Interaction Between Chemokines and Oxidative Stress: Possible Pathogenic Role in Acute Coronary Syndromes

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OBJECTIVES We sought to study the relationships between chemokines and oxidative stress in acute coronary syndrome.

BACKGROUND In view of existing knowledge on the participation of leukocytes and oxidative stress in the pathogenesis of acute coronary syndrome, we hypothesized that chemokines may play a role in recruiting and activating leukocytes in this disorder.

METHODS The levels of chemokines and oxidative stress were studied in 38 patients with stable and 38 with unstable angina and in 20 controls. In separate in vitro experiments the effect of chemokines on reactive oxygen species in monocytes and the effect of antioxidants on chemokine levels in these cells were also studied.

RESULTS 1) Angina patients had raised serum levels of chemokines in both cross-sectional and longitudinal testing, with particularly high levels of interleukin (IL)-8, monocyte chemotactic protein (MCP)-1 and macrophage inflammatory peptide (MIP)-1-alpha in unstable disease. 2) T cells, and particularly monocytes, seem to contribute to the raised IL-8, MCP-1 and MIP-1-alpha levels in unstable angina. 3) Concomitantly, and significantly correlated with MCP-1 and IL-8 levels, stable and particularly unstable angina patients had decreased plasma levels of antioxidants and increased lipid peroxidation, suggesting enhanced oxidative stress. 4) Monocyte chemotactant protein-1 enhanced the generation of O$_2^-$ in monocytes from unstable angina patients, and the antioxidant glutathione-monoethyl ester suppressed the production of IL-8 and MCP-1 in these cells.

CONCLUSIONS Our findings suggest an interaction between chemokines and oxidative stress in unstable angina. This interaction may represent a vicious circle involved in the pathogenesis of acute coronary syndromes. (J Am Coll Cardiol 2001;37:485–91) © 2001 by the American College of Cardiology

Increasing evidence supports the involvement of inflammation in atherogenesis and in the pathogenesis of acute coronary syndrome. Thus, activated monocytes, T cells and granulocytes have been demonstrated in patients with unstable angina (1,2), and extensive infiltration of blood-derived macrophages and T cells into the vessel wall is seen in the active stages of atherosclerosis (3). Chemokines are a family of inflammatory cytokines characterized by their ability to cause directed migration of leukocytes, and raised levels are found in several inflammatory disorders (4). There is also some evidence suggesting that chemokines, e.g., interleukin (IL)-8 and monocyte chemoattractant protein 1 (MCP-1), may play a role in the pathogenesis of atherosclerosis (5,6). In addition to being potent chemoattractants, several other leukocyte responses such as enzyme secretion and induction of reactive oxygen species (ROS) have been observed in vitro after chemokine stimulation (4,7). Some of these responses may clearly be relevant to the development of coronary artery disease (CAD). In particular, enhanced oxidative stress has been implicated in the pathogenesis of CAD and in the triggering of unstable angina (8). In view of the existing knowledge on the association of leukocyte activation and oxidative stress with atherosclerosis and acute coronary syndrome, we hypothesized that chemokines may play an important role in recruiting and activating leukocytes and in enhancing oxidative stress in these disorders. In the present study we investigated this hypothesis by different experimental approaches in stable and unstable angina patients.

METHODS

Patients and controls. Between January and June 1998 patients undergoing diagnostic coronary angiography in the coronary care unit at our hospital were consecutively registered. Among those fulfilling the criteria of unstable angina (see following text), 38 patients were selected randomly for participation in the study (Table 1). All patients with unstable angina had experienced ischemic chest pain at rest within the proceeding 48 h, with no evidence of myocardial
necrosis by enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. For comparison, 38 patients with stable angina were randomly selected among those attending the Department of Cardiology at our hospital in the same period for diagnostic coronary angiography (Table 1). All these patients had stable effort angina of >6 months duration and a positive exercise test. Exclusion criteria included myocardial infarction within the previous month, ECG abnormalities invalidating ST-segment analyses, thrombolytic therapy the previous month, body temperature <38.0°C or the occurrence of inflammatory disease likely to be associated with acute-phase response (e.g., infections, malignancies or autoimmune disorders). Controls in the study were 20 gender- and age-matched healthy blood donors (15 males and five females, age 53 ± 15 years). Blood collection and isolation of monocytes and T (CD3+) cells for the study was performed as previously described (9,10). Informed consent for participation in the study was obtained from all individuals.

**Superoxide anion (O$_2^-$) assay.** Monocytes (3 × 10$^5$/ml; 200 µl/well) were cultured in 96-well trays (Costar, Cambridge, Massachusetts) in RPMI 1640 with L-glutamine (Gibco, Paisley, United Kingdom) supplemented with 10% fetal calf serum (Myoclone, Gibco) either alone or with different concentrations of MCP-1, IL-8 or macrophage inflammatory peptide 1-alpha (MIP-1-alpha), all from R&D Systems, Minneapolis, Minnesota. After 20 h, O$_2^-$ generation in monocytes was measured by the superoxide dismutase-inhibitable reduction of cytochrome c as previously described (10).

**Chemokine production in vitro.** CD3+ T cells (10$^6$cells/ml) and monocytes (3 × 10$^5$cells/ml) were incubated in 96-well trays (200 µl/well, Costar) in medium alone (RPMI 1640 with 2 mmol/l L-glutamine and 25 mmol/L HEPES buffer; Gibco, supplemented with 10% fetal calf serum) or with stimulants: CD3+ T cells: anti-CD3 monoclonal antibodies (final concentration 1.2 ng/ml; clone SpvT3b) (9) combined with anti-CD28 monoclonal antibodies (final concentration 50 ng/ml; clone 15E8 (402); CLB, Amsterdam, Netherlands) and cross-linking immunomagnetic beads (9); monocytes: lipopolysaccharide (LPS) from E. coli O26:B6 (final concentration 10 ng/ml, Sigma). In some experiments different concentrations of glutathione-monooethyl ester (Bachem, Bubendorf, Switzerland) were added to cultures before stimulation. Cell-free supernatants were harvested after 48 h and stored at −80°C.

**Measurements of chemokines.** Levels of RANTES, MCP-1, MIP-1-alpha, IL-8, ENA-78 and GRO-alpha were determined by enzyme immunoassays (R&D Systems).

**Table 1.** Characteristics of the Study Group

<table>
<thead>
<tr>
<th></th>
<th>Stable Angina (n = 38)</th>
<th>Unstable Angina (n = 38)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>59 ± 11</td>
<td>58 ± 12</td>
<td>0.600</td>
</tr>
<tr>
<td>Gender (males/females)</td>
<td>31/7</td>
<td>29/9</td>
<td>0.576</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>21</td>
<td>47</td>
<td>0.030</td>
</tr>
<tr>
<td>Medication (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>15</td>
<td>22</td>
<td>0.422</td>
</tr>
<tr>
<td>Aspirin</td>
<td>91</td>
<td>89</td>
<td>0.752</td>
</tr>
<tr>
<td>HMG CoA reductase inhibitors</td>
<td>74</td>
<td>53</td>
<td>0.075</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>88</td>
<td>97</td>
<td>0.147</td>
</tr>
<tr>
<td>Long-acting nitrates</td>
<td>41</td>
<td>50</td>
<td>0.462</td>
</tr>
<tr>
<td>Infusion of nitroglycerin</td>
<td>0</td>
<td>17</td>
<td>0.013</td>
</tr>
<tr>
<td>Warfarin</td>
<td>12</td>
<td>14</td>
<td>0.792</td>
</tr>
<tr>
<td>Numbers of affected coronary arteries (%)</td>
<td>44</td>
<td>31</td>
<td>0.132</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.8 ± 0.9</td>
<td>4.9 ± 1.0</td>
<td>0.870</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.1 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>0.031</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.8 ± 1.1</td>
<td>1.8 ± 0.9</td>
<td>0.927</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

HDL = high density lipoprotein; HMG CoA = hepatic hydroxymethylglutaryl coenzyme A.
Parameters of oxidative stress and vitamin levels in plasma. Concentrations of vitamin C, vitamin E and beta-carotene were measured by high-performance liquid chromatography (11). Total (free and esterified) 8-isoprostane (8-iso Prostaglandin F2α) in plasma were analyzed by enzyme immunoassay (Cayman Chemical, Ann Arbor, Michigan). Sample purification and recovery analyses were performed according to the purification protocol provided by the manufacturer.

Statistical analysis. When comparing three groups, one-way ANOVA was followed by Scheffe’s post hoc test for statistical significance. The chemokine data were not normally distributed and were subjected to logarithmic transformation before the ANOVA analysis was performed. For comparisons within the same individuals over time, the Wilcoxon matched pairs test was used. P-values are considered significant when <0.05.

RESULTS

Serum levels of chemokines in angina patients. Levels of all measured chemokines were elevated in both unstable and stable angina patients compared with healthy controls (Fig. 1). Although there were no significant differences in RANTES, ENA-78 or GRO-alpha levels between stable and unstable angina, particularly high IL-8, MCP-1 and MIP-1-alpha levels were found in unstable disease (Fig. 1). In ten patients with unstable angina, blood samples were also available before the patients developed an unstable disease (median time between blood samplings: 12 [range 4 to 20] weeks). Concomitant with the progression from stable to unstable angina, there was a significant rise in IL-8, MCP-1 and MIP-1-alpha (p < 0.005), but not in levels of the other three chemokines (data not shown).

Release of chemokines from monocytes and T cells in peripheral blood. To possibly define the cellular sources of IL-8, MCP-1 and MIP-1-alpha in unstable angina, spontaneous and stimulated release of these chemokines from T cells and monocytes were measured in five patients with stable angina, five with unstable angina and five controls. Monocytes in unstable angina released increased levels of IL-8, MIP-1-alpha and MCP-1 both spontaneously and after LPS stimulation, comparing cells in stable angina patients (p < 0.05) and controls (p < 0.01). Furthermore, anti-CD3/anti-CD28 stimulated T cells from patients with unstable disease released higher levels of MIP-1-alpha comparing the two other groups of individuals (p < 0.05).

Chemokine levels in relation to oxidative stress. We first analyzed plasma levels of 8-isoprostane, a sensitive marker of lipid peroxidation (12), and the antioxidant vitamins beta-carotene, vitamin C and vitamin E in 40 angina patients (20 with stable and 20 with unstable disease) and ten controls. Angina patients had raised 8-isoprostane concentrations compared with controls, with particularly high levels in unstable disease (Fig. 2). Furthermore, vitamin C, vitamin E and beta-carotene levels in unstable angina were significantly decreased compared with both stable angina patients and controls (Fig. 2). In unstable angina, IL-8 and MCP-1 were positively correlated with 8-isoprostane (r = 0.71, p < 0.001; r = 0.65, p < 0.005) and negatively correlated with vitamin E (r = −0.61, p < 0.01; r = −0.69, p < 0.001) and beta-carotene levels (r = −0.57, p < 0.01; r = −0.50, p < 0.05), IL-8 and MCP-1, respectively.

The effect of chemokines on oxidative stress and vice versa. By additional experiments we further examined the relationship between oxidative stress and chemokines in seven patients with stable angina, seven with unstable angina and seven controls. Several significant findings were revealed. First, spontaneous and zymosan-stimulated O2− generation was markedly enhanced in monocytes from unstable, but not from stable angina patients, compared with controls (Fig. 3). Second, MCP-1, but not IL-8 or MIP-1-alpha, markedly enhanced zymosan-stimulated O2− generation in monocytes from both patients and controls, with particularly enhancing effect in unstable angina, when added to monocytes after 20 h of culture in medium alone (Fig. 4C).

When the antioxidant glutathione-monoethyl ester was added to monocyte cultures, there was a dose-dependent reduction in LPS-stimulated release of IL-8 and MCP-1, but not MIP-1-alpha, in both patients and controls, with a particularly suppressive effect in unstable angina (Fig. 4A and B). In stable and particularly in unstable angina such a reduction was also found in the spontaneous release of these chemokines (Fig. 4C and D). Glutathione-monoethyl ester also suppressed MCP-1, but not MIP-1-alpha levels, in stimulated T cells in both patients and controls (data not shown).

DISCUSSION

The present study shows that raised levels of several chemokines characterize angina patients, particularly those with unstable disease. Concomitantly, and significantly correlated with raised MCP-1 and IL-8 levels, stable and particularly unstable angina patients had decreased plasma levels of antioxidants and increased lipid peroxidation, suggesting enhanced oxidative stress in these patients. Finally, MCP-1 was found to enhance ROS generation in monocytes from unstable angina patients and the antioxidant glutathione-monoethyl ester reduced the production of IL-8 and MCP-1 in these cells, suggesting an interaction between chemokines and oxidative stress in unstable angina.

Enhanced levels of CXC- and CC-chemokines in unstable angina. We demonstrate that angina patients have raised levels of both CC and CXC-chemokines with particularly high IL-8, MCP-1 and MIP-1-alpha levels in
unstable disease, possibly reflecting pathogenic processes in these patients. Migration of monocytes into the arterial wall is an early event in atheroma formation, and chemokines may be involved in this process (5). Thus, the selective absence of the MCP-1 receptor CCR2 decreases atherosclerosis formation in apolipoprotein E null mice (13). Moreover, infiltration and activation of circulating T cells and monocytes into the atherosclerotic lesion may also be involved in the triggering of acute coronary syndromes (3). Again, chemokines may play an important role in this immune-mediated plaque destabilization, not only by recruiting activated leukocytes into the plaque (5), but also by directly contributing to plaque rupture and thrombus formation (14,15). We found that monocytes and T cells in unstable angina released increased levels of chemokines. If such activation also exists within the atheroma, our findings may represent pathogenic processes involved in the triggering of acute coronary syndromes.

Figure 1. Serum levels of IL-8 (A), MIP-1-alpha (B), MCP-1 (C), GRO-alpha (D), ENA-78 (E) and RANTES (F) in 38 patients with unstable angina pectoris (AP), 38 patients with stable AP and 20 healthy controls. Horizontal lines represent median values.
MCP-1 enhances ROS generation from monocytes in unstable angina. We found markedly decreased levels of several antioxidants in angina patients, with particularly low levels in unstable disease. This decrease in unstable angina was accompanied by increased lipid peroxidation and enhanced ROS generation in monocytes, suggesting enhanced oxidative stress in these patients. Although several factors may mediate enhanced ROS generation in unstable angina (e.g., oxidized LDL, hypoxia/reoxygenation) (8), our results suggest that chemokines and particularly MCP-1 may be involved. Several cytokines may prime phagocytes for enhanced ROS generation (9), but only certain chemokines may directly induce ROS production.

**Figure 2.** Plasma levels of 8-isoprostane (A), beta-carotene (B), vitamin C (C) and vitamin E (D) in 20 patients with unstable angina pectoris (AP), 20 patients with stable AP and 10 healthy controls. Horizontal lines represent median values.

**Figure 3.** MCP-1 and production of reactive oxygen species in monocytes from seven patients with unstable angina pectoris (AP), seven patients with stable AP and seven healthy controls. (A) The effect of different concentrations of MCP-1 on zymosan-stimulated $O_2^-$ generation when added to cell cultures at the start of the culture period (20 hours before zymosan stimulation). (B) The effect of different concentrations of MCP-1 on $O_2^-$ generation when added to monocytes after 20 h of culture in medium alone. *p < 0.05 and **p < 0.01 versus no addition of MCP-1. Data are given as mean ± SEM. Note: both unstimulated and zymosan-stimulated $O_2^-$ generation after 20 h of culture in medium alone (MCP-1 = 0 ng/ml) were significantly raised in unstable angina patients comparing both those with stable disease and healthy controls (p < 0.01).
Antioxidants impair the release of chemokines from monocytes in unstable angina. Whereas MCP-1 enhanced ROS generation in monocytes from unstable angina patients, glutathione-monoethyl ester markedly suppressed MCP-1 and IL-8 production in these cells. Several studies have demonstrated that oxidative stress may activate the transcription factor NF-kappa-B in various cell types (16). Notably, NF-kappa-B, which recently was found to be activated in leukocytes from unstable angina patients (17), is involved in the induction of IL-8 and MCP-1 in both T cells and monocytes (18,19). Thus, if similar mechanisms also operate in vivo within an atherosclerotic lesion, the interaction between chemokines and oxidative stress may represent a pathogenic loop in unstable angina.

Interaction between chemokines and oxidative stress—possible pathogenic role in plaque rupture. Unstable angina patients had raised levels of chemokines and oxidative stress compared with stable angina patients, and as for spontaneous and MCP-1 induced O$_2^-$ production, there was no overlap between the two patient groups. These findings may be of interest. Thus, enhanced ROS generation within the atherosclerotic plaque may promote apopto-

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REFERENCES


