SERUM LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) ARE MARKEDLY ELEVATED IN PATIENTS WITH WEGENER'S GRANULOMATOSIS

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SUMMARY

Objectives. Necrotizing vasculitis and granuloma formation are the predominant features of Wegener’s granulomatosis (WG). We have investigated the importance of vascular endothelial growth factor (VEGF) in monitoring disease activity in WG.

Methods. Serum VEGF levels were determined in 23 patients with active WG, 21 healthy controls and 25 patients with urinary infection, by ELISA using commercially available antibodies to VEGF.

Results. VEGF levels were enormously elevated in patients with WG compared to both controls and patients with urinary infection (P < 0.0001). Of the 23 patients, 21 (91.3%) had VEGF levels above the cut-off value (3.3 ng/ml, calculated as the mean of the controls +2 s.d.). Further analysis of the data showed that VEGF levels did not correlate with age, sex, incidence of classic antineutrophil cytoplasmic antibodies (c-ANCA) or duration of the disease (P > 0.05), but there was correlation with disease activity (r = 0.51, P < 0.01). VEGF levels were higher in patients with major compared to those with minor disease activity (P < 0.01). However, there was no significant correlation between VEGF levels and the Birmingham scores for vascular activity and damage.

Conclusion. VEGF levels are raised in WG patients compared to normal controls and may be a marker of disease activity. Further studies on serial blood samples from a large cohort of patients with WG and other systemic vasculitides are needed to evaluate the specificity and usefulness of VEGF levels in monitoring disease activity.

Key words: Wegener’s granulomatosis, Vascular endothelial growth factor.

WEGENER’S granulomatosis (WG) is a rare disease of unknown aetiology characterized by necrotizing vasculitis and granulomatous lesions affecting primarily the upper and lower respiratory tract. As the disease progresses, other organs also become involved, most commonly the kidneys, skin, eyes, joints and nervous system [1]. The detection of autoantibodies in the patient’s sera and a good response to immunosuppressive therapy suggest an autoimmune background to the disease. A majority of WG patients have classic antineutrophil cytoplasmic antibodies (c-ANCA) [2], the most common c-ANCA in WG being anti-proteinase 3 antibodies. However, c-ANCA are not specific for WG nor are all WG patients c-ANCA positive. Primary vasculitis and granuloma formation are the predominant features of WG, and are the result of a chronic inflammatory reaction that involves the endothelium. Measurement of the inflammatory activity is not precise; von Willebrand factor (vWF), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) have been used, but may not be reliable [2–7].

Vascular endothelial growth factor (VEGF) is an endothelial cell (EC) mitogen in vitro and a potent angiogenic factor in vivo [8]. It exerts its effects through two endothelial cell receptors, the tyrosine kinases Flk-1/KDR and Flt-1, which are important in the regulation of EC growth and differentiation [9]. VEGF expression is regulated by a variety of hormones, growth factors and cytokines, including interleukin-6 (IL-6), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), prostaglandin E2 and nitric oxide [9]. However, the major inducer of VEGF expression is hypoxia [8]. VEGF expression is detectable in areas where EC are proliferating and also around microvessels in areas where EC are normally quiescent [10]. In homozygous mice deficient for VEGF expression, the formation of blood vessels is severely impaired [11, 12], whereas overexpression of VEGF in quail resulted in hypervascularity and hyper-permeability [13]. VEGF mRNA is expressed in brain, kidney, lung, liver and spleen in the adult rat, with expression being highest in the lung. Continued expression of VEGF in adult animal tissues suggests that it may play a role in the maintenance of normal vessel physiology, which may be disturbed in patients with WG. In view of the above functions of VEGF, we hypothesized that it may be important in the pathobiology of WG. In the present study, we have measured the VEGF levels in the serum from 23 patients with active WG, 21 healthy subjects and 25 patients with urinary infection. The correlation of the serum VEGF levels with the activity of WG was assessed.

METHODS

Patients
Sera were obtained from 23 patients who had been diagnosed as having WG by the American College of Rheumatology 1990 criteria [14] and were receiving standard immunosuppressive medication at the time of blood collection. The diagnosis in each case was confirmed by histological examination of biopsy tissues from the lung, bronchi or kidney. All blood samples from the patients were obtained when the disease was clinically active and was classified as major or minor infection.
disease activity as described previously [15]. Briefly, minor disease activity in WG was defined as active lesions of WG in the upper or lower airways without any evidence of vasculitic activity in other organs. Major disease activity was defined as renal involvement leading to deteriorating renal function with red blood cell casts or biopsy-proven necrotizing glomerulonephritis, pulmonary involvement with impending respiratory failure, cerebral vasculitis, or acute abdominal or massive gastrointestinal haemorrhage due to vasculitis. In addition, vascular activity and damage were also scored using criteria described by the Birmingham group [3–5]. Of the 23 WG patients, 83% (19/23) were c-ANCA positive. Male to female ratios in controls and WG patients were 10:11 and 16:7, respectively. The median duration of disease with major and minor disease activities was 13 yr (range 2–35 yr) and 10 yr (range 1–12 yr), respectively. Treatment at the time of the sample was also recorded. Twenty-one serum samples were taken from age-matched volunteers as normal controls. Sera from 25 hospitalized patients with urinary infection were used as non-vascular disease controls. Thirty-seven rheumatoid arthritis (RA) samples were used as controls for inflammation. Serum samples were aliquoted and stored at −70°C until the assay was performed.

Measurement of VEGF
Measurement of VEGF levels in sera from patients and controls was carried out at the same time. An ELISA for VEGF was established using commercial antibodies against VEGF. The use of enhanced chemiluminescence increased the sensitivity of the assay by 30-fold compared to colorimeric ELISA and had intra-assay variations of <7%. For this assay, 96-well white plates (Dynatech Microfluor, USA) were coated with 100 μl/well of goat anti-VEGF164 antibody (R&D Systems) diluted 1/1000 (1 μg/ml) in 0.1 M carbonate buffer (pH 9.6), and incubated in a humid box overnight at 4°C. The coated plates were blocked with 1% (w/v) bovine serum albumin (BSA), 0.01% (v/v) Tween 20 in 0.1 M phosphate-buffered saline (PBS–TWEEN) for 2 h at room temperature. Duplicate serum samples were added to the plates (100 μl/well, diluted 1/2 in PBS–TWEEN). A standard curve was generated using recombinant human VEGF (R&D Systems) at a range of 0.1–40 ng/ml on each plate. After overnight incubation at 4°C, 100 μl/well of rabbit anti-VEGF antibody (Santa Cruz Biotechnology) were added to the plates at 1/2000 dilution (1 μg/ml) in PBS–TWEEN and incubated for 3 h at 4°C. This was followed by the addition of horseradish peroxidase-conjugated goat anti-rabbit antibody (0.5 μg/ml) (diluted 1/2000 with 1% BSA in PBS–TWEEN), which was incubated with shaking for 30 min at room temperature. Three washes with PBS–TWEEN were carried out between each of the steps. Finally, 100 μl/well of Amerlite chemiluminescence signal reagent (Amershams, UK) were added and the plates were read immediately in a plate reader (Kodak Clinical Diagnostics). The measured values of light emission were converted into absolute concentrations by reference to the VEGF standard curve.

Statistical analysis
Data presented are the mean ± s.e.m. For comparison between groups, Student’s t-test was used. Correlation of VEGF with disease activity, ANCA and other parameters was assessed by the Pearson test. A P value (two-tailed) of ≤ 0.05 was considered significant.

RESULTS

VEGF levels in WG, normal and disease controls
VEGF levels (mean ± s.e.m.) from the 21 normal subjects were 1.02 ± 0.25 ng/ml (range 0.14–3.94). In WG patients, VEGF levels were 34.76 ± 6.78 ng/ml (range 1.2–80.0) and were significantly increased compared to controls (P < 0.0001). With a cut-off value of 3.3 ng/ml (this value being the mean + 2 s.d. of the VEGF levels in normal subjects), 91.3% (21/23) of the WG patients had VEGF levels above the cut-off level (Fig. 1). To assess whether the serum levels of VEGF are affected by infection, sera from 25 patients with urinary infection were used to measure the VEGF levels in normal controls. Thirty-seven rheumatoid arthritis (RA) samples were used as controls for inflammation. Serum samples were aliquoted and stored at −70°C until the assay was performed.

FIG. 1.—Serum levels of VEGF (mean ± s.e.m.) in 23 patients with Wegener’s granulomatosis (34.76 ± 6.78 ng/ml) and 21 healthy subjects (1.02 ± 0.25 ng/ml). Horizontal lines indicate the means. VEGF levels were significantly elevated in WG patients (△, patients with major disease activity; ●, patients with minor disease activity) compared to normal controls. Seven patients with WG had VEGF levels > 80 ng/ml.
levels. The results indicated that serum VEGF was similar in individuals with urinary infection (1.35 ± 0.60 ng/ml, range 0–15.08) and normal individuals (P > 0.05), but was lower than in WG (P < 0.0001). To test the hypothesis that increased VEGF levels are a marker of inflammation rather than a WG-specific marker, VEGF levels were measured in 37 RA patients (14 with high CRP levels and 23 with low CRP levels). The results showed that while VEGF levels in RA (13.59 ± 4.97 ng/ml) were higher than normal controls, the increase was not significant (P > 0.01). When VEGF levels in RA were compared to VEGF levels in WG, they were significantly lower (P = 0.0057). There was no difference in VEGF levels between low (11.07 ± 5.7 ng/ml) and high (17.74 ± 9.02 ng/ml) CRP levels (P > 0.01), indicating that increased VEGF levels are not a marker of inflammation.

**Correlation between VEGF levels and disease activity**

Correlation analysis of the data showed that VEGF levels did not correlate with age, sex, c-ANCA or duration of the disease (P > 0.05), but correlated well with major and minor disease status (r = 0.51, P < 0.01): markedly elevated VEGF levels were observed in patients with major (47.67 ± 9.41 ng/ml) compared to minor disease activity (14.67 ± 4.11 ng/ml) (P < 0.01). All the highest values for VEGF (≥80 ng/ml) belonged to patients with major disease activity (Fig. 1). VEGF data were further analysed for their correlation with vasculitis activity as proposed by the Birmingham group [3–5], wherein scores for activity and damage are allocated. Using Pearson’s correlation test, no significant correlation was detected with either the score for activity (r = 0.15, P = 0.54) or damage (r = 0.27, P = 0.24).

**Correlation of c-ANCA with VEGF and disease activity**

The tests for ANCA were carried out by indirect immunofluorescence using normal human neutrophils [2, 6]. Immunofluorescence, when seen, was in all cases cytoplasmic (the staining pattern characteristic of c-ANCA, where the autoantigen is usually proteinase 3) [16]. Correlation analysis showed that neither the occurrence nor the levels of serum c-ANCA correlated with VEGF concentrations (r = −0.25, P = 0.34 and r = −0.42, P = 0.08, respectively). Furthermore, no significant correlation was established between the occurrence or levels of c-ANCA and disease status assessed as major or minor or by the Birmingham activity scale [3–5].

**Correlation of treatment with VEGF levels**

There was no difference in VEGF levels between patients undergoing treatment and those receiving no treatment. Prednisolone, cyclophosphamide and azathioprine had no effect on VEGF levels in WG patients.

**DISCUSSION**

This is the first report which demonstrates high levels (up to 80-fold increase) of VEGF in serum samples from WG patients. Furthermore, the increased levels of VEGF correlated with the disease activity. These results imply that VEGF may play an important role in the pathobiology of WG.

The source of the elevated VEGF in WG patients is a matter of speculation. The data from urinary infection which showed no increase in VEGF levels ruled out the possibility that the markedly elevated VEGF resulted from infection, such as might occur as a secondary phenomenon in patients with vasculitis. In the absence of immunohistochemistry and in situ data, it is not possible to define the cellular origin of VEGF in WG patients, although the published literature suggested that VEGF is produced by numerous cell types, including macrophages, T cells, kidney cells and keratinocytes [9]. Among these cells, T cells and macrophages are predominantly present in the inflammatory infiltrates in WG and thus may be a source of increased VEGF. Several studies have demonstrated that hypoxia plays a major role in modulating VEGF expression [8, 17]. In glioblastoma multiforme and other tumours with a significant necrotic component, VEGF mRNA is highly expressed in ischaemic tumour cells present in the areas of necrosis [18]. Likewise, in human stroke tissues, in ischaemic and repairing zones, high levels of VEGF protein and mRNA have been reported (R. Issa, personal communication). Ischaemia caused by occlusion of the coronary artery results in a dramatic increase in VEGF mRNA levels in the pig myocardium, which again suggests that local hypoxia is an important inducer of VEGF [19]. Increased VEGF levels in the circulation have also been reported in a number of diseases, such as stroke (R. Issa, personal communication) and solid tumours [20]. Therefore, VEGF does not seem to be specific for a particular disease, but probably is a non-specific marker for vascular disorders in which EC damage/repair occurs.

Until recently, vascular endothelium was regarded purely as the victim of vasculitic processes in WG. It is now becoming apparent that EC play an initiating role in vascular damage. Exposure of EC to cytokines such as IL-1, tumour necrosis factor alpha and lymphotoxin causes increased procoagulant activity, adhesiveness for various inflammatory cells, and the release of prostacyclins and platelet-derived growth factor. Furthermore, upregulation of VEGF may cause increased vascular permeability, which could contribute to the transmigration of the inflammatory cells through the vessel wall and the extravasation of plasma proteins from leaky vessels. The latter may lead to the exposure of potential antigens normally sequestered inside the EC and consequently the production of anti-EC antibodies, often present in WG, which are capable of activating EC and facilitating leucocyte recruitment and adhesion to endothelium [21]. It has also been shown that VEGF augments the expression...
of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on EC [9, 10]. These two adhesion molecules play an important part in the transmigration of T lymphocytes expressing very late antigen-4 (VLA-4), the ligand for VCAM-1, and of macrophages and neutrophils expressing leucocyte function-associated molecule-1 (LFA-1), the ligand for ICAM-1. The observation that inflammatory infiltrates in WG consist mostly of T cells, macrophages and neutrophils provides further evidence that VEGF could promote leucocyte homing to the inflammatory areas.

Inflammatory status in WG and other vasculitides is notoriously difficult to assess, especially when deep organs—kidneys and lungs—are involved and when the level of vasculitis is low. In the current study, the activity of the disease had been classified as major and minor or scored using the Birmingham activity and damage scale [3–5]. Serum levels of VEGF, but not c-ANCA, strongly correlated with major/minor disease activity, but none of the two factors significantly related to the Birmingham scores for activity and damage. This lack of association between c-ANCA titres and clinical activity has also been observed previously by ourselves and other investigators [[7] and our unpublished data.] It is important to have a better indicator for monitoring disease activity to guide therapy; therefore, the potential usefulness of serum VEGF needs to be evaluated in serial blood samples taken from a large number of patients.

In conclusion, when pathological features of WG are taken into account, it is tempting to propose that the excessive serum VEGF is the consequence of tissue necrosis which may mirror the extent of necrotizing vasculitis. The increased VEGF could play a potential pathogenic function by inducing granuloma formation, inflammatory cell homing and EC leakage through its effects on angiogenesis, upregulation of adhesion molecules and vascular permeability. The present preliminary study needs augmenting by examining serial samples of sera in well-characterized examples of systemic vasculitis to determine the value of VEGF in monitoring progress and perhaps in predicting prognosis.

References

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