Macrophage Heterogeneity of Culprit Coronary Plaques in Patients With Acute Myocardial Infarction or Stable Angina

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Abstract

We investigated the polarization states of macrophages in coronary atherectomy tissues retrieved from patients with acute myocardial infarction (AMI, n = 52) or stable angina pectoris (SAP, n = 22). The specimens were analyzed immunohistochemically using antibodies specific to CD11c (M1 marker), CD206 (M2 marker), and to markers of endothelial cells, macrophages, and smooth muscle cells. Baseline characteristics were similar in the 2 groups. The proportion of areas immunopositive for smooth muscle actin was similar, but those positive for CD31 and CD68 were larger in the AMI group compared with the SAP group. In addition, AMI had significantly greater areas immunopositive for CD11c (P = .007) than did SAP, but CD206 (P = .102) positivity was not different in the 2 groups. In conclusion, M1 macrophage infiltration, not M2 macrophage infiltration, was increased in culprit plaques of patients with AMI. Macrophage heterogeneity may therefore be related to plaque instability.
single patient that was responsible for the clinical presentation. Directional coronary atherectomy was performed with a Flexi-Cut catheter (Abbott Laboratories/Guidant Vascular Interventions, Santa Clara, CA) under intravascular ultrasound guidance. The study protocol was approved by the local institutional review committee, and all patients provided written informed consent.

Tissue Preparation

Specimens were formalin fixed and embedded in donor paraffin blocks. Tissue microarrays were produced by reembedding tissues from the preexisting donor paraffin blocks into an array on a recipient paraffin block. Sections from the master block were cut with a microtome, mounted on microscope slides, and used for subsequent staining procedures.

Histologic Analysis

Standard H&E staining was performed to determine cellularity and general morphologic features. The area of each plaque was measured with a microscopic image analysis system (Motic Images Advanced 3.2, Motic, Xiamen, China). Plaques were classified as either atheromatous (ie, with necrotic cores and cholesterol clefs but without connective tissue matrix) or fibrocellular and graded as paucicellular (<30 spindle cells per high-power field), moderately cellular (30-100 spindle cells), or hypercellular (>100 spindle cells). All slides were graded by 2 pathologists (C.S.P. and I.H.) who were blind to the clinical status of the patient. Discrepancies between their findings were resolved by discussion.

Immunohistochemistry and Immunofluorescence Staining

Sections of each tissue specimen were stained with polyclonal antibodies against CD11c (1:200, Abcam, Cambridge, England) and CD206 (1:400, Abnova, Taipei, Taiwan) and clonal antibodies against CD11c (1:200, Abcam, Cambridge, MA), CD68 (1:200, mouse antihuman macrophage antibody clone 1A4, DakoCytomation, Carpinteria, CA), CD31 (1:200, mouse antihuman endothelial cell antibody clone WM59, BD Biosciences, Franklin Lakes, NJ), and CD68 (1:200, mouse antihuman macrophage antibody clone KP-1, DakoCytomation). Staining was performed with the Envision-Plus Immunostaining Kit and 3,3-diaminobenzidine or 3-amino-9-ethylcarbazole as the chromogen, as described by the manufacturer (DakoCytomation). Briefly, samples were incubated with primary antibodies that were diluted in antibody diluent (DakoCytomation) for 1 hour, washed twice (5 min each) with Tris-buffered saline/Tween-20, incubated with secondary antibodies conjugated with horseradish peroxidase–labeled polymer (DakoCytomation) for 1 hour, and washed again. As negative controls, adjacent sections were stained with species- and isotype-matched irrelevant antibodies, including normal rabbit immunoglobulin (Ig) G (Abcam). Samples obtained from human tonsil (CD11c) and human placenta (CD206) were used as positive controls for the corresponding antibodies.

Cell types positive for CD11c (M1 marker) and CD206 (M2 marker)3-5 were identified by immunostaining serial sections with anti-CD11c and CD206 antibodies. The immunopositive area was calculated as the ratio of the area of positively stained regions to the total plaque area. For immunofluorescent staining, fixed sections were hydrated in phosphate-buffered saline (PBS) for 10 minutes at room temperature, incubated with DakoCytomation Protein Block (DakoCytomation) for 5 minutes at room temperature, and washed 3 times in PBS/Tween-20 (PBST). Next, sections were incubated for 60 minutes at room temperature with mouse antihuman CD31 antibody (BD Biosciences), mouse antihuman CD68 antibody (DakoCytomation), mouse antihuman smooth muscle α-actin antibody (DakoCytomation), or rabbit anti-CD11c and CD206 antibodies. After 3 additional washes in PBST, sections were incubated with fluorescein isothiocyanate (FITC)–conjugated antirabbit IgG or allophycocyanin (APC)–conjugated antimouse IgG for 60 minutes at room temperature and then washed 3 times with PBST.

Coverslips were mounted onto glass slides with DAKO fluorescent mounting medium (DakoCytomation). FITC was excited by an argon laser at 488 nm, and APC was excited by a helium-neon laser at 633 nm. Detector slits were configured to minimize crosstalk between channels. Images were collected on a Leica TCS-NT/SP confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with a ×40 objective (model NA 0.75) and a Zoom 1–4X. Images were processed with Leica TCS-NT/SP software (version LCS) and Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Statistical Analysis

Continuous variables were expressed as means ± standard deviations or medians (with interquartile ranges [IQRs]). Categorical variables were expressed as frequencies. Continuous variables were compared with the Student t test or Mann-Whitney U test, and categorical variables were analyzed with the χ2 test. Linear regression analysis was used to correlate areas that were positive for CD11c and CD206 with other areas that were positive for endothelial cell, macrophage, or smooth muscle cell markers. Statistical significance was defined as a 2-sided P value less than .05.

Results

Clinical Characteristics

Baseline characteristics of the 2 groups were similar, except for lipid profiles and medications Table II. The mean patient age was 57.1 ± 9.7 years; 82.4% of the patients were
men, and 20.3% had diabetes mellitus. The median time from symptom onset to reperfusion was 3.8 hours (IQR, 2.0-10.5 hours) for ST-elevation AMI (n = 42) and 36 hours (IQR, 24-48 hours) for non-ST elevation AMI (n = 10). At the time of directional coronary atherectomy, patients with AMI were less frequently taking **-blockers, calcium channel blockers, and statins than patients with SAP.

### Histologic Analysis

Histologic data are summarized in Table 2. The total plaque area was larger in the AMI group than in the SAP group. Plaque types and the relative area of atheroma in the 2 groups did not differ. A thrombus was more commonly observed in the AMI group compared with the SAP group (73.1% vs 18.9%, respectively; **P** < .001).

#### Immunohistochemistry

The relative immunopositivity of the plaque areas for smooth muscle actin was similar in the 2 groups, whereas the areas immunopositive for CD68 and CD31 were significantly larger in the AMI group. In addition, the relative area immunopositive for CD11c was greater in patients with AMI compared with patients with SAP (0.2% [IQR 0%-1.1%] vs 0% [IQR 0-0.1%], respectively; **P** = .007). In contrast, the relative areas immunopositive for CD206 were not different in the 2 groups (0% [IQR 0%-0.2%] vs 0% [IQR 0%-0.1%], respectively; **P** = .102). Likewise, the CD11c/CD68 percent ratio was significantly higher in the AMI group than in the SAP group (4.8% [IQR 0.7%-17.0%] vs 0.6% [IQR 0%-6.9%], respectively; **P** = .011), but the CD206/CD68 percent ratio was not different in the 2 groups (1.2% [IQR 0%-5.4%] vs 0.2% [IQR 0%-3.1%], respectively; **P** = .316). CD68-immunopositive areas were significantly correlated with CD11c (r = 0.67, **P** < .001) or CD206-immunopositive areas (r = 0.34, **P** = .005).

Image 1 illustrates representative immunohistochemical staining patterns for CD68, CD11c, and CD206 in coronary plaques in patients with AMI or SAP. Immunoreactivity with anti-CD68 was strong in both AMI and stable plaques.
Image II Representative immunohistochemical staining of CD68, CD11c, and CD206 in coronary plaques from patients with acute myocardial infarction (AMI; A, B, C) or stable angina pectoris (SAP; D, E, F). Immunohistochemical staining with anti-CD68 antibody (dark brown) reveals strongly positive areas in patients with AMI (A) or SAP (D). CD11c is strongly stained in AMI (B) compared with SAP (E). CD206 staining areas are weak and similar in both AMI (C) and SAP (F) (×400).
In the same CD68-positive areas, CD11c-positive areas were relatively larger in the AMI plaque than in the SAP plaque, whereas CD206-positive areas were similar in the 2. Confocal immunofluorescence staining revealed that CD68 immunoreactivity colocalized with cells positive for CD11c and CD206.

**Discussion**

This study shows that CD11c-immunopositive areas (M1 macrophages), not CD206-immunopositive areas (M2 macrophages), were larger in culprit coronary plaques of AMI compared with SAP. In addition, CD68- (macrophages) and CD31- (endothelial cells) immunopositive areas were greater in the AMI plaques than in the SAP plaques. These findings highlight the differences between AMI and SAP plaques and suggest that macrophage heterogeneity is related to plaque instability.

AMI usually results from plaque rupture and superimposed thrombosis formation. The risk of plaque rupture appears to depend on the quality rather than quantity of plaque, requiring further histopathologic characterization of vulnerable plaques. The site of plaque rupture and thrombosis contains numerous inflammatory cells, indicating that inflammation plays an important role in both plaque vulnerability and thrombogenicity. Inflammation is now recognized as a central feature of vulnerable plaques and is responsible for the development of AMI.

**Image 2** Confocal immunofluorescence staining of coronary plaques from a patient with acute myocardial infarction (AMI) using antibodies against CD68 (green; A, D), CD11c (red; B), and CD206 (red, E). Cells immunopositive for CD68 colocalized with cells positive for CD11c (C) or CD206 (F). Arrows denote colocalized cells (x400).
Macrophages are a diverse population of cells that perform a wide range of biological functions. They are divided into 2 separate polarization states (M1 and M2 forms) and are likely activated along a continuum between these states. M1 macrophages are typically induced by lipopolysaccharide or interferon γ and are potent effector cells that produce proinflammatory cytokines (tumor necrosis factor α, interleukin 6, etc). On the contrary, M2 includes various forms of macrophages that are induced by interleukin 4 or interleukin 13, producing anti-inflammatory and immune-suppressive factors (interleukin 10, transforming growth factor β, etc). Macrophage heterogeneity has been extensively studied in various diseases, demonstrating the different pathologic roles of M1 and M2 macrophages. However, little data are available about the relationship between macrophage polarization and plaque instability. In the current study, M1 macrophage infiltration was dominant in culprit plaques in patients with AMI compared with patients with SAP. Plaque rupture is the most important mechanism underlying the sudden onset of AMI, and the macrophage is considered a key player in plaque rupture. However, it remains uncertain what causes a stable plaque to rupture. Our study shows the diversity and polarization of macrophages in culprit coronary plaques of AMI, suggesting that plaque instability might be caused by imbalance between M1 and M2 macrophages. However, it remains uncertain whether the greater proportion of M1 cells in AMI plaques is a consequence or cause of acute plaque rupture. Further studies are needed to better exploit macrophage heterogeneity as a therapeutic and diagnostic target.

Study limitations should be noted. First, the tissue specimens were obtained from selected lesions in large vessels. Calcified, tortuous, small vessels, and vessels with heavy thrombotic lesions are not suitable for directional coronary atherectomy. Thus, it may not be possible to extrapolate our findings to all culprit lesions of AMI. Second, because of the small specimen sizes, inflammatory cytokines could not be examined. Finally, the number of patients in the study was relatively small.

References
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