Elevated Production of Interleukin-6 Is Associated With a Lower Incidence of Disease-Related Ischemic Events in Patients With Giant-Cell Arteritis

Angiogenic Activity of Interleukin-6 as a Potential Protective Mechanism

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Background—Patients with giant-cell arteritis (GCA) who develop a strong acute-phase response are at low risk of disease-related ischemic events.

Methods and Results—To assess the potential protective role of proinflammatory cytokines in the development of ischemic events in GCA, we measured tissue expression (66 individuals) and/or circulating levels (80 individuals) of interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-6 in patients with biopsy-proven GCA. Tissue expression was determined by quantitative real-time polymerase chain reaction and immunohistochemistry. Circulating cytokines were determined by enzyme-linked immunoassay. We found that patients with disease-related ischemic events had lower IL-6 mRNA levels (5.9±2.1 versus 27.6±7.8 relative units, P=0.013), lower IL-6 immunohistochemical expression scores (1.5±0.9 versus 2.7±1, P=0.001), and lower circulating levels of IL-6 (13.6±2.4 versus 24±4 pg/mL, P=0.002) than patients without ischemic complications. No significant differences were found for either IL-1β or TNF-α. We subsequently investigated direct effects of IL-6 on vessel wall components. We found that IL-6 stimulates endothelial cell proliferation and differentiation into capillary-like structures and induces full angiogenic activity in both ex vivo (aortic ring) and in vivo (chick chorioallantoic membrane) assays.

Conclusions—GCA patients with ischemic complications have lower tissue expression and circulating levels of IL-6 than patients with no ischemic events. IL-6 has relevant direct effects on vascular wall components that might be protective: IL-6 activates a functional program related to angiogenesis that may compensate for ischemia in patients with GCA. (Circulation. 2003;107:2428-2434.)

Key Words: angiogenesis ■ interleukins ■ ischemia ■ inflammation

Giant-cell (temporal) arteritis (GCA) is a granulomatous vasculitis that preferentially involves large and medium-sized arteries. Although arterial inflammation may be substantially widespread, the main classic clinical manifestations of the disease arise from symptomatic involvement of the carotid artery branches.1,2

The inflammatory process eventually leads to vessel obliteration with subsequent ischemia of supplied tissues.1-4 Even though GCA is considered a large and medium-sized vessel arteritis, we have recently shown that small cranial vessels are almost invariably involved,5 and indeed, the most common ischemic complications usually occur in territories supplied by small arteries.1-4 Permanent visual loss is the most characteristic and frequent ischemic complication in GCA, affecting ≈15% of patients in most series.1-4 We have previously noticed that ischemic events tend to occur more frequently in individuals who develop a relatively weak systemic acute-phase reaction.3,6 The reason the intensity of the acute-phase response is able to discriminate between patients at high and low risk of ischemic events is unknown. The systemic acute-phase reaction is driven by proinflammatory cytokines, particularly interleukin (IL)-1, tumor necrosis factor (TNF), and IL-6, which act in complex cascades involving many cell types.7 Proinflammatory cytokines, which are produced in GCA inflammatory lesions,8,9 also have strong autocrine and paracrine functions and may
influence vascular responses such as vessel occlusion or regeneration.

The aim of the present study was to measure the expression of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 in vascular lesions from a large series of patients with biopsy-proven GCA to evaluate whether there is any relationship between tissue expression of inflammatory cytokines and the development of ischemic events. We found that IL-6 expression in temporal artery inflammatory infiltrates and circulating levels of IL-6 were significantly reduced in patients with ischemic complications. We hypothesized that IL-6 might have direct effects on vessel wall components that might contribute to prevent ischemic events. Given our previous findings that the angiogenic response is lower in those patients with GCA who have ischemic events and in patients with a weak systemic inflammatory response, we subsequently investigated the potential role of IL-6 in triggering endothelial cell responses related to angiogenesis and the ability of IL-6 to induce full angiogenic activity in ex vivo and in vivo models.

Methods

Patients

The entire study group consisted of 106 prospectively studied patients (74 women, 32 men) aged 76.4 years (range 57 to 91 years) with biopsy-proven GCA. At the time of diagnosis, 33 patients had transient or permanent disease-related ischemic complications, which are depicted in Table 1. Circulating proinflammatory cytokines were determined in sera from 80 of the study patients (20 with ischemic complications), and cytokine tissue expression was analyzed in frozen temporal artery samples from 66 patients (16 with ischemic events). All samples studied were obtained from either untreated patients or patients who had received a single corticosteroid dose (prednisone 1 mg/kg) the day before excision of the temporal artery sample with the commercially available Micro RNA isolation kit (Stratagene). Enough tissue to obtain sufficient good-quality total RNA to perform the study was available from 31 patients (7 with ischemic events).

Cytokine mRNA Quantification in Temporal Artery Samples

RNA Isolation

Total RNA was obtained from 100 frozen sections (20 μm thick) per temporal artery sample with the commercially available Micro RNA isolation kit (Stratagene). Enough tissue to obtain sufficient good-quality total RNA to perform the study was available from 31 patients (7 with ischemic events).

cDNA Synthesis

One microgram of total RNA was reverse transcribed to cDNA with the SuperScript II First-Strand Synthesis kit (Gibco, Life Technologies) with random hexamers as the priming method. Samples were stored at −20°C until use.

Real-Time Quantitative Polymerase Chain Reaction

Cytokine mRNAs were measured by real-time polymerase chain reaction (PCR) with a specific predeveloped TaqMan target kit from Applied Biosystems as described previously. Values were expressed as relative units.

Immunohistochemical Study

Serial 4- to 6-μm cryostat sections, obtained from frozen temporal arteries of 66 patients (16 with and 50 without ischemic complications), were air-dried and fixed with cold acetone. After several blocking steps, sections were incubated with the primary antibodies diluted in PBS for 30 minutes. Primary antibodies used were polyclonal rabbit anti-human TNF-α (Genzyme) at 1:500 dilution, monoclonal mouse anti-human IL-1β (clone B1 from Genzyme) at 10 μg/mL, and monoclonal mouse anti-human IL-6 (clone 6708.111) from R&D Systems at 10 μg/mL. Immunodetection was performed by an avidin-biotin-peroxidase method (EnVision kit from Dako).

Quantification was performed according to a previously described semiquantitative score based on the percentage of cells positively stained in the granulomatous area at the intima/media junction (Figure 1). After agreement was confirmed in the scoring system, cytokine expression in 2 sections per condition was independently evaluated by 3 investigators (J.H.-R., A.G.-M., and M.-J.E.) who were blinded to the patients’ clinical information, and the average score was considered.
Circulating Cytokine Measurement

Serum IL-1β, TNF-α, and IL-6 concentrations were determined by ELISA in sera from 80 patients (20 with and 60 without ischemic manifestations). Commercially available ELISA kits for TNF-α were obtained from Medgenix, and kits for IL-1β and IL-6 were obtained from R&D Systems. Sera were evaluated in duplicate wells, and the procedure was performed according to the instructions of the manufacturer.

Endothelial Cell Growth Assay

Human umbilical vein endothelial cells (HUVECs) were obtained from freshly delivered umbilical cords and cultured as described previously.13–15 Cells were grown until confluence, passed at a ratio of 1 to 4, and used for experiments after the second to fourth passages. Recombinant IL-6 and recombinant soluble IL-6 receptor (sIL-6R) (both from R&D Systems) were diluted in RPMI 1640 (Invitrogen) with 0.1% bovine serum albumin (Sigma) to make a stock solution of 1 and 10 ng/μL, respectively. Confluent HUVECs were released with trypsin-EDTA (Invitrogen) and resuspended in complete growth medium13 diluted at 50% in plain RPMI 1640. Cells were plated in flat-bottomed 96-well plates at 5000 cells/well, with the addition of recombinant IL-6 and/or recombinant sIL-6R at various concentrations, and incubated at 37°C in 5% CO₂ for 4 days. Then, the supernatant fluid was aspirated, and the cells were fixed and stained with 0.2% crystal violet (Sigma) in 20% methanol for 10 minutes. Wells were washed with distilled H₂O and air-dried. After solubilization in 1% SDS, optical density was measured with an ELISA reader (Titertek Instruments) at 560-nm wavelength. Baseline optical density was evaluated in parallel wells 1 hour after cell plating when cells were completely attached and spread. Each data point was tested in quadruplicate wells. The experiment was repeated 2 times with similar results.

Endothelial Cell Differentiation Into Capillary-Like Structures

Forty-eight-well plates were coated with Matrigel at 4°C (150 μL/well) and incubated at 37°C for 30 minutes to allow polymerization. HUVECs were released with trypsin-EDTA, resuspended in growth medium diluted at 50% in RPMI 1640, and plated onto the Matrigel-coated wells at 15 000 cells per well. Recombinant IL-6 and sIL-6R, alone or in combination, were added to triplicate wells at the time of cell plating. Complete growth medium, which has been shown to be optimal for tube formation, was used as a positive control. After an 18-hour incubation, tubes were fixed and stained with Diff-Quik (Dade Behring) and scored by 2 blinded investigators (M.C.C. and H.K.K.) according to the following score: 0, isolated fragments of tubes; 0.5, interconnected tubes occupying <20% of the well area; 1, interconnected tubes occupying 20% to 50% of the well surface; 1.5, 50% to 70%; and 2, 70% to 100%. In preliminary experiments, visual scoring by experienced investigators was found to be as accurate as measuring tube area with an imaging system.13,15 The experiment was repeated 2 times with comparable results.

Chick Embryo Aortic Ring Ex Vivo Angiogenesis Assay

Aortic arches were removed from 14-day-old chick embryos, cleaned of surrounding connective tissue, and sliced under a magnifying lens. Aortic rings were placed on a Matrigel drop in 48-well plates and covered with an additional drop of Matrigel. Four hundred microliters of serum-free SFM-medium (Invitrogen) with various concentrations of IL-6 and/or sIL-6R was added to each well. Endothelial cell growth supplement (ECGS) at 200 μg/mL was used as a positive control. Rings were incubated overnight at 37°C with 0.5% CO₂ to allow microvessel sprouting from the adventitial layer, which was fully apparent in positive controls after 24 hours. Rings were fixed and stained with Diff-Quik and observed under an inverted microscope. Sprouting was measured by 2 blinded investigators (M.C.C. and H.K.K.) using the following score: 0, no sprouting; 0.25, isolated sprouting; 0.5, sprouting in 20% to 50% of the artery circumference; 1, sprouting in 50% to 75% of the circumference; 1.5, sprouts in 100% of the circumference; and 2, 100% of the artery circumference occupied by sprouts longer than one third of the length of the average radius of the rings. Each condition was tested in 6 wells. The experiment was repeated 3 times with similar results.

Chick Chorioallantoic Membrane In Vivo Angiogenesis Assay

Ten nanograms of recombinant IL-6, 10 ng of sIL-6R, or the combination of both were diluted in 5 μL of distilled H₂O, dried on quartered Thermannox coverslips, applied onto the chick chorioallantoic membrane of 10-day-old chick embryos, and incubated at 37°C for 3 additional days. As a negative control, 5 μL of the cytokine vehicle (0.1% bovine serum albumin in RPMI 1640) diluted in distilled H₂O at the same final dilution was applied similarly. Five microliters of ECGS at 20 μg/mL was used as a positive control. Thirty eggs per condition were prepared to allow 50% to 60% mortality inherent to the procedure and to yield a minimum of 8 to 10 available eggs per condition. The angiogenic response was evaluated in a semiquantitative manner, as described previously.13,15 The experiment was repeated twice with similar results.
Statistical Analysis
The Mann-Whitney U test was used for comparisons, and χ² test and Fisher’s exact test were used for contingency tables.

Results
Proinflammatory Cytokine Expression in Temporal Artery Biopsies
IL-1β, TNF-α, and IL-6 transcripts were detected in all GCA specimens by highly sensitive real-time PCR. Patients with disease-related ischemic events had significantly lower IL-6 mRNA concentrations in their lesions than those with no ischemic complications (Figure 2A). Although patients with vascular occlusive events also tended to have lower TNF-α and IL-1β mRNA levels, differences were not statistically significant.

Because cytokine mRNAs have instability sequences, we next evaluated protein expression by immunostaining in a larger series of patients. IL-6 protein expression predominated at the granulomatous area at the media and intima/media junction (Figure 1). The intensity of IL-6 immunostaining was highly variable among patients, even in those samples that showed a similar degree of histopathological involvement as assessed by hematoxylin counterstaining (Figure 1). IL-6 expression was weaker in patients with ischemic events (Figures 1 and 2B). No significant differences were found in TNF-α or IL-1β expression among patients with or without ischemic complications.

Circulating Levels of Proinflammatory Cytokines
Patients with GCA have elevated serum concentrations of IL-6,6,17 and its biological effects may then extend to sites distant from the inflammatory lesions. As shown in Figure 2C, serum IL-6 concentration was also significantly lower in patients with ischemic complications. TNF-α levels also tended to be lower, but the difference was not significant. Circulating IL-1β was undetectable in 76% of the patients with ischemic events and in 75% of the patients without them. No significant differences were found in detectable IL-1β levels in patients with or without ischemic complications (0.59 pg/mL, range 0-5 pg/mL, versus 2.16 pg/mL, range 0.24-20 pg/mL; P=0.58).

Because IL-6 was the proinflammatory cytokine significantly reduced in patients with ischemic events, both in serum and in tissue, we hypothesized that IL-6 might have direct effects on vascular components that may help to prevent vessel occlusion or compensate for ischemia. We have previously shown that patients who do not develop ischemic complications, besides having a more remarkable acute-phase response,3 have more prominent neovascularization in lesions and stronger angiogenic activity in their serum.13 On the basis of these observations, we next tested the effect of IL-6 on endothelial cell biological responses related to angiogenesis, such as endothelial cell growth and differentiation.

IL-6 Induces a Functional Angiogenic Program on Endothelial Cells
At the range of concentrations found in sera from patients with GCA,6 recombinant IL-6 stimulated early-passage endothelial cell growth in a dose-dependent manner (Figure 3),

Figure 2. A, IL-1β, TNF-α, and IL-6 mRNA concentration in temporal arteries from 31 GCA patients with and without ischemic complications, as determined by real-time quantitative PCR. B, Immunostaining scores for IL-1β, TNF-α, and IL-6 in temporal arteries from 66 patients with GCA according to presence or absence of ischemic complications. C, Circulating TNF-α and IL-6 concentrations in sera from 80 patients with GCA with and without ischemic events.
and this effect was abrogated by the addition of recombinant sIL-6R. At later passages (>4), the growth-stimulatory effect of IL-6 was weaker and the inhibitory effect of sIL-6R more prominent (data not shown), which suggests a higher endogenous production of IL-6 by endothelial cells, as has been demonstrated for IL-1α.18

At the same range of concentrations, IL-6 stimulated the morphological differentiation of cultured endothelial cells into capillary-like structures on Matrigel. Tube-forming activity elicited by IL-6 was also inhibited by sIL-6R (Figure 4). These data demonstrate that IL-6 has potent effects on promoting 2 important processes for angiogenesis (proliferation and tube formation) and that the effect is directly on the cells, because sIL-6 can block both of these activities. We next tested whether IL-6 was active ex vivo and in vivo for angiogenesis.

**IL-6 Stimulates Angiogenesis in Ex Vivo and In Vivo Models**

As shown in Figure 5, IL-6 stimulated microvessel sprouting from the adventitia of cultured aortic rings of chick embryos in a dose-dependent manner. Similar results were obtained in a mammalian model, the rat aortic ring assay (data not shown). These models are particularly relevant to our purposes, given that GCA usually involves large arteries. In addition, IL-6 stimulated angiogenesis in the chick chorioallantoic membrane (Table 2). In contrast with the experiments performed with pure endothelial cell cultures, sIL-6R did not exert an inhibitory activity in either ex vivo or in vivo assays. Moreover, in both assays, although quantitative dif-

![Figure 3](image)

**Figure 3.** Effect of recombinant IL-6 on HUVEC proliferation at passage 2. Graph represents optical density (O.D.; mean±SEM) over time in presence and absence of recombinant IL-6 at 10 ng/mL. Growth-stimulatory effect of IL-6 is abrogated by sIL-6R at 100 ng/mL.

![Figure 4](image)

**Figure 4.** Effect of IL-6 at various concentrations on endothelial cell differentiation into capillary-like structures on Matrigel and inhibition by sIL-6R at 100 ng/mL. Graph represents tube area score in triplicate wells (mean±SEM). Data are pooled from 2 experiments.

![Table 2](image)

**Table 2.** Percentage of Eggs With a Positive Angiogenic Response

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Strongly Positive</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>IL-6</td>
<td>77*</td>
<td>23†</td>
</tr>
<tr>
<td>IL-6+sIL-6R</td>
<td>75†</td>
<td>31‡</td>
</tr>
<tr>
<td>ECGS</td>
<td>100</td>
<td>50</td>
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*P=0.0123 vs control; †P=0.0183 vs control; ‡P=0.0162 vs control.
ferences between IL-6 and the combination of IL-6/sIL-6R were not significant (Figure 5; Table 2), the strongest positivity was observed in samples with the combination of IL-6/sIL-6R (data not shown).

Discussion

In this study, performed in a large series of patients, we found that tissue expression of IL-6, both at the mRNA and protein level, and circulating IL-6 levels were significantly lower in patients with vascular occlusive events than in those who never developed ischemic complications. By contrast, differences in concentrations of IL-1β and TNF-α, which are upstream of IL-6 induction in many inflammatory cascades7 and which are in turn downregulated by IL-6,19 were not significant.

IL-6 and other IL-6 superfamily members, such as oncostatin M and leukemia inhibitory factor, are strong inducers of the acute-phase response.7 Our findings are therefore consistent with our previous observation that patients with ischemic events, who, as we show here, are low IL-6 producers, have a weaker systemic inflammatory reaction. However, the mechanisms through which elevated IL-6 production might protect against ischemic events are unknown.

Most of the studies addressing the biological function of IL-6 have focused on its function as an inducer of the acute-phase response,7 its role in the progression from acute to chronic inflammation,20 and its growth-promoting activity in lymphoproliferative disorders.21 Although IL-1β and TNF-α are known to induce thrombotic and fibrinolytic pathways and have effects on vascular tone regulation,10–12 the biologic responses triggered by IL-6 on endothelial cells or other vascular components are not well known, and studies in cell culture systems have led to conflicting results. Endothelial cells are able to produce IL-6, particularly in response to inflammatory stimuli.22 Responsiveness of cultured endothelial cells to exogenous IL-6 in vitro may then depend on the amount of autocrine IL-6 production by endothelial cells, and as suggested by the present results, this may be influenced by tissue culture conditions. Moreover, although endothelial cells have the signaling machinery necessary for IL-6 responsiveness, surface expression of the IL-6 receptor is not always detectable.23 However, it has been demonstrated that complexes formed by IL-6 and its soluble receptor may interact with membrane associated gp130 and transduce intracellular signals leading to STAT3 activation.23 IL-6/sIL-6 receptor complexes have been shown to elicit proinflammatory responses by endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) expression, and production of chemokines, including macrophage chemotactic protein-1 (MCP-1) and IL-8.23 By contrast, and contrary to IL-1 and TNF-α, they do not induce procoagulant activity on endothelial cells.23

The relative affinity of IL-6 for the membrane-associate receptor compared with the soluble receptor has not been investigated. Similarly, the relative efficacy of transducing intracellular signals driven by IL-6 binding to membrane-bound IL-6 receptors with respect to the signaling capacity of the IL-6/sIL-6R complexes is not known. Differences in affinity and signaling capacity may explain the inhibitory effect of sIL-6R on endothelial cell growth and differentiation in culture and the absence of an inhibitory effect in complex systems such as ex vivo and in vivo angiogenesis assays. Moreover, it is possible that in vivo, induction of additional angiogenic molecules (ICAM-1, MCP-1, and IL-8) may act together with IL-6 to induce angiogenesis.24–26 This induction of additional angiogenic molecules would explain why the sIL-6R does not block IL-6–induced angiogenesis ex vivo and in vivo.

It has been suspected that IL-6 might have angiogenic activity. IL-6 is highly expressed by endothelial cells in processes in which angiogenesis is a prominent finding, such as wound healing and Kaposi sarcoma,27,28 and injection of IL-6–expressing transformed cells into athymic mice gives rise to more vascularized tumors.28 Here, we show that IL-6 is able to induce a functional program related to angiogenesis in endothelial cells and that IL-6 induces angiogenesis in ex vivo and in vivo models. The mechanisms by which IL-6 stimulates angiogenesis extend beyond the scope of the present report, but several growth factors with well-recognized angiogenic activity, such as hepatocyte growth factor, epidermal growth factor, and vascular endothelial cell growth factor, transduce signals that lead to activation of the transcription factor STAT3.29,30 STAT3, also phosphorylated by IL-6–driven signaling pathways, has been demonstrated recently to participate in angiogenic responses in vivo.30

Angiogenic activity of IL-6 might be an important compensatory mechanism for ischemia in GCA. In this regard, we have recently shown that patients with GCA who are able to develop a prominent angiogenic response in their lesions and have higher angiogenic activity in their sera have lower prevalence of ischemic complications.13 Moreover, neovascularization is more remarkable in patients with a strong systemic acute-phase response13 who, as we have also shown, are stronger producers of IL-6.6,9 In addition to its compensatory function, angiogenesis has a proinflammatory role in large and medium-sized vessel vasculitis because newly formed vessels are the main site at which adhesion molecules for leukocytes are expressed31 and the main sites through which leukocytes invade the vessel wall, as suggested by the transmigratory phenotype of microvessel-surrounding leukocytes.31,32 We have shown that patients with a strong systemic inflammatory response who have elevated IL-6 production require higher and longer corticosteroid treatment.6,9 IL-6–induced angiogenesis may then contribute to disease perpetuation, given the proinflammatory effects of inflammation-induced angiogenesis.

Paradoxically, IL-6 itself and IL-6–induced molecules such as C-reactive protein have been considered as markers of an increased risk of vascular occlusive events and death in patients with atherosclerotic disease.33,34 However, the inflammatory process in GCA is much more prominent than the slight and smoldering inflammatory component of atherosclerosis,35 and what we have called a weak systemic inflammatory response in GCA patients is much stronger than that found in patients with atherosclerotic disease. In GCA, vascular occlusion is usually produced by intimal hyperplasia, a process that, even though it shares some pathogenic mechanisms with atherosclerosis, is different from plaque
formation and rupture. Elevated levels of C-reactive protein and IL-6 may reflect increased inflammatory activity in atherosclerotic lesions and therefore a more active process that may lead to plaque instability. In addition, IL-6–induced products, such as matrix metalloproteases, or even angiogenesis may contribute to plaque instability and rupture, with its ensuing acute complications. In contrast, highly elevated levels of proinflammatory cytokines or proinflammatory cytokine–induced products such as acute-phase proteins may help to preserve lumen patency or may have a compensatory role by inducing angiogenesis in heavily inflammatory vasculopathies with a high risk of occlusive events, such as GCA.

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