>
<td>0.164</td>
</tr>
<tr>
<td>Stent size (mm)</td>
<td>0.169</td>
<td>0.144</td>
</tr>
<tr>
<td>Stent-artery ratio</td>
<td>0.182</td>
<td>0.123</td>
</tr>
<tr>
<td>Peak dilation pressure (atm)</td>
<td>0.098</td>
<td>0.686</td>
</tr>
<tr>
<td>Reference diameter (mm)</td>
<td>0.165</td>
<td>0.149</td>
</tr>
<tr>
<td>Lesion length (mm)</td>
<td>0.153</td>
<td>0.171</td>
</tr>
<tr>
<td>Baseline MLD (mm)</td>
<td>−0.145</td>
<td>0.186</td>
</tr>
<tr>
<td>Acute gain (mm)</td>
<td>0.215</td>
<td>0.091</td>
</tr>
<tr>
<td>Increase in CD11b expression (48 h/baseline)</td>
<td>0.423</td>
<td>0.002</td>
</tr>
<tr>
<td>Increase in 8B2 binding (48 h/baseline)</td>
<td>0.547</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

MLD indicates minimal lumen diameter.
provided considerable insight into the roles of Mac-1, there are no reports in humans supporting the notion that platelets deposited at sites of arterial injury are capable of local leukocyte integrin activation. Our study provides data that are consistent with a wealth of previous experimental work indicating that vascular injury modulates the activation status of Mac-1 and, furthermore, that this activation is a determinant of late lumen loss after stenting.

Roles of PMNs in Neointimal Hyperplasia

Libby et al. proposed a cascade model of restenosis biology in which inflammatory cells, including PMNs and monocytes, influence smooth muscle cells and extracellular matrix deposition by producing a variety of mediators, including cytokines, chemokines, and growth factors. In animal models of vascular injury, leukocytes are recruited as a precursor to intimal thickening. Quantitative immunohistochemical analysis of coronary atherectomy specimens from humans has shown inflammatory cells to be far more prevalent in restenotic than de novo lesions and to comprise substantial lesional volume. Rogers et al. have collected autopsy specimens of stented human coronaries and found that 1 month after stent implantation, 30% of neointimal cells are smooth muscle cell in origin and 69% of all neointimal cells are monocyte/macrophage in origin. Moreover, in animal models, vessel wall inflammatory cell number is associated with cell proliferation and intimal thickening.

In the present study, we simultaneously analyzed monocyte CD11b expression. However, we did not find any significant changes in monocyte CD11b expression after PCI (data are not shown). There may be several reasons for this negative finding. First, receptor expression changes may be more difficult to observe by whole-blood flow cytometric analysis given the smaller number of monocytes and reduced levels of Mac-1 expression in monocytes compared with PMNs (~3 to 5-fold lower). Second, we may have missed the optimal time point for sampling CD11b expression by limiting blood sample collection to 4 time points over 48 hours.

Potential Limitations

Our study is limited to flow cytometric analysis of Mac-1 expression and activation. We did not directly assess leukocyte recruitment to the injured vessel wall. Although Mac-1 expression and activation are independent predictors of late loss and restenosis after stenting, assigning a pathophysiological role to Mac-1 in the vascular injury and repair response remains speculative, but highly likely in light of prior observations showing that antibody blockade or absence of Mac-1 reduces neointimal thickening after experimental angioplasty. In our study, ticlopidine was used as the post-stent antiplatelet regimen in all patients, raising the possibility that the antiinflammatory action of ticlopidine may have affected Mac-1 expression and activation.

Conclusion: Clinical Implications

In our study, we demonstrated that Mac-1 activation is an early and robust predictor of late lumen loss. Assessing the expression levels of Mac-1 and its activation neoepitope after coronary stenting may be helpful in predicting restenosis risk. Targeting Mac-1 directly seems to be a rational therapeutic strategy for preventing restenosis. In addition, we can envision a significant clinical advantage of this predictor of restenosis given the exciting results of the Immunosuppressive Therapy for the Prevention of Restenosis After Coronary Artery Stent Implantation (IMPRESS) trial, in which patients randomized to antiinflammatory therapy with prednisone or placebo on the basis of C-reactive protein levels (>0.5 mg/dL) 72 hours after stenting had significant reductions in angiographic restenosis (7% versus 33%). B2 binding, detected 10 minutes after stenting, identifies patients with PCI-induced leukocyte activation.

Acknowledgments

This study was supported in part by grants from the Vehicle Racing Commemorative Foundation, Tokyo, Japan. We acknowledge the technical support services of Toshiyuki Miyazaki, PhD, Ohtsuka Tokyo Assay Laboratory, Tokyo, Japan, for flow cytometric analysis. We thank Thomas S. Edgington, MD, PhD, The Scripps Research Institute, La Jolla, Calif, for generously providing the monoclonal antibody 8B2 and critical review of the manuscript. We also thank Daniel I. Simon, MD, Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass, for critical review of the manuscript.

References


Stent-Induced Expression and Activation of the Leukocyte Integrin Mac-1 Is Associated With Neointimal Thickening and Restenosis
Teruo Inoue, Toshihiko Uchida, Isao Yaguchi, Yoshihiko Sakai, Kan Takayanagi and Shigenori Morooka

_Circulation._ 2003;107:1757-1763; originally published online March 24, 2003;
doi: 10.1161/01.CIR.0000060487.15126.56
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/107/13/1757

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/