Clinical Studies

Eosinophils May Be Involved in Thrombus Growth in Acute Coronary Syndrome

Histologic Examination of Aspiration Samples

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Summary

Thrombus aspiration therapy allows for the examination of thrombus and atheroma fragments in acute coronary syndrome (ACS). Inflammatory cells and platelet activation play key roles in thrombus formation in ACS. However, histopathologic evaluation of thrombi in ACS has not been adequately addressed. We performed histologic analysis of tissue samples obtained by thrombus aspiration therapy. We studied 165 samples from patients with ACS. The area of each sample, percentage of red thrombus, and percentage of white thrombus were measured. Samples were stained immunohistochemically with antibodies against macrophages, activated platelets, and interleukin (IL)-5. Seventy-six samples included atheroma fragments. Macrophages, neutrophils, and activated platelets were observed in thrombi and in atheroma fragments. Eosinophil infiltration was also observed predominantly in the area between white thrombus and red thrombus in 106 samples. We categorized all samples into 3 groups according to the grade of eosinophil infiltration (eos-, eos+, eos++ group). Sample area in the eos++ group was greater than that in the eos- group (P < 0.0001). In addition, the percentage of the red thrombus areas in the eos++ group and the eos+ group was greater than that in the eos- group (P < 0.009, P < 0.02, respectively). However, there was no difference in the percentage of white thrombus area between the 3 groups. Staining for IL-5 was identified in inflammatory cells within thrombi. Eosinophils may play an important role in coronary occlusion by promoting thrombus growth. (Int Heart J 2009; 50: 267-277)

Key words: Acute coronary syndrome, Thrombus, Histology, Inflammatory cells, Eosinophils

PLAQUE disruption (ulceration, fissure, erosion) is a key factor in local arterial thrombogenesis.1-3) Platelet activation after plaque disruption, together with

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entrapment of circulating blood cells by the fibrin network, is considered the basic disease process of acute arterial thrombosis, such as acute coronary syndrome (ACS).\(^{3-5}\) Macrophages (monocytes) and neutrophils are reported to play important roles not only in plaque vulnerability but also in thrombus formation and growth in cases of acute arterial thrombosis.\(^{3,6}\) In addition, granule proteins of eosinophils may also promote thrombus formation and growth.\(^{7-9}\)

The recent development of thrombus aspiration therapy has allowed for the investigation of thrombi and atheroma fragments. However, histologic evaluation of inflammatory cells involved in ACS has not been sufficiently thorough. In the present study, we investigated inflammatory cells in thrombi in ACS histologically and immunohistochemically, with a particular focus on the presence of eosinophils.

**METHODS**

**Subjects:** A total of 209 consecutive patients with ACS underwent thrombus aspiration during emergency coronary angiography at Showa University Hospital between June 2001 and September 2005. Tissue samples obtained from 165 patients were used for the present study. Acute myocardial infarction was diagnosed in 157 of 165 patients, and unstable angina of Braunwald class IIIB (rest angina within 48 hours and no creatinine phosphokinase-MB elevation) was diagnosed in the remaining 8. The patient population consisted of 135 men and 30 women. Age ranged from 31 to 94 years (mean, 65.5 ± 12.1 years). All patients received emergency coronary angiography within 12 hours from the onset

| Table. Clinical Characteristics of Patients Who Underwent Thrombus Aspiration Therapy |
|----------------------------------|------------|------------|------------|-----------|
| Patients, n                       | eos- 59   | eos+ 54    | eos++ 52   | P 0.52\(^p\) |
| Male, n                           | 49        | 46         | 40         | 0.53\(^p\) |
| Age, years                        | 64.7 ± 11.8 | 64.9 ± 10.8 | 67.1 ± 13.6 | 0.70\(^p\) |
| ST-segment-elevation MI, n 55     | 52         | 49         | 0.31\(^p\) |
| Unstable angina, n 4              | 2          | 2          | 0.34\(^p\) |
| CK max, IU/L 3187 ± 3017          | 3616 ± 3839| 3289 ± 2343| 0.012\(^p\) |
| Allergic disease, n 0             | 2          | 1          | 0.041\(^p\) |
| Coronary location, n              | RCA 21     | 24         | 1.0 ± 1.2  | 0.10\(^p\) |
|                                 | LMT 1      | 1          | 1.2 ± 1.8  | 0.0046*   |
|                                 | LAD 31     | 22         | 2.4 ± 2.2  | \(\chi^2\) analysis |
|                                 | LCX 6      | 7          | 3          | 0.041\(^p\) |

MI indicates myocardial infarction; CK, creatinine phosphokinase; RCA, right coronary artery; LMT, left main trunk; LAD, left anterior descending artery; LCX, left circumflex artery; WBC, white blood cells; *, One-factor ANOVA and \(^p\), \(\chi^2\) analysis.
of chest pain and/or oppression. The clinical characteristics of the patients are presented in the Table.

**Thrombus aspiration therapy:** Thrombus aspiration therapy was performed in all patients before any interventional devices other than guidewires and thrombectomy devices were used. All patients were administered heparin by injection (10,000 IU), and none received thrombolysis therapy or glycoprotein IIb/IIIa inhibitors (these drugs are not approved in Japan). Thrombus aspiration therapy was carried out with the RESCUE thrombectomy catheter (Boston Scientific Corp.) in 34 patients, with the PercuSurge GuardWire Plus Temporary Occlusion and Aspiration System (Medtronic AVE) in 43 patients, and with the ThromBuster catheter (Kaneka Medics) in 88 patients.

**Histologic and immunohistochemical analyses:** Tissues were placed into 10% buffered formalin immediately after aspiration. After being embedded in paraffin, the samples were cut in order to maximize the cross-sectional area along the long axis. Sections were stained with hematoxylin and eosin and phosphotungstic acid hematoxylin (PTAH) stains. Some sections were observed by electron microscopy.

For immunohistochemical analysis, the following primary antibodies were used: mouse anti-human macrophage monoclonal antibody, 1:100 dilution (CD68; No. M0814; DakoCytomation A/S); rabbit anti-human p-selectin monoclonal antibody, 1:250 dilution (No. M7199; DakoCytomation A/S); rabbit anti-human myeloperoxidase (MPO) polyclonal antibody, 1:3000 dilution (No. A0398; DakoCytomation A/S); and rabbit anti-human interleukin 5 (IL-5) polyclonal antibody, 1:100 dilution (No. 7885; Santa Cruz Biotechnology). Sections were incubated with primary antibody for 1 hour at room temperature after being autoclaved (15 psi, 121°C, 20 minutes) for antigen retrieval. A dextran-based method (EnVision system; DakoCytomation A/S) was used to detect antigens. Horseradish peroxidase activity was visualized with 3,3’-diaminobenzidine tetrahydrochloride, and hematoxylin was used for nuclear staining. As a negative control, 1 section from each case was incubated with purified mouse or rabbit IgG instead of primary antibody.

**Quantitative analysis:** The area of each sample was calculated with WinROOF software (Mitani Corp.). Sample area, percentage of red thrombus (red blood cells and fibrin) area, and percentage of white thrombus (platelets and fibrin) area were calculated. One-way ANOVA was performed for statistical analysis.

**Results**

Samples were obtained from 78 right coronary arteries, 3 left main trunks, 68 left anterior descending arteries, and 16 left circumflex arteries.
cally, 158 samples (95.8%) were of mixed thrombus (white thrombus and red thrombus). White thrombus was composed predominantly of platelets and fibrin, and red thrombus was composed predominantly of red blood cells and fibrin. Atheroma fragments were observed in 76 samples (48.1%); white thrombus was observed adjacent to the atheromatous tissue, and red thrombus was observed adjacent to white thrombus (Figure 1A, B).

**Inflammatory cells within atheroma fragments:** Histologic and immunohistochemical features of atheroma fragments are shown in Figure 1C and D. Not only mononuclear cells but also neutrophils were observed in atheroma fragments.

**Eosinophils within thrombus tissue:** Numerous neutrophils were observed in all 165 thrombus samples. Eosinophils were observed in 106 of the 165 samples (64.2%) (Figure 2); eosinophils were not identified in atheroma fragments. Hematoxylin and eosin staining revealed eosinophilic granules. Eosinophils were

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**Figure 1.** Low-magnification microscopic view of coronary thrombus (A and B). Asterisks indicate atheroma fragments. White thrombus is stained pink, and red thrombus is stained red or dark pink by hematoxylin and eosin staining. Red thrombus is stained blue by PTAH staining. Micrographs of atheroma fragments obtained by thrombus aspiration (C and D). C: Neutrophils (arrowheads) are observed among the inflammatory cells. D: Macrophages, including foam cells, are CD68-positive. (A and B, bars = 1 mm; C, bar = 20 μm; D, bar = 50 μm).
Figure 2. Micrographs, electron micrographs and location of eosinophils within thrombi. A and B, Eosinophils (arrowheads) are observed among other inflammatory cells. C and D, Electron micrographs of eosinophils. Eosinophilic granules were larger than those in neutrophils. E-G, Serial sections of the same sample. Activated platelets are observed at the originating edge of the thrombus (E: p-selectin immunostain). Fibrin net is observed adjacent to platelets in blue (F: PTAH stain). Inflammatory cells are observed predominantly in the fibrin network near the boundary between white and red thrombus (G: hematoxylin and eosin stain). H: High-magnification image of the area in G: Eosinophils (arrowheads) are observed among inflammatory cells (hematoxylin and eosin stain). (A and B, bars = 10 μm; C, bar = 1 μm; D, bar = 2 μm; E-G, bars = 100 μm; H, bar = 20 μm).
identified among neutrophils and erythrocytes within fibrin nets (Figure 2A, B). We also performed electron microscopy (Figure 2C, D). Bilobed nuclei and crystalline bodies within large granules, which are typical for eosinophils, were observed.

The location of eosinophils within thrombi is shown in Figure 2E-H. Immunostaining for p-selectin revealed activated platelets at the originating edge of the thrombus (Figure 2E). Blue PTAH staining revealed fibrin nets adjacent to platelets (Figure 2F). Inflammatory cells, including eosonophils, were observed predominantly within the fibrin network near the boundary between white and red thrombus (Figure 2G, H).

We then performed immunohistochemistry for IL-5, which induces eosinophil migration and proliferation. Mirror sections of thrombus are shown in Figure 3A, B. Eosinophils were observed adjacent to platelets and within fibrin networks among other white blood cells (Figure 3A). IL-5 staining in the cytoplasm of inflammatory cells was observed in the same region (Figure 3B). MPO and CD68 immunostaining are shown in mirror sections (Figure 3C, D). Most neutrophils were positive for MPO staining, and some CD68-positive cells (macrophages) were observed.

Figure 3. Immunostaining for interleukin-5 (IL-5), myeloperoxidase (MPO), and CD68 in mirror sections of thrombotic tissue (A and B, C and D). A: Eosinophils are observed adjacent to platelets and within fibrin networks with neutrophils and mononuclear cells (arrowheads). B: IL-5 immunostaining is observed in the cytoplasm of inflammatory cells in the same region. C: Most neutrophils are MPO-positive. D: CD68-positive cells (macrophages) are observed in the same region (A-D, bars=25 μm).
Relation between eosinophils and aspirated thrombus size: We categorized all samples into 3 groups according to the grade of eosinophil infiltration: the eos++ group comprised samples with > 5 eosinophils per high power field (hpf; × 400) (n = 52), the eos+ group comprised samples with 2-4 eosinophils per hpf (n = 54), and the eos- group comprised samples with 0-1 eosinophils per hpf (n = 59) from more than 5 hpf near the boundary between white and red thrombus. Patient characteristics in the 3 groups are shown in the Table. The percentage of eosinophils in the peripheral blood was increased in the following order: eos-, eos+, eos++ groups. By quantitative analysis, area of sample in the eos++ group was greater than that in the eos- group. In addition, the percentage of red thrombus areas in the eos++ group and the eos+ group was greater than that in the eos- group. However, there was no difference in the percentage of white thrombus area between the 3 groups (one-way ANOVA).

![Figure 4. Results of area measurements among eos-, eos+, and eos++ groups. Sample area was significantly greater in the eos+ and eos++ groups than in the eos- group. The percentage of red thrombus area was significantly less in the eos- group than in the eos+ and eos++ groups. However, the percentage of white thrombus area did not differ between the 3 groups (one-way ANOVA).](image)

With respect to location of the culprit lesion, thrombi obtained from the right coronary artery showed a greater eos++ value than those obtained from the left anterior descending coronary artery. Moreover, the size of sample area was greater in the right coronary artery than left anterior descending coronary artery and left circumflex artery (11.93 ± 11.51 mm², 5.35 ± 3.68 mm², 7.00 ± 3.70 mm², respectively; P < 0.01).

In the present study, thrombus size and the number of eosinophils in the thrombus were not related to the age of the patients (data not shown).
DISCUSSION

The histopathologic findings of the present study suggest that eosinophils play a role in the growth of coronary thrombi.

Although various factors are involved in the formation of arterial thrombi, the most fundamental is a change in the blood vessels. In particular, formation of arterial thrombi involves plaque disruption, and contact between blood and subendothelial tissue caused by separation of endothelial cells may be an essential factor.\(^1,^3\) Thrombus formation begins when platelets adhere to damaged vascular walls, but it is thought that platelet thrombi (white thrombi) alone generally are not large enough for ACS to develop. Some studies suggest that the culprit lesions of ACS do not involve severe coronary stenosis.\(^^{10-12}\)

Thrombus growth might be important for the onset of ACS. Tissue factor, plasminogen activator inhibitor-I, and fibrinogen are considered to play a pivotal role in thrombus growth.\(^3,^4,^{13-16}\) In addition, major basic protein or eosinophilic cationic protein, which is an eosinophil granule protein, activates platelets and promotes thrombus formation by inhibiting the function of thrombomodulin in hypereosinophilic syndrome or allergic diseases.\(^8,^9\) Rohrbach et al\(^7\) reported that major basic protein and eosinophil peroxidase activate platelets. In the present study, eosinophils were most frequently observed near the boundary of white thrombus and red thrombus, which develops after white thrombus. Furthermore, thrombi were significantly larger in patients who showed greater eosinophil infiltration. However, the area of white thrombus consisting mainly of platelets exhibited no significant differences among the three eosinophil grades. The difference in size was considered to be due to the size of the red thrombus. Taking these findings together, we speculate the role of eosinophils in ACS is predominantly in the growth of red thrombus by forming fibrin nets rather than in the platelet aggregation. The relation between granule proteins released from eosinophils and thrombi should be studied further, and the role of eosinophils should be clarified.

In the present study, thrombus size was greater in the right coronary arteries than in the left coronary arteries. Due to differences in flow velocity, coronary diameter and branch morphology, right coronary thrombi tend to be greater than left coronary thrombi.\(^^{17-19}\) Moreover, our data shows the number of eosinophils in a thrombus was greater in patients with a right coronary thrombus. It seems to be reasonable that a large right coronary thrombus includes a large number of eosinophils. However, it is difficult to explain the differences in eosinophil counts between right and left coronary arteries in thrombus. Further investigation is needed to elucidate these differences. Our data also indicate a large thrombus has greater eosinophil counts both in thrombi and peripheral
blood. Thrombus growth might be facilitated in patients with higher eosinophil counts in peripheral blood.

In mixed thrombus in ACS, numerous neutrophils and monocytes are observed. Neutrophil infiltration was also found within arterial sclerotic lesions with macrophages in vascular wall in ACS. In a thrombus, chemotaxis of neutrophils and macrophages may be induced by various chemotaxins released by activated platelets, but eosinophil chemotaxis may also be induced. Recent studies reported that eosinophils adhere to P-selectin, which is expressed on activated platelets, more easily than neutrophils, and eosinophils express more P-selectin ligand than neutrophils. Further, IL-5, which promotes eosinophil chemotaxis and chemotaxin release, was observed in localized inflammatory cells near the boundary between white thrombus and red thrombus, as shown by immunostaining. It is certain that neutrophils play an important role in thrombus growth, but eosinophils may play an important role as well.

Another role of eosinophils may be in relation to coronary spasms. Spastic coronary arteries may be susceptible to thrombus formation. Some clinical studies have shown that Japanese ACS patients experience a higher incidence of coronary spasms than do Caucasians. Although this racial difference may relate to differences in endothelial nitric oxide synthesis or autonomic nervous system activity, the reason for this is not clear. Recent report suggests that coronary spasticity is related to airway hyper-responsiveness. Furthermore, Umemoto, et al reported that peripheral eosinophil counts were significantly greater in patients with severe coronary spasms than in patients with no coronary spasms. In the current study, the number of eosinophils in peripheral blood was increased in the order of eos-, eos+, and eos++ groups. Erdogan, et al reported a significant increase in the number of eosinophils in peripheral blood in patients with unstable angina pectoris compared to that in control subjects. In the present study, we did not perform provocation tests for coronary spasms in ACS patients at disease onset. However, we believe that eosinophils may be involved in ACS by inducing coronary spasms, particularly in Japanese patients.

The present study was performed with thrombi obtained by thrombus aspiration therapy; that is, entire thrombi were not obtained and thrombi can change shape while passing through a catheter. This is a limitation of this study. However, the involvement of eosinophils in thrombus growth is indicated by the many cases in which infiltration of eosinophils in the thrombus was observed and by the relation between the number of eosinophils in a thrombus and thrombus size. Further clarification of the role of eosinophils in thrombus formation may lead to the development of more efficacious or preventative treatments for ACS.
References

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