

Original article

Plasma levels and skin-eosinophil-expression of vascular endothelial growth factor in patients with chronic urticaria

Background: Although chronic urticaria (CU) is often regarded as autoimmune in nature, only less than 50% of sera from CU patients contain histamine-releasing autoantibodies. This suggests that other factors may contribute to its pathogenesis. We evaluated the possible involvement of vascular endothelial growth factor (VEGF), one of the major mediators of vascular permeability, in CU.

Methods: Eighty consecutive adult patients with CU and 53 healthy subjects were studied. VEGF and prothrombin fragment F_{1+2} were measured by enzyme immunoassays. Autologous plasma skin test (APST) was performed in CU patients and, in six of them, skin biopsy specimens were taken from wheals to evaluate the immunohistochemical expression of VEGF and eosinophil cationic protein (ECP).

Results: Plasma VEGF concentrations were higher in CU patients (8.00 ± 0.90 pmol/l) than in controls (0.54 ± 0.08 pmol/l) ($P = 0.0001$) and tended to parallel both the severity of CU and to correlate with F_{1+2} levels. APST was positive in 85.1% of patients. VEGF concentration was significantly higher in APST-positive than in APST-negative patients ($P = 0.0003$). Immunohistochemically, all specimens from patients with CU showed a strong expression of VEGF ($P = 0.002$) that colocalized with ECP, a classic eosinophil marker.

Conclusions: VEGF plasma levels are elevated in CU and parallel the disease severity. This supports a possible role of this molecule in CU pathophysiology. Eosinophils are the main cellular source of VEGF in CU lesional skin.

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The vascular endothelial growth factor (VEGF) is the most potent regulator of angiogenesis presently known, and one of the major mediators of vascular permeability (1, 2). Although it is able to influence other cell types, including hematopoietic stem cells, monocytes, osteoblasts and neurons, VEGF exerts most of its effect on endothelial cells enhancing their proliferation, migration, tube formation, and survival (3). Further, it shows a vasodilator effect through an increase of nitric oxide production by endothelial cells (4).

Abbreviations: APST, autologous plasma skin test; CU, chronic urticaria; ECP, eosinophil cationic protein; TBS, tris buffered saline; TMB, tetramethylbenzidine; VEGF, vascular endothelial growth factor.

There is increasing evidence that VEGF is overexpressed in different conditions including chronic inflammation, fibrosis and cancer. There have been several reports describing the association of VEGF with systemic vasculitis, including Wegener granulomatosis, giant-cell arteritis, and Kawasaki disease (5–7). Moreover, several experimental findings indicate a possible contribution of VEGF in allergic response in asthma (8, 9). The two main sources of VEGF are mast cells and eosinophils (10, 11), two cells which are known to play a role in allergic conditions as well as in chronic urticaria (CU) (12). CU is a quite common disorder, characterized by the appearance of recurrent itchy wheals with or without angioedema for more than 6 weeks. The finding of circulating autoantibodies to the alpha subunit of the high affinity IgE receptor, FcεRI, or to IgE has led to conclude that

CU is an autoimmune disease in a significant proportion of cases (13, 14). The intradermal injection of autologous serum (autologous serum skin test, ASST) has been used as an easily available *in vivo* counterpart of the cumbersome basophil histamine release assay (15). However, functional anti-Fc ϵ RI and/or anti-IgE autoantibodies can be detected only in about one third of cases (16), and several CU sera that do not seem to contain autoantibodies are equally able to induce a clear-cut wheal and flare reaction upon intradermal injection, suggesting the presence of other circulating histamine-releasing factors (17). This view is also supported by experiments carried out using IgG-depleted sera that retain the ability to induce skin autoreactivity (18). Based on these observations, during the last few years we looked at the serum levels of different factors potentially able to induce vasodilation and/or histamine release, including the stem cell factor, substance P, and IL-18 but with little success (19–21). However, we cannot exclude that the rapid degradation of some of these mediators, particularly substance P, may have caused such negative results. Recently, we observed that the prevalence of positive autologous tests rises from about 50% up to 80% if plasma anticoagulated with sodium citrate rather than serum is used for intradermal testing (22). These findings prompted us to investigate the coagulation system and, in effect, we found that patients with CU show signs of thrombin generation due to the activation of the tissue factor pathway of coagulation (22, 23) and that such activation is directly related to the severity of the disorder and may lead to the activation of fibrinolysis (24). Very recently, we were able to show that also in CU the main cell type responsible for the expression of tissue factor in the skin of CU patients is the eosinophil (25), in keeping with the observation that this cell is a major intravascular location for tissue factor storage and exposure (26). In the present study we measured the plasma levels of VEGF in CU and we characterized the cellular source of this molecule.

Subjects and methods

Subjects

Eighty consecutive adult patients (male/female 19/61, mean age $\pm$ SEM 43.4 $\pm$ 14.1) with CU seen at the allergy centers of Ospedale Maggiore Policlinico (Milan, Italy) and Clinica San Carlo (Paderno Dugnano, Milan, Italy) were studied. CU was diagnosed on the basis of the appearance of continuous or recurrent hives with or without angioedema for more than 6 weeks. On the occasion of their first office evaluation, all patients underwent a panel of basic investigations including erythrocyte sedimentation rate, complete blood count and differential, electrophoresis of serum proteins, C3 and C4 complement fractions, antinuclear antibodies, thyrotropin and autoantibodies to thyroglobulin and thyroid peroxidase, in accordance with the guidelines of the Italian Association of Allergologists (27). Stool examination for ova and parasites and *Helicobacter pylori* antigen was performed in selected cases. Patients with physical urticaria were excluded. At the time of blood

sampling the patients had active urticaria, and disease activity was graded from 1 to 3 (slight, moderate and severe) according to the number of wheals present at the time when blood samples were collected, as previously described by Sabroe et al.: 1–10 small (< 3 cm in diameter) wheals = grade 1 (slight); 10–50 small wheals or 1–10 large wheals = grade 2 (moderate); > 50 small wheals or > 10 large wheals = grade 3 (severe) (16).

All patients were being treated with H1 anti-histamines. These drugs (cetirizine, levocetirizine, loratadine, desloratadine or fexofenadine in all cases) were stopped five days before plasma samples for *in vivo* (autologous plasma skin test [APST]) and *in vitro* (VEGF and F₁₊₂) assays were taken. Na citrate-anticoagulated plasma (0.125 mol/l of Na citrate) was used for both APST and *in vitro* assays. Plasma samples from 53 healthy subjects (male/female 14/39, mean age $\pm$ SEM 42.5 $\pm$ 14.7) were used as control. The study protocol conformed to the ethical guidelines of Declaration of Helsinki, and all subjects participating in the study gave their informed consent before participation.

Skin tests

APST was performed by intradermal injection of 0.05 ml of fresh autologous plasma and read at 30 min as previously described (22). Blood samples, collected in 0.125 mol/l of Na citrate, were centrifuged after 15 min at 125 g for 3 min and immediately used for intradermal tests. Intradermal injection of saline solution (0.9% w/v NaCl) was performed as negative control and skin prick test with 10 mg/ml histamine as positive control. Patients showing a wheal with a diameter at least 1.5 mm greater than the control saline solution were considered positive (16).

Vascular endothelial growth factor determination

Plasma VEGF concentration was measured by a sandwich enzyme immunoassay (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instructions. Intra-assay and interassay coefficients of variation were 5% and 7%, respectively. The detection limit of the assay is less than 0.1 pmol/l and the upper limit is 22.2 pmol/l. The assay employs a monoclonal antibody, precoated onto microplate, and an enzyme-linked polyclonal antibody conjugated to horseradish peroxidase, both specific for VEGF. After drawing venous blood from the subjects under examination, plasma was frozen at -80°C until assayed for VEGF concentration.

Prothrombin fragment F₁₊₂ measurement

Prothrombin fragment F₁₊₂, marker of thrombin generation, was measured in plasma samples from 56 CU patients by a sandwich immunoenzymatic assay (Enzygnost F₁₊₂; Behring Diagnostics GmbH, Frankfurt, Germany) according to manufacturer's instructions. Intraassay and inter-assay coefficient of variations were 5% and 8% respectively. The measuring range of the assay is between 20 and 1200 pmol/l.

Immunohistochemical studies

Skin biopsy specimens were taken from wheals lasting less than 12 h in six CU patients. Six biopsy specimens obtained from perilesional normal skin of patients who had undergone surgical excision of different types of skin tumors were used as controls. Furthermore, a skin biopsy of a cutaneous B-cell lymphoma was included among controls as example of noneosinophilic infiltrate.

The tissue samples were fixed in buffered formalin, dehydrated, embedded in paraffin wax, and sectioned. After deparaffining and rehydrating, each tissue section was incubated with a mouse anti-human VEGF monoclonal antibody (American Diagnostica Inc., Greenwich, CT, USA) at room temperature for 45 min, and then washed with tris buffered saline (TBS), pH 7.6, and incubated in biotinylated goat anti-mouse anti-rabbit immunoglobulins (Dako REAL™, Dako Cytomation, Glostrup, Denmark) at room temperature for 30 min. After incubation with the secondary antibody and another washing with TBS, pH 7.6, the sections were incubated with streptavidin conjugated to alkaline phosphatase (Dako REAL™, cod.K5005, Dako Cytomation) at room temperature for 30 min and revealed. A negative control was performed using a pool of mouse immunoglobulins (IgG1, IgG2a, IgG2b and IgM) as primary antibody (Negative Control, Dako Cytomation). Two independent 'blinded' observers evaluated serial sections. VEGF immunoreactivity was scored according to the number of immunoreactive cells per field (200X) as follows: 0 = no immunoreactive cells, 1 = 1–5 immunoreactive cells, 2 = 6–20 immunoreactive cells and 3 = >20 immunoreactive cells.

Colocalization experiments

Colocalization experiments were performed using the eosinophil cationic protein (ECP) monoclonal antibody. For double staining, after VEGF labeling, each slide was immersed in a methyl alcohol solution with 3% of H₂O₂ for 20–30 min in order to block endogenous peroxidase activity. After washing with TBS, pH 7.6, the sections were incubated overnight with a primary antibody (mouse anti-human ECP, 1 : 300, Pharmacia & Upjohn Inc., Bridgewater, NJ, USA) at +4°C, washed again with TBS, pH 7.6, and then incubated in a secondary antibody conjugated to peroxidase. Negative controls were performed using pooled mouse immunoglobulins as primary antibody. Finally, a tetramethylbenzidine (TMB) blue chromogen solution was prepared as indicated by the TMB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA, USA), and used as an enzyme substrate.

Statistical analysis

Results are reported as mean $\pm$ SEM and were log-transformed before analysis. Student's *t*-test for unpaired values was used to assess statistical significance of differences. Since multiple comparisons were performed, the Bonferroni correction of *P*-values was carried out. Differences in the immunohistochemical scores were assessed using the Wilcoxon–Mann–Whitney nonparametric test. A *P*-value of <0.05 was considered statistically significant. The data were analysed using the SPSS statistical package, version 15.00 (SPSS Inc., Chicago, IL, USA).

Results

Mean plasma VEGF level was significantly higher in CU patients (8.00 ± 0.90 pmol/l) than in normal subjects (0.54 ± 0.08 pmol/l) ($P = 0.0001$, Fig. 1). VEGF levels tended to parallel the severity of CU being significantly increased in patients with moderate ($P = 0.01$) and severe urticaria ($P = 0.003$, Fig. 2). APST, performed in 74 CU patients, was positive in 63 patients (85.1%) and negative in 11 (14.9%). VEGF concentration was significantly higher in APST-positive than in APST-negative

patients (9.47 ± 1.03 pmol/l vs 1.95 ± 0.90 pmol/l) ($P = 0.0003$, Fig. 3).

In 56 CU patients we had the opportunity to measure F₁₊₂ plasma levels and we found increased concentration (547 ± 76 pmol/l) (upper limit of normal range: 229 pmol/l). In these patients, a slight tendency to positive correlation was found between F₁₊₂ and VEGF plasma levels ($P = 0.043$).

Histology revealed the presence of an inflammatory infiltrate consisting mainly of lymphocytes and eosinophils, in addition to a variable degree of dermal edema with dilated blood vessels (Fig. 4, panel A). Immunohistochemically, all specimens from patients with CU clearly showed VEGF expression (Fig. 4, panels B and C) that was totally absent in lesional skin of a cutaneous B-cell lymphoma as an example of noneosinophilic infiltrate (Fig. 4, panel D). Statistical analysis, performed on skin specimens from six CU patients and six normal controls, confirmed a significantly higher VEGF score in specimens from patients with CU than in normal controls ($P = 0.002$, Fig. 5). The double staining for VEGF and ECP was performed on the two specimens with the highest VEGF reactivity scores. These experiments showed that VEGF co-localized with ECP, thus indicating that the VEGF-positive cells were eosinophils (Fig. 6). It must be noted, however, that none of the CU patients had peripheral blood eosinophilia.

Discussion

The present study shows that plasma levels of VEGF are significantly higher in CU patients than in normal

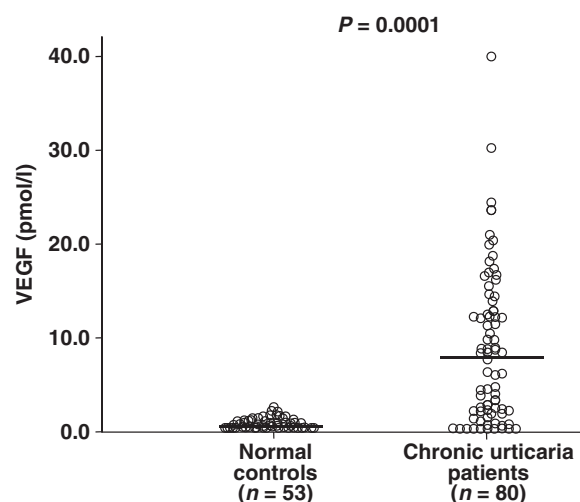


Figure 1. Plasma levels of vascular endothelial growth factor (VEGF) in normal controls and in patients with chronic urticaria. Results are expressed as pmol/l, and horizontal lines represent the mean values. Vascular endothelial growth factor plasma levels were significantly higher in patients with chronic urticaria than in controls.

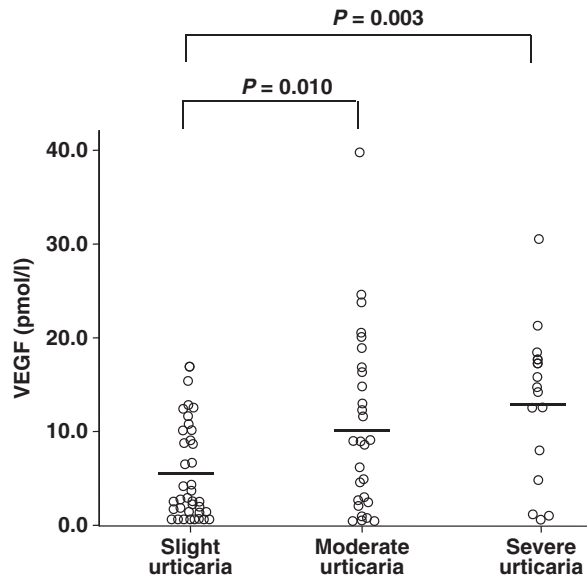


Figure 2. Plasma levels of vascular endothelial growth factor (VEGF) in patients with chronic urticaria divided into three different subsets according to the severity of the disease (slight, moderate, and severe). Results are expressed as pmol/l, and horizontal lines represent the mean values. Vascular endothelial growth factor plasma levels tended to parallel the severity of the disease.

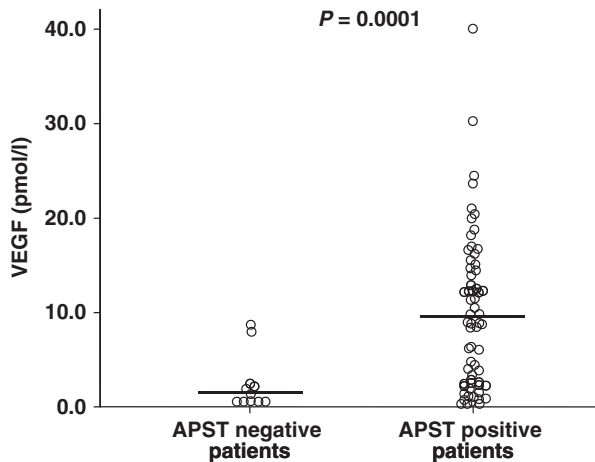


Figure 3. Plasma levels of vascular endothelial growth factor (VEGF) in 74 patients with chronic urticaria divided into two different subsets according to skin reactivity to autologous plasma. Results are expressed as pmol/l, and horizontal lines represent the mean values. Vascular endothelial growth factor plasma levels were significantly higher in 63 patients who were positive on autologous plasma skin test (APST) than in 11 negative patients.

subjects and tend to parallel disease severity. VEGF has vasopermeability and vasodilatory effects which suggest a possible involvement in the pathophysiology of CU. In recent years, it has been demonstrated that CU is often an autoimmune disorder sustained by circulating histamine-

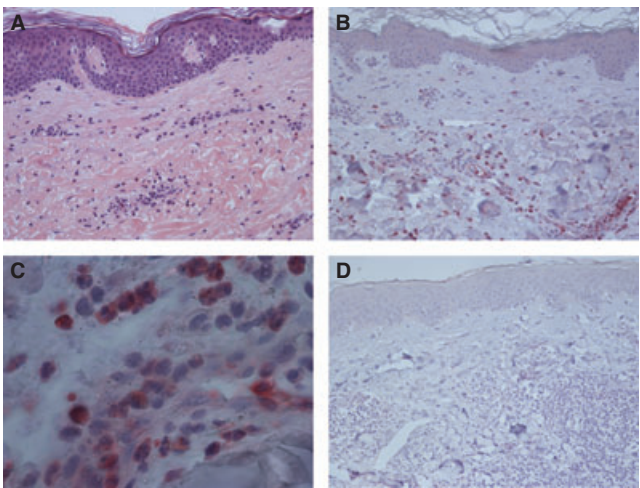


Figure 4. Histological and immunohistochemical studies of chronic urticaria skin. Panel A (hematoxylin-eosin; $\times 200$) reveals dermal edema with a dilated blood vessel and the presence of an inflammatory infiltrate consisting mainly of lymphocytes and eosinophils. Panels B and C [staining for vascular endothelial growth factor (VEGF); $\times 200$ and $\times 1000$] show chronic urticaria skin with strong VEGF expression. Panel D (staining for VEGF; $\times 200$) shows the complete absence of VEGF reactivity in lesional skin of a cutaneous B-cell lymphoma as an example of noneosinophilic infiltrate.

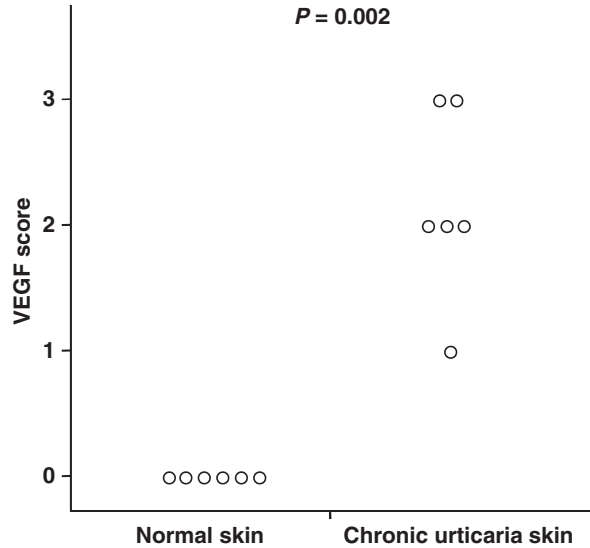


Figure 5. Scores of vascular endothelial growth factor (VEGF) immunoreactivity in tissue samples from chronic urticaria skin lesions (six cases) compared with normal skin (six cases, $P = 0.002$). 0 = No immunoreactive cells; 1 = 1–5 immunoreactive cells; 2 = 6–20 immunoreactive cells; 3 = > 20 immunoreactive cells.

releasing autoantibodies (13, 14). In 1986, Grattan et al. observed that the intradermal injection of autologous serum caused a wheal and flare reaction in about 50% of CU patients (28) and the serum factor causing skin

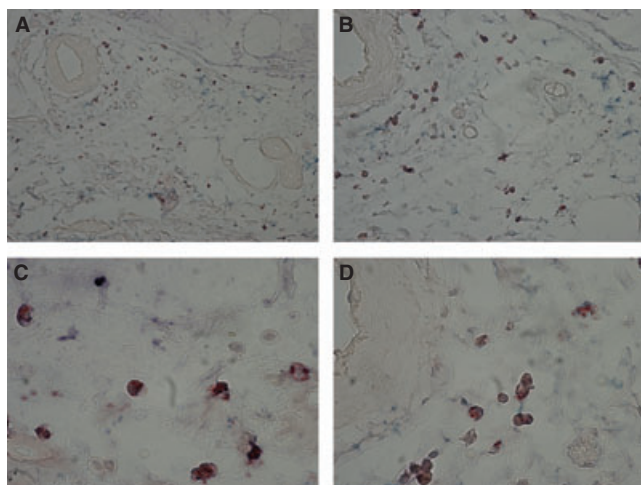


Figure 6. Immunohistochemical double staining for vascular endothelial growth factor (VEGF) (red) and eosinophil cationic protein (blue) in one chronic urticaria patient (panel A $\times 200$, panel B $\times 400$; panels C and D $\times 1000$). Vascular endothelial growth factor co-localizes with eosinophil cationic protein, indicating that the VEGF positive cells are eosinophils.

reactivity was identified as anti-Fc ϵ RI or anti-IgE autoantibodies (29–31). ASST has been indeed considered as an *in vivo* screening test for histamine-releasing autoantibodies (15). However, in subsequent years, a discrepancy emerged between ASST and *in vitro* histamine-releasing activity: sera from only a proportion (less than 50%) of those who score positive on ASST are able to induce histamine release *in vitro* from normal donors' basophils (17). A better concordance between *in-vivo* and *in-vitro* tests can be achieved if sera from patients who score strongly positive on ASST (i.e. a selected subset of patients with CU) are tested on both basophils and mast cells from different donors (32). However, the fact remains that about 50% of CU patients score negative on ASST and that sera from a proportion of those who score positive are unable to induce any histamine release *in vitro*. In a previous study, we observed that histamine-releasing activity *in vitro* is associated with the serum fraction >100 kDa, and hence presumably with functionally active IgG autoantibodies (17). These observations suggest that the presence of circulating autoantibodies in CU patients is almost always associated with autoreactivity on ASST but that there can be autoreactivity in the absence of circulating autoantibodies.

The second discrepancy in the interpretation of ASST/histamine-releasing activity stems from the observations by Fagiolo and co-workers who found that sera from CU patients containing Fc ϵ RI autoantibodies retained their ability to induce a wheal-and-flare reaction upon intradermal injection of autologous serum after depletion of IgG (18). Their findings put the role played by autoantibodies into question and suggested the possible involve-

ment of other factors inducing vasopermeability both in the wheal and flare reaction of ASST and in the pathogenesis of CU.

Recently, we observed that skin reactivity to autologous plasma increased up to 80% of CU patients, suggesting that permeability factors other than histamine-releasing autoantibodies can account for the wheal and flare reaction (22). We supposed indeed that some coagulation factors could contribute to the elicitation of a wheal and flare reaction. We found that the coagulation cascade is activated in CU patients and that this activation starts from the tissue factor pathway, and skin eosinophils represent the main source of tissue factor (23, 25). We hypothesized that thrombin could contribute to the wheal and flare reaction since it can increase vascular permeability and activate mast cells, however it is still unknown whether it has a primary pathogenic role or it simply represents an amplification mechanism. In any case, since this mechanism is not operating in all patients with CU, we considered the possibility that other vasopermeability mediators might be involved. One good candidate was VEGF which, in fact, was elevated in CU patients particularly in those with severe disease. The observation that VEGF levels are higher in APST-positive than in APST-negative patients further supports a role for VEGF in plasma-induced increase of vascular permeability. The VEGF effect may be also indirect through the generation of nitric oxide and platelet-activating factor (4, 33). In our previous studies we observed that plasma anticoagulated with sodium citrate was able to induce a wheal and flare reaction upon intradermal injection whereas heparinized plasma lost this capacity (17, 22, 34). We can now infer that this might be the result of the capacity of heparin to bind VEGF as well as to inhibit coagulation and histamine release. VEGF has been known as a heparin-binding glycoprotein, and *in vivo* large part of VEGF is sequestered by heparan sulphate proteoglycans that act as a reservoir of VEGF that can be mobilized by proteolysis (3).

We have identified eosinophils as source of VEGF in lesional skin of CU patients. In fact, colocalization experiments demonstrated that the cells expressing VEGF also stained for ECP, a classical marker of eosinophils. This observation is consistent with literature data indicating that eosinophils are a source of VEGF and can promote angiogenesis (11). VEGF overexpression has been found in patients with eosinophil-related disorders including asthma, acute eosinophilic pneumonia and angioedema with eosinophilia (9, 35, 36). We previously demonstrated that, in the lesional skin of CU patients, eosinophils are the main source of tissue factor, the initiator of blood coagulation (25). The slight tendency to a positive correlation between VEGF and F $_{1+2}$ levels (marker of coagulation activation) further supports the view that eosinophils have a role in increasing vascular permeability both via VEGF and tissue factor pathways. The role of eosinophils in CU pathophysiology is also

supported by the observation that anti-CD23 autoantibodies are present in about 65% of CU patients and that these antibodies can activate eosinophils which in turn release major basic protein and activate mast cells (37). Our data provide the first demonstration that plasma VEGF levels are increased in CU patients possibly as a consequence of an overexpression by eosinophils in

lesional skin. Although we do not know whether VEGF has a direct causative role in CU, and increased VEGF levels have been found in several allergic and eosinophil-related disorders, we believe that VEGF overexpression is not a nonspecific phenomenon, but that different diseases may share an intermediate mechanism in their pathophysiology.

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