Regulació de la producció de gelatinases (MMP2 i MMP9) pels limfòcits.

Implicació en malalties inflamatòries i síndromes limfoproliferatives

Tesi presentada per

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per a optar al grau de Doctora en Bioquímica

per la Universitat de Barcelona

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ANNEX

ANNEX

Treballs addicionals en el camp de les malalties inflamatòries i de les síndromes limfoproliferatives relacionats amb el tema de la tesi en els quals he participat:

ARTICLES ORIGINALS

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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±2.1 versus 24±2.4 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

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TABLE 1. Ischemic Events in 33 Patients With GCA

Ischemic Event	Clinical Characteristics (n)	Objective Abnormality (n)
Permanent		
Visual loss	Monocular blindness (11) Binocular blindness (3) Campimetric defects (4)	AION (14) Retinal ischemia (1) Normal funduscopy results (2) Funduscopy not done (1)
IV cranial nerve palsy	Diplopia (1)	Normal MRI results (1)
Stroke	Internuclear ophthalmoplegia (1)† Ataxia and dizziness (1)	Mesencephalus infarct (1)† Mesencephalus, protuberance, and cerebellum infarcts (1)
Transient		
Visual loss	Amaurosis fugax (8)	Normal funduscopy (6) Retinal ischemia (1) Funduscopy not performed (1)
Double vision	Transient diplopia (4)	Normal physical examination (4)
Tongue ischemia	Tongue pain (2)*	Tongue cyanosis (2)*

AION indicates anterior ischemic optic neuropathy.
*One of these patients also developed unilateral AION.
†This patient also developed unilateral AION.

influence vascular responses such as vessel occlusion or regeneration.[10–12]

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,[13] 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 or serum extraction. Preliminary experiments showed no significant differences in any of the parameters examined in the present study between untreated patients and patients who had received just 1 prednisone dose. Temporal artery specimens excised for diagnostic purposes from the above patients were embedded in OCT, rapidly frozen in isopentane prechilled in liquid nitrogen, and stored at −80°C until processing.

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.[9] 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).[9] 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.

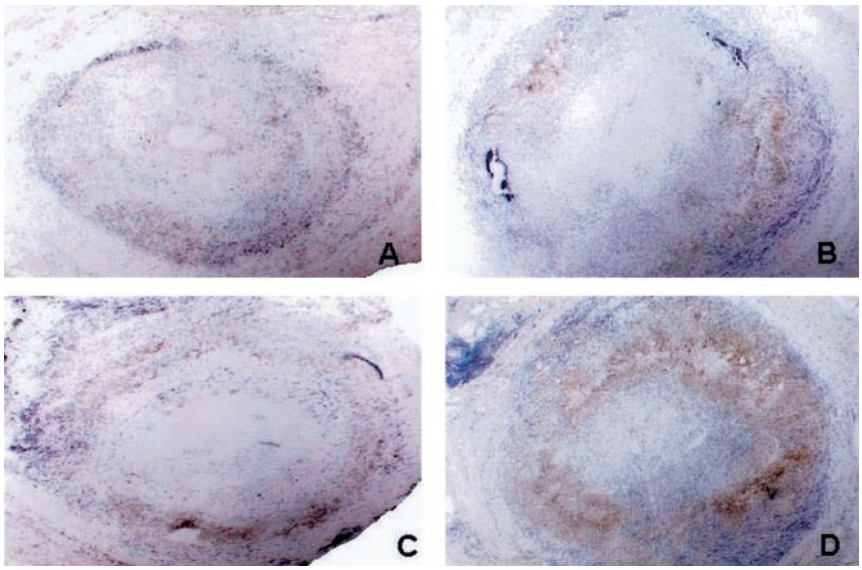

Figure 1. Immunostaining score for IL-6 expression as defined in Methods. A represents score 1; B, score 2; C, score 3; and D, score 4. A and B are temporal artery sections from patients with disease-related ischemic events. C and D are sections from patients without ischemic complications.

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 medium[13] 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% CO_2 for 1 to 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 H_2O 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, and a representative experiment is shown.

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% CO_2 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 H_2O, dried on quartered Thermanox 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 H_2O at the same final dilution was applied similarly. Five microliters of ECGS at 20 μg/μL 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] The experiment was repeated twice with similar results.

Statistical Analysis

The Mann-Whitney U test was used for comparisons, and χ^2 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,[16] 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 to 5 pg/mL, versus 2.16 pg/mL, range 0 to 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),

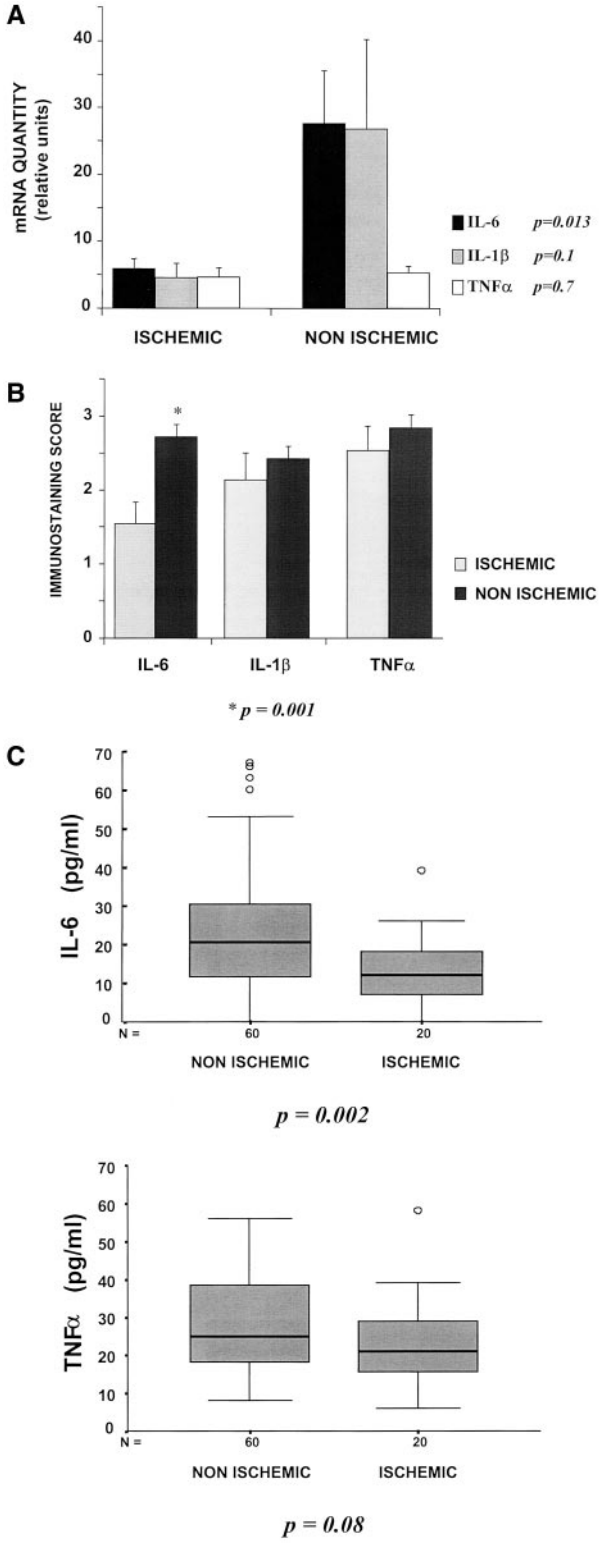

Figure 2. A, IL-1β, TNF-α, and IL-6 mRNA concentration in temporal arteries from 31 GCA patients with and without ischemic complications, as determined by real-time quantitative PCR. B, Immunostaining scores for IL-1β, TNF-α, and IL-6 in temporal arteries from 66 patients with GCA according to presence 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.

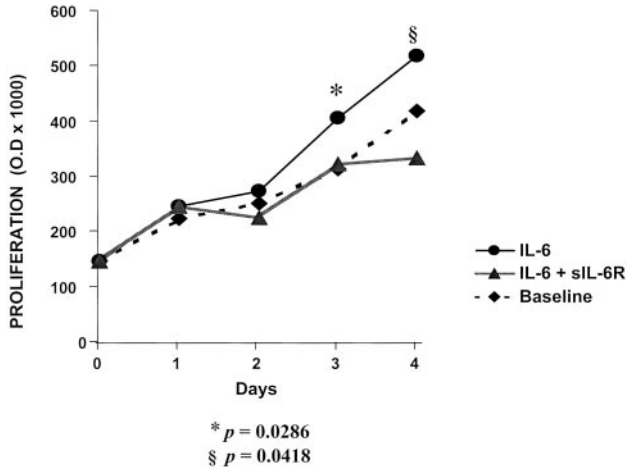

Figure 3. Effect of recombinant IL-6 on HUVEC proliferation at passage 2. Graph represents optical density (O.D.; mean±SEM) over time in presence and absence of recombinant IL-6 at 10 ng/mL. Growth-stimulatory effect of IL-6 is abrogated by sIL-6R at 100 ng/mL.

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.

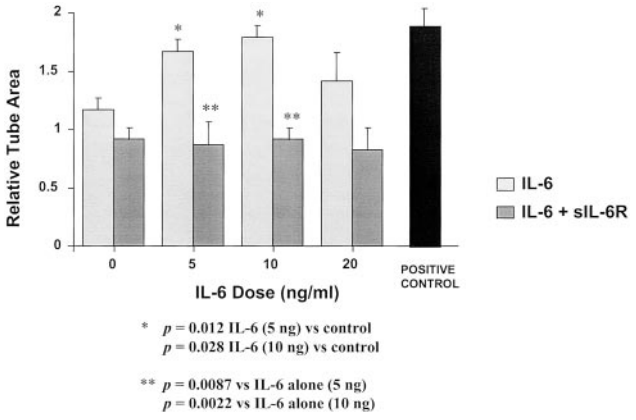

Figure 4. Effect of IL-6 at various concentrations on endothelial cell differentiation into capillary-like structures on Matrigel and inhibition by sIL-6R at 100 ng/mL. Graph represents tube area score in triplicate wells (mean±SEM). Data are pooled from 2 experiments.

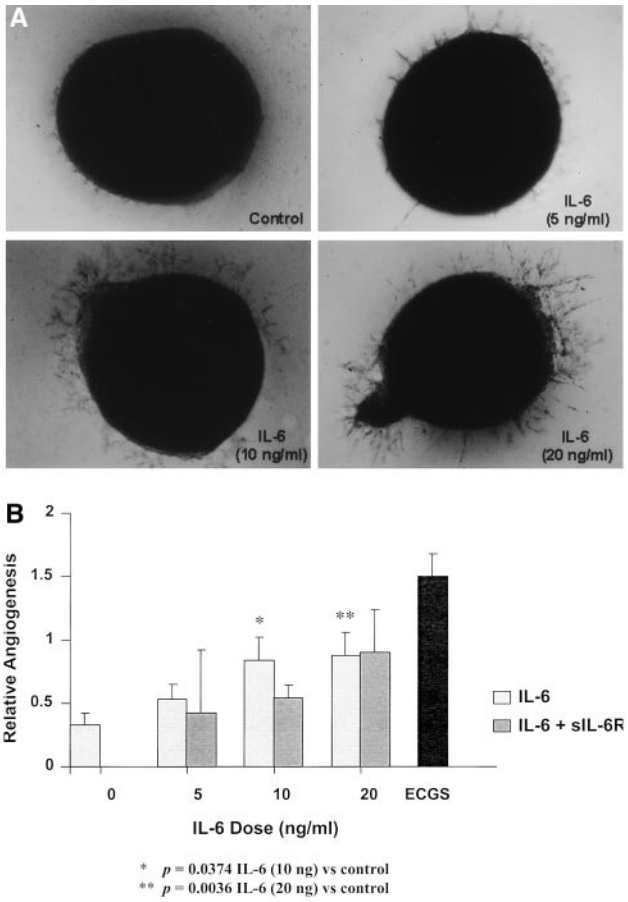

Figure 5. A, Effect of IL-6 at various concentrations on capillary sprouting from chick embryo aortic rings. B, Quantification (mean score±SEM) of capillary sprouting in 6 rings per condition. Data are pooled from 2 experiments.

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-

TABLE 2. Percentage of Eggs With a Positive Angiogenic Response

	Positive	Strongly Positive
Control	27	0
IL-6	77*	23‡
IL-6+sIL-6R	75†	31‡
ECGS	100	50

*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 cascades[7] 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 influence 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 response[13] 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 expressed[31] 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.[1,2,35] 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.[35] In contrast, highly elevated levels of proinflammatory cytokines or proinflammatory cytokine–induced products such as acute-phase proteins[15] 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.

Acknowledgments

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Tissue production of pro-inflammatory cytokines (IL-1β, TNFα and IL-6) correlates with the intensity of the systemic inflammatory response and with corticosteroid requirements in giant-cell arteritis

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Objectives. **To investigate proinflammatory cytokine expression in temporal arteries from patients with giant-cell arteritis (GCA) and to analyse its relationship with the intensity of the initial systemic inflammatory reaction and response to corticosteroid therapy.**

Methods. **Quantification of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and interleukin-6 (IL-6) mRNA by real-time quantitative PCR in temporal artery samples from 36 patients with biopsy-proven GCA and 11 controls. Immunohistochemical detection of IL-1β, TNFα, and IL-6 in temporal artery sections from 74 patients with GCA and 15 controls. Clinical and biochemical parameters of inflammation as well as the time (weeks) required to reach a maintenance prednisone dose < 10 mg/day were recorded.**

Results. **IL-1β (13.8 ± 2.5 *vs* 5.4 ± 1.3 relative units, $P = 0.012$) and IL-6 transcripts (34 ± 13.7 *vs* 7.8 ± 4.5 relative units, $P = 0.034$) were significantly more abundant in patients with a strong systemic inflammatory response compared with those with no inflammatory parameters. Immunohistochemical scores for IL-1β (2.7 ± 0.3 *vs* 1.9 ± 0.2, $P = 0.018$), TNFα (3.2 ± 0.2 *vs* 2.4 ± 0.3, $P = 0.028$) and IL-6 (3 ± 0.2 *vs* 2.1 ± 0.3, $P = 0.023$) were also significantly higher in patients with strong systemic inflammatory reaction. A significant correlation was found between the amount of tissue TNFα mRNA and the time required to reach a maintenance dose of prednisone < 10 mg/day ($r = 0.586$, $P = 0.001$).**

Conclusion. **GCA patients with a strong systemic inflammatory response, who have been previously shown to be more resistant to corticosteroid therapy, have elevated tissue expression of proinflammatory cytokines IL-1β, TNFα and IL-6. High production of TNFα is associated with longer corticosteroid requirements.**

KEY WORDS: Giant-cell arteritis, Vasculitis, Inflammation, Cytokines, Acute phase response.

Giant-cell arteritis (GCA) is a large vessel granulomatous vasculitis preferentially involving large and medium-sized vessels. Although vascular inflammatory lesions can be widespread, most of the classical clinical manifestations of GCA are derived from symptomatic involvement of the carotid artery branches [1, 2]. In addition, GCA is a disease characterized by a strong acute-phase response. About 50% of patients experience fever and 60% weight loss [2]. Chronic anaemia is common and an elevated serum concentration of acute-phase proteins leading to an accelerated erythrocyte sedimentation rate (ESR) is so frequent that it has been considered one of the clinical criteria for the classification of GCA [3]. However, the intensity of the systemic inflammatory response is quite variable among patients. Patients with no constitutional symptoms and a normal or close to normal ESR have been repeatedly reported [4, 5].

We have previously shown that the intensity of the systemic inflammatory response defines clinically relevant subgroups of patients with different prognoses. For incompletely understood reasons, patients with a strong acute-phase response are at low risk of developing vascular occlusive events but are more refractory to therapy, requiring higher cumulative steroid doses and a longer duration of treatment [6, 7]. The mechanisms regulating the intensity of the systemic inflammatory response in GCA have not been investigated.

Accumulated experience in experimental settings and in human disease indicates that the systemic inflammatory response is triggered by pro-inflammatory cytokines, the best studied being interleukin 1 (IL-1), tumour necrosis factor (TNFα) and IL-6 [8, 9]. These cytokines have profound effects on numerous cell types that, in turn, secrete a variety of inflammatory mediators, creating complex networks of interactions and leading to multiple inflammatory cascades [10, 11]. In addition, IL-1 and TNFα have potent effects on vessel wall components, particularly endothelial cells, and may influence mechanisms involved in vessel occlusion and repair [12, 13]. Pro-inflammatory cytokines IL-1, TNFα and IL-6 are known to be produced in temporal artery lesions from patients with GCA [14–18], but the clinical impact of cytokine tissue expression has not been investigated.

The aim of our study was to measure IL-1β, TNFα and IL-6 production in temporal artery samples from a large series of

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patients with biopsy-proven GCA in order to investigate the relationship between the magnitude of cytokine expression in lesions and clinically relevant findings such as the intensity of the systemic inflammatory reaction, which generates the general feeling of sickness, and response to corticosteroid therapy.

Patients and methods

Patients

The entire study group consisted of 74 patients (25 men and 49 women) with an average age of 77 yr (range 58–91) with biopsy-proven GCA. These patients were consecutively selected from those who had fully developed inflammatory lesions involving the three arterial layers of their temporal arteries. Patients whose samples exhibited only adventitial involvement, even enough to establish the histological diagnosis of GCA, were excluded from the study in order to avoid variations in cytokine expression mostly determined by the density of the inflammatory infiltrates. Sixteen patients (22%) had received prednisone (1 mg/kg per day) for an average of 2 days (range 1–5) and the remaining 58 (78%) were untreated at the time of the temporal artery excision. As control samples we included 15 histologically normal temporal arteries from 15 patients (10 women and 5 men) with an average age of 76 yr (range 52–87) in whom a surrogate diagnosis was obtained. The ultimate diagnoses in these patients were isolated polymyalgia rheumatica (5 patients), non-vasculitic ischaemic optic neuropathy (2 patients), self-limited constitutional symptoms with anaemia (2 patients), temporomandibular osteoarthritis (1 patient), chronic otitis media (1 patient), slowly resolving pneumonia (1 patient), headache associated with persistent pyelonephritis (1 patient) and headache associated with cutaneous infection (2 patients).

Clinical manifestations were prospectively recorded. Special attention was paid to the intensity of the systemic inflammatory response which was evaluated according to the following parameters: fever or low-grade fever (confirmed elevation of body temperature above 37°C), patient-reported weight loss of $\geq$4 kg, ESR $\geq$85 mm/h, and haemoglobin concentration $<$110 g/l, all determined before starting treatment.

These parameters were selected because they have clinical impact, they are objective and readily obtainable at first evaluation [6, 7]. In addition, these items have been demonstrated to be useful in defining clinically relevant subgroups of patients with different outcomes, as demonstrated in previous publications [6, 7]. In these previous studies the number of inflammatory parameters, no matter which, presented by every patient was found to be more clinically significant in classifying patients according to the intensity of the systemic inflammatory response than any individual inflammatory marker [6, 7]. Patients with 0–2 inflammatory parameters were considered to have a weak systemic inflammatory reaction, whereas patients with 3–4 inflammatory markers were considered to have a strong systemic inflammatory response.

All patients were treated by the authors (JHR, MCC and JMG) according to uniform criteria. The initial prednisone dose was 1 mg/kg per day (up to 60 mg/day) for 1 month and was subsequently tapered at a rate of 5–10 mg/week. Reductions below 20 mg/day were slower and individualized. A rate of 2.5 mg every 3 months was attempted. When the ESR rose above 50 mm/h and clinical symptoms appeared or haemoglobin fell below 110 g/l, or when clear and worsening symptoms occurred with a normal or slightly elevated ESR, a disease flare was considered. When the ESR rose with no clinical symptoms or anaemia, the maintenance prednisone dose was held until the ESR returned to normal or a flare could be defined. When a disease flare occurred, prednisone was increased 10 mg above the previous effective dose. At the end of the study, 31 patients had a follow-up long enough to attempt a reduction of the prednisone dose below 10 mg/day. Time (weeks) required to reach a maintenance dose less than 10 mg/day, cumulative prednisone dose at that point, and the number of relapses during the first 18 months of follow-up were recorded.

This study was approved by the Internal Review Board of our institution (Hospital Clínic, University of Barcelona), and all patients signed informed consent.

Cytokine mRNA quantification

RNA isolation. Surgically excised temporal artery biopsies were embedded in optimal cutting temperature (OCT), quickly frozen in isopentane prechilled in liquid nitrogen and stored at –80°C until used. Total RNA was obtained from 100 serial sections (20 μm thick) per sample using the commercially available Micro RNA isolation kit (Stratagene, La Jolla, CA) following the instructions of the manufacturer. In order to construct the standard curves for real-time polymerase chain reaction (PCR), RNA was also isolated from different cell lines using TRIzol® (Gibco, Life Technologies, Gaithersburg, MD). Both procedures are based on the Chomczynski method [19].

cDNA synthesis. Total RNA (1 μg) was reverse transcribed to cDNA using the SuperScriptTM II First-Strand Synthesis kit (Gibco) employing random hexamers as the priming method. Reaction conditions were carried out according to the manufacturer's recommendations. Samples were stored at –20°C until use.

Real-time quantitative PCR. Cytokine mRNAs were measured by real-time PCR using specific Pre-Developed TaqManR Target kits from Applied Biosystems (Foster City, CA) [20]. The cytokine target probe and an 18S ribosomal RNA control probe, used as endogenous control, were covalently linked to a different reporter dye (FAM or VIC). A multiplex PCR reaction was carried out with 1 μl of the temporal artery cDNA sample, TaqManR Universal PCR Master Mix, and primers and probe from the target and the endogenous control in a final reaction volume of 25 μl, as recommended by the manufacturer. Each cDNA sample was tested twice. After an initial denaturation step at 95°C for 10 min, 40 cycles were performed as follows: 95°C for 15 s and 60°C for 1 min. The reaction was monitored by measuring the fluorescence signal after each cycle with ABI PrismTM 7700 Sequence Detection system (Applied Biosystems). For TNFα expression, a standard curve was constructed using serial dilutions of cDNA obtained from the Jurkat cell line; for IL-6, with cDNA obtained from the U-937 cell line; and for IL-1β, with cDNA obtained from peripheral blood mononuclear cells of a healthy human donor stimulated with phorbol myristate acetate and calcium ionophor (Calbiochem, CN Biosciences, Inc., Darmstadt, Germany). Standard curves were constructed by plotting the log of standard dilutions *vs* the threshold cycle (CT) values, CT being the fractional cycle number at which the fluorescence passes a fixed threshold. The cytokine mRNA concentration in arterial samples was calculated referring the sample CT to the standard curve, and normalized with the corresponding value of the endogenous control CT. Values were expressed as relative units.

Immunohistochemical study

Serial 4–6-μm cryostat sections were obtained from frozen temporal arteries of the 74 patients and 15 controls described above. In all cases, one section was stained with haematoxylin and eosin to ensure that all samples included had a similar degree of histopathological involvement. The remaining sections were immunostained with the following antibodies: polyclonal rabbit anti-human TNFα (Genzyme, Minneapolis, MN), monoclonal mouse anti-human IL-1β (clone B1 from Genzyme), and monoclonal mouse anti-human IL-6 (clone 6708.111) from R&D Systems (Minneapolis, MN). Preliminary studies were carried out to determine the optimal concentration of the antibodies

providing the strongest specific staining with the lowest background. Anti-TNFα antiserum was used at a 1/500 dilution and purified monoclonal antibodies anti-IL-1β and anti-IL-6 were used at 10 μg/ml.

Temporal artery sections were air-dried and fixed with cold acetone. After several blocking steps, sections were incubated with the primary antibodies diluted in phosphate-buffered saline for 30 min. Immunodetection was carried out with an avidin–biotin–peroxidase system with the EnVision kit from Dako (Carpinteria, CA), according to the instructions of the manufacturer. Peroxidase activity was visualized by 0.02% 3–3' diaminobenzidine (Sigma, St Louis, MO) and 0.05% hydrogen peroxide. All sections were slightly counterstained with Harris' haematoxylin. In each procedure, previously immunostained arteries were included to ensure consistency and additional sections were incubated with just the secondary antibody and detection system to exclude non-specific binding.

Quantification was performed according to the following semiquantitative scores based on the percentage of cells positively stained at the granulomatous area at the intima–media junction: 0, no staining; 1, $\leq$25%; 2, 26–50%; 3, 51–75%; and 4, 76–100% (Fig. 1). After confirming agreement in the scoring system, cytokine expression in two sections per condition was independently evaluated by three investigators (JHR, AGM and MCC) who were blinded to the patients' clinical information. Slides without optimal immunostaining quality were discarded and the final quantification was performed on 67 cases for IL-1β, 59 cases for TNFα and 54 cases for IL-6.

Statistical analysis

The Mann–Whitney U-test was applied to quantitative data. The χ^2-test for trend was used for contingency tables and Pearson coefficient for correlations.

Results

Pro-inflammatory cytokine expression in GCA lesions

Good-quality tissue RNA was obtained from 36 GCA patients and 11 controls. Using highly sensitive real-time PCR, IL-1β, TNFα and IL-6 transcripts were detected in all GCA specimens but also in control samples, although at lower levels. As shown in Table 1, TNFα and IL-1β transcripts were significantly more abundant in patients than in controls. IL-6 levels were also higher in patients, but the difference was not statistically significant. In the control group of histologically normal temporal artery biopsies, no differences in cytokine mRNAs were found between samples from patients with polymyalgia rheumatica compared with specimens from patients with other diagnoses.

Histologically normal arteries did not exhibit significant immunostaining for pro-inflammatory cytokines. By contrast, IL-1β, TNFα and IL-6 were remarkably expressed in all 74 GCA specimens included. All three cytokines clearly predominated in the granulomatous area at the intima–media junction. Adventitial expression was also observed in most cases. The intensity of cytokine expression was highly variable among patients, even those disclosing a similar extent of inflammatory infiltrates (Figs 1 and 2). As described [21], cells with a macrophage appearance were

Table 1. IL-1β, TNFα and IL-6 mRNA levels (relative units) in temporal artery samples from GCA patients and controls

	Controls ($n=11$)	Patients ($n=36$)	P
IL-1β	7.6 ± 3.3	12 ± 2.1	0.041
TNFα	0.9 ± 0.2	5.1 ± 0.7	< 0.0001
IL-6	13.4 ± 6.2	20.6 ± 5.5	0.36

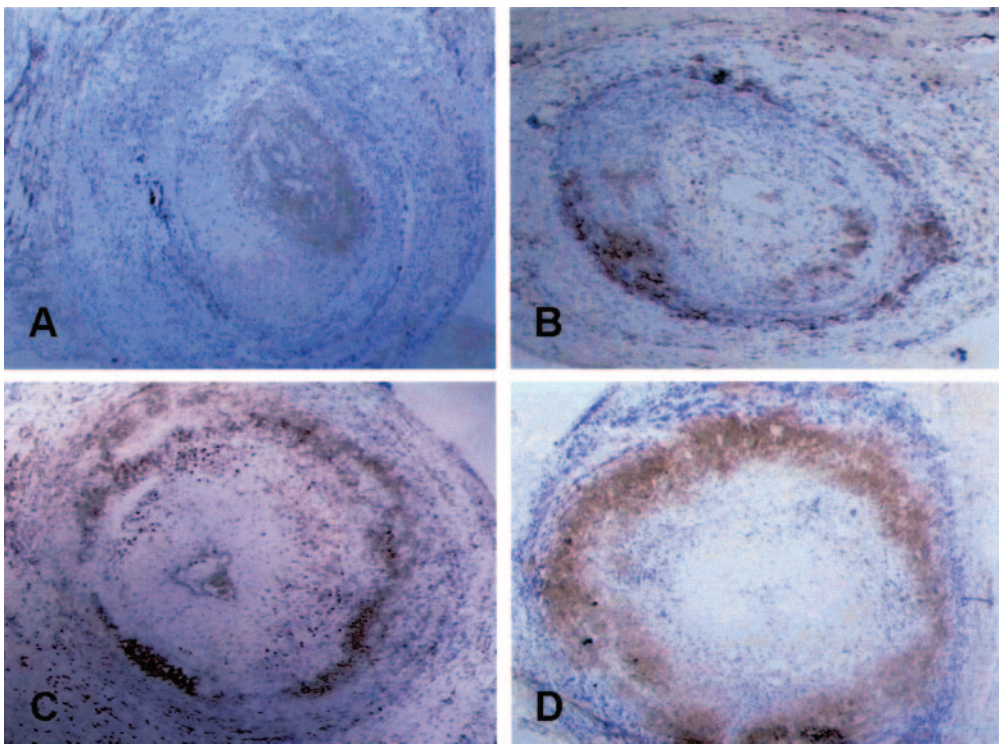

Fig. 1. Immunostaining scoring system. Pictures represent scores for IL-1β expression. (A) Score 1 ($\leq$ 25% positive cells) in a patient with no inflammatory parameters. (B) Score 2 (26–50% positive cells) in a patient with one inflammatory parameter.(C) Score 3 (51–75% positive cells) in a patient with three inflammatory parameters. (D) Score 4 (76–100% positive cells) in a patient with four inflammatory parameters.

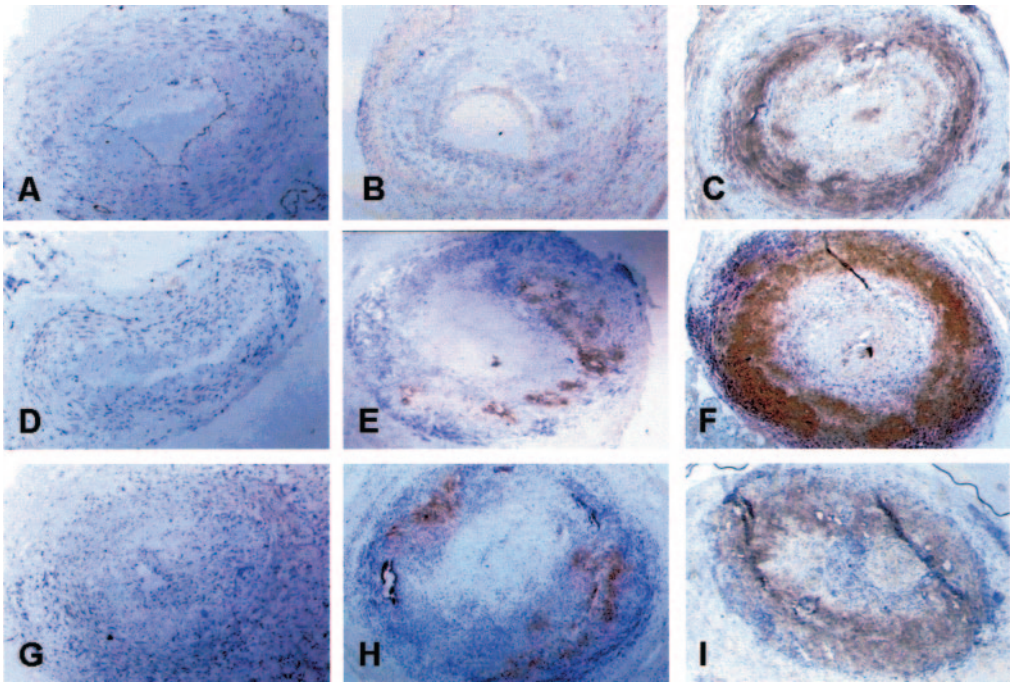

FIG. 2. Immunostaining for IL-1β, TNFα and IL-6 in patients with a weak (B, E, H) and a strong (C, F, I) systemic inflammatory response. A, B and C correspond to IL-1β; D, E and F, to TNFα; and G, H and I, to IL-6 expression. A, D and G are normal temporal arteries.

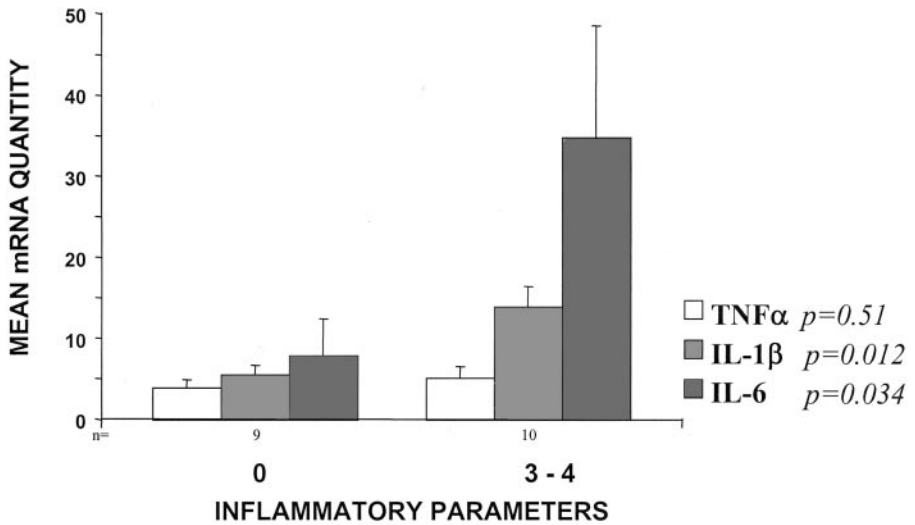

FIG. 3. IL-1β, TNFα and IL-6 mRNA concentrations in temporal artery specimens from patients with GCA according to the number of inflammatory parameters. Bars represent mean ± S.E.M.

the main cell type expressing pro-inflammatory cytokines. In some cases, particularly in those displaying the highest scores, smooth muscle cells, which have been demonstrated to be able to produce pro-inflammatory cytokines [22], also stained positive (Figs 1 and 2).

Correlation between tissue production of pro-inflammatory cytokines and the intensity of the acute-phase response

The amount of tissue cytokine mRNA was different in patients at the edges of the spectrum: patients with a strong systemic inflammatory reaction (3–4 inflammatory parameters) had more abundant IL-1β and IL-6 transcripts compared with those with no inflammatory parameters (Fig. 3). Differences in TNFα mRNA between both groups did not achieve statistical significance. Since cytokine mRNAs have instability sequences at the 3′ untranslated region and significant amounts of cytokine transcripts may not undergo translation into protein [10, 23], we subsequently evaluated protein expression by immunohistochemistry.

Patients with a strong systemic inflammatory response (3–4 inflammatory parameters) had significantly higher scores for all cytokines than patients with no inflammatory parameters (Figs 2 and 4A). When the whole series of patients including the complete spectrum of the intensity of the systemic inflammatory reaction was considered, patients with a strong acute-phase response (3–4 inflammatory parameters) had significantly higher scores for TNFα and for IL-6 than patients with a weak systemic inflammatory reaction (0–2 inflammatory parameters) (Fig. 4B). A similar

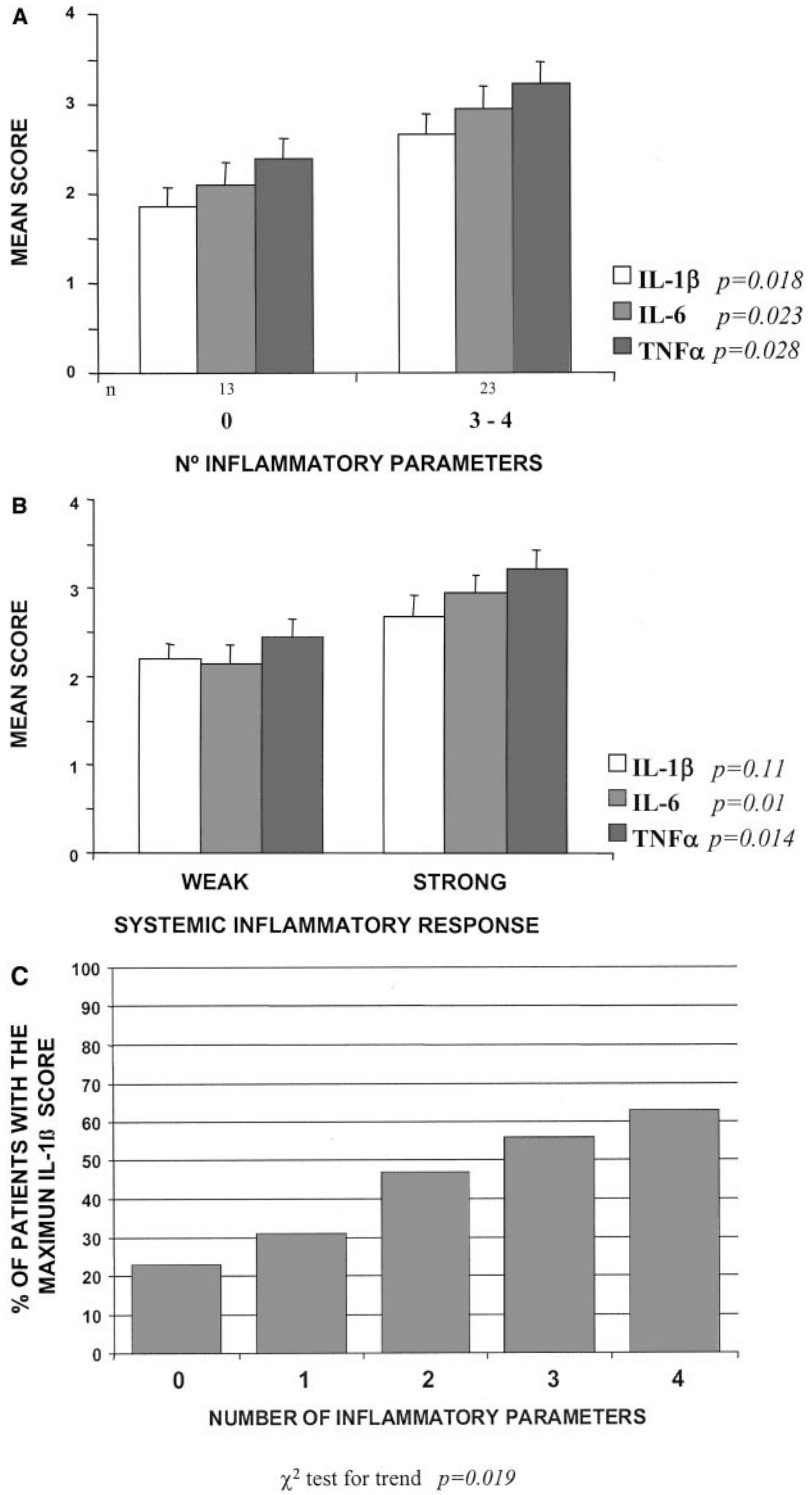

FIG. 4. (A) Immunostaining scores in patients representing the edges of the spectrum: patients with no inflammatory parameters compared with patients with a strong systemic inflammatory response (3–4 inflammatory parameters). (B) Immunostaining scores for IL-1β, IL-6 and TNFα in the whole series of 74 patients with GCA according to the intensity of the systemic inflammatory response (weak: 0–2 inflammatory parameters, strong: 3–4 inflammatory parameters). Bars represent mean $\pm$ S.E.M. (C) Percentage of patients reaching the maximum score for IL-1β, according to the number of inflammatory parameters.

trend was observed for IL-1β expression in the whole series but the difference was not significant. However, as shown in Fig. 4C, the percentage of patients displaying the maximum score for IL-1β increased significantly according to the number of inflammatory parameters.

Correlation between cytokine expression and response to therapy

Since we have previously shown that patients with an initial strong systemic inflammatory response have higher and longer

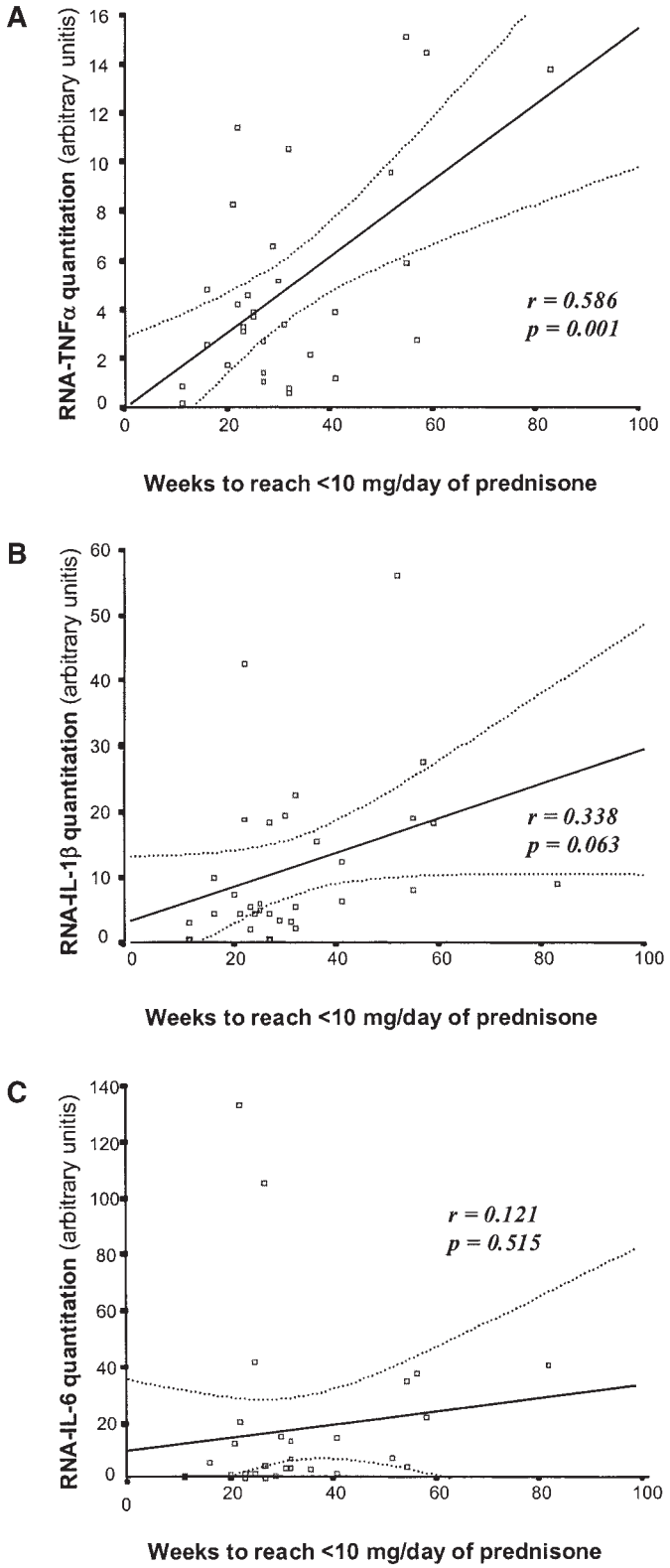

FIG. 5. Correlation between tissue mRNA levels of TNFα (A), IL-1β (B) and IL-6 (C), and the time required to reach a maintenance prednisone dose < 10 mg/day.

corticosteroid requirements [7], we investigated whether there was any correlation between cytokine expression in tissue at the moment of diagnosis and the subsequent duration of treatment. Thirty-one patients had a follow-up long enough to have attempted a prednisone dose reduction to less than 10 mg/day, according to current treatment schedules [7, 24]. In these patients, a significant correlation was found between TNFα transcripts and the time required to achieve a maintenance dose of prednisone less than 10 mg/day (Fig. 5). A similar trend was also observed for IL-1β, although it did not reach statistical significance (Fig. 5). A significant correlation was also found between TNFα mRNA and the cumulative prednisone dose ($r = 0.532$, $P = 0.002$). Among the 29 patients who completed a follow-up of 18 months, six patients experienced two or more relapses, whereas the remaining patients relapsed once or did not relapse at all. TNFα mRNA concentrations tended to be higher in the six patients with multiple relapses but the difference did not reach statistical significance (8.88 relative units, range 1.40–15.04, *vs* 4.27 relative units, range 0.60–11.37; $P = 0.095$), possibly owing to the low number of recurrent relapsers in this series.

Discussion

In this study we found that pro-inflammatory cytokines IL-1β, TNFα and IL-6 are remarkably expressed in temporal artery lesions from patients with GCA, particularly at the granulomatous areas.

Although normal temporal arteries did not display significant immunostaining for pro-inflammatory cytokines, cytokine mRNA was detected in all specimens. Several reasons may account for the notable cytokine gene expression observed in histologically normal temporal arteries which, in contrast, did not contain significant amounts of cytokine protein, as assessed by immunohistochemistry. First, the surgical procedure itself is an injury and ligation of small tributaries or the main artery may convey ischaemia–reperfusion events. Both phenomena are well-known inducers of pro-inflammatory cytokine mRNAs [25, 26], but may not last long enough to allow their translation into proteins. Second, temporal artery specimens are bloody and contain vascularized surrounding connective tissue which may include activated peripheral blood mononuclear cells, given that normal temporal artery biopsies are often obtained from patients with inflammatory conditions in whom the diagnosis of GCA may be suspected [14, 27]. Cytokine production by activated circulating white blood cells may then be detected by highly sensitive PCR. Finally, pro-inflammatory cytokines are subjected to a tight post-transcriptional regulation [23]. Even if some baseline constitutive expression takes place in normal arteries, it may not necessarily be translated into a significant amount of protein. All these limitations must always be taken into account when evaluating cytokine mRNAs in temporal arteries.

Tissue expression of pro-inflammatory cytokines, both at the mRNA and at the protein level, were highly variable among patients, even those disclosing a similar degree of histopathological involvement, indicating variability among patients in the ability to produce cytokines. Previous studies addressing pro-inflammatory cytokine expression in GCA have studied only mRNA [14, 15] or have included a small number of cases focusing on the cellular and topographic distribution of cytokine expression with no attempts to correlate cytokine production with disease expression or outcome [16–18]. At the mRNA level, IL-1β and IL-6 were more abundant in patients with a strong systemic inflammatory response compared with those with no inflammatory parameters. At the protein level, IL-1β, TNFα and IL-6 expression was higher in patients with a strong systemic inflammatory reaction.

Depending on the way results were analysed, differences in cytokine expression were more significant for one cytokine with respect to the others. This is not surprising given that the final intensity of the systemic inflammatory response is determined by many factors. In addition to cytokine transcriptional and post-transcriptional regulation, proteasome- mediated cytokine

turnover, cytokine processing by converting enzymes, cytokine receptor regulation, soluble cytokine receptors, decoy receptors and natural cytokine antagonists play in concert to determine the ultimate functional availability of a given cytokine [28, 29]. Moreover, pro-inflammatory cytokines produced in inflammatory lesions act in complex networks involving many cell types which, in turn, produce a second wave of inflammatory mediators [8, 9]. The responsiveness of target tissues, such as liver, bone marrow, hypothalamus and adipose tissue, to pro-inflammatory cytokines and to second-wave mediators may also determine the intensity of the acute-phase response [8, 9]. Finally the anatomical extent of inflammatory lesions in a typically segmental disease might also determine the net amount of pro-inflammatory cytokine production. In spite of such complexity, and the fact that we correlated a global response with data obtained from just a few millimetres of tissue, our findings suggest that the amount of locally produced pro-inflammatory cytokines in GCA lesions is a significant determinant of the intensity of the systemic inflammatory reaction in this disease.

In a previous study, we showed that patients with a strong systemic inflammatory response who had, indeed, elevated levels of circulating TNFα and IL-6 required higher corticosteroid doses and a longer duration of treatment [7]. In the present study we also found a correlation between tissue production of TNFα and, to a lesser extent, IL-1β and the time required to achieve a maintenance prednisone dose less than 10 mg/day, indicating that patients who are able to produce larger amounts of TNFα and IL-1β in lesions develop a long-lasting disease, more refractory to therapy.

Corticosteroids are highly effective in controlling GCA clinical manifestations and in preventing ischaemic complications [1, 2]. However, corticosteroid requirements are highly variable among individuals. While some patients do remarkably well and easily achieve sustained remissions, other patients frequently relapse and require unacceptably high corticosteroid doses to control disease activity [7, 24, 30]. Our findings suggest that elevated tissue production of pro-inflammatory cytokines define a group of patients with an intense systemic inflammatory response who are more refractory to therapy. Whether persistent disease is determined by elevated levels of these cytokines or both phenomena are impelled by other factors remains to be elucidated, but an intense production of pro-inflammatory cytokines is likely to maintain inflammatory cascades leading to persistent clinical manifestations. The observation that IL-1 receptor antagonist knock-out mice develop large vessel vasculitis suggests that pro-inflammatory cytokines have a significant role in maintaining vessel inflammation [31].

Previous attempts to identify agents with corticosteroid-sparing effects on patients with GCA have led to conflicting results [32, 33]. Although not devoid of serious side-effects, anti-cytokine therapies have successfully expanded the therapeutic scope for several chronic inflammatory diseases. Our findings suggest that patients with GCA might potentially benefit from anti-cytokine therapies, particularly those with a strong systemic inflammatory reaction who, according to our results, produce higher levels of pro-inflammatory cytokines and are more refractory to conventional therapies.

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Response to thalidomide in multiple myeloma: impact of angiogenic factors

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Abstract

Thalidomide has antiangiogenic and immunomodulatory effects, mediated by several cytokines such as vascular endothelial growth factor (VEGF), fibroblastic growth factor (FGF-2), hepatocyte growth factor (HGF), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha). Although extramedullary plasmacytomas (EMP) have a high vascularization, the response of these patients to thalidomide is controversial. Thirty-eight patients with refractory/relapsed MM were treated with thalidomide. Eleven patients had EMP when therapy was initiated. Serum specimens were obtained in patients before treatment was started and at the time of maximum response in responding patients or at thalidomide discontinuation in non-responders. Serum levels of VEGF, HGF and FGF-2 were determined in 18 patients whereas IL-6 and TNF-alpha were measured in 19 patients. Sixteen of the 38 patients (42%) responded to thalidomide. The response rate was significantly higher in patients without EMP (59% vs 0%, $p = 0.0006$). VEGF serum levels were significantly higher in responding patients. In contrast, baseline serum levels of HGF were significantly lower in responders. Neither VEGF nor HGF serum levels showed correlation with the presence of EMP. Baseline TNF-alpha serum levels were significantly lower in responding patients and in those without EMP. The serum levels of FGF-2 and IL-6 did not correlate with response to treatment or presence of EMP.

Keywords: Angiogenesis; Myeloma; Response; Thalidomide

1. Introduction

Thalidomide is an antiangiogenic agent that produces a response rate of 32–64% in patients with refractory/relapsed multiple myeloma (MM) [1–7]. The antiangiogenic effect of thalidomide is considered as one of the possible mechanisms of action in patients with MM [8], which can be mediated by several cytokines such as vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (FGF-2), hepatocyte growth factor (HGF) as well as tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) [9–14]. However, the exact mechanism of action is not known. In this regard, there is evidence that thalidomide can also act through other mechanisms such as: (1) direct inhibition of myeloma cell growth, (2) modulation of adhesion molecules, or (3) induction of interferon gamma and interleukin-2 secretion by CD-8 cells [8]. Interestingly, although extramedullary plasmacytomas (EMP) have a

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high vascularization, controversial results on the response of these patients to thalidomide have been reported [15,16].

The objectives of the present study are: (1) to ascertain whether or not serum levels of different angiogenic cytokines are associated with disease response to thalidomide as well as the possible change in cytokine levels after therapy and (2) to investigate the cytokine's serum levels in patients with and without EMP.

2. Results

2.1. Response to treatment

Sixteen out of the 38 patients (42%) responded to thalidomide. Eight (21%) achieved a partial response and 8 (21%) a minimal response. The response rate was significantly higher in patients without extramedullary involvement (59% vs 0%, $p = 0.0006$). In fact, none of the 11 patients with extramedullary plasmacytomas responded. The median duration of the response was 8.2 months (1–36+).

2.2. Correlation of cytokine serum levels with treatment response and soft-tissue plasmacytomas

The comparison between cytokine serum levels before treatment with thalidomide in responders vs non-responders is shown in Table 1. Serum cytokine levels in patients with or without EMP are summarized in Table 2. Results of cytokine measurements after treatment in responders vs non-responders are depicted in Table 3. Patients who responded to thalidomide had significantly higher serum levels of VEGF than those who did not achieve a response, both before and after treatment. No significant correlation was found between VEGF serum levels and the presence or absence of extramedullary involvement. VEGF serum levels did not decrease after thalidomide therapy.

Baseline HGF serum levels were significantly lower in responders than in non-responders and showed a trend towards lower levels in patients without EMP when compared with patients who had soft-tissue plasmacytomas. There were no differences between groups after treatment. HGF serum levels showed no significant change after treatment.

Baseline TNF-alpha serum levels were significantly lower in responding patients and in those without soft-tissue plasmacytomas when compared with those of non-responders and with those of patients with EMP, respectively. Serum levels of TNF-alpha increased in all patients after treatment, although only patients who achieved a response or did not have EMP had a significant increase (from 23.6 ± 9.5 to 29.4 ± 8.9, $p = 0.028$ in responding patients and from 25.1 ± 9.4 to 30.5 ± 8.8, $p = 0.03$ in patients without EMP).

Serum levels of FGF-2 and IL-6 did not show any correlation either with response to treatment or with extramedullary involvement.

3. Discussion

A number of studies have shown that increased bone marrow angiogenesis is associated with a faster disease progression and poor prognosis in patients with multiple myeloma [9–11,13,14,19,20]. Moreover, solitary bone plasmacytomas have also an increased angiogenic activity and patients with a high neovascularization have a significantly higher risk of progression to MM [21]. In fact, clinical and experimental studies suggest that angiogenesis is crucial in the pathogenesis of MM [11,12,22]. With this background, antiangiogenic agents,

Table 1
Baseline serum levels of cytokines (mean ± SD) in responders vs non-responders

Cytokine (pg/mL)	Response to treatment		*p* value
	Yes ($n = 10$)	No ($n = 8$)	
VEGF	448 ± 350	154 ± 102	0.05
HGF	805 ± 255	1468 ± 789	0.02
FGF-2	8.5 ± 7.9	6.1 ± 4.3	NS
	($n = 11$)	($n = 8$)	
IL-6	34.2 ± 33	28.6 ± 24.1	NS
TNF-alpha	23.6 ± 9.5	40.6 ± 15.6	0.014

Table 2
Comparison of baseline serum levels of cytokines (mean ± SD) in patients with and without extramedullary plasmacytomas

Cytokine (pg/mL)	Extramedullary plasmacytomas		*p* value
	No ($n = 12$)	Yes ($n = 6$)	
VEGF	384 ± 350	183 ± 102	NS
HGF	882 ± 295	1535 ± 920	0.09
FGF-2	7.9 ± 7.7	6.3 ± 3.2	NS
	($n = 13$)	($n = 6$)	
IL-6	33.3 ± 32	28.6 ± 23.6	NS
TNF-alpha	25.1 ± 9.4	43.2 ± 17.5	0.03

Table 3
Cytokine's serum levels after treatment (mean ± SD) in responders vs non-responders

Cytokine (pg/mL)	Response to treatment		*p* value
	Yes ($n = 10$)	No ($n = 8$)	
VEGF	471 ± 361	145 ± 116	0.02
HGF	943 ± 487	1361 ± 1104	NS
FGF-2	16.2 ± 11.1	17.9 ± 21.5	NS
	($n = 10$)	($n = 6$)	
IL-6	85.1 ± 165	49.3 ± 55.8	NS
TNF-alpha	29.4 ± 8.9	48 ± 21.4	0.06

particularly thalidomide, are being currently used in the treatment of this disease. Thalidomide, when administered as a single agent, produces a response rate ranging from 32 to 64% in patients with refractory/relapsed disease [1–7]. Although the rationale for the use of thalidomide in MM was its antiangiogenic potential, little is still known on its precise mechanisms of action as antimyeloma agent. In addition, angiogenesis is a not yet fully established multistep process in which different cytokines and growth factors appear to play an important role [23]. Angiogenesis has been usually assessed by bone marrow or tumor microvessel density estimation [24,25]. However, it can be also evaluated by measuring serum levels of different angiogenic cytokines [13,14,20]. In fact, serum levels of VEGF, FGF-2 and HGF have been correlated with disease stage [26]. In addition, Sezer et al. [13] reported that response to conventional chemotherapy resulted in a significant decrease in serum levels of the above-mentioned angiogenic factors. We studied serum levels of these potent angiogenic cytokines and growth factors as possible predictors of response to thalidomide in patients with relapsed/refractory MM. In addition, these cytokines were determined at the time of maximum response or at drug discontinuation in non-responders in order to explore whether or not response was associated with a decline in angiogenic cytokines. We found that higher levels of VEGF were associated with a significantly higher probability of response. In contrast, responding patients had a significantly lower serum HGF level than non-responders. We found no differences in FGF-2 serum levels between responders and non-responders. Our results are in disagreement with those reported by Neben et al. [27] who found that high levels of FGF-2 predicted the response to thalidomide in both the univariate and the logistic regression analysis, while VEGF serum levels had no influence on response. It is noteworthy that in our serial measurements, the serum levels of VEGF, FGF-2 and HGF did not show any significant change after thalidomide therapy. The lack of decrease in angiogenic cytokines after thalidomide treatment has also been reported by Neben et al. [28]. In this regard, Kumar et al. [25] have reported that in patients with MM the degree of bone marrow angiogenesis did not significantly change after conventional treatment or even after complete remission following high-dose therapy/stem cell support [24]. It has been postulated that in responding patients with MM the angiogenesis process could be perpetuated through the secretion of angiogenic cytokines by residual cells [25].

In the present series none of the 11 patients with soft-tissue plasmacytomas responded to thalidomide. Although soft-tissue plasmacytomas are tissues with a high vascularization, there is no clear correlation between the presence of EMP and the serum levels of angiogenic cytokines. In fact, only TNF-alpha shows a significant correlation with the presence of EMP. The lack of response to thalidomide in patients with extramedullary involvement to thalidomide is in agreement with the finding, in tumor mouse models, that thalidomide is less potent than IMiDs in suppressing both tumor growth and angiogenesis [29]. All the above suggest that the apoptotic effect of thalidomide on plasma cells is higher in the bone marrow than in extramedullary locations.

4. Patients and methods

4.1. Patients and treatment

From November 1999 to December 2002, 38 patients (20 males, 18 females, median age 63 years) with refractory/relapsed MM were given thalidomide as a single agent. Eleven patients had extramedullary plasmacytomas when treatment with thalidomide was initiated. Treatment was started at a single nightly dose of 200 mg and escalated by 100 or 200 mg every 2 weeks, depending on the patient's tolerance, up to a maximum of 800 mg/day. The median dose administered was 400 mg (range, 200–800).

4.2. Evaluation of response

The response was assessed according to the European Group for Bone and Marrow Transplantation (EBMT)/International Bone Marrow Transplant Registry (IBMTR)/Autologous Blood and Marrow Transplant Registry (ABMTR) criteria [17].

4.3. Methods

Serum levels of the following angiogenic cytokines and growth factors were determined: VEGF, HGF, FGF-2, TNF-alpha and IL-6. Serum specimens were obtained in patients before treatment with thalidomide was started. A second specimen was obtained at the time of maximum response in responding patients or atthalidomide discontinuation in those patients who showed no response. There were samples available before and after treatment for VEGF, FGF-2 and HGF in 18 patients and for IL-6 and TNF in 19 patients.

4.4. Angiogenic cytokines and growth factor measurements

Venous blood was drawn by venopuncture during fasting in the morning. The serum was rapidly separated after coagulation. Aliquots of serum frozen were at −20 °C until assayed. Cytokines and growth factors were measured by enzyme-linked-immunoassay (ELISA). Commercially available ELISA tests for TNF-alpha

and IL-6 were obtained from Medgenix (Fleurus, Belgium) and kits for VEGF, HGF and FGF-2 from R&D Systems (Minneapolis, USA). Sera were evaluated in duplicate and the procedure was performed according to the instructions of the manufacturer.

4.5. Statistical methods

The χ^2, Mann–Whitney's U or Student's t test was used to assess the statistical significance of comparison between different patient characteristics, response to therapy and serum cytokine levels. Survival was estimated using Kaplan and Meier method [18]. Variations in pre- and post-treatment cytokine levels were analyzed by means of Wilcoxon's test.

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Extramedullary multiple myeloma escapes the effect of thalidomide

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A B S T R A C T

Background and Objectives. Thalidomide is an antiangiogenic drug that produces a response rate ranging from 32 to 64% in patients with refractory/relapsed multiple myeloma (MM). However, the efficacy of thalidomide in patients with soft-tissue plasmacytomas is controversial. The aim of this study was to assess the response rate to thalidomide in patients with advanced MM and to correlate the response rate with the presence of extramedullary involvement.

Design and Methods. Thirty-eight patients with refractory/relapsed MM were treated with thalidomide. Eleven patients had extramedullary involvement when therapy was initiated. The response rate was evaluated according to the criteria of the European Group for Blood and Marrow Transplantation.

Results. Sixteen of the 38 patients (42%) responded to thalidomide. The response rate was significantly higher in patients without extramedullary involvement (59% vs 0%, p=0.0006). Although four of the 11 patients with extramedullary involvement had a serological response, a progression of the soft-tissue masses was observed in all of them.

Interpretation and Conclusions. Thalidomide is effective in patients with advanced MM. However, extramedullary disease does not respond to thalidomide, as delivered in this series. The mechanisms to explain different response to therapy depending on tumor homing warrant further investigation.

Key words: multiple myeloma, plasmacytomas, thalidomide.

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Multiple myeloma (MM) accounts for about 10% of hematologic malignancies and 1% of all malignant diseases. This disease is clinically characterized by lytic bone lesions, anemia, hypercalcemia, renal function impairment, recurrent bacterial infections and extramedullary involvement, all this leading to a median survival of about three years. Extramedullary involvement by the disease (i.e., palpable or radiographically visualized masses) has been reported in 15% to 20% of patients at diagnosis and in an additional 15% during the course of the disease.[1,2] Several studies have shown an increased microvessel bone marrow density in patients with MM,[3] the degree of bone marrow angiogenic activity being correlated with disease progression. The anti-angiogenic properties of thalidomide provided the rationale for its use in patients with MM. When given as a single agent, this drug produces a response rate ranging from 32 to 64% in patients with refractory/relapsed disease.[4-11] Although plasmacytomas are tissues with high neovascularization,[12,13] controversial results on the response to thalidomide in these patients have been reported.[12,14] Furthermore, cases of extramedullary or bone marrow progression despite a good serological response have been recognized.[5,15,16] The latter observations would support a bone marrow microenvironment-mediated mechanism the effect of thalidomide in MM. We report the results of treatment with thalidomide in 38 consecutive patients with refractory/relapsed MM, eleven of which had extramedullary involvement.

Table 1. Characteristics of patients with extramedullary plasmacytomas (EMP).

Patient Number	*Age/ Sex*	*Platelet count (×10⁹/L)*	*LDH* (UI/L)*	*Previous HDT*	*Serum M-protein g/L (Start /After)*	*Urine M-protein g/24h (Start/After)*	*M-protein response*	*Evolution EMP* (Start/After)*	*Overall response*
2	59/F	96	392	No	8.7-ND	0.05–0.014	Progression	Yes /New	Progression
9	52/M	97	329	Yes	43.7–61.8	2–2.1	Progression	Yes/New@	Progression
12	80/M	214	–	No	39.2–34.2	ND	No change	Yes/No change	No change
14	65/F	180	405	Yes	7.2-11	1.9–7.4	Progression	Yes /@	Progression
19	61/M	88	359	No	63–45.1	1.5–1.9	MR	Yes /@	Progression
25	64/F	315	246	Yes	10.2–10.5	2.5–4.7	Progression	Yes /@	0Progression
28	57/M	173	521	Yes	7–10.5	0.01–0.01	Progression	Yes / New@	Progression
29	77/M	147	914	No	18.3–16.2	0.02–0.09	No change	Yes / No change	No change
30	41/M	86	–	No	25.4–10.4	9–4.8	MR	Yes /@	Progression
31	66/F	123	305	No	61.4–38.8	0.1–0.05	MR	Yes/New	Progression
38	49/M	251	–	Yes	21.7-12	ND-ND	PR	Yes /New@	Progression
3	48/F	322	–	Yes	34.2–16.1	ND	PR	No/New	Progression
17	51/F	220	379	No	14-18	0.2–0.6	Progression	No/New	Progression

*PR: partial response; MR: minimal response; ND: not done; New: appearance of new soft-tissue plasmacytomas; @:increase in size of soft-tissue plasmacytomas: *normal values < 450 UI/L.*

Design and Methods

Patients

From November 1999 to December 2002, 38 consecutive patients from a single institution (20 males, 18 females, median age 63 years) with previously treated and progressive MM were given thalidomide treatment as a single agent. The M-protein type was IgG in 25 cases, IgA in 7, light chain in 5 and IgM in 1. The type of light chain was κ in 23 patients and λ in 15. The median time from the first chemotherapy to treatment with thalidomide was 41 months (range 6-165). The median number of prior chemotherapy regimens was 2 (range 1 - 4). Fifteen patients (40%) had relapsed after an autologous stem cell transplantation (SCT). Eighteen patients had refractory disease (refractory relapse 14, primary resistance 4) while the remaining 20 patients had untested relapse. Eleven patients had extramedullary plasmacytomas when treatment with thalidomide was initiated (Table 1).

Treatment

Thalidomide was started at a single nightly dose of 200 mg. The dose was escalated by 100 or 200 mg every 2 weeks, depending on the patient´s tolerance, up to a maximum of 800 mg/day. The median dose administered was 400 mg (range, 200 to 800). Eight patients received a dose of 600 mg or higher and six reached the upper dose limit of 800 mg. In three patients, treatment with thalidomide was prematurely discontinued because of severe toxicity. In patients who achieved a response, the dose was gradually reduced to a maintenance daily dose of 100 mg. No prophylactic anticoagulation was given.

Evaluation of response

The response was assessed according to the European Group for Bone and Marrow Transplantation (EBMT)/ International Bone Marrow Transplant Registry (IBMTR)/ Autologous Blood and Marrow Transplant Registry (ABMTR) criteria.[17] The plasmacytomas were evaluated by measuring changes in its size and the appearance of new soft-tissue masses. Given the locations of plasmacytomas in most cases the evaluation of response was made by physical examination. CT scans and/or MRI were only performed when clinically indicated. All patients who started thalidomide treatment were included in this analysis. Thus, the results were analyzed on an intention-to-treat basis.

Statistical methods

Fisher's exact test and Mann-Whitney´s U test were used to assess the statistical significance of comparisons between different patients' characteristics and response to therapy. The duration of response was estimated using the Kaplan and Meier method.[18]

Results

Response to treatment

Sixteen out of the 38 patients (42%) responded to thalidomide (95% CI 26-59%). Eight (21%) achieved a partial response and 8 (21%) a minimal response. Three of the patients categorized as having a minimal response had almost a partial response since the serum M-protein decrease was higher than 40%. The median time to maximal response was 80 days (range 37 – 133). The response rate was significantly higher

Table 2. Location of soft-tissue plasmacytomas before and after thalidomide therapy.

Patient number	*Before thalidomide therapy*	*After thalidomide therapy*
2	*Frontoparietal mass 6×7 cm*	*Frontoparietal, frontal*
9	Pre-esternal 5×4 cm	Pre-sternal 7×8 cm, para-sternal 5×4 cm, frontoparietal 4×4 cm, frontotemporal 2×2 cm
12	Paravertebral D1	No change
14	Parietal mass 6x5 cm	Skull parietal mass 8×8 cm
19	Frontal 6x6 cm	Increased size
25	Frontoparietal 6x4 cm	Frontoparietal 6×8 cm
28	Right thoracic wall mass arising from 5th rib, skin, trunk 14×7×12 cm	Thoracic wall mass 24×12 cm, multiple skin nodules
29	Disseminated skin nodules of variable size	No change
30	Parietal, multiple cutaneous nodules, sphenoids, cavernous sinus	Increased size
31	Parietooccipital mass 3×3 cm	Parieto-occipital (no change), frontal 3×3 cm, infrascapular 6 cm
38	Multiple cutaneous nodules	Cutaneous nodules, paravesical mass, liver masses
3	Absence	Retro-orbital mass 2×3 cm, breast masses, skin nodules
17	Absence	Pre-sternal mass

in patients without extramedullary involvement (59% vs 0%, p= 0.0006).

The characteristics of patients with extramedullary involvement are detailed in Table 1. The location of soft-tissue plasmacytomas before and after thalidomide therapy is shown in Table 2. As can be observed, except one patient (case 12) who had a paravertebral mass only shown by CT scan examination the remaining 10 patients had palpable soft masses. In seven patients the soft-tissue plasmacytomas likely arised from underlying bone lesions (skull, ribs, vertebrae, sternum) while four patients had multiple cutaneous nodules. None of the eleven patients with extramedullary plasmacytomas responded to thalidomide. Although one of these patients showed a decrease in the size of soft-tissue involvement, this response did not last the 6 weeks required by the EBMT criteria.[17] This patient had relapsed after autologous SCT, with an increase in serum M-protein and appearance of soft-tissue plasmacytomas in trunk and left leg. One month after treatment with thalidomide had been started, the trunk plasmacytoma disappeared and a >75% reduction in his left-leg plasmacytoma was noted. However, this response was transient (3 weeks' duration) with reappearance of cutaneous masses as well as a huge paravesical mass and multiple hepatic plasmacytomas. Of interest, this patient had achieved an stable partial serological response despite extramedullary progression. Three of the remaining patients with extramedullary plasmacytoma achieved minimal response according to the serum M-protein decrease but showed progression of their extramedullary disease. On the other hand, two patients without extramedullary involvement when therapy with thalidomide was started, developed soft-tissue plasmacytomas while on thalidomide treatment. One of these patients had achieved a serological partial response with a serum M-protein decrease from 34 g/L to 16 g/L, but developed multiple extramedullary plasmacytomas in her left orbit, left breast and skin, whereas the other patient showed a progressive increase in M-serum protein size along with the appearance of soft-tissue plasmacytomas. Also of interest, one of our long-term responders with very limited skeletal involvement at initiation of thalidomide, in whom the serum M-protein had decreased from 51 g/L to 22 g/L, relapsed with hypercalcemia and extensive skeletal disease leading to several pathological fractures of long bone while her M-protein remained stable at 22 g/L.

Of note, the dose of thalidomide given to patients with extramedullary involvement was significantly higher than that in patients without soft-tissue plasmacytomas (median 550 mg /day vs 400 mg/day,

p=0.048). There were no significant differences regarding age, gender, M-protein type, amount of serum M-protein, proportion of bone marrow plasma cells or presence of lytic bone lesions between patients with or without extramedullary plasmacytomas. Nine out of the 16 patients who had achieved a response have relapsed so far. The median duration of the response was 15.9 months (range 1-43+).

Toxicity

About 75% of patients complained of somnolence, fatigue or constipation. Less frequent side effects were mild distal tremor, dizziness and paresthesia. Five patients developed generalized skin rash and one patient had ampollous lesions in both feet. Thalidomide was discontinued in this patient because of progressive disease with development of new soft tissue plasmacytomas. Thalidomide were prematurely discontinued in another 3 patients because of severe adverse effects: one patient developed severe facial angioedema 6 days after thalidomide initiation and other had a sudden cardiac arrest 15 days after starting therapy due to a ventricular arrhytmia and was successfully resuscitated. Finally, a third patient developed two episodes of syncope due to Mobitz type I atrioventricular block and the treatment was discontinued. No cases of deep venous thrombosis or thromboembolism were observed.

Discussion

A number of studies have shown that increased bone marrow angiogenesis is associated with faster disease progression in patients with multiple myeloma.[19-25] Moreover, solitary bone plasmacytomas have increased angiogenic activity.[13] Of interest, patients with marked neovascularization in solitary plasmacytomas have a significantly higher risk of progression to MM.[13] Both clinical and experimental studies suggest that angiogenesis is crucial in the pathogenesis of MM.[21,26,27] On this background, anti-angiogenic agents, particularly thalidomide, are being used in the treatment of MM. Thalidomide, when administered as a single agent, produces a response rate ranging from 32 to 64% in patients with refractory/relapsed disease.[4-11] Although the rationale for using thalidomide in MM was its antiangiogenic potential, its precise mechanism of action is still not fully understood. The overall response rate of 42% reached in this series falls within the expected rate in relapsed/refractory patients treated with thalidomide9 but the duration of the response was longer than that reported in other studies.[9,11]

There was, however, a clear difference in the response rate of those patients without and with extramedullary plasmacytomas (59% vs 0%, p=0.0006). Interestingly, in this series a serological response (i.e. decrease in the M component) was observed in four patients with soft-tissue masses but in none of them this was accompanied by a decrease in the size of the extramedullary plasmacytomas. One patient with no extramedullary plasmacytomas at initiation of thalidomide developed retro-orbital and multiple subcutaneous masses while in serological response and another patient, in whom no decrease in M-protein level was observed, developed a presternal mass shortly after initiation of thalidomide. Furthermore, one patient with long-lasting response to thalidomide had a relapse with multiple pathologic fractures in long bones due to extensive osteolytic lesions although she had no increase in her serum M-protein level. The results from this study confirm and extend the data on the lack of efficacy of thalidomide in patients with MM and extramedullary involvement reported in a smaller series of patients by our group[14] and adds to other reports pointing out to the same concept.[5,15] Recently it has been recognized that relapses may occur under thalidomide maintenance with an increase in bone marrow plasma cells and no increase in the M-protein size.[16] The reasons for the poor response of extramedullary plasmacytomas to thalidomide are unknown. It should be emphasized that the dose of thalidomide given to patients with extramedullary involvement was significantly higher than that given to those with no extramedullary involvement. However, Biagi *et al.*[12] reported three patients who had a predominantly extramedullary relapse after allogeneic transplantation and all three responded to thalidomide. Although based on a small number of cases, these authors postulated that the efficacy of thalidomide on extramedullary involvement after allogeneic transplantation could be different to that in patients who had received only conventional chemotherapy.[12] In any event, data from other groups with larger series of patients are needed to confirm our observation. In a tumor mouse model, thalidomide was shown to be less potent in suppressing tumor growth that its immunomodulatory analogs -IMiDs-28. Thus, IMiDs more efficiently decreased the development of tumors after malignant cell inoculation and induced greater tumor regression in already established tumors than did thalidomide.[28] Morever, the effect of thalidomide on angiogenesis in tumors induced in mice was lower than that achieved with IMiDs.[28] The above *in vivo* experiments are in line with our clinical observation on the lack of efficacy of thalidomide in extramedullary plasmacytomas, supporting the concept that tumor cell homing in different tissues may influence response therapy. The mechanisms explaining the different responses depending on the tumor

location warrant futher investigation.[29] In fact, the introduction of thalidomide in the treatment of MM has constituted a major first step forward in the investigation of innovative therapies, such as IMiDs[30] or the proteasome inhibitor bortezomib,[31] which target not only the malignant plasma cell but also the microenvironment.[32] Whether or not these new agents, with anti-angiogenic/pro-apoptotic mechanisms of action, will also show different efficacy depending on the myeloma cell homing will need to be carefully investigated in future trials.

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Mechanisms

Increased Levels of Atherosclerosis Markers in Salt-Sensitive Hypertension

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Background: Salt sensitivity in essential hypertension is associated with both endothelial dysfunction and increased cardiovascular risk. We evaluated several serum markers of atherosclerosis and endothelial function in a group of essential hypertensive patients classified on the basis of their salt sensitivity.

Methods: Forty-three patients were classified as having salt-sensitive (20 subjects) or salt-resistant (23 subjects) hypertension on the basis of their 24-h blood pressure (BP) response from low salt (50 mmol/d) to high salt (250 mmol/d) intake. Endothelium-dependent and independent responses were measured in the forearm previously to salt manipulation. High-sensitivity C-reactive protein (CRP), soluble intercellular adhesion molecule type 1 (sICAM-1), soluble vascular cell adhesion molecule type 1 (sVCAM-1), e-selectin, p-selectin, interleukin-6 (IL-6), monocyte chemotactic protein type 1 (MCP-1), matrix metalloproteinases types 1, 2, and 9 (MMP-1, MMP-2, and MMP-9), and the tissue inhibitor of metalloproteinases type 1 (TIMP-1) were measured in serum on the last day of both low salt and high salt intakes.

Results: Compared to salt-resistant patients, salt-sensitive hypertensives showed age-adjusted increased levels of p-selectin ($P = .006$), e-selectin ($P = .042$), and MCP-1 ($P = .036$), although differences in e-selectin were not maintained after adjustment for BP values. Moreover, salt-sensitive subjects exhibited decreased serum levels of MMP-9 ($P = .007$) and increased levels of TIMP-1 ($P = .045$). No differences in serum CRP, sICAM-1, sVCAM-1, or IL-6 were observed between salt-sensitive and salt-resistant patients. Finally, maximal acetylcholine-induced vasodilation (319% ± 153% *v* 414% ± 178% increase in forearm blood flow; $P = .022$ age-adjusted) was significantly impaired in salt-sensitive hypertensives.

Conclusions: Serum markers of inflammation, especially selectins and chemokines, as well as markers of vascular remodeling, and endothelium-dependent vasodilation are altered in salt-sensitive hypertension. These alterations could help to explain the greater target organ damage and cardiovascular risk observed in salt-sensitive subjects. Am J Hypertens 2006;19:87–93 © 2006 American Journal of Hypertension, Ltd.

Key Words: Cell adhesion molecules, inflammation, extracellular matrix, endothelium, dietary sodium, sodium-dependent hypertension, atherosclerosis.

Various epidemiologic and interventional studies have demonstrated a clear relationship between salt intake and hypertension.[1] However, blood pressure (BP) response to increased dietary salt is heterogeneous among individuals, a phenomenon known as salt sensitivity.[2] Although salt sensitivity is well established in experimental and human hypertension, the pathophysiologic mechanisms leading to such individual susceptibility remain unresolved.[2] It has been suggested that abnormalities in renal sodium regulation,[3] in the renin-angiotensin system,[4] in the sympathetic nervous system,[5] and in the transmembrane sodium transport,[6] are all involved in the pathogenesis of salt sensitivity.

Two independent groups have suggested that the presence of salt sensitivity is associated with an increased incidence of death or cardiovascular complications,[7,8] representing an accelerated atherosclerotic process in the vascular wall. Moreover, salt-sensitive patients tend to be nondippers,[9] and more frequently exhibit left ventricular hypertrophy[10] and microalbuminuria.[11] In addition, salt-sensitive hypertension is accompanied by more pronounced endothelial dysfunction.[12]

This endothelial dysfunction may be the pathophys-

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iologic link between salt sensitivity and accelerated atherosclerosis. It is well known that the intact endothelium not only contributes to vasodilation, but also has antiagregant, anti-inflammatory, and antiproliferative properties[13] and that endothelial dysfunction is associated with an increased risk of atherosclerotic events.[14] Several acute phase reactants, soluble adhesion molecules, chemokines, and metalloproteinases are altered in patients at risk of cardiovascular events, such as hypertensives,[15–18] diabetics,[19] or those with left ventricular hypertrophy.[20,21] Moreover, in patients with coronary heart disease some of these serum markers have prognostic implications.[22–24]

One previous study[25] measured soluble intercellular and vascular cell adhesion molecules and e-selectin in patients classified on the basis of salt sensitivity, finding an elevation of the latter. We hypothesized that not only inflammation, but also metalloproteinases, could be altered in salt-sensitive patients compared to salt-resistant ones. Thus, the aim of the study was to compare levels of soluble adhesion molecules, selectins, chemokines, and metalloproteinases in essential hypertensive patients classified on the basis of salt sensitivity.

Methods

Patient Selection

The study population included 43 nontreated, newly diagnosed essential hypertensive patients consecutively recruited from the Hypertension Unit, Hospital Clinic, Barcelona, Spain. There were 29 men and 14 women with a mean age of 40 years (range, 26 to 58 years). Subjects with hypercholesterolemia (total cholesterol >6 mmol/L [230 mg/dL]), diabetes mellitus, impaired renal function (serum creatinine >132 μmol/L [1.5 mg/dL]), or previous history of coronary or cerebrovascular disease were excluded from the study, as were patients who drank more than 40 g of ethanol per day, those under chronic treatment with nonsteroidal anti-inflammatory drugs, and women taking oral contraceptives or estrogen replacement therapy.

Assessment of Salt Sensitivity

All patients gave informed consent. The protocol was approved by the Ethics Committee of the Hospital Clinic and by the Spanish Health Authority (Protocol F.I.S. 00/0435). Essential hypertensive patients were placed on a baseline low salt diet containing 50 mmol of Na^+ during 14 days. This diet was supplemented in a random, single-blind fashion by placebo tablets during 7 days (low salt period) and by NaCl tablets, 200 mmol/d (high salt period) during another 7 days. Thus, total NaCl intake during the high salt period was 250 mmol/d.

On the last day of both low salt and high salt periods, 24-h ambulatory BP monitoring (ABPM) was performed with an automated, noninvasive oscillometric device (SpaceLabs 90217; SpaceLabs Inc., Redmon, WA). Blood pressure was registered automatically at 15-min intervals for 24 h. Salt-sensitive hypertension was defined as a significant increase ($P < .05$; >4 mm Hg) of 24-h mean BP from low to high salt intake.[4–6,9,10,12]

Measurement of Endothelial Function

These procedures have been previously described in detail.[12,26] Briefly, before dietary manipulations forearm endothelium-dependent and independent vasodilation were determined by measuring forearm blood flow by strain-gauge venous plethysmography (EC5R-Hokanson, Bellevue, WA) at baseline (0.9% saline infusion), after increasing doses of acetylcholine (from 0.15 to 15 μg/100 mL forearm tissue/min), and after increasing doses of sodium nitroprusside (from 1 to 4 μg/100 mL of forearm tissue/min). The acetylcholine dose–response curve was repeated after the addition of 100μg/100 mL of forearm tissue/min of the nitric oxide (NO) synthase inhibitor L-NMMA (*N*-monomethyl-L-arginine).

Endothelium-dependent vasodilation (EDV), endothelium-independent vasodilation (EIV), and the effect of L-NMMA on acetylcholine response were presented as a percentage increase in forearm blood flow above the baseline.[26]

Measurement of Atherosclerosis Markers

Fasting venous blood samples were drawn on the last day of both low salt and high salt periods between 7 AM and 9 AM. Sera were obtained by centrifugation and stored at −80°C until analysis. High-sensitivity C-reactive protein (hs-CRP) was determined by immunonephelometry (Dade-Behring S.A., Barcelona, Spain). Adhesion molecules, selectins, chemokines, and metalloproteinases were determined in duplicate wells using commercially available ELISA assays from R & D Systems, Minneapolis, MN (soluble vascular cell adhesion molecule type 1 [sVCAM-1], soluble intercellular adhesion molecule type 1 [sICAM-1], e-selectin, p-selectin, interleukin-6 [IL-6], and monocyte chemotactic protein type 1 [MCP-1]) or reagents from Amersham Biosciences (matrix metalloproteinases types 1, 2, and 9 [MMP-1, MMP-2, MMP-9] and tissue inhibitor of metalloproteinases type 1 [TIMP-1]).

Statistical Analysis

Values are expressed as mean ± SD or median (interquartile range). At least, 20 patients in each group (salt sensitive and salt resistant) were needed to detect a 25% difference in serum atherosclerosis markers assuming a common standard deviation of 25% of the mean with a 80% statistical power and 0.05 α error.

Differences between biochemical markers obtained at low and high salt intakes in salt-sensitive and salt-resistant patients were calculated by analysis of variance with the repeated measures design (for CRP and MMP-1 after log

Table 1. Baseline clinical characteristics of salt-sensitive and salt-resistant essential hypertensive patients

Parameter	Salt sensitive (n = 20)	Salt resistant (n = 23)	P
Sex (M/F)	12 / 8	17 / 6	.515
Age (y)	42.8 ± 7.6	36.9 ± 7.9	.016
Weight (kg)	79.8 ± 17.5	80.9 ± 15.8	.829
BMI (kg/m^2)	28.7 ± 7.9	27.5 ± 5.2	.522
Smokers (%)	15	26	.234
Systolic BP (mm Hg)	162.6 ± 16.2	157.8 ± 10.6	.247
Diastolic BP (mm Hg)	96.9 ± 7.9	92.4 ± 6.3	.039
Urinary albumin excretion (mg/24 h)	7.03 [3.7–15.6]	4.6 [2.5–17]	.382

BMI = body mass index; BP = blood pressure. Values as mean ± SD, or median [interquartile range].

transformation). The effect of both salt sensitivity and dietary salt manipulation was tested for all variables. Moreover, differences between salt-sensitive and salt-resistant subjects were adjusted by age and 24-h mean BP obtained at both low salt and high salt intakes (covariates). Differences in endothelium-dependent and independent responses between salt-sensitive and salt-resistant subjects were analyzed by means of one-way ANOVA adjusted by age. The relationship between serum markers and changes in 24-h BP was assessed by a stepwise linear regression analysis.

Results

General Characteristics of Essential Hypertensive Patients

Table 1 shows the clinical characteristics of the 43 essential hypertensive patients included in the study. No differences were observed in terms of gender distribution, body mass index (BMI), and baseline office systolic BP between salt-sensitive and salt-resistant essential hypertensive patients. However, salt-sensitive patients were slightly older and had higher diastolic BP values.

Diagnosis of Salt-Sensitive Hypertension

Salt-sensitive hypertension was diagnosed in 20 patients in whom 24-h mean BP significantly increased ($P < .05$) when switched from low to high salt intake. The mean increase in 24 h mean BP was 10.7 ± 4.5 mm Hg (from 101 ± 11 mm Hg at the end of the low salt period to 112 ± 11 mm Hg at the end of the high salt period) (Table 2). The remaining 23 patients were diagnosed as having salt-resistant hypertension. The change in 24 h mean BP was −0.9 ± 4.1 mm Hg (from 101 ± 10 mm Hg to 100 ± 11 mm Hg). Table 2 also shows systolic and diastolic BP and heart rate during low and high salt intakes in salt-sensitive and salt-resistant essential hypertensive patients.

To check adherence of patients to dietary changes, urinary sodium excretion was measured at the end of both periods (Table 2).

Differences in Endothelium-Dependent and Independent Response Between Salt-Sensitive and Salt-Resistant Hypertensive Subjects

As mentioned previously, EDV and EIV were estimated as the percentage increase in forearm blood flow in response to acetylcholine or sodium nitroprusside. As shown in Fig. 1, salt-sensitive patients exhibited an impaired maximal acetylcholine-induced vasodilation (319% ±153% *v* 414% ± 178%; $P = .022$ adjusted by age), compared to salt-resistant hypertensives. Moreover, the effect of L-NMMA infusion on this endothelium-dependent response was blunted in the former group (72% *v* 170%; $P = .002$). No differences were observed in endothelium-independent response (417% ± 139% *v* 394% ±131%; $P = .550$) between both groups of patients.

Differences in Serum Inflammatory Markers Between Salt-Sensitive and Salt-Resistant Subjects During Low and High Salt Intakes

Table 3 shows values of inflammatory markers in salt-sensitive and salt-resistant patients measured at low and high salt intakes. No effect of dietary salt changes was observed in any of the measured parameters, but the comparison of salt-sensitive and salt-resistant patients revealed differences in the inflammatory markers measured. Thus, age-adjusted p-selectin ($P = .006$) and MCP-1 ($P = .036$) were significantly higher in salt-sensitive hypertensive patients and this significance was maintained after adjustment for BP differences. Values of e-selectin were also higher in salt-sensitive subjects ($P = .042$), although differences lost their statistical significance after adjustment for BP differences.

Table 2. Twenty four-hour blood pressures, heart rate, and urinary Na^+ excretion during low and high salt intakes in salt-resistant and salt-sensitive essential hypertensive patients

	Salt sensitive (n = 20)		Salt resistant (n = 23)		Total (n = 43)	
Parameter	Low Salt	High Salt	Low Salt	High Salt	Low Salt	High Salt
24-h systolic BP (mm Hg)	130.8 ± 13.8	145.6 ± 14.3	132.4 ± 10.7	132.2 ± 9.7	131.6 ± 12.1	138.2 ± 13.6
24-h diastolic BP (mm Hg)	86.9 ± 10.0	95.6 ± 9.3	86.8 ± 9.6	86.0 ± 9.1	86.9 ± 9.7	90.3 ± 10.2
24-h mean BP (mm Hg)	101.1 ± 11.1	111.8 ± 11.2	101.3 ± 10.2	100.3 ± 10.8	101.2 ± 10.5	105.3 ± 12.3
24-h heart rate (beats/min)	77.3 ± 9.4	79.9 ± 7.2	77.9 ± 6.2	77.9 ± 6.2	77.6 ± 7.8	78.7 ± 6.6
24-h urinary Na^+ excretion (mmol)	47.5 ± 23.9	217 ± 47	49.0 ± 18.7	219 ± 63	48.3 ± 21.1	218 ± 55

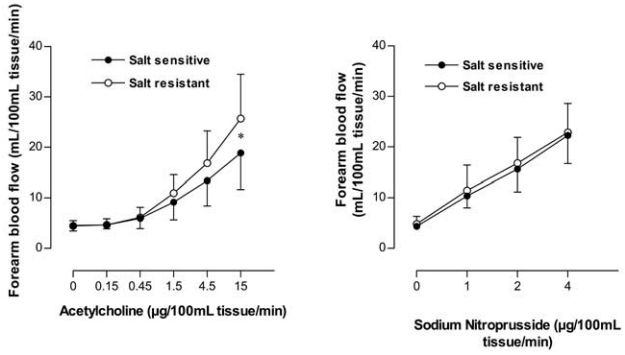

FIG. 1 Increase in forearm blood flow in response to increasing doses of acetylcholine infusion (**left**) or sodium nitroprusside (**right**) in 43 essential hypertensive patients classified on the basis of blood pressure response to sodium intake (salt sensitivity). Salt-sensitive patients present an impaired endothelium-dependent vasodilation with respect to salt-resistant subjects (*P = .022). No differences are observed in endothelium-independent vasodilation.

Differences in Metalloproteinases Between Salt-Sensitive and Salt-Resistant Subjects During Low and High Salt Intakes

Table 4 shows the values of the three metalloproteinases measured (MMP-1, MMP-2, and MMP-9) and their tissue inhibitor (TIMP-1) in salt-sensitive and salt-resistant subjects during low and high salt intakes. No effect of dietary salt changes was observed in any of these parameters, but age-adjusted values of MMP-9 were significantly lower (P = .007) and TIMP-1 significantly higher (P = .045) in salt-sensitive, compared to salt-resistant patients. These differences were maintained after adjustment for mean BP.

Correlation Between Atherosclerosis Markers and Changes in BP Induced by High Salt Intake

The correlation between atherosclerosis markers and salt sensitivity was also assessed by a stepwise multiple linear regression using the 24-h mean BP increase with salt intake as the dependent variable, and atherosclerosis markers (the mean of both measures) as independent variables. We found that after age adjustment, p-selectin ($\beta = 0.035$; P = .028), sVCAM-1 ($\beta = 0.037$; $P < .001$), and MMP9 ($\beta = -0.017$; P = .029) were associated with a BP increase induced by dietary salt (adjusted $R^2 = 0.602$).

Discussion

This study shows that salt sensitivity in essential hypertension is associated with increased circulating levels of atherosclerosis markers. These biomarkers include inflammatory mediators, such as selectins and MCP-1, and a lack of equilibrium between metalloproteinases and their tissue inhibitor TIMP-1, favoring the latter. These findings were demonstrated by comparing salt-sensitive and salt-resistant hypertensives before and after BP adjustment. These

Table 3. Serum inflammatory markers during low and high salt intake in salt-sensitive and salt-resistant hypertensive patients

	Salt Sensitive		Salt Resistant		*P* (effect of salt intake)	*P* (effect of salt sensitivity)		
	Low Salt	High Salt	Low Salt	High Salt		Adjusted by Age	Adjusted by Age and Mean BP at Low Salt	Adjusted by Age and Mean BP at High Salt
CRP (mg/dL)	0.56 ± 0.39	0.55 ± 0.46	0.36 ± 0.27	0.46 ± 0.29	.504	.274	.232	.242
sICAM-1 (ng/mL)	205 ± 83	204 ± 95	209 ± 50	199 ± 46	.673	.587	.448	.880
sVCAM-1 (ng/mL)	359 ± 92	353 ± 64	327 ± 73	349 ± 103	.540	.590	.227	.810
p-Selectin (ng/mL)	200 ± 62	193 ± 57	140 ± 51	142 ± 71	.780	.006	.011	.014
e-Selectin (ng/mL)	44.1 ± 13.0	44.3 ± 12.6	35.8 ± 12.5	37.7 ± 14.4	.524	.042	.072	.092
IL-6 (pg/mL)	2.48 ± 0.73	2.63 ± 1.03	2.17 ± 0.68	2.13 ± 0.57	.728	.254	.064	.124
MCP-1 (pg/mL)	314 ± 56	340 ± 50	266 ± 80	276 ± 97	.300	.036	.042	.022

Table 4. Metalloproteinases and their tissue inhibitor measured during low and high salt intake in salt-sensitive and salt-resistant hypertensive patients

	Salt Sensitive		Salt Resistant		*P* (effect of salt intake)	*P* (effect of salt sensitivity)		
	Low Salt	High Salt	Low Salt	High Salt		Adjusted by Age	Adjusted by Age and Mean BP at Low Salt	Adjusted by Age and Mean BP at High Salt
MMP-1 (ng/mL)	12.5 ± 7.5	17.5 ± 17.2	13.4 ± 11.5	14.3 ± 13.3	.212	.462	.526	.998
MMP-2 (ng/mL)	1057 ± 154	1253 ± 154	1005 ± 217	1170 ± 316	.079	.419	.186	.276
MMP-9 (ng/mL)	185 ± 87	215 ± 92	339 ± 142	321 ± 107	.660	.007	.019	.026
TIMP-1 (ng/mL)	668 ± 152	688 ± 178	549 ± 197	545 ± 210	.964	.045	.033	.018

results strengthen the idea that endothelial dysfunction plays a role in the pathogenesis of salt-sensitive hypertension, as indicated by previous results from our group showing impaired endothelial-dependent vasodilation in the forearm circulation of salt-sensitive hypertensive patients,[12] which have been confirmed in the present study. Increased levels of atherosclerosis markers and impaired endothelium-dependent vasodilation may help to explain the greater frequency of target organ damage[10,11] and the poorer prognosis in hypertensive subjects classified as having salt-sensitive hypertension.[7,8]

Increased salt intake is related to the development of hypertension[27] and confers an increased risk of cardiovascular disease to individuals who are more salt sensitive.[7,8] Salt-sensitive hypertension is also related to increased target organ damage, including left ventricular hypertrophy,[10] renal impairment,[11] and vascular endothelial dysfunction.[12] This association between salt sensitivity and endothelial dysfunction was the basis of the hypothesis of this study, which was that abnormal endothelium in salt-sensitive hypertensive patients may be responsible for enhanced vascular inflammation leading to greater cardiovascular risk.

We found that salt-sensitive hypertensive patients have increased levels of several inflammatory markers, especially selectins and MCP-1. Moreover, p-selectin was associated with a BP increase with salt intake in the multivariate analysis.

E-selectin is produced in endothelial cells, whereas p-selectin is found in both platelets and endothelial cells. They are markers of either endothelium or platelet activation and they both act in the first steps of the atherosclerotic process by favoring rolling of leukocytes to the endothelial surface.[28,29] Increased serum p-selectin levels have been related to the development of future cardiovascular events in healthy women.[30] Moreover, e-selectin levels are associated with poor prognosis in patients with coronary artery disease.[22] The higher levels of both p-selectin and e-selectin found in salt-sensitive hypertensives in the present study are in agreement with a previous study from Ferri et al,[25] who also found increased serum e-selectin in salt-sensitive patients. In that study levels of p-selectin were not measured.

We also found increased serum MCP-1 in salt-sensitive patients. The MCP-1 is responsible for monocyte recruitment and trafficking, has been found in human and animal atherosclerotic lesions, and has recently been associated with a poor prognosis in patients with acute coronary syndromes.[23] The MCP-1 production clearly increases in the presence of endothelial dysfunction and classic atherosclerotic risk factors,[31] of which both situations are more common in salt-sensitive subjects.[10,12]

We found no difference in levels of soluble adhesion molecules (sICAM-1 and sVCAM-1) or IL-6 between patients with or without salt-sensitive hypertension, although in the multiple linear regression analysis sVCAM-1 was associated with the BP increase with dietary salt. Although they have been associated with hypertension[15,16] and cardiovascular disease,[22] they probably do not represent biomarkers of a more subtle cardiovascular risk related to the presence of salt sensitivity in hypertension. In a previous study of salt-sensitive hypertension, no differences in sICAM-1 and sVCAM-1 were observed between patients with salt-sensitive or salt-resistant hypertension.[25]

Finally, we found that, compared to salt-resistant, salt-sensitive hypertensive patients had lower levels of MMP-9 and higher levels of the tissue inhibitor of metalloproteinases TIMP-1. The MMP-9 was also inversely associated with BP increase with salt in the multivariate analysis. Matrix metalloproteinases are produced by macrophages and smooth muscle cells and play an important role in vascular remodeling.[32] Two studies[17,18] have found lower levels of various metalloproteinases (MMP-1, MMP-2, and MMP-9), and increased levels of the tissue inhibitor (TIMP-1)[17] in essential hypertensive patients compared to normotensive subjects. Moreover, TIMP-1 has been related to left ventricular hypertrophy in essential hypertensives.[21] The present study also confirms that, in salt-sensitive hypertensive subjects, the relationship between extracellular matrix degradation (as represented by low MMP-9 levels) and the inhibition of such degradation (as represented by high TIMP-1 levels) is skewed to the latter, probably representing more pronounced collagen deposition in the vascular wall.

The present study has some limitations due to the low number of patients included, the fact that salt-sensitive patients were somewhat older and more severe hypertensive compared to salt-resistant ones, and the evaluation of target organ damage did not include the measurement of left ventricular mass. However, all the differences between salt-sensitive and salt-resistant patients were maintained after adjustment by age and BP. Moreover, the relationship between salt sensitivity and left ventricular hypertrophy has been previously reported.[10,11]

In conclusion, salt-sensitive hypertension is related to several abnormalities in biomarkers of atherosclerosis and remodeling, including inflammation (high levels of selectins and chemokines) and extracellular matrix degradation (low MMP-9 and high TIMP-1). Salt-sensitive hypertension is also characterized by an impaired endothelial dysfunction. These alterations could help to explain the greater target organ damage and cardiovascular risk observed in salt-sensitive subjects.

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ASSOCIATION BETWEEN INCREASED CCL2 (MCP-1) EXPRESSION IN LESIONS AND PERSISTENCE OF DISEASE ACTIVITY IN GIANT-CELL ARTERITIS.

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ABSTRACT

Objective: Patients with giant-cell arteritis (GCA) usually respond dramatically to corticosteroid treatment. However recurrences are frequent and corticosteroid requirements are highly variable among patients. The aim of our study was to identify genes potentially involved in disease persistence.

Methods: Gene expression was explored with cDNA arrays in temporal artery biopsies from 6 GCA patients with relapsing disease and 6 patients who easily achieved sustained remission. Differentially expressed genes of interest were subsequently analyzed by quantitative real-time PCR and immunohistochemistry in temporal artery biopsies from 35 patients with biopsy-proven GCA and 9 controls.

Results: CCL2 (MCP-1) was up-regulated in temporal artery samples from relapsing individuals. In the extended series of patients, CCL2 mRNA concentration in lesions was significantly higher than in controls (31 ± 15.6 vs 0.44 ± 0.10, $p = 0.0001$). In addition, CCL2 was more abundant in patients who experienced 2 or more relapses during the first year compared to those who endured sustained remission (127 ± 82 vs 11 ± 5.5, $p = 0.0233$) and correlated with the cumulated prednisone dose ($r = 0.533$, $p = 0.0024$). CCL2 mRNA concentration correlated with IL-1β ($r = 0.45$, $p = 0.02$), TNFα ($r = 0.47$, $p = 0.013$), and IL-6 ($r = 0.52$, $p = 0.0053$) mRNA. However, circulating CCL2 determined by ELISA was decreased in patients with strong systemic inflammatory response, suggesting that reduction in circulating CCL2 may reinforce the local gradient in lesions.

Conclusion: Increased CCL2 (MCP-1) expression in lesions is associated with persistence of disease activity in GCA.

INTRODUCTION

Patients with GCA usually experience a dramatic relief of their symptoms with corticosteroid treatment (1). However, corticosteroid requirements are highly variable among patients (2). While some patients easily enter sustained remission with relatively short corticosteroid courses, others suffer from a relapsing disease requiring remarkable cumulated corticosteroid doses with their ensuing adverse effects (1, 2).

We have previously shown that the intensity of the acute phase response is empirically associated with different disease outcomes (2). Patients with weak systemic inflammatory response have higher risk of developing disease-related ischemic events (3) but achieve more rapidly a sustained remission, suffer from fewer relapses, and require lower cumulated corticosteroid doses (2). By contrast, patients with strong systemic inflammatory response have more refractory disease and require more prolonged corticosteroid treatment (2). Associated with this persistent activity these patients have higher tissue production and circulating levels of pro-inflammatory cytokines, and a more prominent angiogenic response with stronger expression of endothelial cell adhesion molecules in their lesions compared to patients with weak systemic inflammatory reaction (2, 4-7). These observations support the concept that some patients would develop an inflammatory process which would easily evolve to a healing stage with higher risk of ischemic complications, perhaps facilitated by the scarring process, whereas other patients would develop persisting disease with sustained inflammatory cascades leading to a more refractory and relapsing outcome.

Pro-inflammatory cytokines IL-1β, TNFα, and IL-6 may contribute to determining these different disease outcomes in GCA. As mentioned, their tissue expression correlates with the intensity of the systemic inflammatory response (4). In

addition, TNFα and, to a lesser extent, IL-1β mRNA levels in lesions correlate with subsequent corticosteroid requirements (4). Based on their known biologic functions, pro-inflammatory cytokines may directly maintain inflammatory cascades leading to persistent disease activity (8-9). However, pro-inflammatory cytokines may be downstream products regulated by other factors with stronger impact on the fate of the disease or may be co-ordinately regulated with other mediators which are also relevant in determining disease expression and outcome.

In this study, we used cDNA arrays to screen for differential gene expression in tissue samples from untreated patients with different subsequent disease outcomes in order to identify additional genes with potential prognostic significance. We characterized CCL2 (MCP-1) as a significant factor associated with disease persistence and identified other potentially relevant genes that deserve further study. Some of the differentially expressed genes have previously unknown functions in inflammation or in vascular biology.

PATIENTS AND METHODS

Patients

We consecutively selected 2 groups of patients from our prospectively recorded database of patients with biopsy-proven GCA. The essential requirement for all patients was having a temporal artery biopsy excised prior to the administration of corticosteroids, and stored frozen at – 80^{o}C in guanidine-thiocyanate (GTC) to preserve RNA. For group 1, we selected 6 patients with weak systemic inflammatory response (≤ 2 inflammatory parameters as described) (2, 3) who had tolerated corticosteroid reduction with no relapses, successfully achieving a stable maintenance prednisone dose < 10 mg/day in less than 35 weeks. Four of the patients had ischemic events at the time of diagnosis. For group 2, we selected 6 patients with strong systemic inflammatory reaction (3-4 inflammatory parameters). All these patients required more than 50 weeks to achieve a stable maintenance prednisone dose < 10 mg/day and none of them developed ischemic complications. The main clinical characteristics of these patients are summarized in table 1.

RNA extraction from temporal artery biopsies

Temporal artery biopsies were obtained for diagnostic purposes. Part of the specimen was snap frozen in isopentane pre-chilled in liquid nitrogen and stored at – 80^{o}C. This fragment was used for histopathologic examination and immunohistochemical studies.

About 0.3-0.5 cm of the biopsies were frozen in GTC and stored at -80^{o}C. These samples were thawed in TRIzol reagent (Life Technologies) and RNA extraction and purification were performed according to the manufacturer's instructions. The yield was

2.75 μg RNA ± 0.77 (mean ± SEM) per patient in group 1 and 2.15 μg RNA ± 0.58 per patient in group 2. RNAs from patients in each group were pooled in order to have enough RNA to hybridize the cDNA arrays after DNAse treatment. Pooling RNA was also performed to minimize non-specific changes in the expression of genes modulated by ischemia or involved in tissue repair, due to unavoidable variations either in tissue manipulation during the excision, duration of the surgical procedure, or delay in tissue processing.

cDNA array hybridization

We used Human Atlas 1.2 I nylon cDNA arrays (Clontech, BD Biosciences) containing 1,176 known genes (list available at http://bioinfo.clontech.com/atlasinfo) .

Pooled RNA samples were treated with DNAse to remove contaminating genomic DNA and 2 μg of total RNA per condition were ^{32}P-labeled using MMLV reverse transcriptase and 3' primers corresponding to the genes represented on the Atlas 1.2 I array (Atlas Pure Labeling System protocol, Clontech). After the recommended blocking steps and purification of the labelled probes, cDNA array membranes were hybridized overnight at 68^{o}C with 25 x 10^{6} cpm of each labelled probe. Membranes were then stringently washed and exposed for 6 days to a Phosphor Imager screen. With this exposure time, low abundance transcripts may not be detected. Hybridization of control and housekeeping cDNAs was equivalent in both filters and contamination by genomic DNA was excluded by internal controls. Negative controls (plasmid and phage DNA) did not provide signal, excluding non-specific binding. After subtraction of background and global normalization, the intensity of the radioactive signals was compared using the Atlas-Image software (Clontech).

Real-time quantitative RT-PCR measurement of cytokines in temporal artery samples

CCL2 (MCP-1) was one of the genes differentially expressed in both groups of patients. CCL2 mRNA was subsequently measured in a wider series of 35 consecutively diagnosed patients with biopsy-proven GCA, who had been diagnosed and treated by the authors according to a previously reported schedule (2) and prospectively followed for at least 1 year. Patients were unselected; all of them fulfilled the ACR classification criteria for GCA, and encompassed the entire classical spectrum of disease manifestations. TNFα, IL-1β, and IL-6 cytokine mRNAs were also measured for comparison purposes. One μg of total RNA obtained from temporal artery samples was reverse transcribed to cDNA with the Superscript^{TM} II First Strand Synthesis kit (Life Technologies), using random hexamers as a priming method. Quantitative real -time PCR amplification of the above cytokine cDNAs was performed using Pre-Developed TaqMan Target kits from Applied Biosystems (Foster City, CA), as described (4, 5). The reaction was monitored by measuring the fluorescence signal after each cycle with ABI Prism TM 7700 Sequence Detection System (Applied Biosystems). Values were expressed as relative units. CCL2 mRNA was also quantified in 9 normal temporal artery biopsies obtained from patients in whom the disease was initially suspected but not confirmed, being subsequently diagnosed with other conditions. The ultimate diagnoses in these patients were: isolated polymyalgia rheumatica (2 patients), anemia related to diabetic nephropathy (2 patients), monoclonal gammopathy (1 patient), non-vasculitic anterior ischemic neuropathy (1 patient), temporomandibular osteoarthritis (1 patient), ophthalmoplegia due to orbital myositis (1 patient), and acute lymphoblastic leukaemia (1 patient).

Immunohistochemical detection of CCL2 and its cell receptor CCR2 in temporal artery biopsies

Expression of CCL2 and its receptor CCR2 was subsequently analyzed by immunohistochemistry in temporal artery sections from 50 patients and 9 normal controls. Seven out of the 50 GCA samples exhibited only adventitial inflammation whereas in the remaining 43 specimens inflammatory infiltrates extended through the media and the intima

Serial 4-6 μm serial cryostat sections were air dried, fixed with cold acetone, and incubated with the following primary monoclonal antibodies for 30 minutes at room temperature: mouse anti-human CCL2 (clone 23002 from R&D Systems at 5 μg/ml) and mouse anti-human CCR2 (clone 48607.121 from R&D Systems at 10 μg/ml). Immunoreactivity was detected with a HRP-bound polymer coupled to a secondary anti-mouse antibody (Envision system from Dako). Peroxidase activity was visualized by 0.02% 3-3´diaminobenzidine (Sigma) and 0.05% hydrogen peroxide. Mouse immunoglobulin (Dako) at the same concentration as the primary antibodies and incubated with the detection system served as negative control. All sections were slightly counterstained with Harris' haematoxylin. Samples were stratified according to the intensity and extension of immunostaining from 0 to 4, being 0 negative and 4 the maximum staining observed. Sections were evaluated by 2 investigators (MCC and JHR) blinded to the clinical data.

CCL2, TNFα and IL-6 detection in sera from patients with GCA

Circulating levels of CCL2 were determined in 56 patients (40 females and 16 males) with biopsy-proven GCA using enzyme-linked immunosorbent assays (ELISA) (R&D Systems), following the instructions of the manufacturer. These patients had been

prospectively evaluated at diagnosis, treated and followed by the authors according to a predefined schedule (2). Serum samples were obtained before starting corticosteroid treatment in 47 patients and the remaining 9 had received a single prednisone dose (60 mg) the day before serum collection. Eighteen age and sex- matched healthy donors were used as controls. Each sample was tested in duplicate wells. TNFα and IL-6 were also measured by ELISA (R&D Systems) in sera from 48 of these patients and in 15 controls for comparison purposes.

Statistical analysis

Mann-Whitney U test was applied to qualitative data. Fisher exact test was used for contingency tables and Pearson or Spearman's rank coefficients for correlations. Time required to achieving a stable maintenance prednisone dose <10 mg/day was analyzed by the Kaplan-Meier survival analysis method and compared by the log rank test.

RESULTS

Patients with different disease outcomes exhibit differentially expressed genes in their temporal artery lesions

Among the 1,176 genes screened, 14 were differentially expressed in both groups of patients. Since the hybridization conditions of the arrays favoured specificity rather than sensitivity, it is possible that additional relevant genes were not detected. In fact, expression of the pro-inflammatory cytokines IL-1β, TNFα, and IL-6, as well as endothelial adhesion molecules, which we have previously found to be up-regulated in GCA (4, 7), were barely or not detected.

The known biological functions of some of the up-regulated genes, suggest that their increased expression may be related through different pathways to the persistence of inflammatory activity. Among them, the chemokine CCL2 may contribute to the perpetuation of the inflammatory process through the recruitment and activation of monocytes which, when becoming tissue macrophages, are significant effector cells in GCA (10). As shown in Table 2, other genes differentially expressed in this screen were transcriptional factors or transcriptional regulators involved in inflammatory responses (11), genes involved in cell migration (10, 12-14), cell survival (15) and genes expressed during cell response to stress (16, 17). Intriguingly, some genes involved in bone remodelling (18) and in neural transmission (19) or neural development (21) were also differentially expressed. The function of these genes in either inflammation or vascular biology is unknown.

To validate the array findings, mRNA from one of the genes differentially expressed, CCL2 was measured by quantitative real-time PCR in 3 individual samples

from group 1 and 4 individual samples from group 2. In agreement with the cDNA array results, CCL2 transcripts were more abundant in patients in group 2 (178 relative units ± 117) (mean ± SEM) than in group 1 (1.59 relative units ± 0.94).

CCL2 (MCP-1) gene expression in vascular inflammatory lesions from patients with GCA

Since CCL2 was over-expressed in the pooled samples from patients with a more refractory outcome, and given its potential contribution to the perpetuation of lesions, we investigated CCL2 expression in temporal artery samples from a larger series of patients. Since pro-inflammatory cytokines have been demonstrated to induce CCL2 in different cell types, IL-1β, TNFα, and IL-6 expression was also assessed in the same samples.

CCL2 mRNA could be detected in normal biopsies although at low levels, indicating baseline CCL2 expression in normal temporal arteries. However CCL2 transcripts were much more abundant in GCA samples (31.4 ± 15.6 relative units) (mean ± SEM) than in normal specimens (0.44 ± 0.10 relative units) ($p = 0.0001$) (figure 1A). Twenty-six out of the 35 patients studied had received no treatment at the time of the temporal artery excision, whereas 9 had received prednisone at 1 mg/Kg for a median of 2 days (range 1-6). No significant differences were observed in CCL2 values between untreated patients and patients who had received corticosteroids. These findings indicate that, contrarily to what has been observed for pro-inflammatory cytokines and endothelial adhesion molecules (5, 7) corticosteroid treatment for less than one week is not sufficient to significantly down regulate CCL2 expression**.**

A significant correlation was found between CCL2 and IL-1β ($r = 0.45$, $p = 0.02$), TNFα ($r = 0.47$, $p = 0.013$), and IL-6 ($r = 0.52$, $p = 0.0053$) transcripts,

supporting an interrelated regulation of these cytokines in GCA. Interestingly, and as observed for IL-6 (7), CCL2 transcripts were significantly less abundant in samples from patients with disease-related ischemic events (3.1 ± 1.5 vs 39.7 ± 20, p = 0.0243) (figure 1B).

CCL2 mRNA levels were significantly higher in patients who suffered ≥ 2 relapses than in patients who did not relapse during the first year of treatment (figure 1C) and correlated with the cumulated dose of prednisone during this period of time (r = 0.533 , p = 0.0024). In addition, the time required to achieve a stable maintenance dose of prednisone < 10 mg/day, was significantly longer in patients with CCL2 mRNA levels higher than 3 relative units (figure 1D). These data suggest that CCL2 expression may be involved in the persistence of inflammatory activity in GCA.

Immunohistochemical detection of CCL2 (MCP-1) and its receptor CCR2 in temporal artery samples.

We next investigated the expression of CCL2 protein and its receptor, CCR2, in temporal artery lesions from 50 patients and from 9 normal controls. CCL2 protein was detected mainly in vascular smooth muscle cells (VSMC) and in infiltrating leukocytes of macrophage appearance (figure 2). This distribution is in accordance with a previously published study performed by other investigators in a small series of patients (22). CCR2 expression was also observed in infiltrating leukocytes and in VSMC (figure 2). Interestingly, in samples with only initial adventitial inflammation, adventitial infiltrating leukocytes strongly expressed CCR2, whereas in full-blown lesions, CCR2 expression predominated in the granulomatous areas (figure 2)**.** Given that inflammatory cells are thought to reach and invade the temporal artery wall through the adventitial vasa vasorum, this distribution suggests a relevant role for CCL2 /CCR2

interactions in the recruitment of inflammatory cells into GCA lesions. A significant correlation was observed between CCL2 and CCR2 immunohistochemical scores, suggesting coordinated regulation of CC2L and its receptor ($r = 0.29$, $p = 0.012$). Four out of the 9 normal temporal arteries also exhibited low immunoreactivity for CCL2 in accordance with the results obtained with real-time PCR (figure 2).

Immunoshistochemical scores at the media, where VSMC were the main cell type, were significantly higher in patients than in controls and in patients with inflammatory infiltrates extending through the three layers compared to those with only adventitial inflammation (Figures 3 A and 3B). This finding suggests, in agreement with in vitro studies with cultured cells, that paracrine induction by proinflammatory cytokines or by other mediators released by inflammatory cells is required for efficient CCL2 expression by VSMC in GCA (24).

Circulating CCL2 (MCP-1) in patients with GCA

Serum CCL2 concentrations were similar in patients than in age and sex-matched healthy donors (137 ± 11 pg/ml vs 206 ± 53 pg/ml, $p = 0.26$). However, when patients were stratified according to the severity of the acute phase response, prospectively evaluated at the time of diagnosis (2, 3), a significantly negative association with CCL2 levels was observed (figure 4A). We have previously shown that serum IL-6 and to a lesser degree TNFα, correlate with the intensity of the systemic inflammatory response (2). Although a negative correlation between individual CCL2 and IL-6 or TNFα values did not reach statistical significance, serum TNFα and IL-6 concentrations showed a positive association with the intensity of the acute phase response in the same cohort of patients, following a pattern opposite to CCL2 (figures 4B and 4C).

DISCUSSION

In this study, we show that patients with weak systemic inflammatory response who have higher prevalence of ischemic complications and a better response to corticosteroid treatment have different gene expression in their lesions than patients with strong systemic inflammatory reaction who are more refractory to therapy. Among the 1,176 genes analyzed, differential expression of 14 genes was significantly detected. Although their potential role in the pathogenesis of GCA needs to be investigated more extensively, the characterized functions of many of these genes suggest their relationship to the persistence of vascular inflammation (10-21).

Interestingly, one of the most significantly up-regulated genes was CCL2, a potent chemotactic factor for monocytes and activated Th1 lymphocytes, the most prominent cell types in GCA inflammatory infiltrates (10). CCL2 is known to be expressed by vessel wall components, such as VSMC and endothelial cells, and may contribute to the continuous recruitment and activation of Th1 lymphocytes and monocytes (23, 24). Although CCL2 mRNA and protein expression were detected at low levels in VSMC from normal temporal arteries, a much stronger expression was detected in inflamed arteries from patients with GCA. In addition, CCL2 expression by VSMC was more intense in patients with inflammatory lesions extending through the media than in those with solely adventitial inflammatory infiltrates suggesting that inflammatory mediators released by infiltrating leukocytes have a major role in amplifying vascular CCL2 expression. In fact, a significant correlation between CCL2 and proinflammatory cytokine mRNAs was observed in an extended series of patients. CCL2 can be also expressed by activated macrophages (25) and, in fact, the strongest

immunostaining for CCL2 was observed in the granulomatous areas. This may constitute an amplificatory feed-back mechanism through which activated macrophages promote the recruitment of additional monocytes. Supporting this concept, MRP-8, a S-100 related molecule expressed by early recruited phagocytes, was also up-regulated in relapsing patients. We have previously shown that MRP-8 is mainly expressed by infiltrating cells located at the adventitia and in surrounding vasa vasorum, the sites through which inflammatory cells are thought to penetrate the vessel wall (15). Up-regulated MRP-8 may reflect an increased presence of freshly recruited monocytes. The elevated expression of RhoA, Rho B, and PRL-1, pivotal elements in cell migration would also represent the active influx of inflammatory cells into the vessel wall (13, 14) and down-regulation of PTP-C2, a molecule promoting apoptosis of haematopoietic cells, might, in turn, prolong survival of infiltrating leukocytes (16).

CCL2 may act by multiple mechanisms to maintain inflammation. In addition to its chemotactic effects on leukocytes, CCL2 is also chemotactic for endothelial cells and may stimulate angiogenesis, a potent amplificatory mechanism in chronic inflammatory diseases (26). The pro-inflammatory role of CCL2 has been demonstrated in animal models of inflammatory diseases (27-29) and is thought to contribute to the inflammatory component of atherosclerosis and graft vasculopathy (30-31).

Similar to IL-6 (5), CCL2 expression was associated with lower risk of ischemic events. The mechanisms underlying this observation are not clear at present. CCL2 may be co-ordinately regulated with other protective factors or may have a protective function by itself. As mentioned, and similarly to IL-6, CCL2 has angiogenic properties (5, 26) and may help to prevent ischemia by contributing to compensatory neovascularization.

Our patients did not exhibit elevated levels of circulating CCL2 compared to age and sex-matched controls. This was unexpected since other investigators have reported elevated concentrations of CCL2 in patients with GCA (22) and in patients with other chronic inflammatory conditions such as rheumatoid arthritis, psoriatic arthritis, and Kawasaki disease (32-34), although there is no complete agreement in these studies. In the previously existing study performed in patients with GCA, a young population, aged 43 (range 33-59) was used as control and it has been demonstrated that, as with other inflammatory molecules, circulating CCL2 increases with age (35). Other chronic inflammatory disorders usually occur in younger individuals and differences from healthy controls may be more apparent.

Interestingly, when patients where stratified according to the number of inflammatory parameters, CCL2 concentration in sera was negatively related to the intensity of the systemic acute phase response. It has been demonstrated that there is an increased population of circulating activated monocytes in GCA which actively produce IL-6 (36). Elevated serum concentrations of TNFα and IL-6 may then reflect an increased number of activated monocytes. CCR2 is up-regulated in activated monocytes and may sequester circulating CCL2, as it has been demonstrated in experimental models (31). Similar to other chemokines, local CCL2 concentrations are more functionally relevant than circulating levels since CCL2 acts in an autocrine/paracrine manner determined by a local gradient, facilitated by interactions with proteoglycans. Reduced circulating CCL2 may help to maintain an efficient local gradient at the sites of inflammation.

Several strategies addressed to block CCL2 expression or biological activity are being developed as potential new therapeutic interventions. These are being tested in animal models of atherosclerosis, graft vasculopathy, and chronic inflammatory

diseases (27, 29). Our findings indicate that CCL2 expression is associated with persistent disease activity and might contribute to the perpetuation of inflammatory lesions in GCA, suggesting that GCA may also be considered among the diseases that could potentially benefit from CCL2 blockade. However, definitive confirmation of the role of CCL2 in disease persistence can be only obtained from clinical trials.

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Table 1. Clinical characteristics of the patients included in the cDNA array study

	Group 1 (Relapsing patients) N(%) or mean ± SEM)	Group 2 (Remitting patients) N(%) or mean ± SEM)	p value
Ischemic events*	4 (67%)	0 (0%)	§
Fever	1 (17%)	5 (83%)	§
Weight loss	3 (50%)	5 (83%)	§
ESR (mm/h)	62 ± 9	117 ± 6	0.0022
Hb (gm/L)	121 ± 3	92 ± 4	0.0022
Time (weeks) †	23 ± 3	77 ± 32	0.0087
Cumulated prednisone (mg) ‡	4881 ± 174	7796 ± 663	0.0159

* Bilateral blindness (1), unilateral blindness (1), amaurosis fugax (1), transient tongue ischemia (1)

† Time (weeks) required to achieve a stable maintenance dose of prednisone < 10 mg/day.

‡ During the first year of treatment.

§ Number is too small for statistical evaluation of a contingency table.

Table 2. Genes differentially expressed in temporal artery samples from relapsing patients (group 2) compared to remitting patients in (group 1).

Gene	GeneBank Accession #	Status	Fold Change	Function	References
PRL-1	U48296	Up	94 x	Increases migration and invasiveness	16
TREB 36	X55544	Up	10.6 x	Transcription factor	-
RAD 52	U12134	Up	31 x	DNA repair	21
Neuronatin	U250033	Up	29 x	Brain developmental gene (protects from injury)	24
PCAF 65β	AF069736	Up	16.8 x	Transcriptional activity regulator	14, 15
MRP8	X06234	Up	174 x	Expressed by early phagocytes	18
CCL2	M24545	Up	53.5 x	Chemotaxis	13
RhoA	L25080	Up	2 x	Cell migration	17
RhoB	X06820	Up	2.9 x	Cell migration	17
HSP 27	M11717	Up	2.87 x	Stress protein	20
PTP-2C	L08807	Down	Undetected	Decreases hematopoetic cell survival	19
NK-3R	M89473	Down	53.5 x	Neurotransmission	23
OSF	U63717	Down	Undetected	Bone remodelling	22
PNAT/MNAT	X14672/ X17059	Down	Undetected	Drug resistance Aminoacid transporter	-

FIGURE 1

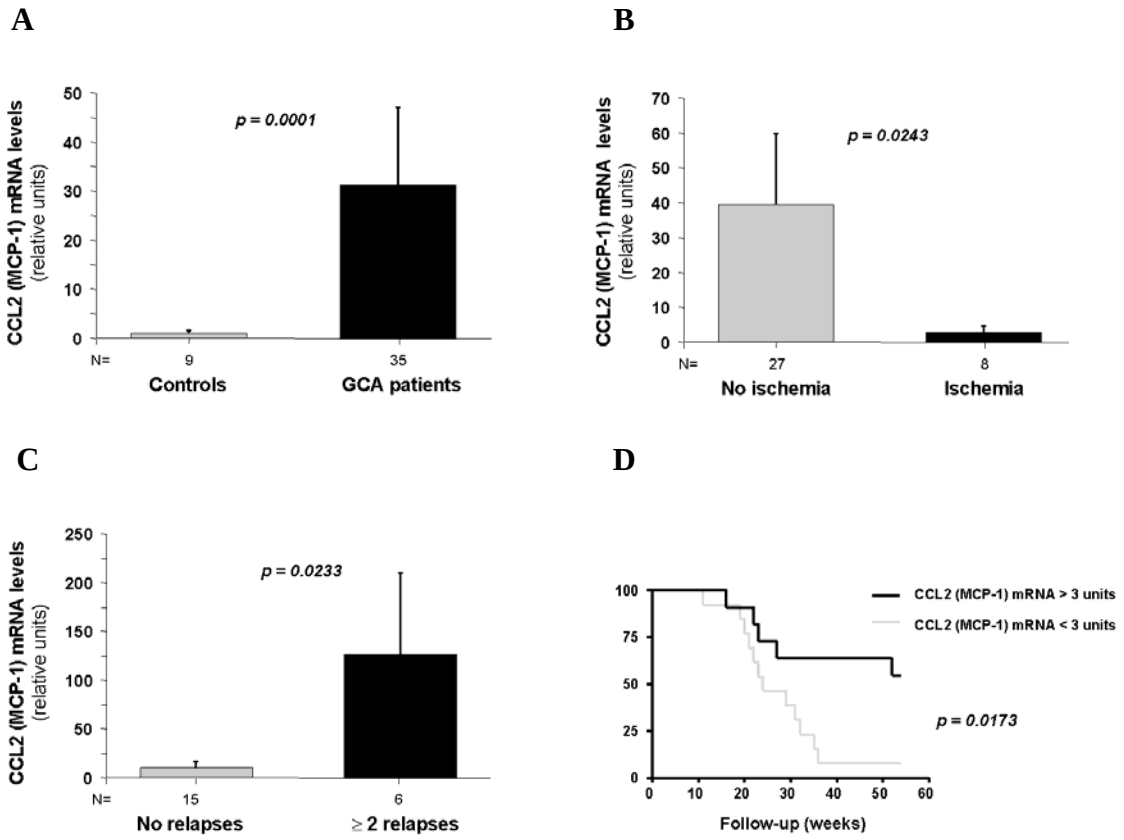

Figure 1: A) CCL2 (MCP-1) mRNA levels in temporal artery samples from 35 patients compared to 9 controls. B) CCL2 (MCP-1) mRNA concentrations in temporal artery biopsies from patients with or without disease-related ischemic complications at diagnosis. C) CC2L (MCP-1) mRNA levels in patients who presented 2 or more disease flares during the first year of follow-up compared to those who did not relapse. Bars represent mean (x) ± standard error of the mean (SEM). D) Percentage of patients requiring a maintenance dose ≥ 10 mg prednisone/day over time (total follow-up 53 weeks) according to the CCL2 mRNA concentration in their biopsies.

FIGURE 2

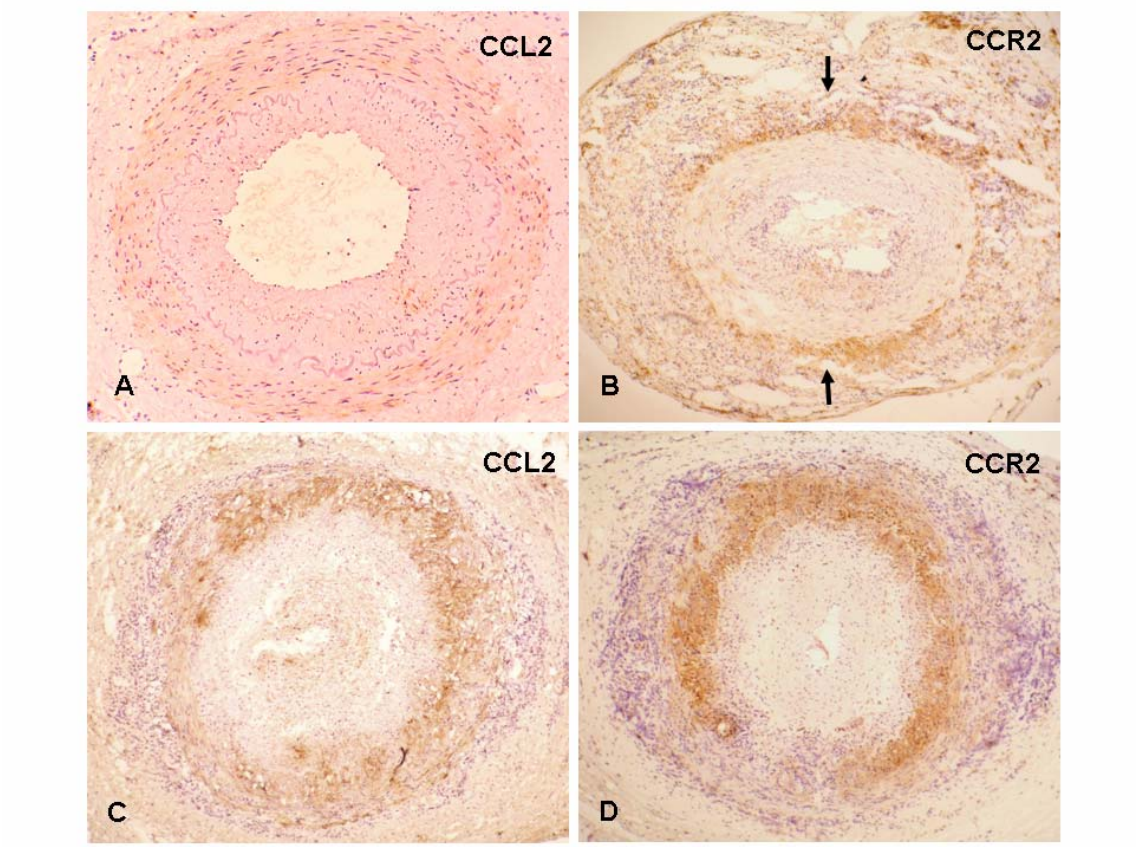

Figure 2: Expression of CCL2 (MCP-1) and CCR2 in GCA lesions. A) Slight CCL2 expression at the medial layer in a normal temporal artery from a control individual. B) CCR2 expression in infiltrating leukocytes at the adventitia (arrows) in a temporal artery with incipient adventitial inflammation. C) CCL2 expression predominates at the the granulomatous area in fully developed lesions. A substantial proportion of infiltrating leukocytes are negative. D) CCR2 expression at the granulomatous area in full-blown GCA lesions.

FIGURE 3

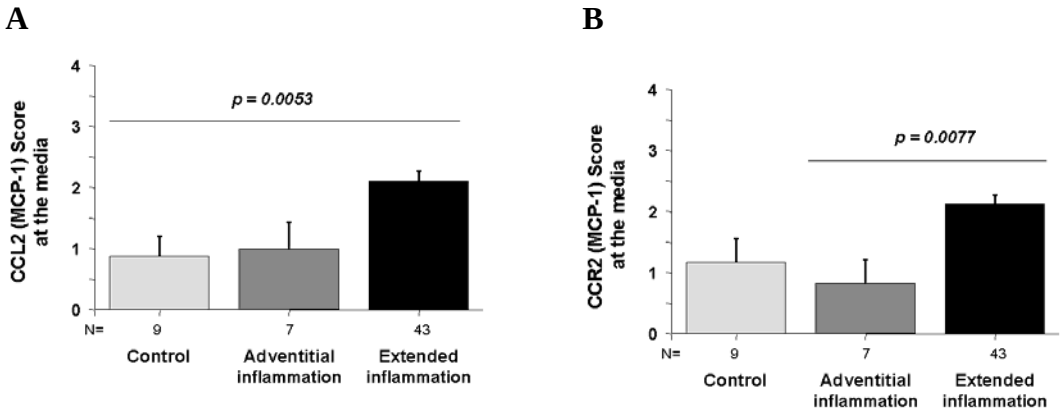

Figure 3: CCL2 (MCP-1) (A) and CCR2 (B) immunostaining scores in the media in temporal artery biopsies from controls, in specimens from patients displaying only adventitial involvement and in samples from patients disclosing inflammatory infiltrates extending through the artery wall.

FIGURE 4

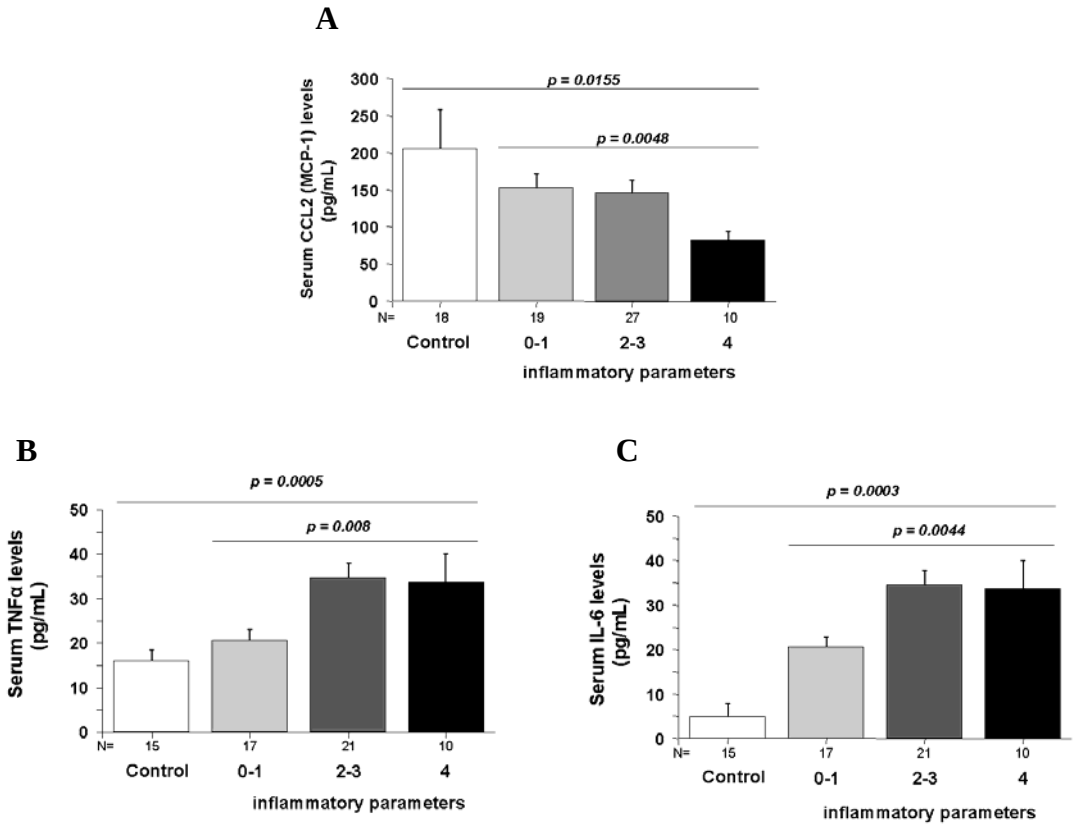

Figure 4: Serum concentration of CCL2 (MCP-1) (A), TNFα (B), and IL-6 (C) in patients with GCA stratified according to the intensity of the systemic inflammatory response. Inflammatory parameters are fever (T^a >37^{o}C), weight loss (≥ 4 Kg), anemia (Hb < 110 gm/L) and ESR ≥ 85 mm, as previously defined (2, 3).

Endothelial Cells, Antineutrophil Cytoplasmic Antibodies, and Cytokines in the Pathogenesis of Systemic Vasculitis

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Endothelial cells play a pivotal role in the pathogenesis of systemic vasculitis. Endothelial cells have significant proinflammatory activities, amplifying and perpetuating the inflammatory process and contributing to vessel regeneration and repair. Significant contributions have improved the understanding of additional ways through which antineutrophil cytoplasmic antibodies (ANCA) may potentiate neutrophil- and monocyte-mediated endothelial cell activation and damage. Signaling pathways mediating ANCA effects have been delineated, and new animal models have demonstrated the pathogenic role of ANCA in the development of systemic vasculitis. Significant efforts have identified anti-endothelial cell antibody specificities and elucidated mechanisms through which these antibodies may promote endothelial cell activation and injury. New ways to assess in vivo endothelial cell damage and dysfunction also have been developed. In addition, besides being a relevant compensatory mechanism for ischemia, angiogenesis may have important proinflammatory functions in vasculitis. The potential relevance of bone-marrow–derived endothelial cell precursors in neovascularization has begun to be appreciated.

Introduction

The vascular endothelium wrapping the inner surface of blood vessels forms a major and ubiquitous system exerting crucial biologic activities in physiologic and pathologic conditions. Among diseases in which endothelial cells play a major role, the vasculitides, inflammatory diseases of blood vessels, are especially relevant because the vascular tree itself is the main target for inflammation and injury [1].

The role of endothelial cells in the pathogenesis of vascular inflammation is multifactorial. Endothelial cells may be a major target for injury; however, endothelial cells have prominent proinflammatory functions and are crucial players in orchestrating vascular response to inflammation, a complex array of functional changes in vessel wall components that occur in response to a variety of cytokines, growth factors, and other inflammatory mediators released by infiltrating leukocytes. Besides magnifying the inflammatory process, vascular response to inflammation leads to vessel remodeling and regeneration, and, eventually, vessel occlusion, which is the cause of some of the most devastating complications in patients with systemic vasculitis [1].

As discussed later in detail, most of the immunopathogenic mechanisms thought to be involved in the development of systemic vasculitis are able to damage endothelial cells. When acting sublethally, the same mechanisms are able to elicit proinflammatory activities on endothelial cells, contributing to amplify the inflammatory reaction.

The Endothelial Cell As A Target for Injury

Endothelial cells can be damaged through a variety of mechanisms during the development of inflammatory cascades, leading to vascular inflammation. These include, through various complex and often combined mechanisms, infectious agents, complement-mediated damage driven by immune complexes, antineutrophil cytoplasmic antibodies (ANCA), and anti-endothelial cell antibodies (AECA) [1].

Infectious agents

Endothelial cells, as well as other vascular components, particularly smooth muscle cells, can be injured by pathogens. Infectious agents classically known to cause vasculitis in humans include bacteria (*ie,* tuberculosis, syphilis,

Salmonella, and infectious endocarditis) and fungi (*ie, Aspergillus*). These agents are thought to invade blood vessels by contiguous or hematogenous spreading or septic emboli [2].

Some intracellular pathogens may directly infect endothelial cells and trigger vasculitic lesions. These include rickettsiae and herpesvirus family members, particularly cytomegalovirus (CMV), varicella-zoster, and herpes simplex viruses [2,3]. Infections by rickettsiae and serious infections by herpesvirus family members in immunocompromised hosts frequently convey vasculitic lesions. In this setting, endothelial cells may be lysed by active replication of intracellular pathogens or may be the target of immune-mediated cytotoxic damage. In animal models, chronic infection by herpesvirus family members (CMV and murine-gamma [γ] herpesvirus 68) preferentially targets smooth muscle cells in medium- and large-sized vessels. Their ability to cause medium- and large-vessel vasculitis has been proven in interferon-γ–deficient mice [3]. However, their contribution to the pathogenesis of large-vessel vasculitis in humans has not been demonstrated.

Additional viruses with a demonstrated role in the pathogenesis of systemic vasculitis in humans are hepatitis B virus, hepatitis C virus (HCV), and HIV [2,4]. Endothelial damage in hepatitis B virus– and HCV-induced vasculitis is thought to be mediated by immune complexes. Pathogenic mechanisms involved in HIV-associated vasculitis are probably heterogeneous and, in some cases, may include the participation of opportunistic infections [2,4].

Immune complex–mediated endothelial cell damage

Experimental models of Arthus phenomenon and serum sickness demonstrated, more than three decades ago, the potential of immune complexes to damage the vessel wall. This was the first identified immunopathogenic mechanism able to cause vascular inflammation and injury. Complement-mediated lysis and neutrophil-mediated damage appear to be the main mechanisms underlying endothelial cell injury in immune complex–mediated vasculitis. Henoch-Shönlein purpura, polyarteritis nodosa, and cryoglobulinemic vasculitis are the clinicopathologic entities best fitting in this pathogenic model. Vasculitic lesions from patients with Henoch-Shönlein purpura typically disclose immunoglobulin (Ig) and complement deposition, particularly IgA, and, in cryoglobulinemic vasculitis, Ig of the same isotype as circulating cryoglobulins can be detected in involved vessels [4]. In HCV-associated mixed cryoglobulinemia, immune complexes containing cryoglobulins and HCV antigens have been detected in lesions [4]. The membrane attack complex C5b-9, the final product of the complement activation cascade, has been detected in vascular lesions of necrotizing vasculitis, such as classical polyarteritis nodosa and vasculitis complicating Sjögren's syndrome (Fig. 1) [1].

Antineutrophil cytoplasmic antibodies and endothelial cell injury

The strong association between the presence of ANCA with specificity for proteinase 3 (PR3) and Wegener's granulomatosis, and, to a lesser extent, between ANCA with specificity for myeloperoxidase (MPO) and microscopic polyangiitis, have highly suggested that ANCA directly participates in the pathogenesis of vascular inflammation and injury [5]. Moreover, although their clinical use in monitoring and making therapeutic decisions in individual patients remains controversial, active patients usually have higher ANCA levels than patients in remission and relapses are often preceded by reappearance or increase in ANCA titers.

As discussed later, many in vitro experiments, as well as several animal models, support a significant, although complex and multifactorial, role of ANCA in inducing and amplifying neutrophil-mediated endothelial injury in ANCA-associated systemic vasculitis. Although there is much evidence supporting the ability of ANCA to produce vessel damage, some unanswered questions still await a satisfactory explanation. The consistent failure in detecting ANCA in glomerular and vasculitis lesions is one of the most intriguing unanswered questions. There also are animal models of autoimmune reactions spontaneously generating ANCA and never developing lesions similar to those appearing in ANCA-associated vasculitis in humans [5].

Physiologic roles of antineutrophil cytoplasmic antibodies targets

Proteinase-3 and MPO are enzymes with important physiologic roles in neutrophil biology. When neutrophils are primed with cytokines, such as tumor necrosis factor–alpha or interleukin (IL)-8 (CXCL8), PR3 and MPO are translocated from the azurophilic granules of neutrophils to the cell membrane [5,6]. Recent contributions indicate that granulocyte-macrophage colony-stimulating factor and transforming growth factor–beta also are able to translocate PR3 to the cell surface [7]. Membrane translocation and release of neutrophil enzymes is part of the physiologic response of neutrophils to inflammatory mediators and probably has an important function in host defense against injury. Circulating PR3 and MPO have been detected in patients with ANCA-associated vasculitis and also in patients with systemic lupus erythematosus, polyarteritis nodosa, subjects with renal failure caused by other conditions, patients with atherosclerosis, and even at low concentrations in healthy individuals [8,9]. Similarly, increased surface expression of PR3 in circulating neutrophils has been observed in Wegener's granulomatosis and also in other diseases, such as sepsis, rheumatoid arthritis, and other inflammatory conditions [7]. In Wegener's granulomatosis, strong PR3 surface expression, which would make neutrophils more readily available for ANCA recognition, is associated with a more relapsing outcome [10•]. It has been recently shown that healthy individuals also may have spontaneous expression of membrane PR3 in their

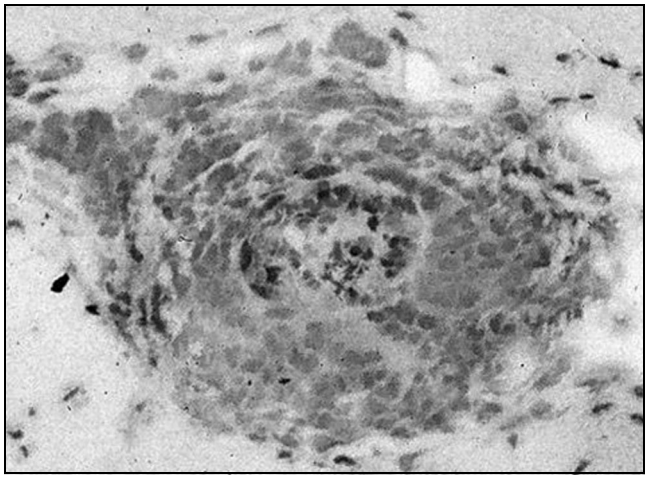

Figure 1. Immunohistochemical detection of the membrane attack complex C5b-9 in a medium-sized artery in classical polyarteritis nodosa (*arrow*). Alkaline phosphatase anti–alkaline phosphatase technique, using the monoclonal antibody aE11 (Dako, Glostrup, Denmark).

neutrophils [7]. Spontaneous expression may be high, low, or bimodal, and this pattern appears to be genetically determined because it remains stable over time and matches in homozygous twins [11]. Although high constitutive membrane expression may be a normal phenotype, the development of Wegener's granulomatosis, as well as other inflammatory diseases, such as rheumatoid arthritis, appears to be significantly more frequent in individuals with strong surface expression of PR3 [7]. A polymorphism in the promoter region of PR3 (-564 A/G) has been demonstrated to be associated with higher PR3 expression, and it is more frequent among patients with Wegener's granulomatosis [12•]. Similarly, a polymorphism (-436 G/A) in the MPO gene is associated with an increased risk of relapse in patients with MPO-ANCA–associated vasculitis [9].

Proteinase-3 expression can be detected in ANCA-associated and other glomerulonephritis [5]. Taken together, these observations indicate the participation of neutrophil enzymes recognized by ANCA in physiologic response against injury and in a variety of chronic inflammatory processes.

Effects of antineutrophil cytoplasmic antibodies on proteinase-3 and myeloperoxidase enzymatic activity

As mentioned, PR3 and MPO enzymatic activity have an important role in neutrophil physiologic functions. As with other proteolytic systems, their activity needs to be tightly regulated to avoid uncontrolled damage, because MPO and PR3 have a remarkable destructive potential. Lung perfusion with PR3 has been shown to cause emphysema in hamsters. PR3 is able to deactivate by cleavage C1 inhibitor and, consequently, may enhance complement-mediated damage. In vitro, PR3 has been demonstrated to be able to detach endothelial cells from their substrate [5,7,13]. MPO catalyzes the production of hypochlorous acid, which reacts with a variety of cell substrates, promotes low-density lipoprotein oxidation, and deactivates protease inhibitors, indirectly facilitating proteolytic cascades [9].

Antineutrophil cytoplasmic antibodies binding to their targets may modulate enzyme activity [5]. Studies performed with overlapping peptides have disclosed that epitopes more frequently recognized by ANCA are located near the catalytic site of PR3 [14••], and, in fact, ANCA are able to prevent endothelial cell detachment and lysis induced by PR3 in vitro [13,14••]. However, ANCA binding to PR3 prevents enzyme inactivation by the natural inhibitor alpha-1-antitrypsin, indirectly enhancing its proteolytic activity [5]. The resulting balance of this apparently opposite influence of ANCA on PR3 enzymatic activity may vary along the course of the disease. It has been shown that epitopes recognized by ANCA may vary according to disease activity in Wegener's granulomatosis. The recognition of epitopes within or close to the catalytic site would be more frequent in ANCA detected in patients in remission [15••]. Because shifting in epitope recognition may have functional relevance, this fact may explain, in part, why some patients with significant ANCA titers may endure sustained remission [14••,15••].

Effects of antineutrophil cytoplasmic antibodies on neutrophil activation

The first evidence that ANCA may substantially amplify neutrophil-mediated injury was provided by Falk *et al.* [6] in 1990. ANCA recognition of membrane-translocated MPO or PR3 in primed neutrophils induces and amplifies neutrophil degranulation and respiratory burst, which, according to subsequent contributions by other authors, may actually result in increased endothelial cell damage. Released enzymes, including PR3 and MPO, can bind to the surface of unprimed neutrophils, facilitating subsequent recognition by ANCA and amplifying ANCA-potentiated neutrophil activation [16].

It has become clear that specific recognition of membrane-associated MPO or PR3 by the $F(ab')_2$ fragment, as well as Fc interaction with Fc receptors FcγRII and FcγRIII, participate in these phenomena. The signaling pathways mediating neutrophil stimulation by ANCA have begun to be delineated. ANCA binding transduces several signals, including calcium mobilization, protein kinase C activation, tyrosine phosphorylation, and mitogen-activated kinase (MAPK) activation [17•]. ANCA binding also activates heterotrimeric guanosine triphosphatases, probably through $F(ab')_2$-specific recognition, because G proteins are not known to be activated by FcγR-mediated mechanisms [18•,19•]. Although $F(ab')_2$ and Fc binding stimulate different signaling pathways, they all lead to Akt activation, which participates in pathways mediating neutrophil degranulation [20•]. ANCA-mediated Fc signaling may be of clinical relevance, because patients with Wegener's granulomatosis carrying the NA1 allele of the FcγRIIIb gene may have more severe renal disease. Recent studies indicate that ANCA stimulation of neutrophils

homozygous for this alelle elicits a stronger release of alpha-defensins, further supporting the importance of ANCA binding to Fcγ RIIIb receptors in enhancing neutrophil functions [21].

Since this initial discovery, many other neutrophil functions have been shown to be modulated by ANCA. ANCA also induce accelerated neutrophil apoptosis. Apoptotic neutrophils express high levels of ANCA targets in their membrane. It has been suggested that ANCA recognition of their targets on apoptotic neutrophils facilitates their opsonization and clearance via macrophages with the subsequent release of proinflammatory cytokines that maintain and amplify the inflammatory process [22].

When acting sublethally, ANCA binding to membrane-associated enzymes potentiates a number of additional neutrophil functions, with amplifying effects on the inflammatory response. ANCA potentiate neutrophil chemotactic response to f-met-leu-phe peptide, nitric oxide production through nitric oxide synthase–independent pathways, and production of tissue factor, potentially facilitating thrombosis in ANCA-associated vasculitis [5,23]. ANCA increase neutrophil adhesion to tumor necrosis factor-alpha–stimulated endothelial cell monolayers in flow conditions increasing their ability to penetrate into tissues [24].

Antineutrophil cytoplasmic antibodies targets are expressed by cells of the monocytic lineage and ANCA binding also may modulate monocyte functions. ANCA may enhance release of reactive oxygen species by monocytes, predominantly through Fc-mediated mechanisms [20•] and also may upregulate leukocyte integrins, particularly CD18, and the lipopolysaccharide-receptor CD14 [25•]. In addition, ANCA binding to monocytes induces expression of proinflammatory cytokines, such as IL-18 [25•]. Studies with gene expression microarrays have shown that circulating leukocytes from patients with ANCA overexpress a variety of proinflammatory genes, including cyclooxygenase-2 and genes involved in monocyte differentiation, such as differentiation-dependent gene-2 [26••]. These results have been confirmed in vitro by treating peripheral blood leukocytes with ANCA. The set of genes upregulated by ANCA IgG and $F(ab')_2$ portion are distinct, but partially overlapping [26••].

Effects of antineutrophil cytoplasmic antibodies on endothelial cells

The ability of enhanced neutrophil activation by ANCA to damage endothelial cells has been demonstrated in different settings by several authors. Among the relevant products released by activated neutrophils are the ANCA targets MPO and PR3. PR3 can bind to endothelial cell membrane, and MPO and PR3 can be internalized by endothelial cells. PR3 has been demonstrated to promote apoptosis by cleaving nuclear factor-kappaB through mechanisms other than those mediated by elastase and caspases [5,27].

When acting sublethally, PR3 induces a number of proinflammatory functions on endothelial cells, including chemokines IL-8 (CXCL8) and monocyte chemotactic protein-1 (CCL2), which will prime and recruit additional neutrophils and monocytes [5,28]. ANCA binding to endothelial cell–bound PR3 further amplifies inflammatory reactions by enhancing endothelial cell expression of adhesion molecules for leukocytes [28]. In addition, PR3 promotes a prothrombotic phenotype of endothelial cells by inducing tissue factor [5]. All of these effects may likely amplify vessel damage in ANCA-associated vasculitis.

Animal models

Much evidence indicates that ANCA can potentiate neutrophil functions that may result in vessel inflammation and injury in vivo. Early animal models demonstrated that ANCA induction enhanced vessel inflammation and damage promoted by other stimuli, but the potential of ANCA themselves to fully induce lesions was not completely proven. In this regard, infusion of MPO-ANCA was demonstrated to increase lesions in animal models of glomerulonephritis induced by other mechanisms, such as mercurium chloride or sub-nephritogenic doses of anti-glomerular basement membrane antibodies [5]. Brower *et al.* (quoted in Day *et al.* [5]) induced antibodies against MPO in Brown Norway rats, but local perfusion of H_2O_2 and neutrophil extracts was necessary for inducing crescentic glomerulonephritis.

Recently, Xiao *et al.* [29••] succeeded in inducing crescentic glomerulonephritis, vasculitis, and pulmonary capillaritis resembling ANCA-mediated disease in humans by transferring splenocytes obtained from MPO knockout mice immunized with human MPO into immunodeficient Rag 2 -/- mice. Less severe lesions were obtained by injection of purified MPO-ANCA than by splenocyte transferring, suggesting that cell-mediated immune responses play a significant role in ANCA-mediated diseases [29••]. Pulmonary vasculitis and granuloma-like structures also have been induced in Wistar rats by injecting cytoplasmic ANCA–positive IgG fraction purified from serum of patients with Wegener's granulomatosis [30•].

Anti-endothelial cell antibodies

Circulating AECA have been detected in several vasculitis including Wegener's granulomatosis, microscopic polyangiitis, Kawasaki disease, thromboangiitis obliterans, and Behçet's and Takayasu's disease [1,31]. AECA also have been detected in autoimmune diseases that can be complicated with vasculitic phenomena, such as systemic lupus erythematosus, rheumatoid vasculitis, and inflammatory bowel disease [1,31]. Some AECA, such as those detected in Kawasaki disease, recognize cytokine-inducible molecules, whereas others, such as those detected in Wegener's granulomatosis and microscopic polyangiitis, recognize constitutive endothelial cell antigens. In most cases, AECA titers correlate with disease activity [1,31]. In vitro studies have shown that some, but not all, AECA may trigger complement activation or antibody-dependent cellular

cytotoxicity. Other antibodies are able to activate endothelial cells and induce nuclear factor-kappaB activation and subsequent expression of IL-6, von Willebrand factor antigen, and endothelial adhesion molecules for leukocytes, mechanisms that could contribute to vessel inflammation and occlusion [31]. The generation of AECA via idiotypic manipulation in a mouse model led to the development of perivascular inflammatory infiltrates [31]. According to the antigenic heterogeneity of different vascular beds, AECA specific for vessels of different sizes have been described; AECA from patients with Behçet's disease preferentially react against endothelial cells from small vessels, whereas AECA obtained from patients with Takayasu's disease are more specific for endothelial cells obtained from larger vessels (Fig. 2) [31]. The specificity of AECA seems to be highly heterogeneous and largely unidentified, and their precise pathogenic role has not been fully characterized [32•]. Recently, one of the antigens recognized by AECA present in sera from patients with Behçet's disease and other vasculitis has been identified as alpha-enolase by proteomics technology. Alpha-enolase is expressed by neutrophils and is one of the specificities recognized by ANCA from patients with Wegener's granulomatosis, other ANCA-associated vasculitis, and other conditions, such as inflammatory bowel disease [33••].

It is highly unlikely that AECA play a primary pathogenic role in systemic vasculitis, and they probably appear as a consequence of antigen modification because of endothelial cell activation or damage mediated by other mechanisms. However, according to the mentioned properties, AECA probably contribute to amplify vascular inflammation and endothelial cell injury.

In vivo evidence of endothelial cell damage

Abnormalities in endothelial cell morphology have been classically observed by histopathologic examination in systemic vasculitis. Luminal endothelial cells in inflamed vessels appear swollen, and staining for endothelial cell markers, sharply defined in normal vessels, decreases and discloses a blurry aspect, indicating abnormalities in the expression and distribution of constitutive molecules [34]. As the inflammatory process proceeds, luminal endothelium eventually disappears and, in obsolescent lesions, vessels are occluded by intimal hyperplasia or fibrotic tissue subsequent to thrombus organization (Fig. 3).

Recently, Woywodt *et al.* [35••] demonstrated elevated numbers of circulating, presumably detached, endothelial cells in patients with active systemic vasculitis, significantly higher than those found in patients in remission, healthy control subjects, and in patients with infection or other inflammatory conditions. Some of the circulating cells disclose early apoptosis markers, but the great majority of patients exhibit a necrotic and prothrombotic phenotype.

Evidence of diffuse endothelial dysfunction, as assessed by endothelium-dependent relaxation, has been observed in systemic vasculitis and improves with treatment [36••,37]. Endothelial cell dysfunction may be, at least in part, a reversible functional change that is secondary to widespread effects of circulating proinflammatory cytokines, which may induce functional changes, even in endothelial cells from noninvolved vessels [36••,37]. However, speculation that inflammation-induced endothelial dysfunction may contribute to increased vascular morbidity, observed in the long-term follow-up of patients with systemic vasculitis, is tentative.

Proinflammatory Functions of Endothelial Cells

Most of the described immunopathogenic mechanisms able to damage endothelial cells, when acting sublethally, are able to induce proinflammatory activities. Furthermore, endothelial cells, along with other vessel wall components, actively react in response to a variety of mediators produced locally by infiltrating leukocytes. Endothelial cells at distant sites also may respond to increased circulating cytokines with systemic effects. Endothelial cells are able to amplify inflammatory cascades by three main mechanisms—cytokine and chemokine production, adhesion molecule expression, and angiogenesis.

Cytokine and chemokine production

In an inflammatory setting, endothelial cells are able to produce a wide array of cytokines, chemokines, and growth factors. A variety of cytokines have been identified in inflammatory infiltrates in several systemic vasculitis, including giant-cell arteritis, Churg-Strauss syndrome, ANCA-associated vasculitis, and Kawasaki disease (Fig. 4) [38–40]. Although infiltrating leukocytes are the major producers of these mediators, endothelial cells are able to produce proinflammatory cytokines and may contribute to the systemic acute phase reaction, which is characteristically prominent in many systemic vasculitides compared with other chronic inflammatory disorders.

Endothelial cells are able to produce chemokines, such as IL-8 (CXCL8), RANTES (regulated on activation, normal T cell expressed and secreted; CCL5), Gro-alpha (CXCL1), and monocyte chemotactic protein-1 (CCL2), among others [41]. Chemokines selectively attract leukocyte subpopulations bearing specific receptors, and some of them have been demonstrated to be expressed in vasculitis lesions [39,40]. Chemokine production via endothelial cells and other vascular components may contribute to tissue tropism typically observed in systemic vasculitis. By attracting specific leukocyte subsets, endothelial cell production of chemokines probably contributes to maintain and amplify vessel inflammation [41]. Several immunopathogenic mechanisms contributing to systemic vasculitis increase cytokine and chemokine production by endothelial cells. These include direct infection [2], complement activation products [1], and ANCA binding

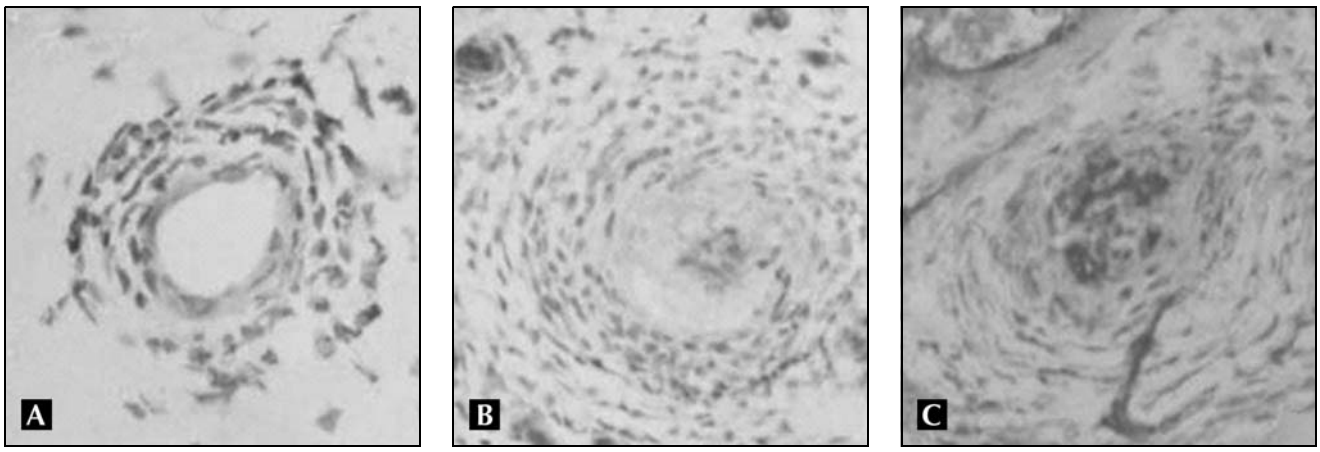

Figure 2. Phenotypic differences among endothelial cells from different vascular beds are obvious, even in morphology. **A**, Cultured endothelial cells obtained from human aorta. **B**, Cultured endothelial cells obtained from a human umbilical vein. **C**, Cultured microvascular endothelial cells from adipous tissue. Phase-contrast inverted microscopy.

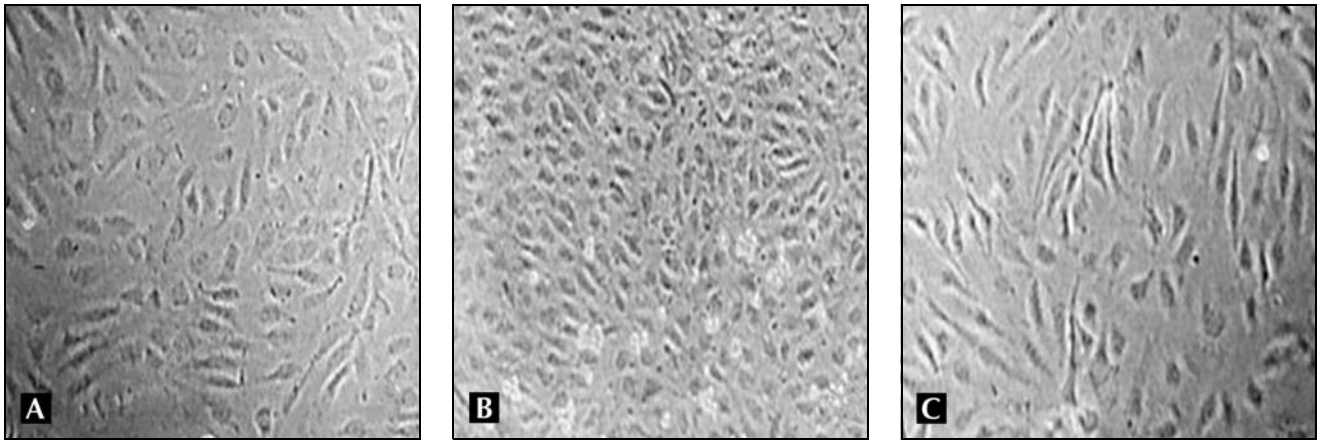

Figure 3. Changes in endothelial cell appearance at different stages in classical polyarteritis nodosa. **A**, Luminal endothelial cells appear well-defined in early lesions, with slight inflammatory infiltrates and a preserved lumen. **B**, In advanced lesions, endothelial cells stain blurry, whereas sharply defined small neovessels appear at the periphery. **C**, In obsolescent lesions, the vascular lumen disappears, and it is replaced by fibrous tissue. Sharply defined neovessels replace the former lumen. Endothelial staining using biotinylated Ulex Europaeus lectin (Dako, Glostrup, Denmark) and streptavidin-peroxidase complex. (*From* Coll-Vinent *et al.* [34]; with permission.)

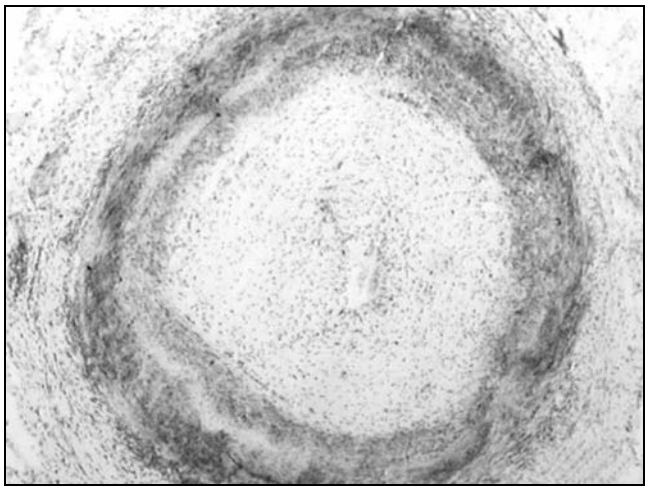

Figure 4. Strong tumor necrosis factor–alpha expression by infiltrating macrophages in giant cell arteritis. Immunostaining with a polyclonal rabbit anti-human tumor necrosis factor–alpha (Genzyme Corporation, Cambridge, MA). Avidin-biotin-peroxidase method.

to target enzymes bound to endothelial cell surface [5]. As mentioned, PR3 interaction with endothelial cells increases endothelial production of IL-8 (CXCL8) and monocyte chemotactic protein-1 (CCL2) [28].

Expression of endothelial adhesion molecules

Endothelial cells play a pivotal role in recruiting circulating leukocytes into tissues by expressing adhesion molecules. On exposure to proinflammatory cytokines, endothelial cells express selectins E and P, which mediate initial interactions with circulating leukocytes in post-capillary venules. Subsequently, constitutive intercellular adhesion molecule-1 (ICAM-1) is upregulated and vascular cell adhesion molecule-1 (VCAM-1) is induced on endothelial cells. Endothelial adhesion molecules, as well as constitutive ICAM-2 and platelet endothelial cell adhesion molecule-1, serve as ligands for leukocyte integrins, which mediate firm adhesion of leukocytes, transmigration through the endothelial cell junctions, and

subsequent progression through the basement membrane and the interstitial matrix [1].

Most of the primary immunopathogenic mechanisms that are thought to play a role in the pathogenesis of blood vessel inflammation in vasculitis have been shown to influence adhesion molecule expression or function. In vitro studies have shown that complement activation products induce adhesion molecule expression by cultured endothelial cells. C1q induces E-selectin, ICAM-1, and VCAM-1, and C5a has been shown to upregulate P-selectin expression [1]. Adhesion molecule expression and function is required for immune complex and complement-mediated vessel damage in vivo [1].

Antineutrophil cytoplasmic antibodies binding to endothelial cell membrane–associated PR3 or related epitopes on the endothelial cell surface may induce E-selectin and VCAM-1 expression by endothelial cells [1]. In an inflammatory context, PR3 released by neutrophils in the vicinity of endothelial cells is able to induce endothelial cell ICAM-1 expression [28]. In vitro studies have shown that AECA binding to endothelial cells induces endothelial adhesion molecule expression [32•].

In vasculitis, activated lymphocytes and macrophages actively produce IL-1, tumor necrosis factor–alpha, and interferon-γ, the main inducers of endothelial cell adhesion molecules. Expression of these cytokines by infiltrating cells has been clearly demonstrated in giant cell arteritis and in lesions from patients with microscopic polyangiitis (Fig. 4) [1,38].

Overexpression of endothelial adhesion molecules has been demonstrated in inflammatory vascular lesions of cutaneous leukocytoclastic vasculitis, Kawasaki disease, Behçet's disease, giant cell arteritis, and polyarteritis nodosa [1,34,42,43•]. In all of them, expression of inducible adhesion molecules E-selectin and VCAM-1 by endothelial cells can be detected at some point, and constitutive endothelial expression of ICAM-1 is usually increased compared with normal vessels. In glomerular lesions of ANCA-associated vasculitis, VCAM-1 and ICAM-1 expression can be observed at the glomerular tuft, as well as in tubular epithelial cells and peritubular capillaries [1,34,42,43•].

Changes in cell adhesion molecule expression at different stages have been investigated in cutaneous leukocytoclastic vasculitis and in polyarteritis nodosa. In cutaneous leukocytoclastic vasculitis, endothelial adhesion molecule expression occurs in the luminal endothelium [42]. In early lesions, E-selectin expression is prominent, whereas ICAM-1 and VCAM-1 predominate in evolved lesions. This pattern closely follows the kinetics of induction of endothelial adhesion molecules in vitro. E-selectin expression correlates with infiltration by neutrophils and T lymphocytes expressing the skin-homing carbohydrate cutaneous lymphocyte antigen, which interacts with E-selectin [42]. In late lesions, infiltration by neutrophils and cutaneous lymphocyte antigen expressing cells decreases and other T lymphocyte subsets and macrophages predominate [42]. In medium-sized vasculitis, such as classical polyarteritis nodosa, the luminal endothelium expresses constitutive or inducible adhesion molecules at early stages [34]. As the inflammatory process proceeds, the luminal endothelium is damaged and the vascular lumen is occluded by intimal fibrosis. At this point, endothelial cell adhesion molecules are strongly expressed by adventitial neovessels. In scarring lesions, adhesion molecule expression decreases along with the density of infiltrating leukocytes [34]. In kidney lesions of ANCA-associated vasculitis, glomerular expression of ICAM-1 and VCAM-1 also declines in sclerotic glomeruli [1]. In large-vessel vasculitis, such as giant cell arteritis, adhesion molecule expression occurs in neovessels at the adventitia and within the granulomatous lesions, mainly at the intima-media junction. These observations suggest that, in large- and medium-sized vessels, infiltrating leukocytes do not come from the vascular lumen. Rather, inflammatory cells invade the vessel wall through the adventitial vasa vasorum and neovessels [43•].

Inmunohistochemical studies usually disclose a close topographic relationship between endothelial expression of adhesion molecules and expression of their ligands by infiltrating leukocytes, suggesting that interactions mediated by adhesion molecules participate in the development of inflammatory infiltrates in vasculitis [34,42,43•]. The functional relevance of interactions mediated by adhesion molecules in the pathogenesis of vessel inflammation has been investigated in in vitro studies exploring adhesion of T lymphocytes to glomeruli in tissue sections from patients with renal vasculitis and in animal models [1]. In a murine model of systemic vasculitis induced by immunization against *Mycobacterium butyricum*, the administration of blocking monoclonal antibodies and the application of vital microscopy have demonstrated the important participation of interactions mediated by selectins and by alpha-4 integrins in leukocyte adhesion and transmigration through postcapillary venules. Consistently, ICAM-1 deficiency considerably reduces the development of vasculitis in MRL/lpr mice and blocking E-selectin ligands or alpha-4 integrins prevents the development of beta-glucan–induced granulomatous vasculitis [1]. Although none of these models satisfactorily represents specific human vasculitic syndromes, these findings underline the functional importance of interactions mediated by adhesion molecules in the development of vascular inflammation.

Angiogenesis

Neovascularization is a prominent finding in inflammatory lesions of systemic vasculitis. In small-vessel vasculitis, neovascularization usually occurs in the adventitial layer or surrounding tissues. In large-vessel vasculitis, neovessels also appear within the inflammatory infiltrates, particularly at the granulomatous areas at the intima-media

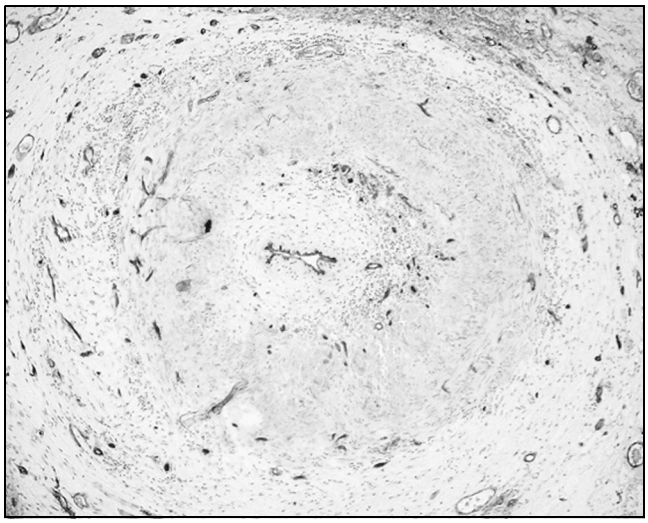

Figure 5. Marked neovascularization in giant-cell arteritis. Neovessels accumulate within granulomatous infiltrates (*arrow*). Endothelial staining with biotinylated Ulex Europaeus lectin (Dako, Glostrup, Denmark) and streptavidin-peroxidase.

junction (Fig. 5) [44••]. The authors have proposed that angiogenesis may play a dual role in systemic vasculitis. Angiogenesis may compensate for ischemia; however, angiogenesis may amplify and maintain the inflammatory process, given the proinflammatoy functions of endothelial cells.

Angiogenesis may be an important compensating mechanism to avoid ischemia. The relevance of angiogenesis as a compensatory mechanism is illustrated by interferon-alpha, a potent angiogenesis inhibitor that may decrease vascularization and worsen ischemic complications in severe cryoglobulinemic vasculitis [45]. Similarly, in giant cell arteritis, the intensity of the angiogenic response in temporal artery samples inversely correlates with the development of ischemic complications [46••]. Moreover, serum angiogenic activity is reduced in patients with ischemic events. This finding indicates that the ability to develop an efficient angiogenic response may be an individual characteristic and may extend to vascular territories more relevant than superficial temporal arteries in which the biopsies are usually performed. With contrast-enhanced magnetic resonance imaging, highly vascularized inflammatory tissue has been demonstrated around the optic nerve in a patient with long-lasting giant cell arteritis [47].

As mentioned, angiogenesis also may have a prominent proinflammatory function. Besides providing oxygen and nutrient supply to the inflammatory process, newly formed vessels intensively express adhesion molecules for leukocytes providing new sites through which leukocytes may invade the vessel wall. In addition, new vessels widen the endothelial surface providing and additional source of cytokines, chemokines, and growth factors, amplifying the inflammatory process.

The authors have shown that, in patients with giant cell arteritis, the angiogenic response in temporal artery lesions, as well as serum angiogenic activity, is more prominent in patients with a strong systemic inflammatory response who are at lower risk of ischemic complications and also are more refractory to therapy, further underlying the dual function of angiogenesis in giant cell arteritis [44••]. The authors also have shown that patients with a strong systemic inflammatory response have higher tissue expression and circulating levels of the angiogenic cytokine IL-6. Furthermore, patients with ischemic complications have lower tissue expression and serum levels of IL-6 than patients with no vascular events [46••].

It has been recently demonstrated that neovascularization in pathologic conditions occurs through angiogenesis derived from preexisting vessels and from vasculogenesis derived from hematopoetic precursors—hemangioblasts. This has been demonstrated in several settings, including tumors and atherosclerotic vascular disease, in humans and in animal models [48]. Recently, increased numbers of circulating progenitors identified as CD34-positive cells have been found in patients with systemic vasculitis [49••,50•]. Circulating progenitors increase even further in patients with vasculitis during the first weeks after corticosteroid therapy. This finding suggests an effect of corticosteroids in mobilizing bone marrow progenitors and indicates an important role of endothelial cell precursors in vessel and tissue remodeling and repair in patients in early remission.

Conclusions

Endothelial cells are active and dynamic players in multifactorial inflammatory cascades leading to vessel inflammation in systemic vasculitis. Although endothelial cells are a clear target for all the major immunopathogenic mechanisms that are believed to participate in the development of vascular inflammation and injury, endothelial cells themselves have major proinflammatory functions and actively participate in vessel and tissue repair. Over the recent years, a remarkable advance has been made in the understanding of the cellular interactions and molecular mechanisms through which these complex interplays take place. The authors expect that continuous progress in the field will lead to new diagnostic tools, improved ways to monitor disease activity, and new modalities of therapeutic intervention.

Acknowledgments

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We apologize for omitting citation to many seminal contributions to the various subjects addressed in this review. Because of space limitations and priority given to most recent publications, many outstanding articles have been replaced by contemporary reviews through which the interested reader can reach the original papers.

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- •• Of major importance

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