A Decline in Platelet Activation and Inflammatory Cell Infiltration is Associated with the Phenotypic Redifferentiation of Neointimal Smooth Muscle Cells after Bare-metal Stent Implantation in Acute Coronary Syndrome

Masashi Nakagawa¹, Takahiko Naruko², Yoshihiro Ikura¹, Ryushi Komatsu², Yoko Iwasa¹, Chizuko Kitabayashi¹, Takeshi Inoue³, Akira Itoh², Minoru Yoshiyama⁴, and Makiko Ueda¹

¹Department of Pathology, Osaka City University Graduate School of Medicine, Osaka, Japan
²Department of Cardiology, Osaka City General Hospital, Osaka, Japan
³Department of Pathology, Osaka City General Hospital, Osaka, Japan
⁴Department of Cardiology and Internal Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan

Aim: This immunohistochemical investigation was to analyze the relationship between platelet activation/aggregation, inflammatory cell infiltration, the differentiation state of neointimal smooth muscle cells (SMCs), expression of platelet-derived growth factor (PDGF), and endothelial cell regeneration at sites of bare-metal stents (BMS) in patients with acute coronary syndrome (ACS).

Methods: Sixteen coronary arteries after stenting were obtained at autopsy from ACS patients. Serial frozen sections were stained with antibodies against SMCs (1A4, HHF-35, CGA-7), macrophages, neutrophils, endothelial cells, GP IIb/IIIa, P-selectin, PDGF-B, and PDGF-β receptor.

Results: Up to 12 days after BMS, the stent sites contained P-selectin-positive activated platelets with neutrophil infiltration. From 24 to 55 days after BMS, parts of the platelet thrombi were still positive for P-selectin, and infiltration of neutrophils and macrophages was also found. Neointimal SMCs at these stages stained positive with 1A4 but negative with CGA-7, and PDGF-B and PDGF-β receptor were expressed in macrophages and SMCs. At sites from 3 months onward, platelet thrombi and neutrophil infiltration were not detected, and the neointima contained increased numbers of highly differentiated SMCs with CGA-7 positivity. The P-selectin-positive area was positively correlated with the neutrophil count and macrophage-positive area (neutrophils, r = 0.86, p < 0.0005; macrophage, r = 0.66, p < 0.05). In contrast, the P-selectin-positive area was negatively correlated with the HHF-35-negative area and CGA-7-negative area (HHF-35, r = -0.90, p < 0.0001; CGA-7, r = -0.82, p < 0.005).

Conclusion: These observations suggest that P-selectin-positive platelet thrombi in the neointima are positively associated with the inflammatory cell infiltration and reversely associated with the phenotypic redifferentiation of neointimal SMCs after BMS in ACS patients.


Key words: Stents, Neointima, Platelets, P-selectin, Pathology

Introduction

In-stent restenosis remains a recognized clinical problem. Recently, the use of drug-eluting stents (DES) has significantly reduced restenosis rates compared with bare-metal stents (BMS), however, although rare, late stent thrombosis has emerged as a se-
vere complication after DES owing to its high mortality.\textsuperscript{2} Daemen \textit{et al.} reported that acute coronary syndrome (ACS) is an independent risk factor for late stent thrombosis after DES in their large registry with 4 years of follow-up.\textsuperscript{3} Recent pathological data also suggested a significantly increased risk of late thrombotic complications in patients treated with DES for acute myocardial infarction (AMI)\textsuperscript{4}, therefore, BMS is recommended for patients with AMI.\textsuperscript{5}

Previous studies have shown that platelet aggregation and inflammatory reactions occur at the site of percutaneous coronary intervention (PCI)-related injuries.\textsuperscript{5,6} In our previous study based on coronary specimens after BMS implantation, abundant neointimal proliferation with a concentric layer of neovascularization at sites of in-stent restenosis suggested a role for the organization of thrombosis.\textsuperscript{7} The platelet glycoprotein (GP) IIb/IIIa receptor has been identified as the key mediator of platelet aggregation.\textsuperscript{8} Platelet activation is an important step leading to platelet aggregation.\textsuperscript{9} P-selectin is a component of the α-granule of unstimulated platelets, and is rapidly redistributed to the plasma membrane when platelets are activated.\textsuperscript{9-12} Clinically, the presence of activated platelets has been detected in patients with ACS by flow cytometric analysis using a monoclonal anti-P-selectin (CD62P) antibody (clone 1E3).\textsuperscript{13} On platelet activation, P-selectin also mediates the adherence of activated platelets to neutrophils and monocytes.\textsuperscript{12,14} Moreover, the inhibition of P-selectin-mediated leukocyte recruitment prevents the development of neointimal formation, adventitial inflammation, and vascular shrinking in a rat balloon-injury model.\textsuperscript{15} These previous data strongly suggest that platelet activation and platelet-leukocyte association are important in the process of neointimal formation at the site of BMS in patients with ACS.

Neointimal tissue proliferation at the site of PCI-related injuries is dominated by smooth muscle cells (SMCs).\textsuperscript{16} Previous experimental and human studies have demonstrated that dedifferentiation and redifferentiation of SMCs occur during the evolution of neointimal thickening, as revealed by changes in the cytoskeletal phenotype of SMCs.\textsuperscript{17-19} On the basis of experimental and human studies, it has been also suggested that platelet-derived growth factor (PDGF) is one of the growth factors involved;\textsuperscript{20-23} however, in patients with ACS, no systematic studies have focused on immunohistochemical characteristics of the differentiation state of neointimal SMCs at the site of BMS.

In the present study, therefore, we immunohistochemically investigated the association among GP IIb/IIIa- and P-selectin positivity, inflammatory cell infiltration, the differentiation state of neointimal SMCs, and regeneration of endothelial cells during the process of neointimal formation after BMS implantation in patients with ACS. We also examined the immunolocalization of PDGF-B and PDGF-β receptor at the sites of BMS in these ACS patients. This study was based on frozen tissue samples, because monoclonal antibodies against GP IIb/IIIa and P-selectin work well on frozen sections only.

**Materials and Methods**

**Coronary Tissue Specimens**

The study was conducted using 16 coronary artery segments after BMS implantation obtained as frozen samples at autopsy from 15 patients with AMI (n = 12) and unstable angina pectoris (UAP) (n = 3), who had undergone emergency coronary stenting. The relevant clinical data are summarized in Table 1. The mean age of the patients (11 men and 4 women) was 74 years (range 46 to 86 years). In these patients, the interval between coronary stenting and death ranged from 7 days to 5 years. After administration of 5,000 IU heparin and conventional wire crossing, predilatation with a balloon before stenting, or direct stent implantation was performed. A Palmaz-Schatz stent (Johnson & Johnson Interventional System) was inserted into two sites. One coronary artery received a Terumo stent (Terumo Co.). Two coronary arteries received GRII stents (Cook Inc.) and two coronary arteries received S670 stents (Medtronic Inc.). The remaining 9 arteries contained Multi-Link stents (Advanced Cardiovascular Systems). All patients with BMS were treated initially with heparin and then maintained on aspirin (81 mg) and ticlopidine (200 mg), which is the standard regimen in Japan. GP IIb/IIIa inhibitors were not used in this study, as these agents are not available in Japan. All autopsies were performed within 3 hours after death. The site of BMS was identified by comparing heart specimens with angiograms after stenting. The coronary arteries were removed from the epicardial surface in the fresh state, and the site containing the stent was cut with scissors into 5-mm segments. Stent fragments on the cut surface of each of the 5-mm segments were removed under a dissection microscope, and from each coronary artery containing the stent, one or two 5-mm segments were snap-frozen and stored at −80°C. The remaining 5-mm segments were fixed in 20% buffered formalin. These frozen samples were sectioned serially at 7-μm thickness. Each time a portion was encountered that still contained stent fragments, these fragments were removed carefully, again by cut-
Clinical data relevant to this study of 15 patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Reason for Stenting</th>
<th>Stent Site</th>
<th>Interval Stent/Death</th>
<th>Stent Type</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79/M</td>
<td>AMI</td>
<td>LAD</td>
<td>7 days</td>
<td>Multi-Link</td>
<td>VF</td>
</tr>
<tr>
<td>2</td>
<td>69/M</td>
<td>AMI</td>
<td>LAD</td>
<td>9 days</td>
<td>Palmaz-Schatz</td>
<td>Subacute thrombosis</td>
</tr>
<tr>
<td>3</td>
<td>81/F</td>
<td>AMI</td>
<td>LAD</td>
<td>12 days</td>
<td>GR II</td>
<td>Cardiogenic shock</td>
</tr>
<tr>
<td>4</td>
<td>75/M</td>
<td>AMI</td>
<td>RCA</td>
<td>12 days</td>
<td>Palmaz-Schatz</td>
<td>Subacute thrombosis</td>
</tr>
<tr>
<td>5</td>
<td>69/M</td>
<td>AMI</td>
<td>RCA</td>
<td>24 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>6</td>
<td>78/F</td>
<td>AMI</td>
<td>LAD</td>
<td>28 days</td>
<td>Terumo</td>
<td>Retroperitoneal hemorrhage</td>
</tr>
<tr>
<td>7</td>
<td>77/M</td>
<td>AMI, OMI</td>
<td>LAD</td>
<td>35 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>8</td>
<td>80/F</td>
<td>UAP</td>
<td>LAD</td>
<td>35 days</td>
<td>GR II</td>
<td>CHF**</td>
</tr>
<tr>
<td>9</td>
<td>67/M</td>
<td>AMI</td>
<td>LAD</td>
<td>39 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>10</td>
<td>80/F</td>
<td>AMI, OMI</td>
<td>LAD</td>
<td>55 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>11</td>
<td>46/M</td>
<td>AMI</td>
<td>LMT</td>
<td>3 months</td>
<td>S670</td>
<td>CHF*</td>
</tr>
<tr>
<td>12</td>
<td>80/M</td>
<td>UAP</td>
<td>RCA (proximal)</td>
<td>6 months</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>13</td>
<td>80/M</td>
<td>UAP</td>
<td>RCA (distal)</td>
<td>6 months</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>14</td>
<td>56/M</td>
<td>AMI</td>
<td>LAD</td>
<td>2 years</td>
<td>Multi-Link</td>
<td>Renal failure</td>
</tr>
<tr>
<td>15</td>
<td>86/M</td>
<td>AMI</td>
<td>RCA</td>
<td>5 years</td>
<td>S670</td>
<td>Pneumonia</td>
</tr>
</tbody>
</table>

AMI, acute myocardial infarction; LAD, left anterior descending; RCA, right coronary artery; LMT, left main trunk; LCX, left circumflex; Muti-Link (Advanced Cardiovascular Systems); Palmaz-Schatz (Johnson & Johnson Interventional System); GR II, (Cook Inc.); Terumo (Terumo Co); S670 (Medtronic Inc); VF, ventricular fibrillation; CHF*, congestive heart failure related to initial infarction; CHF**, congestive heart failure due to multivessel disease.

Immunohistochemical Staining

The primary monoclonal antibodies used for the identification of SMCs were anti-SMC actin markers; 1A4 (DAKO, Glostrup, Denmark), HHF-35 (DAKO), and CGA-7 (Enzo Laboratories, NY). In this study, the differentiation state of SMCs within the neointima at the site of BMS was evaluated using two anti-actin markers, HHF-35 and CGA-7, according to our previous studies. Highly differentiated SMCs stained positive with both HHF-35 and CGA-7, whereas dedifferentiated SMCs stained negative with both HHF-35 and CGA-7, and intermediatedifferentiated SMCs stained positive with HHF-35 but negative with CGA-7. To identify GP IIb/IIIa, a monoclonal antibody against GP IIb (CD41, DAKO) was used. Activated platelets were detected with a monoclonal antibody against P-selectin (CD62P, clone 1E3; DAKO). The CD62P antigen is a 140kDa transmembrane glycoprotein present on activated platelets. In unstimulated platelets, CD62P is stored in the membranes of secretory granules and shows no surface immunostaining with anti-CD62P. When platelets are activated by agonists such as thrombin, the granules rapidly fuse with the plasma membrane, which will express CD62P on the surface. To identify neutrophils, the following antibodies were used: CD66b (80H3; Beckman Coulter, CA), elastase (NP57; DAKO) and myeloperoxidase (MPO-7; DAKO). The remaining cellular components were analyzed using monoclonal antibodies against macrophages (EBM11; DAKO), and endothelial cells (vWF; DAKO). For PDGF-B identification, a mouse monoclonal antibody (PGF-007) was used (kindly provided by Mochida Pharmaceutical Co, Inc., Japan); its specificity has been reported. For identification of the PDGF-β receptor, a rabbit polyclonal antibody (PDGFR-β (958)) was used (Santa Cruz Biotechnology, Inc., CA.). Nonimmune mouse IgG serum (DAKO) served as a negative control. Sections were incubated at 4°C overnight or for 1 hour at room temperature, and then subjected to a three-step staining procedure, using the streptavidin-biotin complex method (SABC) for detection. Peroxidase activity was visualized with 3-amino-9-ethyl-carbazole (10 minutes, room temperature), and the sections were faintly counterstained with hematoxylin.

For the simultaneous identification of GP IIb/IIIa and P-selectin, double immunostaining was performed based on two primary antibodies of a different IgG subclass, as reported previously. In this staining, alkaline phosphatase was visualized with fast blue

Table 1. Clinical data relevant to this study of 15 patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Reason for Stenting</th>
<th>Stent Site</th>
<th>Interval Stent/Death</th>
<th>Stent Type</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79/M</td>
<td>AMI</td>
<td>LAD</td>
<td>7 days</td>
<td>Multi-Link</td>
<td>VF</td>
</tr>
<tr>
<td>2</td>
<td>69/M</td>
<td>AMI</td>
<td>LAD</td>
<td>9 days</td>
<td>Palmaz-Schatz</td>
<td>Subacute thrombosis</td>
</tr>
<tr>
<td>3</td>
<td>81/F</td>
<td>AMI</td>
<td>LAD</td>
<td>12 days</td>
<td>GR II</td>
<td>Cardiogenic shock</td>
</tr>
<tr>
<td>4</td>
<td>75/M</td>
<td>AMI</td>
<td>RCA</td>
<td>12 days</td>
<td>Palmaz-Schatz</td>
<td>Subacute thrombosis</td>
</tr>
<tr>
<td>5</td>
<td>69/M</td>
<td>AMI</td>
<td>RCA</td>
<td>24 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>6</td>
<td>78/F</td>
<td>AMI</td>
<td>LAD</td>
<td>28 days</td>
<td>Terumo</td>
<td>Retroperitoneal hemorrhage</td>
</tr>
<tr>
<td>7</td>
<td>77/M</td>
<td>AMI, OMI</td>
<td>LAD</td>
<td>35 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>8</td>
<td>80/F</td>
<td>UAP</td>
<td>LAD</td>
<td>35 days</td>
<td>GR II</td>
<td>CHF**</td>
</tr>
<tr>
<td>9</td>
<td>67/M</td>
<td>AMI</td>
<td>LAD</td>
<td>39 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>10</td>
<td>80/F</td>
<td>AMI, OMI</td>
<td>LAD</td>
<td>55 days</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>11</td>
<td>46/M</td>
<td>AMI</td>
<td>LMT</td>
<td>3 months</td>
<td>S670</td>
<td>CHF*</td>
</tr>
<tr>
<td>12</td>
<td>80/M</td>
<td>UAP</td>
<td>RCA (proximal)</td>
<td>6 months</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>13</td>
<td>80/M</td>
<td>UAP</td>
<td>RCA (distal)</td>
<td>6 months</td>
<td>Multi-Link</td>
<td>CHF**</td>
</tr>
<tr>
<td>14</td>
<td>56/M</td>
<td>AMI</td>
<td>LAD</td>
<td>2 years</td>
<td>Multi-Link</td>
<td>Renal failure</td>
</tr>
<tr>
<td>15</td>
<td>86/M</td>
<td>AMI</td>
<td>RCA</td>
<td>5 years</td>
<td>S670</td>
<td>Pneumonia</td>
</tr>
</tbody>
</table>
BB (blue, CD41) and peroxidase with 3-amino-9-ethylcarbazole development (red: P-selectin).

Quantitative Methods

The area occupied by HHF-35-, CGA-7-, EBM11-, P-selectin-, and GPIIb/IIIa-positive cells was quantified by computer-aided planimetry, and expressed as a percentage of the area occupied by neointimal tissue. The numbers of CD66b-positive neutrophils were counted in the neointima, and expressed as the number of cells per mm² of the neointima. Morphometric analysis was performed by a single investigator who was blinded to the patients’ characteristics and histological classifications. Data are shown as the mean ± SD. Intraobserver variability was determined on the basis of triplicate measurements. The mean ± SD differences among measurements were 3.8 ± 1.4%. For comparisons of 2 groups of individuals, the Mann-Whitney U test was used in all circumstances. When comparing 3 groups of individuals, the nonparametric Kruskal-Wallis test was used. Pearson correlation coefficients were calculated to assess the relation between 2 parameters. Values of p < 0.05 were considered significant.

Results

At the earliest stage up to 12 days after BMS, stent sites showed thrombus formation with minimal cellular reaction. At 7 days and 9 days after BMS, all thrombi stained positive for GP IIb/IIIa and P-selectin, and neutrophil infiltration was found around these P-selectin-positive platelet thrombi (Fig. 1). In two lesions at 12 days after BMS, the stent sites contained GPIIb/IIIa-positive platelet thrombi (Fig. 2B). At these sites, P-selectin-positive platelet aggregates were detected in areas close to the luminal surface (Fig. 2C), and neutrophil infiltration was detected in regions near or around these P-selectin-positive platelet aggregates (Fig. 2D). Within GPIIb/IIIa-positive platelet thrombi, the deeper layers of thrombi contained abundant macrophages, whereas only occasional macrophages were seen on the luminal side areas of thrombi with P-selectin positivity (Fig. 2E). Moreover, lacerated areas around the stent struts showed cellular reactions (Fig. 2F). Immunohistochemically, the cellular response at this stage was composed of abundant macrophages (Fig. 2G) and actin-negative spindle-shaped cells, which stained negative with actin markers 1A4, HHF-35, and CGA-7 (Fig. 2H).

From 24 to 35 days after BMS, the stent sites showed a distinct layer of neointimal tissues composed of SMCs mixed with GP IIb/IIIa-positive platelet thrombi and inflammatory cells (Fig. 3). In these early stages after BMS, parts of the platelet thrombi were still positive for P-selectin (Fig. 3C), and double immuno staining for GP IIb/IIIa and P-selectin exhibited distinct double staining for GP IIb/IIIa and P-selectin (Fig. 3D). Neutrophil infiltration was found in limited areas around P-selectin-positive platelet thrombi (Fig. 3E). Neointimal tissues at these stages showed an accumulation of macrophages (Fig. 3F) around the ar-

Fig. 1. Nine days after BMS implantation
Fig. 2. Twelve days after BMS implantation

A, Hematoxylin–eosin stain. Cross section shows preexistent atherosclerotic plaque (AS), media (M), stent struts (arrowhead), and mural thrombi. The lacerated area indicated by the arrow is shown in higher magnification in F–H. B, Anti-GP IIb/IIIa antibody reveals the presence of GP IIb/IIIa-positive platelet thrombi. C, Anti-P-selectin antibody reveals that P-selectin-positive platelet aggregates are located in areas close to the luminal surface. D, Staining with the anti-neutrophil CD66b antibody. Neutrophil infiltration is detected in regions near or around P-selectin-positive platelet aggregates. E, Anti-CD68 antibody shows that the deeper layers of platelet thrombi contain abundant macrophages, while only occasional macrophages are seen in the luminal side areas of platelet thrombi. F, Hematoxylin–eosin stain. Serial section, 0.4 mm remote from the site shown in A. Cellular reaction (asterisk) is seen at the laceration site adjacent to the media (M). Inset: Detail of the cellular reaction, taken from the area indicated by the asterisk. G, Staining with the anti-CD68 antibody. The cellular reaction is composed of CD68-positive macrophages and spindle-shaped cells (arrows) that are negative for CD68. H, Staining with the anti-1A4 antibody. These spindle-shaped cells are negative with the actin marker 1A4. SMCs within the media stain positive with 1A4. Bar: A–E, 500 μm; F, 200 μm; G, H, 100 μm.

Fig. 3. Twenty-eight days after BMS implantation.

A, Hematoxylin–eosin stain. Cross section shows distinct neointimal proliferation (asterisks), atherosclerotic plaque, media, and stent struts (arrowheads). The neointimal area indicated by the arrow is shown in higher magnification in B–H. B, Anti-GP IIb/IIIa antibody reveals the presence of GP IIb/IIIa-positive platelet thrombi within the neointima. C, Anti-P-selectin antibody reveals that parts of the platelet thrombi are positive for P-selectin. D, Double immunostaining for GP IIb/IIIa (blue) and P-selectin (red). Parts of the thrombi exhibit double staining (purple) (arrows), indicating that these are activated platelets with P-selectin positivity. E, Anti-neutrophil CD66b antibody reveals neutrophil infiltration in regions around P-selectin-positive platelets. F, Anti-CD68 antibody shows an accumulation of macrophages around the areas of GP IIb/IIIa-positive platelet thrombi in the neointima. G, Staining with SMCs (1A4). Neointimal tissue is composed mainly of SMCs. H, Staining with SMCs (HHF-35). Occasional SMCs within the neointima stain positive with HHF-35. The area containing GP IIb/IIIa-positive platelets and inflammatory cells shows the presence of dedifferentiated SMCs, and negative staining with HHF-35. I, Staining with SMCs (CGA-7). At this stage, neointimal SMCs are negative with CGA-7. Bar: A, 1,000 μm; B–H, 200 μm.
eas of GP IIb/IIIa-positive platelet thrombi (Fig. 3B). With regard to the differentiation state of neointimal SMCs in these early stages, most SMCs within the neointima stained positive with 1A4 (Fig. 3G), but HHF-35 positivity was occasionally found in neointimal SMCs (Fig. 3H), and almost all neointimal SMCs were negative with CGA-7 (Fig. 3I). In this neointimal lesion containing abundant macrophages, occasional SMCs stain positive with HHF-35. H. Staining with SMCs (CGA-7). In this neointimal lesion containing abundant macrophages, occasional SMCs stain positive with HHF-35. J. Staining with SMCs (CGA-7). In this lesion, some SMCs within the neointima are also positive with CGA-7. Bar: A, 1,000 μm; B–C, 500 μm; D, 100 μm; E–J, 200 μm.

At 3 months after BMS, staining for GP IIb/IIIa (Fig. 5B) and P-selectin (Fig. 5C) showed no platelet thrombi within the neointima. There were no neutrophils in the neointimal tissues (Fig. 5D), and the deeper layer of the neointima, close to the stent struts, revealed a distinct accumulation of macrophages (Fig. 5E). At this stage, most SMCs within the neointima stained positive with both 1A4 and HHF-35 (Fig. 5F), and these neointimal SMCs also showed increased staining positivity with CGA-7 (Fig. 5G). In the present cohort, regeneration of vWF-positive endothelial cells covering the neointima was first identified 3 months after BMS, and subendothelial staining for vWF was found at this stage (Fig. 5I). In neointimal lesions from 6 months onward, there were no platelet thrombi with GP IIb/IIIa and P-selectin positivity (Fig. 6B, 6C). Neutrophils were...
not present within the neointimal tissues (Fig. 6D), while some macrophages were identified in the deeper layers of the neointima, close to the stent struts (Fig. 6E). At these stages, from 6 months onward, the neointima consisted predominantly of highly differentiated SMCs showing staining positivity with 1A4 (Fig. 6F), HHF-35 (Fig. 6G), and CGA-7 (Fig. 6H), indicating that the vast majority of neointimal SMCs regained a differentiated state (redifferentiation). In these lesions, a complete lining of regenerated endothelial cells was identified at the luminal surface of the neointima (Fig. 6I).

**PDGF-B and PDGF-β Receptor Expression**

At the earliest stage up to 12 days after BMS, P-selectin-positive activated platelets stained positive for PDGF-B (Fig. 7A), but negative for PDGF-β receptor (Fig. 7E). At sites ranging from 24 to 55 days after BMS, staining positivity for PDGF-B and PDGF-β receptor was found in accumulated macrophages, in dedifferentiated SMCs (negative with both HHF-35 and CGA-7), and in intermediately differentiated SMCs (positive with HHF-35 but negative with CGA-7) within the neointima (Fig. 7B, 7F). At 3 months after BMS, most SMCs within the neointima, which were positive with HHF-35 but negative with CGA-7, showed staining positivity for PDGF-B and PDGF-β receptor. In addition, accumulated macrophages in the deeper layer of the neointima, close to the stent struts, stained positive with PDGF-B and PDGF-β receptor (Fig. 7C, 7G). However, at 6 months after BMS, the neointima consisted predominantly of highly differentiated SMCs (positive with both HHF-35 and CGA-7). At this stage, PDGF-B and PDGF-β receptor expression within the neointima decreased markedly, and only occasional SMCs or macrophages in the neointima showed staining positivity for PDGF-B and PDGF-β receptor (Fig. 7D, 7H). In neointimal lesions ranging from 1 year to 5 years after BMS, there was no staining positivity for PDGF-B and PDGF-β receptor.

**Morphometric Analysis**

Four sites at the earliest stage (at 7–12 days after
BMS) were excluded, because they revealed only minimal cellular reaction. In the remaining 12 sites after BMS, we performed subgroup analysis with 3 groups, based on the interval of stenting and death (Group I: 24–35 days after BMS (n = 4), Group II: 39–55 days after BMS (n = 2), Group III: > 3 months after BMS (n = 6)). Morphometric results are shown in Fig. 8, 9. Quantitative analysis demonstrated that the percentages of GP IIb/IIIa-positive and P-selectin-positive areas, and the number of CD66b-positive neutrophils in
group III were significantly lower than in either group I or group II (GP IIb/IIIa, I versus III, \( p < 0.005 \); and II versus III, \( p < 0.01 \); P-selectin, I versus III, \( p < 0.005 \); and II versus III, \( p < 0.01 \); neutrophils, I versus III, \( p < 0.005 \); and II versus III, \( p < 0.01 \)). The percentage of macrophage-positive area was also significantly lower (macrophages, \( p < 0.05 \)) in group III than in group I. In contrast, the percentages of the HHF-35-positive and CGA-7-positive areas in group III were significantly higher than in either group I or group II (HHF-35, I versus III, \( p < 0.05 \); and II versus III, \( p < 0.05 \); CGA-7, I versus III, \( p < 0.01 \); and II versus III, \( p < 0.05 \)) (Fig. 8).

The percentage of the P-selectin-positive area was positively correlated with the number of CD66b-positive neutrophils and the percentage of the macrophage-positive area (P-selectin versus CD66b-positive neutrophils, \( r = 0.86, p < 0.0005 \); P-selectin versus macrophage-positive area, \( r = 0.66, p < 0.05 \), Fig. 9A, 9B). In contrast, the percentage of the P-selectin-positive area was negatively correlated with the percentages of the HHF-35-positive and CGA-7-positive areas (P-selectin versus HHF-35, \( r = -0.90, p < 0.0001 \); P-selectin versus CGA-7, \( r = -0.82, p < 0.005 \), Fig. 9C, 9D).

**Discussion**

To the best of our knowledge, the present study, based on the analysis of frozen sections and using immunohistochemical single and double-staining techniques, is the first to demonstrate the relationship between the aggregation of activated platelets, neutrophil and macrophage accumulation, phenotypic changes of SMCs, PDGF-B and PDGF-\( \beta \) receptor expression, and the regeneration of endothelial cells during the evolution of neointimal tissue proliferation at the BMS site in ACS patients.
Previous studies, using conventional formalin-fixed or methanol-Carnoy-fixed sections, documented thrombus formation at the early stages of neointimal formation after coronary angioplasty or stenting in humans \(^{5,7,18}\), but their functional significance was not further elucidated. Recently, accumulating evidence has suggested that an association between activated platelets and leukocytes plays a pivotal role in inflammatory processes \(^{27,28}\). P-selectin, a membrane glycoprotein in platelets, is expressed on the surface of platelets only after cellular activation \(^{9,12}\). Thus, P-selectin is a convincing marker of platelet activation, and can be detectable immunohistochemically on frozen sections. Our previous study, based on the analysis of frozen sections and using the same anti-P-selectin antibody as the present study, reported that the aggregation of P-selectin-positive activated platelets plays an important role in the progression of inflammatory processes in the active phase of ulcerative colitis in humans \(^{29}\). The present study, using frozen sections, clearly demonstrates the presence of P-selectin-positive activated platelets in neointimal lesions at early stages (<55 days) after BMS, in coronary arteries obtained from patients with ACS. Grewe et al. reported that in human coronary specimens after BMS, the early neointima contains thrombotic materials, including red blood cells and aggregated thrombocytes \(^{30}\). Farb et al. also investigated human coronary specimens after BMS, using conventional formalin-fixed sections, and described that platelet-rich thrombi were not found in coronary sections more than 30 days after BMS \(^{6}\); however, in the present study, P-selectin-positive platelet thrombi were still seen in a patient with ACS, 55 days after BMS. The differences observed between our study and that of Farb et al. may be related to different patient characteristics or methods of the

Fig. 9

(A, B) Graphs showing that the percentage of the P-selectin-positive area is positively correlated with the number of CD66b-positive neutrophils and the percentage of the macrophage-positive area (P-selectin versus CD66b-positive neutrophils, \(r = 0.86, p < 0.0005\), P-selectin versus macrophage-positive area, \(r = 0.66, p < 0.05\)). (C, D) Graphs showing that the percentage of the P-selectin-positive area is negatively correlated with the percentage of the HHF-35-positive area and the CGA-7-positive area (P-selectin versus HHF-35, \(r = 0.90, p < 0.0001\), P-selectin versus CGA-7, \(r = 0.82, p < 0.005\)).
identification of platelet thrombi. Our study, based on frozen sections and immunohistochemical techniques, suggests that prolonged activation and aggregation of platelets may occur at BMS sites in ACS patients.

In the present study, our immunohistochemical analyses further revealed the close positional association of P-selectin-positive activated platelets and neutrophils in early neointimal lesions after BMS in ACS patients. Moreover, our morphometric analyses clearly demonstrated that the percentage of the P-selectin-positive area and the number of CD66b-positive neutrophils were significantly higher in lesions in the early stages after BMS, and that the percentage of the P-selectin-positive area was positively correlated with the number of CD66b-positive neutrophils. These findings suggest that a close association between activated platelets and neutrophils plays a role in the local inflammatory process in the early stages of neointimal formation after BMS in human coronary arteries. The pathophysiologic role of platelet-neutrophil interaction during the progression of neointimal proliferation has been documented in experimental models and clinical studies. Kumar et al. demonstrated that P-selectin is involved in processes leading to neointimal proliferation by mediating leukocyte recruitment and the interaction between platelets and leukocytes. Moreover, Inoue et al. suggested, based on flow cytometric analysis, that cellular interactions between platelets and neutrophils may be related to the progression of neointimal proliferation after BMS in patients with stable angina pectoris. Welt et al. also described that neutrophils play a causative role in neointimal hyperplasia after balloon injury in rabbit iliac arteries. These previous data and our present findings support the hypothesis that platelet-neutrophil association plays an important role in the early stages of neointimal proliferation after BMS in ACS patients.

This study also provides new data with regard to the relationship between the platelet-neutrophil association and the differentiation state of neointimal SMCs at BMS sites in ACS patients. In the early stages after BMS, P-selectin-positive activated platelets with infiltration of neutrophils were identified in the neointimal lesion after BMS; however, at later stages, from 3 months onward, P-selectin-positive platelets and neutrophils could no longer be detected within the neointima; at these stages, the neointima contained increased numbers of highly differentiated SMCs with CGA-7 positivity. Our quantitative analyses unequivocally revealed that the percentage of the P-selectin-positive area was positively correlated with the number of neutrophils and the percentage of the macrophage-positive area, and was negatively correlated with the percentages of the HHF-35-positive area and the CGA-7-positive area. These results strongly suggest that a gradual decline of the aggregation of activated platelets and platelet-leukocyte association during the evolution of the neointima after BMS is closely related to the phenotypic redifferentiation of neointimal SMCs in patients with ACS. The background of these observations remains unclear; however, it could be hypothesized that activated platelets and infiltrated inflammatory cells play a role in the control of the redifferentiation of neointimal SMCs.

It has been suggested that PDGF-B acts as an extremely efficacious repessor of the expression of smooth muscle α-actin. Thus, PDGF expression is important not only for SMC proliferation and migration but also for the phenotypic cytoskeletal modulation of SMCs. Our previous studies, using in situ hybridization and immunohistochemical techniques, showed that PDGF mRNA, PDGF-B protein, and PDGF-β receptor are expressed in neointimal lesions only at early stages after coronary angioplasty injuries in humans. The present study of ACS patients clearly demonstrated that at the earliest stage after BMS, PDGF is expressed in P-selectin-positive activated platelets. This finding is in agreement with previous studies, which revealed a close relationship between platelet activation and release of PDGF. Our observations in lesions with neointimal proliferation after BMS further showed that the expression of PDGF-B and PDGF-β receptor in the neointima decreased markedly in the later stage, 6 months after BMS; at this stage, neointimal SMCs differentiated into a highly differentiated state. These findings suggest that the decline in the expression of PDGF-B and PDGF-β receptor during the evolution of the neointima relates to the state of differentiation of neointimal SMCs; therefore, it is conceivable that the phenotypic redifferentiation of neointimal SMCs after BMS in patients with ACS may be, at least in part, the effect of a decrease in PDGF in the process of neointimal proliferation.

The present study, moreover, shows the time course of reendothelialization at the site of the neointima after BMS in ACS patients. In our previous immunohistochemical study, we demonstrated partial endothelial cell regeneration 64 days after BMS and complete restoration of the endothelial cell lining in lesions, 12, 15, and 21 months after BMS. However, because of the limited number of autopsied cases studied, our previous study could not clarify the detailed time course of reendothelialization at the BMS site. The present study provides additional immunohisto-
chemical data regarding the reendothelialization after BMS in ACS patients. In this study, 3 months after BMS, the lesion showed regeneration of endothelial cells covering the neointima with subendothelial staining for vWF; however, the sites from 6 months onward revealed a complete lining of regenerated endothelial cells at the luminal surface of the neointima. Our previous ultrastructural study suggested a gradual maturation of regenerated endothelial cells during the evolution of the neointima after angioplasty injuries in human coronary arteries. Therefore, it is possible that the subendothelial vWF staining seen 3 months after BMS may relate to the presence of immature regenerated endothelial cells, characterized by large gaps between cells with an incomplete basement membrane. It is also possible that subendothelial staining for vWF may relate to increased vWF biosynthesis in regenerating endothelial cells, as suggested by previous experimental studies. In the present study, moreover, the regeneration of endothelial cells appeared to coincide with an increase in the number of highly differentiated SMCs with CGA-7 positivity in neointimal lesions of ACS patients. These observations are endorsed by the previous experimental study by Kocher et al. and by our human studies using coronary specimens after angioplasty, which suggested a relation between phenotypic redifferentiation of neointimal SMCs and the regeneration of endothelial cells.

Study Limitations

With regard to the attempt to reconstruct a time course of the pathological changes after BMS in human coronary arteries, the main limitation is the small number of autopsied cases studied; however, it is not easy to acquire suitable coronary specimens after BMS from autopsied cases for reliable immunohistochemical studies. In the present study, we used frozen tissue samples, because some monoclonal antibodies work well on frozen sections only; therefore, we consider that the quality of data is sufficiently high to warrant our conclusion. Further studies with large numbers of cases are needed to validate our observations.

Conclusion

The present study demonstrates that P-selectin-positive platelet aggregation and platelet-neutrophil association are key events in the early stages of neointimal proliferation at the site of BMS in human coronary arteries of ACS patients. Furthermore, this strongly suggests that a gradual decline in the aggregation of activated platelets with inflammatory cells during the evolution of neointimal thickening after BMS implantation is closely related to the phenotypic redifferentiation of neointimal SMCs in ACS patients.

References

12) Johnston GI, Cook RG, McEver RP: Cloning of GMP-140, a granule membrane protein of platelets and
endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell, 1989; 56: 1033-1044
18) Ueda M, Becker AE, Naruko T, Kojima A: Smooth muscle cell de-differentiation is a fundamental change preceding wound healing after percutaneous transluminal coronary angioplasty in humans. Coron Artery Dis, 1995; 6: 71-81
37) Bosmans JM, Kockx MM, Vrints CJ, Bult H, De Meyer GR, Herman AG: Fibrin(ogen) and von Willebrand factor deposition are associated with intimal thickening after balloon angioplasty of the rabbit carotid artery. Arterioscler Thromb Vasc Biol, 1997; 17: 634-645