Correlation between Monocyte and T-lymphocyte Activation Markers in Patients with Acute Coronary Syndrome

Yoon-Ho Choi, MD, Won-Ha Lee, PhD, Yoon Lee, MS, Jin Koo Kim, MD, Sung-Youn Lee, MD, and Jeong-Euy Park, PhD

SUMMARY
Evidence suggesting the involvement of activated monocytes and T-lymphocytes in the acute phase of coronary artery disease (CAD) has been increasing. But a detailed analysis of a correlation between monocyte and T-lymphocyte activation markers in CAD has not yet been done. We analyzed plasma C-reactive protein (CRP) levels and the expression levels of CD14 and CD11b on monocytes and the percentage of HLA-DR+ T-lymphocytes in 25 patients with acute coronary syndrome (ACS), 12 stable angina (SA) patients, and 23 control subjects using flow-cytometry. The expression of CD14 by monocytes was increased significantly in ACS patients (activation index = 38.7 ± 2.5, mean ± SEM) in comparison to the control subjects (8.0 ± 1.9) and the SA patients (16.9 ± 3.9) (\(p < 0.001\) and \(p < 0.01\), respectively). The expression of CD11b by monocytes of ACS patients (4.6 ± 0.6) was also increased significantly in comparison to control subjects (2.2 ± 0.1) and the SA patients (2.2 ± 0.3) (\(p < 0.001\) for both). Also, a significantly higher percentage of HLA-DR positive T-lymphocytes (19.2 ± 1.8 vs 13.5 ± 1.2%, \(p < 0.05\)) was observed among ACS patients in comparison to control subjects. Significant increases in plasma CRP levels were also detected in ACS patients. Furthermore, there were statistically significant correlations among these activation markers. These results indicate that activation of inflammatory cells may play a role in the pathogenesis of ACS. The correlation between the activation status of monocytes and T-lymphocytes indicates that the activation of these immune cells is linked in such a way that activation of one type of cell may lead to the activation of another type of cell. (Jpn Heart J 2000; 41: 605-615)

Key words: CD14, CD11b, HLA-DR, Unstable angina, Stable angina, Inflammation, Flow cytometry

INCREASING evidence suggests that inflammatory changes and activation of immune cells are involved in the acute phase of atherosclerosis.\(^1\) The atherosclerotic plaques of AMI and UA patients are considered active plaques and vulnera-
ble to either rupture or fissure which can lead to thrombus formation. In contrast, SA patients show a reduction in their inflammatory status, and their lesions are in an inactive stage. Markers of systemic inflammation, such as C-reactive protein (CRP), serum amyloid A, and IL-6 were reported to be increased in UA patients. These markers have been shown to have prognostic value with respect to future coronary events and in-hospital complications. In monocytes, the expression level of adhesion molecules was shown to be elevated in ACS patients. Furthermore, monocytes isolated from UA patients were shown to be in an activated state with respect to the expression of a tissue factor (TF) which has a pro-coagulating activity.

T-lymphocytes expressing activation markers such as very late activation antigen-1 (VLA-1) and HLA-DR have been reportedly found in atherosclerotic plaques. These T-lymphocytes release cytokines such as interferon (IFN)-γ which causes the plaques to become unstable. In peripheral blood of UA patients, the proportion of HLA-DR positive T-lymphocytes was shown to be increased when compared to SA patients and control subjects. Also, T-lymphocytes isolated from UA patients have been shown to activate monocytes isolated from normal individuals, while T-lymphocytes isolated from normal subjects or stable effort angina patients did not activate monocyte procoagulant activity. This suggests that the activation of T-lymphocytes may cause the activation of monocytes and that the activation status of T-lymphocytes and monocyte is linked in UA patients.

Here, we investigated the level of CD14 and CD11b expression on monocytes and the percentage of HLA-DR positive cells among T-lymphocytes and B-lymphocytes in AMI and UA patients. We then compared the data to those of SA patients and healthy control subjects.

**MATERIALS AND METHODS**

**Patients and blood samples:** Twenty-five ACS and 12 SA patients and 23 control subjects were studied. Heparinized peripheral blood samples from ACS patients were drawn in the emergency room (ER) and coronary care unit (CCU) within 15 hours after the first onset of chest pain and after obtaining informed consent. All patients had angiographically proven coronary artery disease (CAD). The diagnosis of AMI was based on the typical electrocardiographic changes and clinical and laboratory data. Unstable angina was defined as either angina pain of recent onset, that is within 6 weeks, brought on by minimal exertion, or a more severe, prolonged or more frequent anginal attack superimposed on a chronic effort
angina. Stable angina was defined as effort angina with a stable pattern over the previous 3 months with angiographically documented significant coronary artery disease. The control subjects, selected from individuals who came to the health promotion center for periodic medical check-ups, were healthy individuals without any previous history or diagnosis of CAD.

Flow cytometric analysis: Whole blood samples were stained within 30 minutes after sampling to prevent artificial activation of the cells. Flow cytometric analysis of lymphoid cells was performed on an FACS-vantage (Becton-Dickinson, Mountain View, CA, USA). Briefly, whole blood (50 µl) was mixed with 0.5 µg of fluorochrome-conjugated antibodies in 50 µl of staining buffer (1× PBS containing 0.5% bovine serum albumin and 0.1% NaN₃) and incubated for 20 min on ice. The PE-anti-CD14 and FITC-anti-CD11b monoclonal antibodies were purchased from Caltag Laboratory (Burlingame, CA, USA). FITC-anti-HLA-DR and PE-anti-CD3 monoclonal antibodies were purchased from Pharmingen (San Diego, CA, USA). After incubation, the blood samples were sequentially treated with 100 µl of erythrocyte lysis solution (CAL-LYSE lysing solution, Caltag Lab.) for 10 minutes and with 1 ml of distilled water for 10 minutes to lyse the RBCs and fix WBCs. Stained cells were suspended in 2% paraformaldehyde in PBS and kept in a 4°C refrigerator until analysis. Upon flow cytometric analysis, the data on 30,000 cells were collected and analyzed. The monocyte and lymphocyte peaks were electronically isolated by recording the dual-histogram of a 90° light scatter versus a forward angle light scatter. For the monocytes, CD14 and CD11b expression levels are represented by an activation index which is the ratio of the mean fluorescence intensity (MFI) of specific staining to the background MFI. For the analysis of T- and B-lymphocytes, blood cells were double stained with PE-anti-CD3 / FITC-anti-HLA-DR and CD3 positive lymphoid cells (for T-lymphocytes) and CD3 negative lymphoid cells (for B-lymphocytes) were analyzed for the expression of HLA-DR antigen. Only cells expressing fluorescence levels higher than 99% over background staining were defined as HLA-DR positive cells.

Measurement of plasma levels of CRP: Plasma prepared from heparinized blood was stored in a −70°C freezer in aliquots until analysis. CRP levels were measured by immunoturbidimetry using CRP-Latex™ (DENKA SEIKEN CO. Tokyo, Japan) and a Hitachi 747-100 (Hitachi Corp. Japan) automatic chemical analyzer. The detection limit of the assay was < 0.1 mg / l.
Statistical analysis: Because the observed values did not follow a normal distribution curve, comparisons between groups were carried out employing a nonparametric test (Kruskal-Wallis test). Spearman's correlation analysis was used to analyze the interrelationship between the activation markers. All probability values reported are two-tailed, with values of \( p < 0.05 \) considered statistically significant.

RESULTS

ACS patients included 7 AMI and 18 UA patients. Fifteen out of the 25 ACS patients were newly diagnosed without any previous history of CAD. Table I shows the clinical and demographic characteristics of each study group.

After the activation indices of monocytic CD14 and CD11b were measured by flow cytometry, we found both activation markers were significantly increased in ACS patients when compared to those of SA and normal control subjects (Figures 1A and B). The activation indices for these activation markers in SA patients, however, were not significantly different from those in the normal control group.

We then analyzed the percentage of HLA-DR positive cells among

<table>
<thead>
<tr>
<th>Table I. Characteristics of the Study Populations</th>
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<tr>
<td>Group</td>
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<tr>
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</tr>
<tr>
<td>Age (yrs)</td>
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<tr>
<td>Sex (male / female)</td>
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<tr>
<td>BMI (kg / m²)</td>
</tr>
<tr>
<td>Total Cholesterol (mg / dL)</td>
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<tr>
<td>HDL (mg / dL)</td>
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<tr>
<td>LDL (mg / dL)</td>
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<td>Smoking (current / ex / non-smoker)</td>
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<td>Diabetes Mellitus (n)</td>
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<td>Previous medications (n)</td>
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<td>Acetylsalicylic acid</td>
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<tr>
<td>HMG-CoA reductase inhibitor</td>
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<tr>
<td>β-blockers</td>
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<td>Nitrates</td>
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Age, BMI, total cholesterol, HDL and LDL levels are expressed as mean ± SD. n.a. = not available.
T- and B-lymphocytes. The percentage of HLA-DR positive T-lymphocytes was increased in ACS patients but not in SA patients when compared to normal control subjects (Figure 1C). The percentage of HLA-DR positive B-lymphocytes, however, was not increased in ACS patients. SA patients tended to have a lower HLA-DR positive percentage of B-lymphocytes, which resulted in a statistically significant difference when compared with ACS patients but not when compared with normal control subjects (Figure 1D).
Table II. The Correlation among Plasma CRP Levels, Monocytic CD14 Activation Indexes, Monocytic CD11b Activation Indexes, and Percentage of HLA-DR Positive T Cells in Total Study Population.

<table>
<thead>
<tr>
<th></th>
<th>CRP</th>
<th>CD14</th>
<th>CD11b</th>
<th>HLA-DR+ T cells (%)</th>
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<tbody>
<tr>
<td>CRP</td>
<td>-</td>
<td>0.4584</td>
<td>0.3615</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt; 0.0018)</td>
<td>(&lt; 0.0024)</td>
<td>(&lt; 0.0202)</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>-</td>
<td>0.7887</td>
<td>0.4340</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt; 0.0001)</td>
<td></td>
<td>(0.0007)</td>
<td></td>
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<tr>
<td>CD11b</td>
<td>-</td>
<td></td>
<td>0.4039</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(0.0018)</td>
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HLA-DR+ T cells (%) -

Calculations were based on Spearman correlation analysis. Numbers represent Spearman correlation coefficient. Numbers in parentheses represent p values.

Figure 2. Analysis of correlation between activation markers. Activation indices of monocytic CD14 and CD11b and percentage of HLA-DR positive T-lymphocytes were compared with each other in the total study population. Numbers represent p values and the numbers in parentheses represent Spearman correlation coefficients.
When we looked for correlations between these activation markers, we found a strong association between monocytic CD14 and CD11b expression (Figure 2). The HLA-DR positive T-lymphocyte percentage was significantly correlated with the CD14 and CD11b activation indices. The HLA-DR positive B-lymphocyte percentage, however, did not reveal a significant correlation with the other markers (data not shown).

We then tested plasma levels of CRP in our study population. Plasma CRP levels were significantly increased in ACS patients (2.20 ± 0.18 mg/dl, mean ± SEM) compared to normal control subjects (0.01 ± 0.006) (p < 0.001). In contrast, the plasma CRP levels of SA patients (0.05 ± 0.01) were not significantly different with normal control subjects. We then analyzed the correlations among these activation markers (Table II). Plasma CRP levels were significantly associated with the activation indexes of monocytes CD14 (p = 0.0018) and CD11b (p = 0.0024) and the percentage of HLA-DR positive T cells (p = 0.02).

**DISCUSSION**

Our results provide the first evidence that the activation status of T-lymphocytes and monocytes is linked. Activation markers of monocytes and T-lymphocytes were significantly elevated in ACS patients. In contrast, activation markers on T-lymphocytes and monocytes in SA patients were not increased in comparison to control subjects. Since the atherosclerotic plaques in ACS patients are unstable and those in SA patients are in an inactive stage, the distribution of activation markers on monocytes and T-lymphocytes correlates well with the unstable versus stable nature of the plaques.

The activation of T-lymphocytes was observed in atherosclerotic plaque. Hansson, et al. demonstrated the presence of T-cells expressing very late activation antigen-1 (VLA-1) and HLA-DR, which are synthesized by T cells only in an activated state. Furthermore, at least some of the activated T-cells in the atherosclerotic plaques appear to respond specifically to oxidized-low density lipoprotein (oxi-LDL) which is a known inducer of foam cell transformation of monocytes. All these observations suggest that the presence of activated T-lymphocytes is the result of specific immune responses to atherogenic components. In unstable angina patients, activated T-cells were also detected in circulation. Both CD4+ and CD8+ circulating T-lymphocytes were shown to be activated with respect to the expression of HLA-DR. Cytokines derived from activated T-lymphocytes, such as Interferon (IFN)-γ, are known to
induce instability in plaque.\textsuperscript{11,15-17} Furthermore, it has been reported that T-lymphocytes isolated from unstable angina patients can activate the pro-coagulating activity of monocytes isolated from normal individuals.\textsuperscript{18} In contrast, monocytes isolated from unstable angina patients, incubated with T-lymphocytes isolated from normal or SA patients, did not have any increasing effect on the pro-coagulating activity. The results thus suggested that T-lymphocytes might control the responses of monocytes to atherogenic stimuli. Considering these previous reports, it is likely that the correlation we saw in our study between monocytic and T-lymphocyte expression of activation markers could be the consequence of an initial activation of T-lymphocytes which then lead to the activation of monocytes.

An increasing number of reports indicate that the activation of monocytes is involved in the acute phase of UA and AMI. First, an increase in total leukocyte count is associated with a risk of future CAD.\textsuperscript{19,20} Furthermore, the Paris Prospective study II indicated that a high monocyte count was strongly associated with the risk of coronary heart disease after adjustment for tobacco use and the other classical risk factors for the disease.\textsuperscript{21} Monocytes isolated from coronary sinus blood in UA patients expressed significantly higher levels of CD11b / CD18 than monocytes isolated from the aorta, while the monocytes of SA patients or normal subjects did not exhibit this difference in the expression level of CD11b between monocytes isolated from coronary sinus vs. aorta.\textsuperscript{22} Peripheral blood monocytes isolated from AMI patients also expressed significantly higher amounts of lymphocyte function associated antigen-1 (LFA-1), CD11b / CD18, very late activating antigen-4 (VLA-4), and intercellular adhesion molecule-1 (ICAM-1) than control subjects.\textsuperscript{6} Since a rapid increase in leukocyte adhesion to endothelial cells is one of the first events of an acute inflammatory response and part of the pathogenesis of vascular diseases, the increase in adhesion molecules in the peripheral as well as the coronary tree in ACS patients provides clear evidence for monocytes having an important role in the acute phase of CAD.

Monocytes are potent activators of blood coagulation because of their ability to synthesize the tissue factor (TF). Both the TF-mediated coagulation activity and the expression level of TF on the monocyte surface were found to be increased in ACS patients\textsuperscript{8,9,23} This activation of monocytes appears to be linked to the increased responsiveness of ACS patients to external stimuli. Ott, \textit{et al}. reported increases in the monocytic CD11b / CD18 expression levels, procoagulant activity, and systemic level of IL-6 in AMI patients, but not in SA patients, following percutaneous translu-
Engagement of the CD11b/CD18 activates monocytes to express tissue factor and TNF-α, which thus affects local thrombotic pro-coagulation. Our observation of the activation of monocytic CD11b expression is in agreement with previous reports. The activation of monocytic CD14 in ACS patients is a novel finding.

It is likely that an increase in activation markers in ACS patients is related to systemic inflammation. Haverkate, et al. analyzed the serum concentrations of CRP and serum amyloid A protein in stable and unstable angina patients and reported that the serum level of CRP is a good indicator of future coronary events within two years in both stable and unstable angina patients. Further studies confirmed that baseline CRP levels are an independent risk factor for future coronary events in normal or angina patients and the increase in CRP levels after acute myocardial infarction is related to the frequency of short term coronary events. As expected, we found a strong correlation between plasma CRP levels and the activation status of immune cells.

Currently it is not known whether the activation of monocytes and T-lymphocytes is a consequence of an acute phase reaction in ACS patients or the cause of the acute phase. ACS patients, in contrast to stable angina patients or normal control subjects, have myocardial ischemia and unstable atherosclerotic plaques. It is likely that pro-inflammatory cytokines such as IL-6 and TNF-α, which are known to be induced by cardiac myocytes during ischemic injury in ACS patients, may activate circulating immune cells. Alternatively but not exclusively, it is also possible that a relatively small erosion or fissuring of an atherosclerotic plaque can lead to the temporary formation of thrombi, which may then lead to the activation of these inflammatory cells. Activated immune components may in turn enhance the unstable nature of plaques by secretion of pro-atherogenic cytokines and the expression of adhesion molecules and activation markers.

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REFERENCES


