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Impact of primitive cells in intracoronary thrombi on lesion prognosis: temporal analysis of cellular constituents of thrombotic material obtained from patients with acute coronary syndrome

Hiroshi Iwata,1 Masataka Sata,1,2 Jiro Ando,1 Hideo Fujita,1 Toshihiro Morita,1 Daigo Sawaki,1 Masao Takahashi,1 Yoichiro Hirata,2 Shuichiro Takanashi,3 Minoru Tabata,3 Yasunobu Hirata,1 Ryozo Nagai1

ABSTRACT

Background Clinical evidence suggests that intracoronary thrombus formation is associated with a high incidence of late restenosis after successful coronary intervention in patients with myocardial infarction (MI). However, little is known about the mechanism by which intracoronary thrombi play pathological roles.

Methods and Results We analysed the cellular constituents of 108 thrombi aspirated from coronary lesions with a thrombectomy device in 62 patients who underwent emergent coronary intervention for ACS as their onset of chest pain (correlation coefficient $\rho = 0.683$, $p < 0.01$), aspirated thrombotic materials revealed that the content of platelets, as determined by immunostaining for CD42a, had a negative correlation with the time after the onset of chest pain (correlation coefficient $\rho = -0.683$, $p < 0.01$). Immunofluorescent staining for CD34 and breast cancer-resistant protein-1 (bcrp-1) detected primitive cells in intracoronary thrombi. Furthermore, the ratio of CD34-positive cells in intracoronary thrombi had a significant positive correlation with restenosis at follow-up coronary angiography (correlation coefficient $\rho = 0.76$, $p < 0.01$).

Conclusions The findings of this study indicate that the early accumulation of primitive cells in platelet aggregates may play a role in neointimal growth after successful coronary intervention in patients with acute coronary syndrome.

It is well known that most of coronary thrombi are precipitated by rupture of soft and vulnerable plaques and platelet aggregation plays an important role initially in their evolution. Thrombus removal using mechanical thrombectomy devices for reducing the thrombotic burden has been thought to be advisable not only for procedural success, but also for better long-term outcome in patients undergoing coronary angioplasty for acute coronary syndromes (ACS).1 Lesions with a high thrombotic burden are prone to increased procedural risk.2–4 Furthermore, a number of clinical studies has demonstrated that the presence of intracoronary thrombus detected by angiography and/or intravascular ultrasonography increases the risk of long-term restenosis after angioplasty.5 Autopsy studies have revealed that coronary thrombi have a layered structure, with thrombus material of differing ages, indicating that they are formed successively from repeated mural deposits that cause progressive luminal narrowing over an extended period of time.6 However, it remains unclear how intracoronary thrombi promote restenosis after successful intervention in patients with ACS.

Evaluation of intracoronary thrombi in ACS patients had been relatively difficult because only post-mortem specimens were available. Recent progress in mechanical thrombectomy and distal protection devices3–4 had made it possible to aspirate fresh intracoronary thrombotic material for histological analysis.8

In the present study, the cellular constituents of intracoronary thrombotic material aspirated from patients with acute or recent ST-elevation myocardial infarction (MI) were analysed in accordance with the clinical time course. Our findings suggested that the accumulation of primitive cells in the thrombotic material may play a role in the pathogenesis of lesion progression after successful coronary intervention for ACS.

MATERIALS AND METHODS

Patient population

One hundred and eight thrombus samples from 62 patients were successfully obtained and analysed. The subjects were patients hospitalised at the University of Tokyo Hospital with the diagnosis of acute or recent ST-segment elevation MI who underwent percutaneous coronary intervention including thrombectomy. They were diagnosed according to their symptoms accompanied by ST-segment elevation on the ECG and/or a positive result of qualitative analysis of cardiac troponin T. After diagnosis, all patients received intravenous administration of 130 units/kg of unfractionated heparin in addition to oral administration of 200 mg aspirin if they did not have any contraindication.

Procedures and devices

The femoral artery approach with a 7 or 8 French sheath was selected in all patients. After engaging the guiding catheter into the coronary artery, a conventional 0.014-inch guide wire was employed to cross the target lesion. After morphological lesion examination with intravascular ultrasound, a thrombectomy device, such as the Thrombuster.
Acute coronary syndromes

Table 1 Patients’ characteristics

<table>
<thead>
<tr>
<th>Table 1 Patients’ characteristics</th>
<th>18/44</th>
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</thead>
<tbody>
<tr>
<td>Female/male (number)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>68.0±19.3</td>
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<tr>
<td>Body mass index</td>
<td>23.6±3.5</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>49 (79.0)</td>
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<tr>
<td>Diabetes, n (%)</td>
<td>20 (31.5)</td>
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<tr>
<td>Lipid disorder, n (%)</td>
<td>17 (25.1)</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>117.2±75.4</td>
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<td>Low-density lipoprotein cholesterol (mg/dl)</td>
<td>114.2±33.7</td>
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<tr>
<td>Smoking history, n (%)</td>
<td>43 (69.4)</td>
</tr>
<tr>
<td>Diagnosis, n (%)</td>
<td>48 (77.5)</td>
</tr>
<tr>
<td>Target vessel, n (%)</td>
<td>24 (38.7)</td>
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<tr>
<td>Procedures, n (%)</td>
<td>35 (56.5)</td>
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<tr>
<td>Only thrombectomy</td>
<td>3 (4.8)</td>
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<tr>
<td>Mean time between onset and thrombectomy (h)</td>
<td>17.8 (0.5-72)</td>
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<td>Follow-up coronary angiography, n (%)</td>
<td>57 (96.6)</td>
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<tr>
<td>Major adverse cardiac events, n (%)</td>
<td>11 (18.6)</td>
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<tr>
<td>Cardiac death, n (%)</td>
<td>1 (1.7)</td>
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<tr>
<td>Myocardial infarction, n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Target lesion revascularisation, n (%)</td>
<td>10 (16.9)</td>
</tr>
</tbody>
</table>

AMI, acute myocardial infarction; LAD, left anterior descending artery; LCx, left circumflex branch; LMCA, left main coronary artery; POBA, plain old balloon angioplasty; RCA, right coronary artery; RMI, recent myocardial infarction.
†One patient died due to uncontrollable congestive heart failure.
§All implanted stents were bare-metal stents.

RESULTS

Characteristics of patients and procedures

The patients’ characteristics are summarised in table 1. Forty-four of 62 patients were men. The mean age was 68.0±19.3 years; 31.5% of patients had diabetes, 79.0% had hypertension and 74.8% had dyslipidaemia. Of the affected vessels, 41.9% were the left anterior descending artery, 17.7% the left circumflex branch and 40.3% the right coronary artery. In 35 patients (56.5%), stents were directly implanted. All patients were implanted with bare metal stents except three patients who archived good re-flow by thrombectomy alone without any additional intervention. The mean time between the onset of symptoms and thrombectomy in this study was 17.8±18.9 h, which widely ranged from 0.5 to 72 h.

Major adverse cardiac events occurred in 11 of 59 patients. One patient who presented with cardiogenic shock due to left main coronary artery occlusion died of uncontrollable heart failure. Ten patients needed target lesion revascularisation.

Immunohistochemical analysis of thrombotic material

The macroscopic findings of the aspirated thrombotic material are shown in figure 1. These were highly variable in colour, size, configuration and quantity among patients (figure 1A), and even among aspirates from one patient (figure 1B(1)–(3)). H&E staining of thrombotic material demonstrated the structure of thrombi (figure 2). They were generally composed of three parts;
nucleated cell clusters (figure 2A), organised fibrin with infiltration of a few nucleated cells (figure 2B) and clusters of erythrocytes (figure 2C, arrows). The proportion of each component was related to the colour and shape of the sample. There was no significant correlation among the numbers of CD34, HAM56, CD45-positive cells. Immunofluorescent staining (figure 3) revealed that the infiltrated cells were mostly white blood cells expressing CD45 and/or HAM56, a marker for macrophages. Although the thrombotic material included various amounts of lipid detected by Oil-red-O staining (figure 4), the content of triglyceride showed no relationship with the serum triglyceride/cholesterol levels or body mass index.

Figure 5 shows the results of immunofluorescent and immuno-histochemical studies of SMα-actin and platelets (CD42a). The areas positive for each antibody were basically distinctive. CD42a was mainly expressed in organised fibrin, whereas SMα-actin-positive cells were mainly located in the area of clusters of nucleated cells (figure 5A). Notably, the proportion of the CD42a-positive area had a significant negative correlation with the time passed between the onset of symptoms and...
thrombectomy (correlation coefficient: $-0.59, p<0.01$) (figure 5B). The proportion of SMα-actin-positive area tended to have a positive correlation with the time after onset (correlation coefficient: $0.29, p=0.025$) (data not shown).

Moreover, immunostaining with antibodies against smooth muscle cell (SMC) lineage markers demonstrated that a very limited number of nucleated cells expressed highly differentiation markers of SMC, such as smooth muscle myosin heavy chain isoform 1 (SM1), calponin and caldesmon (see supplementary figure 1a, available online only). Conversely, considerable number of cells expressed cytokines that are known as promoting neointima hyperplasia, such as tumour necrosis factor alpha, platelet-derived growth factor B and matrix metalloproteinase 2 (see supplementary figure 1b, available online only).

To characterise further the cellular constituents in thrombi, histological evaluation was performed focussing on primitive stem cells. Among the nucleated cells in the thrombus samples, some cells expressed stem cell markers, such as CD34 and bcrp1/ABCG2, which is known to be an essential cell surface co-transporter of side-population cells$^{10}$ (figure 6A). The number of CD34-positive cells in the blood was compared with that in intracoronary thrombi in five patients. The mean proportion of CD34-expressing cells in 100,000 alive nucleated cells was $0.165\pm0.034\%$ in the blood aspirated from the occluded coronary artery. In comparison, it was significantly greater in thrombus samples from the same patients ($2.67\pm0.687\%, p=0.019$), although different assays for the detection of CD34-positive cells were employed. Regarding the correlation between the proportion of CD34-positive cells and clinical outcomes, the proportion of CD34-positive cells ($2.58\pm0.35\%$, range $0.05$–$8.5\%$) showed a significant correlation with percentage diameter stenosis at follow-up coronary angiography calculated by quantitative coronary analysis (correlation coefficient $0.57, p<0.01$) (figure 6B). On the other hand, no significant correlation was found with the number of CD45 and HAM56-positive cells. Similarly, analysis of the two patient groups ‘restenosis +’ ($\geq60\%$ diameter stenosis at follow-up angiography) and ‘restenosis −’ ($<60\%$) showed that the proportion of CD34-positive cells was significantly greater in the restenosis+ group than in the restenosis− group ($5.10\pm0.66\%$ vs $1.88\pm0.24\%, p<0.01$). No significant difference in the mean CD45 and HAM56-positive ratio was observed between the two groups (figure 6C). Conversely, when the patients were divided into two groups, low CD34 ($<2.5\%, n=34$) group and high CD34 ($\geq2.5\%, n=23$) group, percentage diameter stenosis at follow-up angiography was significantly greater in the high CD34 group, despite there being no significant difference in clinical, procedural and angiographical characteristics. The proportion of CD34 cells showed no relation with pre and post-procedural percentage diameter stenosis (figure 6D).

**DISCUSSION**

Our study demonstrated that: (1) the cellular constituents of thrombotic material in patients with ST-elevation acute MI or recent MI varied according to the time since the onset of
symptoms; (2) thrombotic material included primitive stem cells expressing stem cell markers; (3) the proportion of primitive stem cells was significantly greater in thrombotic material than in peripheral blood; and (4) the number of CD34-positive primitive cells in intracoronary thrombi positively correlated with the degree of lesion progression after coronary intervention, whereas no correlation was found between the number of macrophages/monocytes and lesion prognosis.

It is well known that the presence of intracoronary thrombus increases the risk of long-term restenosis after angioplasty. Moreover, a recent study examining aspirated intracoronary thrombus demonstrated that the prognosis of patients with ST-elevation MI varied in accordance with pathological findings of thrombus. However, the pathophysiological role of the intracoronary thrombi effect on local as well as clinical outcomes remains largely unknown.

There remains a great deal of controversy regarding the origin of cells in vascular remodelling. Although it had been widely believed that intimal SMC are derived from the medial SMC that undergo migration into the intima, on the contrary, recent reports suggest that vascular stem cells resident in the circulating blood or local lesions might give rise to cells that express some markers of SMC in both animal models and humans. In addition, haematopoietic stem cells are known to have paracrine and autocrine properties in conditions promoting intimal cell proliferation and differentiation.

The present study demonstrates primitive stem cells in thrombotic material in patients with ACS. In addition to CD34-positive cells, Bcrp1/ABCG2-positive cells indicating very primitive cells were found (side-population cells). Furthermore, flow cytometric and histological analyses showed that the proportion of CD34-positive cells within thrombi was significantly greater than that in blood drawn from the occluded coronary artery. These findings indicate that primitive cells expressing stem cell markers greatly accumulate into intracoronary thrombi.

Although a recent trial with intracoronary infusion of primitive bone marrow cells for patients with ST-elevation MI showed beneficial effects including the prevention of cardiac remodelling, there remains concern that these cells might promote neointimal proliferation. Bartunek et al reported a high rate of in-stent restenosis or re-occlusion after intracoronary injection of CD133-positive cells in ACS patients. Kang et al reported that more than half of MI patients who were administered granulocyte colony-stimulating factor alone or purified CD34-positive cells mobilised by granulocyte colony-stimulating factor showed restenosis. Moreover, the CD34-positive cell-capture stent trial indeed showed long-term safety and efficacy in patients with stable and unstable angina; however, to date, the efficacy of that CD34-capture stent in MI patients is still unknown. Furthermore, we previously showed a significantly greater elevation in the number of peripheral CD34-positive cells in patients with restenosis at chronic time points after coronary stenting with bare metal stents than in patients without stenosis. Taken together, these data indicate that circulating or local CD34-expressing primitive cells might play a role in neointimal hyperplasia. The results of the present study suggest that the infiltration of a greater number of CD34-positive cells is an indicator of excessive neointima formation. Although the precise roles of these cells remain unclear, it is possible that the primitive cells observed in intracoronary thrombi may play a role in the pathogenesis of restenosis.

Previous reports suggested that enhanced infiltration of macrophages in atherosclerotic lesions was associated with a high incidence of restenosis after angioplasty. In contrast, no significant correlation was observed between the number of macrophages/monocytes and lesion progression in this study. This discrepancy apparently results from the difference in the lesions analysed. We
could count the macrophages only within the aspirated intracoronary thrombi, whereas most of the previous studies performed immunohistochemical analysis on the cross-sections of the coronary arteries obtained during atherectomy. Moreover, recent evidence suggests that macrophages/monocytes consist of heterogeneous subpopulations with different immunological functions.26 The subpopulation of macrophages in thrombotic materials might differ from that in atherosclerotic plaques. Therefore, this study might at least partly indicate that in patients with MI, immunohistological analysis of intracoronary

Figure 5 Characterisation of cells observed in thrombotic material. (A) Immunostaining for CD42a (green) and \(\alpha\)-smooth muscle actin (SM\(\alpha\)-actin, red). Immunostaining with anti-CD42a and SM\(\alpha\)-actin antibody shows that the expression of these two molecules was generally distinct. CD42a was mainly expressed in the area of organised fibrin as determined by H&E staining. On the other hand, a part of the nucleated cells expressed SM\(\alpha\)-actin in the area of cell infiltration. The co-expression of SM\(\alpha\)-actin and CD42a was seldom observed. (B) Negative correlation between time passed since onset to thrombectomy and the proportion of the CD42a-positive area. There is a significant negative correlation between the time since onset and the proportion of the CD42a-positive area. The longer the time passed after the onset until thrombectomy, the fewer platelets and fibrin were observed.
thrombi focussing on primitive cells might be an attractive tool for the prediction of lesion prognosis.

**POTENTIAL STUDY LIMITATIONS**

As thrombectomy devices always indiscriminately aspirate intracoronary material, the possibility cannot be excluded that the material aspirated through these devices includes other components, such as ruptured vessel wall, the content in lipid cores and thrombi that are thought to be present for an extended time. In that respect, studies examining material aspirated through thrombectomy devices may be more imprecise than autopsy-based studies for examining ‘intracoronary thrombus’ itself. Moreover, as aspirated samples were highly variable even within the same patient, potential sampling error could not be excluded. However, thrombectomy devices enable us to examine samples by real-time analysis. Therefore, this method could be considered suitable for evaluating events that change dramatically with the time course, such as evolving thrombi with the recruitment of cells with primitive cell phenotypes. Larger scale clinical studies as well as detailed and quantitative evaluation of expressions of inflammatory cytokines/growth factors are required to confirm the precise role of primitive cells observed in coronary thrombi in lesion progression after successful intervention in patients with ACS.

**Figure 6**  Primitive cells observed in thrombotic material. (A) Immunofluorescent images of thrombotic material stained for markers of primitive cells. In the clusters of nucleated cells, there was a cell that was positive for both CD34 and Bcrp1/ABCG2. (B,C) Positive correlation between the CD34-positive ratio in thrombotic material and lesion progression (restenosis) after coronary intervention. As shown in the upper panel, the number of CD34-positive cells varied among samples. A significant positive correlation was observed between the proportion of CD34-positive cells and the percentage diameter stenosis calculated by quantitative coronary analysis at follow-up coronary angiography (correlation coefficient 0.57, p<0.01). (D) The proportion of CD34-positive cells was significantly greater in patients with restenosis (percentage diameter stenosis (%DS) ≥60) than in patients without restenosis. There was no significant difference between the two groups in the proportion of CD45 or HAM-positive cells. (D,E) Patients were divided into a ‘low CD34 group’ (<2.5%) and ‘high CD34 group’ (≥2.5%) according to the proportion of CD34-positive cells. Percentage diameter stenosis was compared between the groups before intervention (pre %DS), after intervention (after %DS), and at follow-up coronary angiography (follow-up %DS). Percentage diameter stenosis at follow-up angiography was significantly greater in the high CD34 group than in the low CD34 group (45.3±7.4 vs 16.0±2.2%).


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**Competing interests** None.

**Ethics approval** This study was conducted with the approval of the ethics committee of the University of Tokyo.

**Provenance and peer review** Not commissioned; externally peer reviewed

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