Increased Secretion of Tumor Necrosis Factor-α and Interferon-γ by Mononuclear Leukocytes in Patients With Ischemic Heart Disease

Relevance in Superoxide Anion Generation

K. Vaddi, DVM, PhD; F.A. Nicolini, MD; P. Mehta, MD; J.L. Mehta, MD, PhD

Background There is growing evidence for a pathogenic role for cytokines in atherosclerosis. The presence of certain cytokines has been documented in human atherosclerotic vessels. This study was designed to investigate cytokine production by mononuclear leukocytes from patients with ischemic heart disease.

Methods and Results We measured kinetics of secretion of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) by mononuclear leukocytes from 8 control subjects, 10 patients with stable angina pectoris, and 10 patients with unstable angina pectoris. Mononuclear leukocytes were isolated and incubated with or without the plant lectin mitogen concanavalin A for 48 hours. TNF-α and IFN-γ secretion were measured by ELISA. The effect of TNF-α and IFN-γ on superoxide radical generation by neutrophils was also examined. Secretion of both TNF-α and IFN-γ by mononuclear leukocytes increased progressively over 48 hours, and it was consistently higher (P<.02) in patients compared with control subjects. A similar increase in cytokine secretion was observed in patients with stable or unstable angina pectoris. In addition, there was no relation between the severity of coronary artery disease by angiography and cytokine secretion. Basal neutrophil superoxide radical generation was increased in patients with ischemic heart disease, and incubation with cytokines failed to further stimulate superoxide generation in these patients.

Conclusions Similar increases in cytokine secretion by mononuclear leukocytes in stable or unstable angina pectoris indicate that the increased cytokine release is not a nonspecific inflammatory response in acute myocardial ischemia. Increased cytokine secretion in ischemic heart disease may play a role in superoxide radical generation, endothelial injury, deposition and activation of cellular elements on the vessel wall, and possibly in the progression of atherosclerosis.

Key Words • proteins • interferon • ischemia • heart diseases

Cytokines are extracellular signaling proteins secreted by immunocompetent cells as well as nonimmune cells such as vascular endothelium.1 Although these intercellular peptide mediators have long been known to play an important role in the regulation of inflammation by controlling recruitment and activation of various responsive cell populations, it is only recently that cytokines have been proposed to have a role in the triggering and perpetuation of atherosclerosis.2 Among the different cytokines, some are believed to be more relevant than others in the context of atherosclerosis and its complications, such as ischemic heart disease. Among these are interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). These cytokines promote angiogenesis and induce morphological and functional alterations in endothelial cells.3 In addition, these cytokines cause expression of human leukocyte antigen (HLA)4* and other leukocyte adhesion molecules.5 TNF-α also stimulates cyclooxygenase activity6 and promotes adhesion of neutrophils, basophils, eosinophils, and lymphocytes on endothelial cells.7 IFN-γ acts synergistically with TNF-α to increase the expression of intracellular adhesion molecules as well as class I and class II major histocompatibility antigens.8 These cytokines increase the activity of plasminogen activator inhibitor as well as the synthesis of tissue plasminogen activator,9,9 but the overall effect of cytokine secretion appears to be procoagulant. IFN-γ is released locally in the arterial intima during the vascular response to injury and has been shown to regulate vascular smooth muscle proliferation.10,11 IFN-γ is generated by T cells,12 whereas TNF-α is produced by several types of cells, including smooth muscle cells,13 lymphocytes,12,14 and macrophages.14

Hansson et al15 first identified activated T lymphocytes in human atherosclerotic plaques. Barath et al16 localized TNF-α in a majority of human atherosclerotic tissues, not only in the cytoplasm of macrophages, but also in the cytoplasm and cell membranes of smooth muscle cells and endothelial cells.

We hypothesized that secretion of cytokines may be increased in patients with established ischemic heart disease and may contribute to the propagation of coronary atherosclerosis. As such, we examined the cytokine secretory capacity of the mononuclear leukocytes from patients with unstable and stable angina pectoris as well as normal subjects.

Methods

Subjects

All control subjects and patients were men. Ten patients with documented unstable angina pectoris, 10 patients with...
stable angina pectoris, and 8 control subjects participated in this study, which was approved by the Institutional Review Board.

All patients with stable angina had typical exertional chest pain and angiographically documented coronary artery disease (>50% luminal narrowing of one or more major coronary arteries). Unstable angina was defined as an increase in the frequency or duration of chest pain or both in patients with previously documented chest pain or new onset of symptoms suggestive of myocardial ischemia with subsequent angiographic demonstration of coronary artery disease. There was no evidence of acute myocardial infarction as determined by rise in serum creatine kinase or a new q wave in the ECG. The mean ages of patients with stable angina pectoris (63 years), patients with unstable angina pectoris (64 years), and control subjects (60 years) were similar. There was no difference in the extent of coronary artery disease or drug intake in patients with stable or unstable angina (Table 1). None of the patients or control subjects had smoked within 2 hours of blood collection. Blood was collected from patients with unstable angina pectoris within 1 hour of chest pain. Blood from control subjects and patients with stable angina was collected between 9 and 10 AM.

### Blood Collection and Separation of Mononuclear Leukocytes

Peripheral venous blood was collected in heparin (10 U/mL) for isolation of mononuclear leukocyte fraction. The heparinized blood was layered over MonoPoly Resolving Medium (Flow Laboratories) and centrifuged at 500g for 30 minutes at 24°C. Red blood cells were lysed by brief (15 seconds) suspension of the mononuclear leukocyte–rich layer in hypotonic saline solution followed by addition of hypertonic saline. The cells were then removed and washed in Hanks' balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺.

### Mononuclear Cell Culture and Mitogenic Stimulation

Peripheral blood mononuclear leukocytes were suspended in Medium 199 supplemented with 2.5% fetal bovine serum (Sigma Chemical Co) and streptomycin and penicillin. Three aliquots (each in duplicate) of mononuclear leukocytes (each containing 2×10⁶ cells/mL) from each subject were incubated for a period of 0, 4, 12, 24, or 48 hours with or without the mitogen concanavalin-A (Con-A) (10 μg/mL) at 37°C in 95% O₂ and 5% CO₂. The supernatants were collected and stored at −70°C until assay for cytokines. Culture conditions were carefully monitored to maintain sterile atmosphere. At the end of the incubation period, the culture supernatants were checked for endotoxin levels with E-Toxate assay system (Sigma). The supernatants containing any detectable level of endotoxin were discarded.

### Cytokine Assays

A solid-phase ELISA kit (Genzyme Corp) was used to measure the TNF-α and IFN-γ levels in serum as well as mononuclear leukocyte culture supernatants. This kit uses a sandwich ELISA principle. All samples (0.1 mL) were measured for cytokine secretion in duplicate. Levels of TNF-α and IFN-γ were expressed as nanograms per 10⁶ cells and units per 10⁶ cells, respectively.

### Neutrophil Superoxide Generation

Neutrophils from study subjects were isolated by density gradient centrifugation in MonoPoly Resolving Medium as described above. Neutrophils were incubated in the presence of recombinant TNF-α or IFN-γ (Genentech) for 30 minutes, and the amount of superoxide radicals generated by neutrophils was determined by measuring the superoxide dismutase–inhibitable formation of ferricytochrome C. In brief, neutrophils at a concentration of 1×10⁶ cells/mL were placed in a shaking water bath at 37°C in HBSS with Ca²⁺ and Mg²⁺ containing 0.1 mol/L ferrocyanochrome C and cytochalasin B 5 μg/mL. Cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL) for 10 minutes at 37°C. The generation of superoxide radicals was calculated as the difference in the absorbance between aliquots of cells incubated without and with superoxide dismutase 100 μg/mL as described earlier.17

### Statistical Analysis

The data within subgroups (control subjects and patients with unstable angina pectoris or stable angina pectoris) were compared by one-factor ANOVA, and the differences between groups were analyzed by Scheffé’s F test with an Apple Macintosh IIcx and the statistics program STATVIEW 512+.

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**Table 1. Characteristics of Patients With Ischemic Heart Disease**

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects (n=8)</th>
<th>Patients With Stable Angina Pectoris (n=10)</th>
<th>Patients With Unstable Angina Pectoris (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60±4</td>
<td>64±7</td>
<td>63±10</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>25</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Previous myocardial infarction, %</td>
<td>0</td>
<td>90</td>
<td>20</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>25</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Serum cholesterol, mg/dL</td>
<td>164±14</td>
<td>218±31</td>
<td>221±21</td>
</tr>
<tr>
<td>White blood cells, 10⁶ cells/mL</td>
<td>7.8±3.0</td>
<td>8.8±3.1</td>
<td>8.1±2.1</td>
</tr>
</tbody>
</table>

Medication, % of patients

<table>
<thead>
<tr>
<th></th>
<th>Aspirin</th>
<th>Calcium blockers</th>
<th>β-Adrenergic blockers</th>
<th>Extent of coronary artery disease, % of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>1 Vessel</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40</td>
<td>70</td>
<td>2 Vessels</td>
</tr>
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<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3 Vessels</td>
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<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>40</td>
<td></td>
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</tbody>
</table>

NA indicates not applicable. Values are mean±SD.
**Table 2. Constitutive Release of TNF-α and IFN-γ**

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Patients With Stable Angina</th>
<th>Patients With Unstable Angina</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α, ng/10⁶ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.66±0.38</td>
<td>1.55±0.60*</td>
<td>1.14±0.39*</td>
</tr>
<tr>
<td>48 h</td>
<td>1.09±0.40</td>
<td>2.24±0.63*</td>
<td>1.43±0.42*</td>
</tr>
<tr>
<td>IFN-γ, U/10⁶ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>31±11</td>
<td>80±15*</td>
<td>73±21*</td>
</tr>
<tr>
<td>48 h</td>
<td>33±8</td>
<td>87±15*</td>
<td>87±32*</td>
</tr>
</tbody>
</table>

24 h and 48 h refer to duration of incubation of mononuclear leukocytes. Values are mean±SD.

*P<.05 vs control subjects.

value of P<.05 was considered significant. All data are presented as mean±SD.

**Results**

**Cytokine Secretion From Mononuclear Leukocytes**

The basal or “constitutive” release of cytokines, particularly TNF-α, from mononuclear leukocytes exhibited a modest increase over the 48-hour period of observation. Stimulation of mononuclear leukocytes with Con-A resulted in marked stimulation of TNF-α and IFN-γ over the 48-hour period, particularly after 24 and 48 hours of incubation. Therefore, data obtained only at these time points are presented.

The constitutive release of TNF-α was greater (P<.05) from mononuclear leukocytes from patients with stable or unstable angina than from those of control subjects. Similarly, the constitutive release of IFN-γ was increased (P<.05) in patients with stable or unstable angina compared with control subjects. These differences were evident at 24- and 48-hour periods of incubation of cells (Table 2).

The secretion of TNF-α and IFN-γ by mononuclear cells increased upon stimulation with Con-A over the 48-hour period. This increase was observed in control subjects and patients with ischemic heart disease. Con-A-stimulated mononuclear leukocytes from stable angina pectoris patients exhibited greater (P<.02) secretion of TNF-α than the cells from normal control subjects at 24 hours of incubation (mean, 3.5 ng/10⁶ cells versus 1.7 ng/10⁶ cells, P<.02, Fig 1). Similarly, greater secretion of TNF-α was apparent after 48 hours of stimulation (mean, 5.2 ng/10⁶ cells versus 2.2 ng/10⁶ cells, P<.02, Fig 1). Increased secretion of TNF-α was also observed in Con-A-stimulated mononuclear leukocytes from patients with unstable angina after 24 or 48 hours of incubation (mean levels, 4.5 and 5.7 ng/10⁶ cells, respectively, both P<.02 versus control subjects, Fig 1). No significant differences with respect to the secretion of TNF-α by mononuclear leukocytes were observed between patients with stable and unstable angina pectoris.

Studies on the secretion of IFN-γ by mononuclear leukocytes also indicated increased secretion in patients with ischemic heart disease compared with control subjects. The increased secretion of IFN-γ by Con-A-stimulated mononuclear leukocytes was apparent at 24 hours of stimulation in patients with stable angina pectoris compared with the control subjects (mean, 160 versus 107 U/10⁶ cells, P<.02). Similarly increased secretion of IFN-γ was observed after 48 hours of stimulation (mean, 186 versus 115 U/10⁶ cells, P<.02, Fig 2). In patients with unstable angina pectoris, a similar increased secretion of IFN-γ from mononuclear leukocytes was observed compared with control subjects (mean, 185 and 208 U/10⁶ cells at 24 and 48 hours, respectively, Fig 2). Again, there was no difference in the ability of mononuclear leukocytes from stable and unstable angina pectoris patients to secrete IFN-γ.

Secretion of TNF-α or IFN-γ did not correlate with the extent of coronary artery disease (r=.093 and r=.166, respectively, both P=NS). Also, there was no correlation between cytokine secretion and drug intake (β-blockers, calcium blockers, or aspirin), presence of hypertension, or cigarette smoking (all P=NS).

**Effect of Cytokines on Neutrophil Superoxide Radical Generation**

Incubation of neutrophils with TNF-α (10 ng/10⁶ cells) resulted in a modest enhancement of superoxide radical generation by neutrophils from control subjects. Incubation with IFN-γ (25 ng/10⁶ cells; specific activity of IFN-γ, 1 ng=50 U) also resulted in enhanced super-

![Fig 1](http://circ.ahajournals.org/)

Fig 1. Plot showing secretion of tumor necrosis factor-α (TNF-α) by mononuclear leukocytes. The TNF-α secretion is greater (P<.02) by mononuclear leukocytes from patients with stable angina or unstable angina at both 24 (left) and 48 (right) hours of incubation with concanavalin-A. There is no difference in TNF-α secretion by mononuclear leukocytes from patients with stable angina or unstable angina.
oxide radical generation by these neutrophils (both $P<.05$ versus control).

Neutrophils from patients with stable or unstable angina pectoris generated significantly more ($P<.01$) superoxide radicals compared with the normal control subjects even without any exogenous cytokine exposure (mean, 15.7 versus 3.5 nmol/10^6 cells per 10 minutes). However, neutrophils from patients with stable or unstable angina pectoris did not show any further increase in the superoxide generation when these cells were incubated with either TNF-α or IFN-γ (Fig 3).

**Discussion**

Monocytes are present on the vascular endothelial cells in the early stages of atherosclerosis. These cells subsequently transform into macrophages, migrate into the subendothelial layers, and probably contribute to the process of atherogenesis. Mononuclear cells in general are important sources of cytokines; neutrophils, on the other hand, appear to play an important role in myocardial reperfusion injury, probably via release of proteolytic enzymes and free oxygen radical species. The interaction between mononuclear cells and neutrophils along with platelets and vascular tissues may be important in the initiation and propagation of coronary arterial and myocardial injury.

In this study, we found that mononuclear leukocytes from patients with stable as well as unstable angina pectoris secrete significantly more TNF-α and IFN-γ than do the mononuclear cells from healthy but elderly control subjects. Similar mononuclear cell cytokine secretory capacities in both patient groups indicate that there is an inherent difference in the cytokine secretory abilities of mononuclear cells from patients with ischemic heart disease and those from control subjects. The increase in cytokine secretion is not an acute-phase reaction, since similarly enhanced cytokine secretion was observed in patients with stable angina pectoris, in whom there is no ongoing inflammation, and those with unstable angina. Lack of correlation with the number of diseased coronary arteries implies that cytokine secretion does not correlate with the extent of atherosclerosis. Modest elevation of cytokine secretion in some control subjects may reflect underlying subclinical atherosclerosis in the elderly control population (mean age, 60 years).

Although there has been speculation relative to the role of these cytokines in ischemic heart disease, there are very few studies showing alterations in their secretion in patients with chronic stable or acute unstable angina pectoris. Barath et al described localization of TNF-α in 88% of human atherosclerotic tissue sections and its absence in all six tissue sections that were classified as normal. A recent study in pigs shows that macrophages accumulate in significant numbers in the ischemic myocardium and release TNF-α, which may be responsible for subsequent micronecrosis and angiogenesis. Since both new vessel formation and central necrosis are known biological effects of TNF-α, it has been suggested that this cytokine may be involved in the evolution of atheroma after the initial ischemic event. A recent study by Frye et al shows release of large amounts of TNF-α in the coronary sinus blood of patients with heart transplants. These patients are

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**Fig 2.** Plot showing secretion of interferon-γ (IFN-γ) by mononuclear leukocytes. The IFN-γ secretion is greater ($P<.02$) by mononuclear leukocytes from patients with stable angina or unstable angina at both 24 (left) and 48 (right) hours of incubation with concanavalin-A. There is no difference in IFN-γ secretion by mononuclear leukocytes from patients with stable angina or unstable angina.

**Fig 3.** Bar graph showing effect of cytokines on neutrophil superoxide radical generation. Neutrophil superoxide radical generation increases significantly ($P<.05$) when cells from normal control subjects ($n=8$) are incubated with tumor necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) for 30 minutes. However, no such stimulation of superoxide radical generation is observed in neutrophils from patients with ischemic heart disease (IHD, $n=15$, 8 with stable angina and 7 with unstable angina). Note that the values of superoxide radical generation by neutrophils are higher ($P<.01$) in IHD patients compared with control subjects before treatment of cells with cytokines.
known to develop extensive and early coronary atherosclerosis. Our observations of increased secretion of TNF-α and IFN-γ by circulating mononuclear cells from patients with ischemic heart disease may relate to propagation of atherosclerosis by promoting intravascular coagulation and cell adhesion.

Although different cytokines have been shown to induce a variety of morphological and functional changes in the endothelial cells, the pragmatic importance of these changes is still unclear. However, some of these effects need special mention in the context of ischemic heart disease. Both IFN-γ and TNF-α synergize to induce nitric oxide production. 22 Although nitric oxide in physiological amounts is a potent vasodilator species, release of large amounts of nitric oxide also exerts tissue-damaging effects. 23,24 Induction of leukocyte adhesion molecules on the vascular endothelial cells is another and very important effect of cytokines. 5 The adhesion markers, such as endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1, have been described as the cellular ligands that bind leukocytes to the activated endothelial cells. 25 Thus, cytokines, such as IFN-γ and TNF-α, may augment inflammation and immunologic processes. 26 A recent report demonstrates expression of these adhesion molecules in the atherosclerotic human coronary artery plaques. 27 Increased adhesion of leukocytes to endothelium and subsequent transmigration is a consequence of expression of the adhesion molecules in the study on the role of cytokines in promoting transendothelial neutrophil passage, Moser et al 28 found a time- and dose-dependent enhancement in the junctional penetration of monolayers by neutrophils after exposure to the cytokines IL-1 and TNF-α, whereas transmigration potentiation was not observed as a consequence of mere neutrophil attachment to endothelial cells.

Activated neutrophils are known to produce free oxygen radicals, such as superoxide and hydrogen peroxide, which are aimed at destruction of target cells 29,30 and inactivation of invading organisms. 31 The free oxygen radicals released by these activated neutrophils form an important basis for the initiation of vessel wall injury, particularly during tissue reperfusion after a period of ischemia. 32 There are numerous reports of association of neutrophil number and activation with myocardial ischemic events as well as severity of outcome from myocardial ischemia. 33,34 Previous studies from our laboratory have demonstrated release of large amounts of elastase from neutrophils in active ischemic heart disease. 35 Neutrophils from patients with stable and unstable angina pectoris exhibited increased chemotactic activity and leukotriene B4 generation, whereas those from patients with unstable angina showed pseudopod formation and granule extrusion on transmission electron microscopy. It was suggested that these changes may relate to ongoing neutrophil activation in vivo.

In the present study, we observed a modest but definite stimulation of superoxide radical generation by normal control neutrophils after their exposure to cytokines. This is in keeping with the studies by Rao et al, 36 who showed that TNF-α and IFN-γ cooperate in inducing the generation of reactive oxygen intermediates and expression of chemotactic receptors in differentiated HL-60 cells. Activation of human neutrophil functions, as indicated by phagocytic ability and antibody-dependent cell-mediated cytotoxicity by IFN-γ as well as TNF-α, has also been documented. 37 Neutrophils from patients with ischemic heart disease in the present study, before their exposure to cytokines, released almost three times as much superoxide radical as did the neutrophils from normal subjects. Upon exposure to cytokines, no further superoxide radical generation by neutrophils from patients was observed, indicating maximal generation at rest. These observations of significantly higher levels of superoxide radical generation and lack of further enhancement after exposure to cytokines in patients with ischemic heart disease indicate maximal stimulation of oxidative activity in these neutrophils.

The mechanism of neutrophil activation by cytokines is not known. However, the current working hypothesis is that cytokines stimulate synthesis and release of platelet-activating factor, which in turn activates neutrophils. 38 On the basis of our data, it seems plausible that enhanced cytokine secretory capacity of the mononuclear cells in ischemic heart disease is a basis for the activation of neutrophils. This activation leads to enhanced synthesis of cyclooxygenase and lipoxygenase products of arachidonic acid metabolism, resulting in the production of metabolites that further stimulate cytokine secretion in a positive feedback mechanism. 39

van der Wal et al 40 recently examined thrombosed coronary artery segments from 20 patients and identified macrophages, and to a lesser extent T lymphocytes, as the dominant cells at the immediate site of either rupture or superficial erosion in each instance. There was abundant expression of HLA-DR antigens on the inflammatory cells and adjacent smooth muscle cells, suggesting an active inflammatory reaction in atherosclerosis. These authors also observed deposition of neutrophils in deep fissures. In an accompanying editorial, 41 Buja and Willerson thought that these important observations, if confirmed, would provide new ways of thinking about mechanisms involved in plaque disruption.

The present study provides evidence of ongoing release of proinflammatory and procoagulant cytokines by stimulated as well as unstimulated mononuclear cells from patients with ischemic heart disease. The increased release of TNF-α and IFN-γ, and possibly other cytokines, may have a bearing on increased free radical generation, endothelial injury, recruitment of neutrophils in the ischemic-reperfused tissues, and possibly the progression of coronary atherosclerosis.

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