Preference Toward a T-Helper Type 1 Response in Patients With Coronary Spastic Angina

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Background—Coronary artery spasm plays an important role in the pathogenesis of ischemic heart diseases such as unstable angina (UA) and acute myocardial infarction. Nitric oxide (NO) plays an important role in coronary artery spasm. We previously reported a deficiency in NO activity in the spasm arteries of patients with coronary spastic angina (CSA). Others have reported that NO influences the immune response. Therefore, we investigated the balance between T-helper type 1 (Th1) and 2 (Th2) responses in patients with CSA by evaluating the frequencies of interferon (IFN)-γ–producing T cells and interleukin (IL)-4–producing T cells in the peripheral blood of such patients.

Methods and Results—Peripheral blood mononuclear cells were collected from 50 consecutive patients with CSA, 23 consecutive patients with UA, 36 patients with stable angina (SA), and 21 patients with chest pain syndrome (CPS). Cytokine-producing CD4+ T cells were quantified by 3-color flow cytometry after stimulation with phorbol myristate acetate and ionomycin. UA and CSA were associated with a significant increase in the frequency of CD4+ T cells that produced IFN-γ, whereas these conditions caused no significant difference in the frequency of CD4+ T cells that produced IL-4. Culturing with an NO donor compound for 24 hours before stimulation inhibited the increase in the frequency of CD4+ T cells that produced IFN-γ.

Conclusions—We demonstrated that there was a preference toward the Th1-type response in patients with CSA and that T cells showed a reduced Th1-type response after being treated with NO. (Circulation. 2003;107:●●●-●●●.)

Key Words: vasospasm • inflammation • lymphocytes

Recently, unstable angina (UA) has been shown to be associated with an inflammation. Patients with UA show increased frequencies of lymphocyte and monocyte activation in the peripheral blood and elevated levels of acute-phase proteins, which are predictive of the clinical outcome. Liuizzo et al recently reported that monocyte activation in UA represents a downstream effect of an altered T-cell response characterized by the preferential production of interferon-γ (IFN-γ). They found that IFN-γ–producing T cells in the peripheral blood were more frequent in patients with UA than in patients with stable angina (SA). There is now increasing evidence that coronary spasm is implicated in the pathogenesis not only of variant angina but also of UA and acute myocardial infarction.

Nitric oxide (NO) plays an important role in coronary artery spasm, and in this regard, we previously reported that a deficiency in NO activity plays an important role in its pathogenesis. Recently, the potential for NO to act as an immunoregulator altering T-helper type 1 (Th1)/T-helper type 2 (Th2) cytokine production has been demonstrated. In the present study, we investigated the balance between Th1- and Th2-type responses in patients with coronary spastic angina (CSA) by evaluating the frequencies of IFN-γ–producing T cells (Th1 cells) and interleukin (IL)-4–producing T cells (Th2 cells) in the peripheral blood of such patients.

Methods

Study Population

We studied 130 patients who underwent diagnostic catheterization (89 men and 42 women, mean age ± SD 66±11 years) between May 2001 and October 2002. Fifty consecutive patients (28 men and 22 women, mean age 65±9 years) with CSA were included in the present study. All had spontaneous attacks associated with ST-segment elevation or depression on the ECG more than once per week. While coronary arteriography was being conducted, coronary spasm (total or subtotal occlusion) was demonstrated angiographically during an anginal attack of chest pain, and ST-segment changes were observed when an intracoronary infusion of acetylcholine was used to induce spasm, as reported previously. Acetylcholine 20 to 100 μg was injected into the left and right coronary arteries at separate times in all patients. This method enables spasm to be provoked separately in both coronary arteries and allows us to evaluate the inducibility of spasm in each coronary artery safely. The
sensitivity and specificity of this method for provoking coronary spasm have been validated by Okumura et al. Patients with CSA were divided into 2 groups: CSA with coronary stenosis and CSA without coronary stenosis. Twenty-three consecutive patients (17 men and 6 women, mean age 69 ± 11 years) with UA were included in this study. UA was defined as chest pain at rest with documented transient ST-segment depression or ST-segment elevation of 0.1 mV in at least 2 contiguous ECG leads. The last spontaneous attack was required to have occurred within a period of 24 hours before entry into the study. Patients with new Q-wave development or with an increase in creatine kinase levels of more than twice the normal upper limit were excluded from the study. We confirmed by coronary arteriography that all patients with UA had significant coronary artery stenosis but no coronary spasm. We studied 36 patients with SA (27 men and 9 women, mean age 71 ± 7 years) who had typical exertional chest discomfort associated with horizontal or downsloping ST-segment depression >1 mm in an exercise test, ≥90% narrowing of the major coronary arteries, and no acetylcholine-induced coronary spasm. Moreover, the patients in this group did not experience any acute events, any worsening of symptoms during the previous 6 months, or any anginal episodes within the week preceding enrollment. There were 21 patients with chest pain syndrome (CPS; 12 men and 9 women, mean age 58 ± 17 years) whose chest pain was not accompanied by ECG changes, coronary organic stenosis, or coronary spasm when an intracoronary injection of acetylcholine was given during coronary arteriography.

No patient was treated with steroids. None had thromboembolism, collagen disease, disseminated intravascular coagulation, advanced liver disease, renal failure, malignant disease, sepsis, other inflammatory disease, valvular heart disease, atrial fibrillation, or malignancy or was using a pacemaker. Written informed consent was obtained from each patient. The study conformed to the guidelines approved by the ethics committee at our institution.

### Blood Samples
Blood samples were obtained from all patients in the recumbent position with a 21-gauge needle for clean venipuncture of an antecubital vein. The first 3 mL of blood was used for biochemical assessment. Subsequently, 3 mL of blood was collected sequentially into evacuated tubes containing 0.3 mL of sodium heparin for flow cytometric analysis.

### Flow Cytometric Analysis for Cytokine Production
The samples with 3 mL of blood were mixed with 3 mL of either nonactivating medium (10% FCS-supplemented RPMI 1640 with 40 μg/mL Brefeldin A [Sigma] or activating medium (nonactivating medium with 40 ng/mL phorbol myristate acetate [Calbiochem] and 4 μg/mL ionomycin [Sigma]) and then incubated for 4 hours at 37°C and 5% CO2. After being washed with ice-cold PBS, cells were recovered by centrifugation, adjusted to 5×10^6 white blood cells per test, and stained with Cy-chrome–labeled anti-human CD4 monoclonal antibody (BD Pharmingen). The fixation and permeabilization of cells were both performed with IntraPrep reagent (Beckman Coulter) according to the manufacturer’s instructions, and intracellular cytokines were stained with FITC-labeled anti-human IFN-γ and phycoerythrin-labeled anti-human IL-4 monoclonal antibodies (Beckman Coulter). The IL-4– and IFN-γ–producing CD4+ T cells were analyzed with a FACScan instrument (Becton Dickinson). Nonspecific staining with the isotype-matched control monoclonal antibody was <1%.

### Effect of NO on Th1- and Th2-Type Response
Peripheral blood mononuclear cells were collected from the whole-blood samples by Ficoll density separation and washed 3 times with RPMI 1640 medium before being cultured. They were cultured for 24 hours with or without 0.3 mmol/L NOC 18, an NO donor compound (Dojindo Laboratory), and then flow cytometric analysis was performed to evaluate cytokine production.

### Statistical Analysis
All data are given as mean ± SD. Comparisons of continuous data among the 4 patient groups were performed with the Kruskal-Wallis test followed by the Dunnett procedure and those between the 2 subgroups (CSA with coronary stenosis and CSA without coronary stenosis; single-vessel group and multivessel group in SA) with the Mann-Whitney U test. The frequencies of cytokine-producing T cells obtained for incubation with NOC 18 were compared with those obtained without NOC 18 by the Wilcoxon signed-rank test. Frequency data among the 4 patient groups and between the 2 subgroups were compared by χ² test. Probability values <0.05 were considered statistically significant.

### Results
#### Patient Characteristics of the 4 Patient Groups
The clinical characteristics of the UA, CSA, SA, and CPS groups are shown in the Table. The CSA, UA, SA, and CPS groups were matched for age, gender, frequency of coronary risk factors, and all lipid levels except HDL cholesterol level.

### Assessment of Frequencies of IFN-γ–Producing T Cells and IL-4–Producing T Cells in the 4 Patient Groups
There was no difference in absolute lymphocyte numbers among the 4 patient groups. After in vitro activation, the frequencies of peripheral CD4+ T cells that stained for IFN-γ were 29.4 ± 12.7% in the UA group, 26.9 ± 9.4% in the CSA group, 18.0 ± 6.0% in the SA group, and 15.5 ± 5.8% in the CPS group. The frequency of IFN-γ–producing T cells was found to be significantly higher in the CSA and UA groups than in the SA and CPS groups ($P<0.01$; Figure 1). Differences in frequencies between the CSA and UA groups and between the SA and CPS groups were not significant. The frequencies of peripheral CD4+ T cells that stained for IL-4 were 3.0 ± 1.4% in the UA group, 3.5 ± 2.0% in the CSA group, 3.1 ± 2.0% in the SA group, and 3.1 ± 1.9% in the CPS group. The differences in frequency among the 4 patient groups were not significant (Figure 1). Frequencies of double-positive cells for IFN-γ and IL-4 were 1.2 ± 0.9% in the UA group, 1.5 ± 1.5% in the CSA group, 1.1 ± 1.2% in the SA group, and 0.9 ± 0.7% in the CPS group, but these differences in frequency were not significant. These cells were counted as both IFN-γ– and IL-4–producing cells.

### Assessment of Frequencies of IFN-γ– and IL-4–Producing T Cells in Subgroups
The frequencies of IFN-γ–producing peripheral CD4+ T cells and IL-4–producing T cells in the CSA with coronary stenosis group ($n=17$) and CSA without coronary stenosis group ($n=33$) are shown in Figure 2. For the former, frequencies were 26.4 ± 10.6% for the CSA with stenosis group and 27.2 ± 8.9% for the CSA without stenosis group, but these differences were not significant. In the case of the latter, frequencies were 3.7 ± 1.9% for the CSA with stenosis group and 3.4 ± 2.1% for the CSA without stenosis group. These differences also were not significant. There were no significant differences in the frequencies of IFN-γ–producing T cells (single-vessel group 17.2 ± 7.7%, multivessel group 18.6 ± 4.0%) or IL-4–producing T cells (single-vessel group 2.7 ± 2.4%, multivessel group 3.5 ± 1.6%) between the single-
vessel disease subgroup (n = 17) and multivessel disease subgroup (n = 19) of the SA group.

**Assessment of Effect of NO on the Frequency of IFN-γ- and IL-4-Producing T Cells**

Mononuclear cells from 10 patients were divided into 2 samples, 1 for culture with the NO donor compound and the other for culture without it. We found the frequencies of IFN-γ–producing T cells to be significantly lower in mononuclear cells cultured with 0.3 mmol/L NOC 18 than in mononuclear cells cultured without NOC 18 (27.5 ± 12.7% vs 34.4 ± 10.8%; P ≤ 0.005; Figure 3). On the other hand, the frequencies of IL-4–producing T cells in mononuclear cells cultured with NOC 18 were not significantly different from those in mononuclear cells cultured without NOC 18 (1.6 ± 0.8% vs 1.8 ± 1.0%; P = 0.647). Thus, culturing with the NO donor compound reduced the frequencies of IFN-γ–producing T cells but did not affect the frequencies of IL-4–producing T cells.

**Discussion**

Coronary lesions infiltrated with immune cells, including macrophages, T lymphocytes, and mast cells, are suspected of contributing to plaque instability.16–18 In this regard, we have reported that in directional coronary atherectomy specimens, the area of macrophage infiltration was larger for patients with UA than it was for those with SA, and tissue factor expression on macrophages was also more frequently observed for patients with UA.19 Liuzzo et al9,20,21 demonstrated that plaque instability in patients with UA is associated with monocytes activated by IFN-γ derived from stimulated T cells. It has also been reported that T-cell–mediated endothelial cell injury is a novel pathway of tissue damage that contributes to plaque destabilization.22 The present study suggests that aberrations of the global immune system are of functional importance to such plaque inflammation not only in patients with UA but also in those with CSA, and we

*Table 1. Patient Characteristics in the 4 Study Groups*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>UA (n=23)</th>
<th>CSA (n=50)</th>
<th>SA (n=36)</th>
<th>CPS (n=21)</th>
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</thead>
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<tr>
<td>Age, y</td>
<td>Mean</td>
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<td>65±9</td>
<td>71±7</td>
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<tr>
<td></td>
<td>Range</td>
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<td>40–81</td>
<td>50–84</td>
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<td>28/22</td>
<td>27/9</td>
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<td></td>
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<td>23</td>
<td>21</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td>9</td>
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<td>11</td>
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</tr>
<tr>
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<td>8</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
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<td>201±24</td>
<td>194±31</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
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<td>48±11</td>
<td>54±13*</td>
<td>44±11</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td></td>
<td>150±107</td>
<td>133±62</td>
<td>140±67</td>
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<tr>
<td>No. of coronary arteries narrowed &gt;75%</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>0</td>
<td>33</td>
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<td>6</td>
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</tbody>
</table>

Values are expressed as mean±SD or number.

*P < 0.05 vs SA; † P < 0.05 vs UA and SA.*

Figure 1. Comparison of frequencies of CD4+ T cells producing IFN-γ (left) and IL-4 (right) among UA, CSA, SA, and CPS groups.

Figure 2. Comparison of frequencies of CD4+ T cells producing IFN-γ (left) and IL-4 (right) between CSA without stenosis and CSA with stenosis groups.
previously reported the presence of inflammation in the coronary circulation of patients with UA and CSA.23–25 The data we present here show that patients with UA and CSA have expanded unusual CD4+ T cells that excessively produce IFN-γ on stimulation. The present data also show that there was a preference toward the Th1-type response not only in patients with UA but also in those with CSA.

Furthermore, we noted that there was no significant difference in the frequency of IFN-γ-producing T cells between CSA patients with stenosis and CSA patients without stenosis. There was also no significant difference in the frequency of IFN-γ-producing T cells between single-vessel disease patients and multivessel disease patients in the SA group. These data indicate that stenosis is not associated with Th1-type response. The association of T-cell repertoire perturbations with UA and CSA raises the possibility that it is associated with an abnormality of the intima. 29 The mechanism of coronary spasm is still unclear, it has been suggested that it is associated with an abnormality of the intima.29 The data from the present study suggested the relation between coronary spasm and T lymphocytes. It has been reported that NO is consumed by vascular NO oxidase and that the endothelium-dependent relaxant response is impaired during inflammation.30 We previously studied the effects of oxidative stress on coronary spasm, finding that the exogenous addition of glutathione improved vasomotor reactivity to acetylcholine in the epicardial coronary arteries of patients with CSA through reduction of oxidative stress.31 Thus, antioxidants may reduce the consumption of NO and improve vasomotor reactivity. Other researchers demonstrated that NO can inhibit the secretion of IFN-γ by Th1 cells but has no effect on IL-4 production by Th2 cells.32 These 3 studies suggest the possibility that the Th1-type response in CSA is caused by a loss of NO, which can inhibit the production of IFN-γ. In a previous study, we reported that NO activity is deficient in patients with CSA.11 Later, Roozendaal et al33 showed that exposure of T cells to an NO donor compound for 24 hours before stimulation resulted in a more pronounced inhibition of IFN-γ secretion and that under these conditions, IL-4 secretion was not inhibited. In the present study, NO decreased IFN-γ production without affecting IL-4-production in CD4+ T cells. This indicates that T cells show a reduced Th1-type response after exposure to NO.

In conclusion, we have demonstrated for the first time that there is a preference toward the Th1-type response in patients with CSA. Our findings suggest that an NO-mediated immune response is associated with coronary spasm.

Acknowledgments

This study was supported in part by a research grant for cardiovascular disease (14C-1 and 14C-4) from the Ministry of Health, Labor and Welfare, a grant-in-aid for scientific research [No. C1)12203001, C(2)13670727, B(2)14770319] from the Ministry of Education, Culture, Sports, Science, and Technology, and a Smoking Research Foundation Grant for Biomedical Research Foundation, Tokyo, Japan.

References


Figure 3. Comparison of frequencies of CD4+ T cells producing IFN-γ (left) and IL-4 (right) between CD4+ T cells cultured with NOC 18 (NO donor compound) and those cultured without NOC 18.


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Circulation. published online April 14, 2003;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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