Tissue and Serum Angiogenic Activity Is Associated With Low Prevalence of Ischemic Complications in Patients With Giant-Cell Arteritis

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Background—Vascular inflammatory lesions from patients with giant-cell arteritis show a remarkable amount of neovascularization, but its clinical implications have never been investigated.

Methods and Results—To assess the clinical relevance of neovascularization in giant-cell arteritis, angiogenesis was measured in temporal artery sections from 31 patients with biopsy-proven giant-cell arteritis by staining endothelial cells with Ulex europaeus lectin. Angiogenesis was highly variable among these patients. Patients without ischemic complications had higher tissue angiogenesis scores than patients with ischemic events (5.69±0.6 versus 2.91±0.6, P=0.003). Angiogenesis was also more prominent in patients with a strong acute phase response (score: 5.31±0.6) compared with those with a weak systemic inflammatory reaction (2.30±0.44; P=0.0007). Serum angiogenic activity was studied in an additional series of 38 biopsy-proven patients. Sera from patients without ischemic events tended to be more active in stimulating human umbilical vein endothelial cell growth (optical density ×1000, 270±15 versus 192±14, P=0.065) and differentiation into capillary-like structures (107±5 versus 84±8 relative units, P=0.0058) than patients with ischemic complications. Sera from patients without ischemic events had more in vivo full angiogenic activity tested in the chick chorioallantoic membrane than sera from patients with ischemic complications.

Conclusion—Inflammation-induced angiogenic activity may play a compensatory role for ischemia in patients with giant-cell arteritis. (Circulation. 2002;106:1664-1671.)

Key Words: angiogenesis ■ inflammation ■ vasculature

Giant-cell arteritis (GCA) is a chronic granulomatous disease preferentially involving large and medium-sized arteries. Vascular inflammation may eventually lead to vessel occlusion, which results in ischemia of the supplied tissues. The most frequent ischemic complication in giant-cell arteritis is visual loss due to anterior ischemic optic neuropathy or, less frequently, central retinal artery occlusion. Total or partial permanent visual impairment occurs in about 16% of patients. Occasionally, stroke, lingual ischemia, or scalp necrosis may also occur. Ischemic manifestations in extracranial territories are rare, but disease-related myocardial infarction and limb or mesenteric ischemia have been reported. We have previously shown that cranial ischemic events frequently appear relatively early in the course of the disease and tend to cluster in certain patients, suggesting that these individuals would be more prone to suffer ischemic complications when developing GCA. In addition, we and others have reported that patients with cranial ischemic complications frequently have a less intense acute-phase response than patients without ischemic events.

Tissue ischemia derived from vessel occlusion may be at least partially compensated by neovascularization. Angiogenesis, which is new vessel formation, is a crucial process in a variety of pathological conditions. In some instances, such as tumor dissemination and chronic inflammatory diseases like rheumatoid arthritis, angiogenesis may contribute to disease progression. In vascular disorders leading to vessel occlusion, such as coronary artery disease or peripheral arteriopathy, neovascularization may have a compensatory role. In fact, patients with coronary artery disease who develop efficient neovascularization have less devastating myocardial infarcts when coronary arteries become occluded. Induction of angiogenesis by gene transfer or locally delivered soluble angiogenic factors has received a great deal of attention as a potential therapeutic intervention in coronary artery disease and in limb ischemia.
The role of angiogenesis in vasculitis has begun to be investigated. We have shown that angiogenesis is observed in vascular inflammatory lesions of patients with GCA,\textsuperscript{13–15} polyarteritis nodosa,\textsuperscript{16,17} and cryoglobulinemic vasculitis,\textsuperscript{18} and that sera from patients with different types of vasculitis, including Wegener’s granulomatosis, Takayasu’s disease, and GCA, have angiogenic activity.\textsuperscript{19}

We have proposed that angiogenesis may have a dual role in vasculitis.\textsuperscript{13,14,19} On one hand, new vessel formation may compensate for ischemia in target organs. On the other hand, neovessels are the main site where adhesion molecules for leukocytes are expressed in GCA and polyarteritis nodosa lesions and, therefore, they seem to be the main sites through which circulating leukocytes are recruited into vascular inflammatory lesions.\textsuperscript{15,17} Thus, angiogenesis may also have a proinflammatory role in vasculitis.

The clinical implications and consequences of angiogenesis in vasculitis have never been addressed. In the present study, we analyzed the relationship between the magnitude of inflammation-driven angiogenesis in temporal artery biopsies from patients with GCA and the development of ischemic complications. Because angiogenesis was evaluated in temporal artery specimens and ischemia usually occurs at distant sites supplied by small branches, we also investigated whether angiogenic activity is a generalized response that extends beyond the boundaries of medium-sized cranial arteries. In this regard, we investigated serum angiogenic activity in an additional series of patients to evaluate its relationship to the development of ischemic events.

Methods

Patients

The study group consisted of 69 patients (19 males and 50 females with an average age of 75 years [range: 57 to 91]) with biopsy-proven GCA. Tissue measurement of angiogenesis was performed in 31 patients (group 1). The results obtained prompted us to subsequently analyze serum angiogenic activity in an additional series of 38 patients with biopsy-proven GCA and active disease in whom serum samples were prospectively collected at the time of diagnosis (group 2). Sera from 11 healthy donors (4 males and 7 females) aged 70.6±8.6 years were also obtained. All samples were stored at −80°C until used.

Clinical findings were prospectively recorded and were comparable in both groups of patients (Table). Special attention was paid to the presence and characteristics of cranial ischemic events and to the intensity of the systemic inflammatory response, which was evaluated according to the presence or absence of fever (including low-grade fever, >37°C), weight loss, <5 kg, hemoglobin <110 mg/dL, and an erythrocyte sedimentation rate ≥85 mm/h. Patients were considered to have a weak acute phase reaction when they met 0 or 1 inflammatory parameters, a moderate acute-phase reaction when they met 2, and a strong acute phase reaction when they met 3 or 4 inflammatory parameters. According to this definition, 22 patients (9 in group 1 and 13 in group 2) had a weak systemic inflammatory response, 17 patients (8 in group 1 and 9 in group 2) had a moderate systemic inflammatory response, and 30 patients (14 in group 1 and 16 in group 2) had a strong systemic inflammatory response. Twenty-nine patients (14 in group 1 and 15 in group 2) suffered permanent or transient disease-related cranial ischemic events (Table). Among patients with ischemic events, 15, 9, and 5 had a low, moderate, and strong systemic inflammatory response, respectively.

Quantitation of Angiogenesis in Temporal Artery Biopsies

Thirty-one temporal artery samples from the patients described above were snap-frozen in isopentane prechilled in liquid nitrogen and stored at −80°C until processing. All frozen temporal artery fragments studied exhibited inflammatory lesions, supporting the histopathological diagnosis of GCA. Artery samples were classified into 4 categories according to the extent of inflammatory infiltrates: 1 when there were only slight inflammatory infiltrates at the adventitial layer; 2 when there were slight inflammatory infiltrates both at the adventitia and at the intima/media junction; 3 when there were extensive inflammatory infiltrates in both areas but the media was relatively preserved; and 4 when inflammatory changes were panarteritic, involving the 3 layers of the artery wall.

Cryostat sections (4 to 6 µm thick) were obtained from each sample, air-dried, and fixed in cold acetone. Endothelial cells were identified with biotinylated Ulex europaeus lectin (Dako, Carpenteria, Calif) and detection was performed with streptavidin coupled to peroxidase (Dako). Peroxidase activity was visualized by 0.02% 3,3′-diaminobenzidine (Sigma) and 0.05% hydrogen peroxide. Ten normal temporal arteries from patients in whom giant-cell arteritis was initially suspected but not confirmed were used as controls. These included 2 males and 8 females with an average age of 73 years (range 55 to 84). Their definitive diagnoses were pneumonia (1 patient), isolated polymyalgia rheumatica (2 patients), systemic vasculitis sparing the temporal artery (1 patient), non-vasculitic ischemic optic neuropathy (2 patients), non-specific headache in a patient with osteoarthritis (1 patient), multiple myeloma (1 patient), self-limited prolonged fever (1 patient), and multifactorial chronic anemia (1 patient).

We designed a semi-quantitative score for angiogenesis measurement. We estimated the number of layers in which microvessels were distributed. The score was constructed by adding the ratio of the artery circumference occupied by microvessels in each layer. Mi-
crovessels were measured in 3 sections per individual and the total value was considered.

The degree of intimal hyperplasia was also quantified as follows: 0 when the thickness of the intimal layer was less than 25% of the distance spanning from the center of the lumen to the internal elastic lamina; 1 when it was between 25% and 50%; 2 when it was between 50% and 75%; 3 when it was more than 75%; and 4 when the lumen was virtually occluded. Scoring was performed by 2 independent observers (Drs Cid and Esteban) blinded to clinical data.

**Endothelial Cell Culture**
Human umbilical vein endothelial cells (HUVECs) were obtained from freshly delivered cords by collagenase A digestion (Worthington Biochemical Co, Lakewood, NJ) as described. The growth medium consisted of RPMI 1640 supplemented with 20% iron-fortified bovine calf serum (Hyclone Laboratories), 200 µg/mL endothelial cell growth supplement (ECGS) (Collaborative Research), 100 µU/mL penicillin-streptomycin, 50 µg/mL gentamicin, 2.5 µg/mL amphotericin B, 2 mmol/L glutamine (Invitrogen), and 5 U/mL sodium hyaluronate (Fisher Scientific). Cells were grown until confluence, passed at a ratio of 1 to 4, and used for experiments after the third or fourth passages.

**Endothelial Cell Growth Assay**
Confluent HUVECs were released with trypsin-EDTA (Invitrogen) and resuspended in serum-free growth medium. Cells were plated in flat-bottom 96-well plates at 5000 cells/well, supplemented with 5% human serum from either patients or controls, and incubated at 37°C in 5% CO₂ for 1 to 5 days. The supernatant fluid was then 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 using 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. After 5 days of culture, cells were confluent in all conditions. Preliminary experiments were performed throughout the 5-day culture to assess the best discriminative time-point, which was found to be day 3. Experiments comparing patients and controls and subgroups of patients were subsequently evaluated at day 3. Each data point was tested in four wells. The experiment was repeated 3 times and a representative experiment is shown.

**Endothelial Cell Differentiation Into Capillary-Like Structures**
Forty-eight well plates were coated with Matrigel (Becton-Dickinson) 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 serum-free medium, and plated onto the Matrigel-coated wells at 15 000 cells per well. Sera from either patients or controls at 2% concentration were added to duplicate wells. After an 18-hour incubation, tubes were fixed and stained with Diff-Quik (Dade Behring), and the total tube area in duplicate wells was measured with an image analysis computer program (National Institutes of Health ImageJ). The experiment was repeated 3 times with comparable results.

**Chick Chorioallantoic Membrane In Vivo Angiogenesis Assay**
A window was opened in shells from 3-day fertilized eggs after removing 5 mL of ovoalbumin through a pinhole. The window was covered with clear tape and eggs were incubated at 37°C for 7 additional days, with daily monitoring to discard dead embryos. Five µL of either control or patient serum was driven on quartered Thermanox coverslips (Nunc), applied onto the chick chorioallantoic membrane (CAM) of 10 day-old chick embryos, and incubated at 37°C for 3 additional days.

We tested 16 individuals in the CAM assay: 4 healthy controls, 6 GCA patients with ischemic events and a low acute phase reaction, and 6 patients with prominent systemic inflammatory response and no ischemic events. Fifteen eggs per patient were prepared to allow for the 30% to 40% mortality inherent to the procedure and to yield a minimum of 8 to 10 available eggs per patient. As a negative control, 5 µL of distilled H₂O was applied similarly. Five µL of ECGS (Collaborative Research) at 1 mg/mL was used as positive control. Preliminary experiments were carried out with various serum volumes and dilutions, and we found that 5 µL of undiluted serum was able to discriminate between patients and controls.

To enhance visual contrast, 30% whipping cream diluted in distilled H₂O was injected into the CAM before examination under a magnifying lens. About 30% of H₂O-treated eggs developed some angiogenic reaction, probably because of manipulation, whereas about 80% of the ECGS-treated eggs displayed a clear angiogenic response.

The angiogenic response was evaluated in a semiquantitative manner. Eggs were considered positive (+) when vessel formation was observed under the sample and were considered strongly positive (+++) when the surface covered by neovessels extended beyond the limit of the dried serum drop.

**Statistical Analysis**
The Mann-Whitney U test was used for statistical analysis. Correlations were calculated with the Spearman correlation coefficient, and the χ² test for trend was used with contingency tables.

**Results**

**Pathological Characteristics**
Histopathological evaluation showed that 2 samples had an inflammatory score of 1, 9 biopsies had a score of 2, 8 arteries had a score of 3, and 12 had a score of 4. Four arteries had no intimal hyperplasia, 2 had a score of 1, 6 had a score of 2, 17 had a score of 3, and 2 had scores of 4. The degree of intimal hyperplasia correlated significantly with the extent of inflammatory infiltrates (r = 0.4, P = 0.028) and tended to be most prominent in panarteritic arteries.

**Angiogenesis in Temporal Artery Biopsies From Patients With GCA**
With Ulex europaeus staining, only a few vasa vasorum were observed at the adventitial layer in normal temporal arteries, and no microvessels were observed within the vessel wall. Neovessels were invariably observed in temporal artery biopsies from patients with GCA and could be identified at the adventitial layer, at the intima/media junction, and within the granulomatous reaction.

The amount of neovessels was highly variable among patient samples (Figure 1). The angiogenesis score correlated with the degree of intimal hyperplasia (r = 0.45, P = 0.012) suggesting, as indicated by other authors, that local conditions, such as hypoxia at the inner part of the artery, might contribute to angiogenesis induction, particularly at the intima/media junction.

Even though ischemic complications usually occur at sites distant from the temporal artery where angiogenesis was measured, angiogenesis scores were significantly lower in GCA patients with disease-related ischemic complications.
compared with those who did not have ischemia (2.91 ± 0.6 versus 5.69 ± 0.6, P = 0.003; Figure 1 and Figure 2A), suggesting that the ability to develop an efficient neovascularization was a generalized capacity that was not dependent on local factors. Supporting that concept was the fact that angiogenesis scores were higher in individuals with strong acute phase response (5.31 ± 0.6) than in patients with low systemic inflammatory reaction (2.30 ± 0.44, P = 0.0007; Figure 2B) who, as we have previously shown, are at higher risk of developing ischemic events.3

**Effect of Sera From Patients With GCA on Endothelial Cell Growth**

To test the hypothesis that the capacity of GCA patients to develop a strong inflammation-induced angiogenic response was not only determined by local conditions but was also a more generalized property, we functionally tested serum for angiogenic activity in an additional series of patients.

Sera from patients with GCA stimulated endothelial cell growth more rapidly than sera from healthy controls (optical density ×1000, 270 ± 15 versus 192 ± 14, P = 0.038). This effect was not specific for human endothelial cells because a similar but less significant trend, was observed with rat fibroblasts (data not shown). Sera from patients with ischemic events tended to stimulate endothelial cell growth less efficiently than sera from patients without ischemic complications, but the difference was not significant (234 ± 19 versus 292 ± 20, P = 0.065). No significant differences in proliferation activity were observed between patients with weak or strong acute phase reaction (232 ± 30 versus 287 ± 23, P = 0.3).
Effect of Sera From Patients With GCA on Endothelial Cell Differentiation Into Capillary-Like Structures

In addition to endothelial cell proliferation, angiogenesis requires other biological activities, such as cell migration, protease secretion, and differentiation into tubular structures with cell polarization and a functional lumen. All these processes are represented in tube-forming activity elicited by the extracellular matrix substrate Matrigel. When plated on Matrigel and in complete growth medium containing 20% bovine calf serum, endothelial cells differentiate into capillary-like structures in 18 hours. At low serum (2%) concentration, tubes do not form a complete and interconnected network (Figure 3A). As we have previously shown, tube forming activity was stimulated by sera from patients with GCA compared with sera from healthy controls (99.4 ± 7.2 relative units versus 77.4 ± 7.2, P = 0.023; Figure 3 and Figure 4). Capillary-like differentiation was increased further by sera from patients who did not present ischemic complications (107.5 ± 5.2 versus 84.8 ± 8.4, P = 0.0058; Figure 4). Sera from patients with a strong acute-phase response tended to stimulate tube formation more efficiently than patients with a weak or moderate systemic inflammatory reaction, but the difference was not significant (107.5 ± 7.2 versus 93.5 ± 5.2, P = 0.086).

Angiogenic Activity in Sera From Patients With GCA

Because sera from patients with GCA stimulated growth and differentiation of endothelial cells in vitro, we next tested whether these sera had complete angiogenic activity in an in vivo model. Sera from GCA patients exhibited full angiogenic activity in the chick CAM assay (Figure 5). As shown in Figure 5 and Figure 6, angiogenic activity was reduced in patients with ischemic events and a weak acute phase response, whereas sera from patients with no ischemic complications and a strong systemic inflammatory response induced an intense angiogenic reaction. A strong angiogenic response, as defined in the Methods section, was observed in 10.7% of the control sera, 17.6% of sera from patients with ischemic events and weak acute phase response, and in 30.8% of sera from patients without ischemic events.

Discussion

Angiogenesis is an important process in chronic inflammatory diseases, where it is thought to exert a potent proinflammatory function. Indeed, neovascularization provides oxygen and nutrient supply to the highly demanding metabolic activity present in inflammatory foci. In addition, growing vessels produce colony-stimulating factors, which have been demonstrated to be able to prolong the half-life of infiltrating leukocytes. Newly formed vessels express adhesion molecules for leukocytes and release chemokines, amplifying leukocyte recruitment into inflammatory infiltrates. Angiogenesis, then, seems to have an active and crucial role in the maintenance, amplification, and perpetuation of chronic inflammatory diseases, and angiogenesis inhibition has been considered as a potential therapeutic strategy in these disorders.

We have proposed that angiogenesis may play a dual role in vasculitis which, in this regard, is unique among chronic inflammatory disorders. Neovessels are the main site where endothelial cell adhesion molecules for leukocytes are expressed in medium-sized and large vessel vasculitis such as...
polyarteritis nodosa and GCA.\textsuperscript{15,17} In addition, in both diseases, leukocytes surrounding adventitial neovessels have a migratory phenotype, suggesting that neovessels are actively recruiting leukocytes into inflammatory lesions.\textsuperscript{15,17} Therefore, as in other chronic inflammatory diseases, angiogenesis seems to have a proinflammatory role in vasculitis. In vasculitis, however, vascular inflammatory lesions eventually lead to vessel occlusion with resulting ischemia of supplied tissues. New vessel formation may then have a beneficial and compensatory role and may help to prevent irreversible tissue damage in ischemic organs. According to this hypothesis, we have previously shown that angiogenesis inhibition with interferon-\(\gamma\) may have devastating effects on ischemic lesions from patients with hepatitis C virus-related cryoglobulinemic vasculitis.\textsuperscript{18}

Using in vitro and in vivo models, we showed that a strong angiogenic response in temporal artery samples and strong angiogenic activity in serum are associated with a reduced prevalence of ischemic complications in patients with GCA, supporting the concept that the angiogenic response has a significant compensatory role in this disease. The angiogenic response in temporal arteries may not be functionally relevant in compensating for ischemia, but neovascularization may be crucial at distal sites in target organs. We have previously shown that small cranial vessels are almost invariably involved in GCA\textsuperscript{26} and, in fact, the most frequent ischemic complication, anterior ischemic optic neuritis, results from occlusion of small arteries supplying the optic nerve.

Angiogenesis is a complex process requiring a series of coordinated steps. Endothelial cells from the parental vessel must degrade the basement membrane, proliferate, migrate through the underlying matrix, and differentiate into tubular structures surrounded by their own new basement membrane.\textsuperscript{7,8,27} A wide array of molecules are crucial in this process, including growth factors stimulating cell proliferation, stabilizing factors such as angiopoietin-1, chemotactic factors stimulating vectorial motility, surface receptors (mainly integrins) for specific sequences in extracellular matrix proteins, and a variety of soluble and membrane-associated proteases.\textsuperscript{7,8,27–31} Angiogenic activity results from the net balance between angiogenic stimulators and angiogenesis inhibitors. Many of these products are produced by macrophages in inflamed tissues, but other cells participating in the inflammatory process, such as granulocytes, mast cells, lymphocytes, platelets, and endothelial cells themselves may also contribute. Several angiogenic factors, including growth factors such as vascular endothelial growth factor, fibroblast growth factor-2, transforming growth factor-\(\beta\), platelet-derived
growth factor, and tumor necrosis factor-α, and chemokines such as monocyte chemotactic protein-1 and interleukin-8 are expressed in GCA inflammatory lesions and might contribute to the angiogenesis observed in the arterial wall (Cid et al, unpublished data, 2002). In addition, elevated levels of angiogenic factors such as vascular endothelial growth factor, fibroblast growth factor-2, and soluble intercellular adhesion molecule-1 are elevated in serum from patients with GCA (Cid et al, unpublished data), but their clinical significance is not known. The presence and the potential counterbalancing role of inhibitory factors have not been investigated in GCA.

Tissue neovascularization and serum angiogenic activity were more prominent in patients with a strong acute phase response. It is interesting that among the endothelial biological activities tested, proliferation was not significantly associated with the intensity of the systemic inflammatory response. We have shown that patients with an intense acute phase response have more elevated levels of tumor necrosis factor-α and interleukin-6. Although tumor necrosis factor-α may be angiogenic in vivo by stimulating other angiogenic responses such as protease production or by activating other cell types, it inhibits proliferation of cultured endothelial cells. We have previously shown that GCA patients with an intense acute phase reaction are less prone to develop ischemic events but are more refractory to therapy, requiring longer duration of treatment and receiving higher cumulative steroid doses. These observations support both the compensatory and proinflammatory role of neoangiogenesis in GCA. The reason why a strong inflammatory response is associated with low prevalence of ischemic events is unknown. As suggested by the present study, concomitant induction of angiogenic factors may be one of the reasons. Both the acute phase response and the release of angiogenic factors may also be downstream events induced by other factors that may influence vascular tone and lumen patency. The acute phase response, driven by proinflammatory cytokines, is a highly conserved aspect of innate immune mechanisms addressed to avoid excessive tissue destruction. In this regard, it is consistent that the acute phase response is associated with angiogenic activity that may be additionally modulated in damaged tissues by local factors regulating receptor expression or signaling pathways. In fact, one of the most typical acute phase proteins, haptoglobin, which is elevated in patients with GCA, has remarkable angiogenic activity.

Therapeutic manipulation of angiogenesis is a focus of active research in many diseases, and some angiogenic and anti-angiogenic factors are the subjects of clinical trials. Angiogenesis modulation is an interesting therapeutic approach in systemic vasculitis. According to our results, angiogenesis inhibition may be harmful in early steps of the disease, when ischemic complications tend to occur, but may have therapeutic usefulness in patients with long-lasting disease in whom angiogenesis blockade may disrupt inflammatory cascades leading to perpetuation of inflammatory lesions.

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