Neutrophil Infiltration of Culprit Lesions in Acute Coronary Syndromes

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Background—Neutrophils in unstable atherosclerotic lesions have not received much consideration, despite accumulating evidence suggesting a link between systemic inflammation and acute coronary syndromes.

Methods and Results—Coronary artery segments were obtained at autopsy from 13 patients with acute myocardial infarction (AMI); 8 had a ruptured and 5 an eroded plaque. Patients (n=45) who had died of noncardiovascular diseases served as reference. Atherectomy specimens were obtained from 35 patients with stable angina pectoris (SAP) and from 32 patients with unstable angina pectoris (UAP). Antibodies against CD66b, elastase, myeloperoxidase, and CD11b identified neutrophils; CD10 identified neutral endopeptidase (NEP). CD66b-positive and NEP-positive neutrophils were counted and expressed as a number per square millimeter of tissue. All specimens with plaque rupture or erosion showed distinct neutrophil infiltration; the number did not differ between ruptured and eroded plaques. However, the number of NEP-positive neutrophils was significantly higher (P<0.0001) in ruptured plaques than in eroded plaques. UAP patients showed neutrophils in 14 of 32 culprit lesions; in SAP only 2 of 35 lesions contained neutrophils. The number of neutrophils and NEP-positive cells in patients with UAP was significantly higher (neutrophils, P<0.0005; NEP-positive cells, P<0.005) than in patients with SAP.

Conclusions—The observations suggest that neutrophil infiltration is actively associated with acute coronary events. The high number of NEP-positive neutrophils in ruptured plaques, compared with eroded plaques, may reflect differences in the underlying pathophysiological mechanisms.

Key Words: myocardial infarction ■ angina ■ inflammation ■ atherosclerosis

Plaque rupture or erosion with mural thrombus formation is considered to represent the most important morphological changes that underlie the transformation of stable coronary lesions into clinically unstable lesions, causing unstable angina pectoris (UAP) or acute myocardial infarction (AMI). The pathomorphological substrate underlying such complicated lesions is heterogeneous with respect to plaque architecture and cellular composition, but the presence of a localized intraplaque inflammatory process is a common denominator.

Presently, a growing body of literature suggests a link between systemic inflammation and acute coronary syndromes. It is of note, therefore, that the presence of neutrophils in unstable lesions has not received much consideration, despite the fact that these cells have been identified at rupture sites and, in general, are among the first phagocytic cells in acute inflammatory responses to tissue injury. Epidemiological studies, moreover, have shown that leukocyte counts in peripheral blood correlated positively with coronary atherosclerotic risk and risk of AMI; the strongest epidemiological association is with neutrophil counts. Indeed, clinical studies have demonstrated that neutrophils are activated in patients with UAP and AMI, and accumulation of neutrophils, with adherence of fibrin-platelet thrombus, occurs at the site of endothelial cell denudation almost instantly after coronary artery bypass grafting.

The major function of neutrophils at sites of tissue injury is complex but can be summarized by stating that they may endocytose foreign material or secrete enzymes, such as elastase and myeloperoxidase. Neutrophils also contain neutral endopeptidase 24.11 (NEP), a membrane protein known to modulate inflammatory responses. NEP can be detected on mature (segmented) neutrophils only. Immature neutrophils are NEP-negative but have a greater chemotactic re-
spontaneous response to activated complement than NEP-positive mature neutrophils.\textsuperscript{9,10} Thus, the presence or absence of NEP on neutrophils may provide some insight into their functional capacity.

The present study has focused on the question of whether there could be a role for neutrophils in the culprit lesions of acute coronary syndromes. The study is based on frozen tissue samples, because monoclonal antibodies against neutrophil markers, as well as the antibody against NEP, work well on frozen sections only.

**Methods**

**Coronary Tissue Specimens**

This study is based on 3 different groups of specimens; the first and second groups of specimens were obtained at autopsy, and the third group of specimens was obtained by atherectomy (Table 1).

**Autopsy**

Coronary artery segments (n=126) were obtained at autopsy from 58 patients; 13 had died of AMI and 45 had died of noncardiovascular diseases. The ages of the patients ranged from 21 to 77 years (noncardiovascular diseases, 61±11; AMI, 51±19; mean±SD). Of those with noncardiovascular diseases, 84% were men; for AMI, 77% were men. The following risk factors were evaluated: cigarette smoking, hypertension as defined by the Joint National Committee V,\textsuperscript{11} diabetes mellitus as defined by the WHO Study Group,\textsuperscript{12} and hypercholesterolemia (cholesterol level >220 mg/dL). Their distribution among patients with noncardiovascular diseases versus those with AMI was as follows: cigarette smoking (27% versus 62%), hypertension (18% versus 46%), diabetes mellitus (9% versus 39%), and hypercholesterolemia (27% versus 38%).

Of the patients who had died of noncardiovascular diseases, 113 segments were obtained. Twenty-six of these contained normal coronary artery with diffuse intimal thickening (AHA classification type I).\textsuperscript{13,14} The other 87 segments contained atherosclerotic lesions and were characterized histologically either as atherosclerotic lesions with hypercellularity (n=26) or as advanced atherosclerotic lesions (n=61), according to our classification described previously.\textsuperscript{13,14} The hypercellular lesion was defined as a cell-rich intimal lesion, predominantly composed of smooth muscle cells (SMCs) and occasional macrophages, but without an extracellular lipid core (“hypercellular lesions” are not recognized by the AHA classification).\textsuperscript{13,14} The advanced atherosclerotic lesions were additionally divided into fibrolipid (type Va; n=31) and fibrous (type Vc; n=30).

**Atherectomy**

The atherectomy specimens were obtained from 67 patients, all of whom underwent atherectomy of the target lesion considered responsible for either stable angina pectoris (SAP) (n=35) or UAP (n=32). The demographic data (SAP versus UAP) were as follows: age (58±11 versus 59±10), male sex (83% versus 81%), cigarette smoking (67% versus 69%), hypertension (45% versus 45%), diabetes mellitus (26% versus 28%), and hypercholesterolemia (58% versus 45%). There were no statistically significant differences between patients with SAP or UAP. Immediately after atherectomy, the tissue specimens were carefully orientated along their longest axis, snap-frozen, and stored at −80°C.

**Immunohistochemistry**

**Single Staining**

The sources and specificity of all antibodies used in this study are summarized in Table 2. Five different antibodies were used to

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**Table 1. Human Coronary Specimens Used in the Study**

<table>
<thead>
<tr>
<th>Origin and Histological Classification of Lesions</th>
<th>AHA Classification</th>
<th>No. of Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autopsy</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Specimens obtained from patients with noncardiovascular diseases</td>
<td>...</td>
<td>113</td>
</tr>
<tr>
<td>Diffuse intimal thickening</td>
<td>Type I</td>
<td>26</td>
</tr>
<tr>
<td>Hypercellular lesion</td>
<td>...</td>
<td>26</td>
</tr>
<tr>
<td>Fibrolipid plaque</td>
<td>Type Va</td>
<td>31</td>
</tr>
<tr>
<td>Fibrous plaque</td>
<td>Type Vc</td>
<td>30</td>
</tr>
<tr>
<td>Specimens obtained from patients with AMI</td>
<td>...</td>
<td>13</td>
</tr>
<tr>
<td>Ruptured plaque</td>
<td>Type VI</td>
<td>8</td>
</tr>
<tr>
<td>Eroded plaque</td>
<td>Type VI</td>
<td>5</td>
</tr>
<tr>
<td>Atherectomy</td>
<td>...</td>
<td>...</td>
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<tr>
<td>SAP (n=35)</td>
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<td>35</td>
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<td>UAP (n=32)</td>
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<td>32</td>
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AHA Classification indicates the American Heart Association histological criteria.\textsuperscript{13,14}
identify neutrophils: anti-CD66b, elastase, myeloperoxidase (monoclonal [MPO-7] and polyclonal [MPO]), and CD11b. NEP was identified by using anti-CALLA (CD10). Nonimmune mouse IgG serum (DAKO, Glostrup, Denmark) served as negative control; human kidney obtained at autopsy was used as positive control.

Sections were incubated at 4°C overnight and then subjected to a 3-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.

Double Immunostaining

The simultaneous identification of SMCs and macrophages was performed on the basis of 2 primary antibodies of a different IgG subclass (1A4/CD68), as reported previously. The enzymatic activity of β-galactosidase for 1A4 was visualized in turquoise (BioGenex Kit, BioGenex) and that of alkaline phosphatase for NEP SS2/36 MAb (IgG1) was visualized in red (New Fuchsin Kit, DAKO). We also performed double immunostainings between macrophages (CD68) and each of the 5 neutrophil markers (CD66b, elastase, myeloperoxidase MPO-7, myeloperoxidase MPO, and CD11b), as well as between CD66b and each of the remaining 4 neutrophil markers, using modifications of procedures reported previously. In both double immunostainings, alkaline phosphatase was visualized with fast blue BB and peroxidase with 3-amino-9-ethylcarbazole development.

To identify neutrophils that express NEP, double immunostainings (NEP/CD66b; NEP/elastase) were performed, according to procedural modifications previously reported. Again, alkaline phosphatase was visualized with fast blue BB (blue: CD66b and elastase) and peroxidase with 3-amino-9-ethylcarbazole development (red: NEP).

Quantitative Methods

Numbers of CD66b-positive neutrophils and NEP-positive cells were counted in the entire tissue sections and expressed as the number of cells per square millimeter of intimal tissue. In atherectomy specimens, neutrophils or NEP-positive cells within thrombi or tissue-attached blood clots were excluded. The tissue area occupied by immunostained macrophages was quantified, using computer-aided planimetry and expressed as a percentage of the total surface area of the tissue section. The morphometric analysis was performed by a single investigator who was blinded to the patients’ characteristics and histological classifications. Data are shown as mean±SD. The 2 groups were compared with an unpaired Student’s t test or with Mann-Whitney U test when the variance was heterogeneous. Statistical comparisons between >3 groups were performed with one-way analysis of variance and post-hoc multiple comparison using Schef- fe’s test. Values of P<0.05 were considered significant.

Results

Neutrophil Identification

The staining pattern of the 5 antibodies (CD66b, elastase, myeloperoxidase MPO-7, myeloperoxidase MPO, and CD11b) used to identify neutrophils in frozen sections differed slightly. Double immunostaining analysis revealed that CD66b positivity was detected in neutrophils but not in macrophages. In contrast, CD11b positivity was found in neutrophils but occasionally also in macrophages. The double immunostainings for CD66b/elastase, CD66b/MPO-7, CD66b/MPO, macrophages/elastase, macrophages/MPO-7, and macrophages/MPO demonstrated that most elastase-positive, MPO-7-positive, and MPO-positive cells within the plaques are neutrophils (Figures 1 and 2).

Autopsy Specimens

Specimens Obtained From Patients With Noncardiovascular Diseases

Normal coronary arteries with diffuse intimal thickening contained no macrophages. In hypercellular lesions, 13 of the 26 lesions were composed almost solely of SMCs, whereas the remaining 13 lesions contained foci of macrophages. In advanced fibrous plaques, 12 of the 30 lesions had a small number of macrophages. However, in advanced fibrolipid plaques, all lesions contained macrophages, albeit to various degrees: only 2 of the 31 lesions contained some neutrophils at the shoulder region. Lesions that contained macrophages but no neutrophils (CD66b and elastase-negative) did not stain positive for MPO-7 or MPO.

NEP expression was not detected in normal coronary arteries with diffuse intimal thickening, hypercellular lesions, and advanced fibrous plaques. In the 2 fibrolipid plaques with neutrophil infiltration, the neutrophils were negative for NEP in one plaque, whereas in the other plaque, occasional neutrophils were positive for NEP.

Specimens Obtained From AMI Patients

Macrophages were abundantly present in both ruptured and eroded plaques (Figures 1A, 1B, 2A, and 2B). Distinct neutrophil infiltration was detected in all specimens with
plaque rupture or erosion (Figures 1C through 1E and 2C through 2E). Double immunostaining (MPO-7/CD66b) demonstrated that most MPO-7–positive cells were neutrophils (Figures 1F and 2F). Double immunostainings for MPO-7 (or MPO) and macrophages revealed that only occasional macrophages were positive (Figures 1G, 1H, 2G, and 2H).

In ruptured plaques, neutrophils were positive for NEP (Figures 3A through 3C), whereas in eroded plaques, most neutrophils lacked NEP positivity (Figures 3D through 3F).

Morphometric Analysis
Morphometric results are shown in Figure 4. The macrophage-positive area was significantly higher ($P<0.0001$) in ruptured and eroded plaques than in normal coronary arteries with diffuse intimal thickening, hypercellular lesions, and advanced fibrous plaques. The number of CD66b-positive neutrophils did not differ between ruptured and eroded plaques; the number of neutrophils in the culprit lesion was not significantly different between AMI patients with PTCA and those without PTCA. The number of NEP-positive cells was significantly higher ($P<0.0001$) in ruptured plaques than in eroded plaques.
Atherectomy Specimens

All 32 lesions obtained from patients with UAP contained abundant macrophages, and 14 (44%) contained neutrophils (Figure 5). Double immunostaining for NEP and neutrophils showed that both NEP-positive cells are neutrophils. D, Section adjacent to Figure 2B, stained with anti-neutrophil CD66b antibody, reveals numerous neutrophils. E, The adjacent section stained with anti-NEP antibody reveals that most neutrophils lack NEP positivity. F, Double immunostaining (neutrophil CD66b, blue; NEP, red) shows that all cells stain blue, indicating that these neutrophils are negative for NEP. Original magnification: A and B, ×110; C, ×444; D and E, ×110; F, ×444.

Discussion

Intraplaque inflammation is presently widely acknowledged to play a crucial role in the changing morphologies of atherosclerotic plaques, with most interest focusing on macrophages and T lymphocytes. However, given the growing notion that systemic inflammation could be involved also, the question arises to what extent local neutrophil infiltration could be involved. Previous studies, using conventional formalin-fixed sections, documented occasional neutrophils within plaques, but their functional significance was not additionally elaborated.2 To the best of our knowledge, the present study, based on frozen sections and using immunohistochemical single and double staining techniques, is the first study systematically analyzing neutrophils in culprit lesions of acute coronary syndromes.

All culprit lesions of patients who had died of AMI had neutrophils within the plaques, although the number varied widely. In contrast, neutrophils were extremely rare in coronary lesions obtained from patients who had died of noncardiovascular diseases; in only 2 of the 87 atherosclerotic lesions neutrophils were identified. Similar observations were made in patients in whom atherectomy material was studied. In patients with UAP, neutrophils within the culprit lesion were detected in 14 of 32 (44%), whereas this was the case in only 2 of 35 (6%) patients with SAP (Figure 6).

![Figure 3](image1.png)

Figure 3. Serial sections taken immediately adjacent to the site shown in Figure 1 (A through C; ruptured plaque) and Figure 2 (D through F; eroded plaque). A, Section adjacent to Figure 1B, stained with anti-neutrophil CD66b antibody, reveals a large number of neutrophils. B, The adjacent section stained with anti-NEP antibody reveals the presence of abundant NEP-positive cells. C, Double immunostaining (neutrophil CD66b, blue; NEP, red) reveals that almost all cells show double staining (purple), indicating that the NEP-positive cells are neutrophils. D, Section adjacent to Figure 2B, stained with anti-neutrophil CD66b antibody, reveals numerous neutrophils. E, The adjacent section stained with anti-NEP antibody reveals that most neutrophils lack NEP positivity. F, Double immunostaining (neutrophil CD66b, blue; NEP, red) shows that all cells stain blue, indicating that these neutrophils are negative for NEP. Original magnification: A and B, ×110; C, ×444; D and E, ×110; F, ×444.

![Figure 4](image2.png)

Figure 4. Graphs showing the macrophage-positive area, expressed as a percentage of the total surface area, the number of neutrophils, and the number of NEP-positive cells, both expressed as absolute numbers per square millimeter of intimal tissue, in diffuse intimal thickening (DIT, n=26), hypercellular lesions (HC, n=26), fibrous plaques (FP, n=30), fibrolipid plaques (FLP, n=31), ruptured plaques (RP, n=8), and eroded plaques (EP, n=5).

![Figure 5](image3.png)

Figure 5. Micrographs of an atherectomy specimen obtained from a culprit lesion in a patient with UAP. A, Fragment of atherosclerotic plaque tissue. Double immunostaining (SMC, turquoise; macrophage, red) reveals areas containing abundant macrophages. The area indicated by the asterisk is shown in higher magnification in B. B, The anti-neutrophil CD66b antibody shows a large number of neutrophils. Original magnification: A, ×96; B, ×233.
These findings contrast markedly with those recently reported by Sugiyama et al.\(^18\) The authors reported that only few neutrophils were found at sites of erosion or rupture. However, they used formalin-fixed paraffin-embedded sections for the study of ruptured and eroded plaques, apparently unaware of the fact that the CD66b antibody only works on frozen samples (in accordance with the data sheet of Coulter, endorsed by our own pilot studies; data not shown). It thus seems that the negative findings by Sugiyama et al\(^18\) relate to their handling of the tissue specimens.

Previous clinical studies have demonstrated intense in vivo neutrophil activation in UAP and AMI.\(^5^–^7\) Mehta et al\(^5\) reported a 15-fold increase in plasma levels of peptide B\(_\beta\), a marker of elastase release, in patients with UAP compared with those in patients with SAP. Biasucci et al\(^6\) measured the index of intracellular neutrophil myeloperoxidase and showed a significant release of myeloperoxidase from neutrophils related to their activation in patients with AMI and UAP. Mazzone et al\(^9\) also revealed increased expression of CD11b/CD18 integrins in neutrophils in UAP patients. These clinical observations together with our present findings suggest that neutrophil activation may be one of the inflammatory components of acute coronary syndromes. Activated neutrophils are known to release a variety of proteolytic enzymes, all of which have the potential for tissue destruction. In particular, neutrophil elastase has been shown to mediate both degradation of basement membrane constituents and endothelial damage.\(^6\) Hence, it is conceivable that neutrophils contribute to the pathogenesis of plaque destabilization in human atherosclerotic plaques.

In this study, 7 of the 13 AMI patients underwent emergency PTCA, and, therefore, one could consider the presence of neutrophils a result of PTCA-related injuries.\(^20\) However, there were no significant differences in numbers of neutrophils within culprit lesions of patients with and without PTCA. Moreover, neutrophils were found in the atherectomy specimens in \(~40\%) of the UAP patients, indicating that neutrophils occur in culprit lesions in UAP and AMI patients, irrespective of a PTCA procedure.

The present study also focused on the presence of NEP.\(^9\) The fact that NEP expression occurs primarily at the fully mature polymorphonuclear stage of neutrophil development is of interest, given our observation that ruptured plaques contain a significantly higher number of NEP-positive neutrophils than do eroded plaques. The pathobiology of this phenomenon remains unknown as yet. However, the observation that plaque erosions predominantly contain NEP-negative neutrophils suggests a rapid and relatively sudden outburst of neutrophils, like that seen in acute and severe inflammatory states.

In conclusion, the distinct presence of neutrophils in atherosclerotic plaques underlying UAP and AMI strongly suggests that neutrophils play a role in mediating destabilization of atherosclerotic plaques.

References


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