

Expression of Tissue Factor by Eosinophils in Patients with Chronic Urticaria

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Key Words

Tissue factor · Eosinophils · Chronic urticaria · Skin

Abstract

Background: Although several cases of chronic urticaria (CU) are currently regarded as autoimmune in origin, associated with histamine-releasing autoantibodies, an activation of blood coagulation via tissue factor (TF) and a strong expression of TF in lesional skin have been described. Eosinophils, which are involved in CU skin lesions, have recently been demonstrated as the major source of TF in human blood. We assessed whether eosinophils are the cellular source of TF in CU skin lesions. **Methods:** Twenty patients with severe CU were studied. Skin biopsy specimens were taken from wheals. The control group consisted of specimens of perilesional normal skin from different types of skin tumours (10) and various skin disorders with non-eosinophilic infiltrates, including leukocytoclastic vasculitis (7), lichen planus (8) and mastocytosis (3). TF expression was evaluated by immunohistochemical methods using an anti-TF monoclonal antibody. Co-localization of TF and eosinophil cationic protein, a classic cell marker of eosinophils, was investigated by double-staining studies using 2 specific monoclonal antibodies in the 4 specimens showing the highest TF reactivity scores. **Results:** All specimens from patients with CU clearly showed TF expression that was absent in all normal control specimens ($p = 0.0001$) and in the skin disorders with non-eosinophilic infiltrates ($p = 0.001–0.0001$). The

double-staining experiments for TF and eosinophil cationic protein clearly showed that the TF-positive cells were eosinophils. **Conclusions:** Eosinophils are the main source of TF in CU lesional skin. This finding highlights the role of these cells in the pathophysiology of CU and might pave the way for new therapeutic strategies.

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Introduction

Although several mechanisms have been described in the pathophysiology of chronic urticaria (CU), this disorder is frequently considered autoimmune in origin and associated with circulating histamine-releasing autoantibodies directed against the α -subunit of the high-affinity IgE receptor (anti-Fc ϵ RI) or against IgE (anti-IgE) [1–3]. However, such autoantibodies, which can be detected in vitro by a functional assay employing basophils from normal donors, are found in only about 25% of CU cases [4]. As a consequence, during the last years some doubts have arisen about the pathomechanisms underlying this disease, and different pathways for histamine release have been sought. One interesting observation has been that many patients, whose sera do not show circulating autoantibodies in vitro, score positive on the intradermal injection of autologous serum [4], an in vivo test which has been considered as a marker of the presence of autoantibodies [5], and that IgG-depleted sera from CU pa-

tients maintain their ability to induce a wheal-and-flare reaction upon intradermal injection [6]. Recently, some evidence of the possible involvement of the coagulation cascade in the pathogenesis of CU has emerged. One study observed that positive autologous skin testing rises up to 80% if autologous plasma instead of autologous serum is injected [7], and that CU patients show elevated plasma levels of prothrombin fragment F_{1+2} , suggesting thrombin generation [7]. Subsequent studies found that CU patients show an activation of the tissue factor (TF) pathway of coagulation cascade [8], and that in patients with a severe disease such activation can be so pronounced to produce an elevation of plasma levels of D-dimer [9]. In one of those studies [8], immunohistochemical experiments showed TF expression in skin specimens from CU patients, but the inflammatory cells responsible for this phenomenon were not identified. It has been shown that eosinophils are the major intravascular source of TF, which is mainly embodied within their specific granules and is rapidly exposed by cell-specific stimulation [10]. With this background, we evaluated eosinophils as the possible cells that express TF within the inflammatory infiltrate of CU skin lesions.

Materials and Methods

Patients

Twenty patients with severe CU (13 women and 7 men, mean age 39 years, age range 18–70 years), referred to the Institute of Dermatological Sciences, IRCCS Foundation Maggiore Policlinico Hospital, Mangiagalli, Regina Elena and University of Milan, 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. Patients with physical urticaria and urticarial vasculitis were excluded. Fifteen patients had more than 50 small wheals (<3 cm) or more than 10 large wheals (>3 cm), while 5 patients were virtually covered with wheals. Patients had not been given systemic corticosteroids and anti-histamines for at least 1 week. Skin biopsy specimens were taken from wheals lasting from 3 to 12 h. Ten biopsy specimens obtained from perilesional normal skin of patients who had undergone surgical excision of different types of skin tumours were used as controls. In addition, various skin disorders with non-eosinophilic infiltrates, including leukocytoclastic vasculitis (7 cases), lichen planus (8 cases) and mastocytosis (3 cases), were also used as negative controls.

The protocol was approved by our Institutional Review Board and all subjects gave informed written consent before participating in the study.

Methods

Immunohistochemical Studies

The tissue samples were fixed in buffered formalin, dehydrated, embedded in paraffin wax and sectioned. After deparaffinizing

and rehydrating, each tissue section was incubated with a mouse anti-human TF monoclonal antibody (American Diagnostica Inc., Greenwich, Conn., 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) 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, K5005; Dako Cytomation) 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. TF immunoreactivity was scored according to the number of immunoreactive cells per field (200×) as follows: 0 = no immunoreactive cells, 1 = 1–5 immunoreactive cells, 2 = 6–20 immunoreactive cells, 3 = >20 immunoreactive cells.

Co-Localization Experiments

Co-localization experiments were performed using the eosinophil cationic protein (ECP) monoclonal antibody. For double staining, after TF labelling, each slide was immersed in a methyl alcohol solution with 3% of H_2O_2 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 and Upjohn Inc., Bridgewater, N.J., 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 blue chromogen solution was prepared as indicated by the TMB substrate kit for peroxidase (Vector Laboratories, Burlingame, Calif., USA) and used as an enzyme substrate.

Statistics

Differences in the immunohistochemical scores were assessed using the Wilcoxon-Mann-Whitney non-parametric test. $p < 0.05$ was considered statistically significant.

Results

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. 1a). Immunohistochemically, all specimens from patients with CU clearly showed TF expression (fig. 1b, c) that was totally absent in leukocytoclastic vasculitis lesional skin (fig. 1d). Statistical analysis confirmed a significantly higher TF score in specimens from patients with CU than in normal controls ($p = 0.0001$) as well as in non-eosinophil-mediated skin disorders such as leukocytoclastic vasculitis ($p = 0.0001$), lichen planus ($p = 0.0001$) and mastocytosis ($p = 0.001$; fig. 2). The double staining for TF and ECP was performed on the 4 specimens showing the highest TF

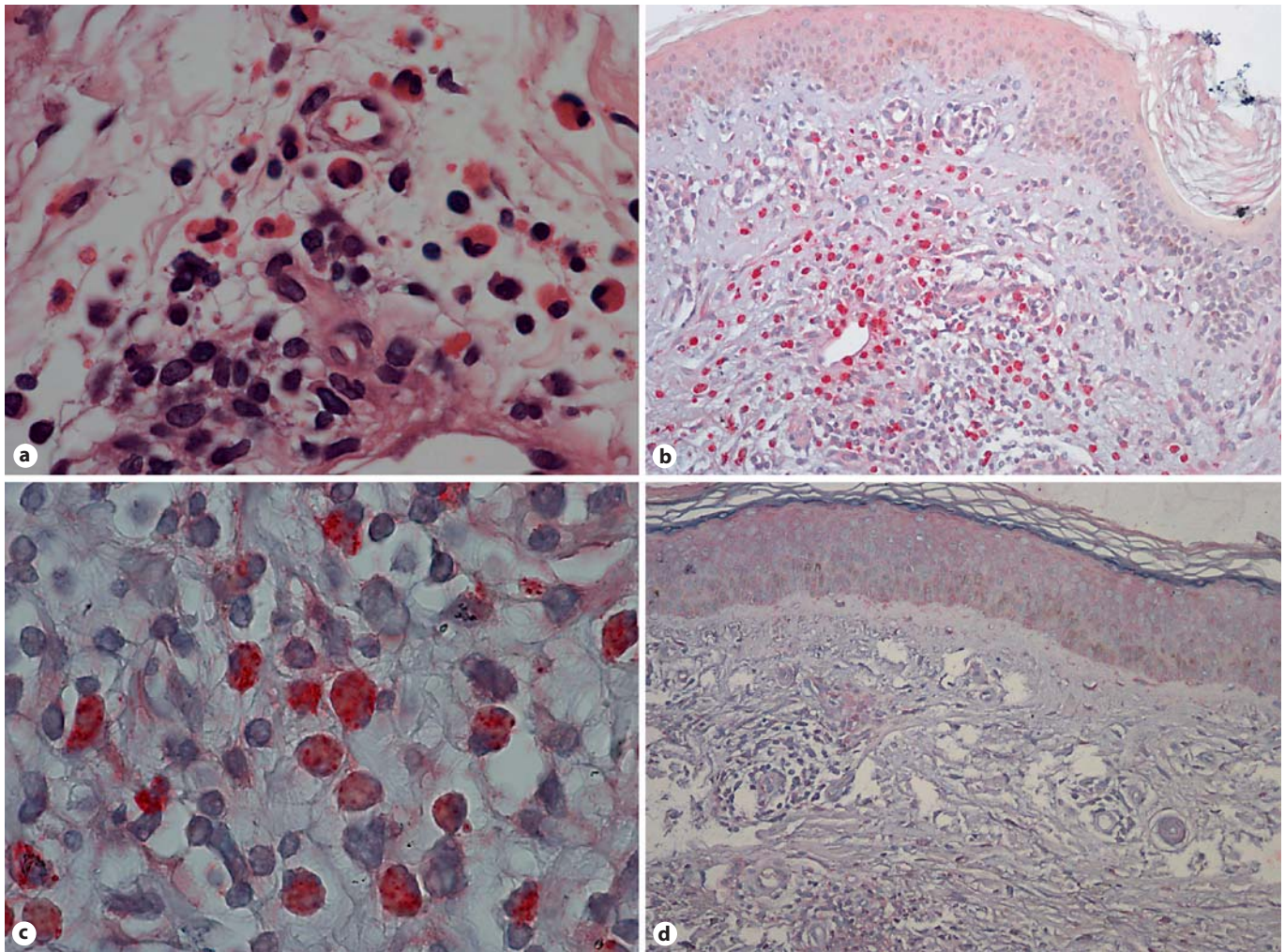


Fig. 1. Histological and immunohistochemical studies of CU and normal skin. **a** Dermal oedema with a dilated blood vessel and the presence of an inflammatory infiltrate consisting mainly of lymphocytes and eosinophils. Haematoxylin and eosin.

$\times 1,000$. **b, c** CU skin with strong TF expression in a granular pattern. Staining for TF. **b** $\times 200$. **c** $\times 1,000$. **d** Complete absence of TF reactivity in leukocytoclastic vasculitis lesional skin. Staining for TF. $\times 200$.

reactivity scores. These experiments showed that TF colocalized with ECP, thus indicating that the TF-positive cells were eosinophils (fig. 3).

Discussion

Along with autoimmune mechanisms mediated by autoantibodies to Fc ϵ RI or to IgE, an activation of the TF pathway of coagulation resulting in thrombin generation seems to occur in many patients with CU, and a strong expression of TF has been found in CU lesional skin [8]. In the present study, we demonstrated that TF is ex-

pressed by eosinophils present in the inflammatory infiltrate of CU skin lesions. These data highlight the importance of eosinophils in CU as a source of TF, in keeping with recent studies showing that eosinophils store TF and rapidly transfer it to the cell membrane during activation [10]. The strong expression of TF in CU lesional skin may be due to eosinophil activation, even if patients with CU virtually never show peripheral eosinophilia, probably because TF specifically facilitates the early transendothelial migration of the eosinophils [10]. In CU patients, biopsy generally reveals a non-necrotizing perivascular mononuclear cell infiltrate consisting primarily of lymphocytes with a variable number of mast cells and mono-

Fig. 2. Scores of TF immunoreactivity in tissue samples from CU skin lesions (20 cases) compared with normal skin (10 cases, $p = 0.0001$), skin lesions from leukocytoclastic vasculitis (7 cases, $p = 0.0001$), lichen planus (8 cases, $p = 0.0001$) and mastocytosis (3 cases, $p = 0.001$). 0 = No immunoreactive cells; 1 = 1–5 immunoreactive cells; 2 = 6–20 immunoreactive cells; 3 = >20 immunoreactive cells.

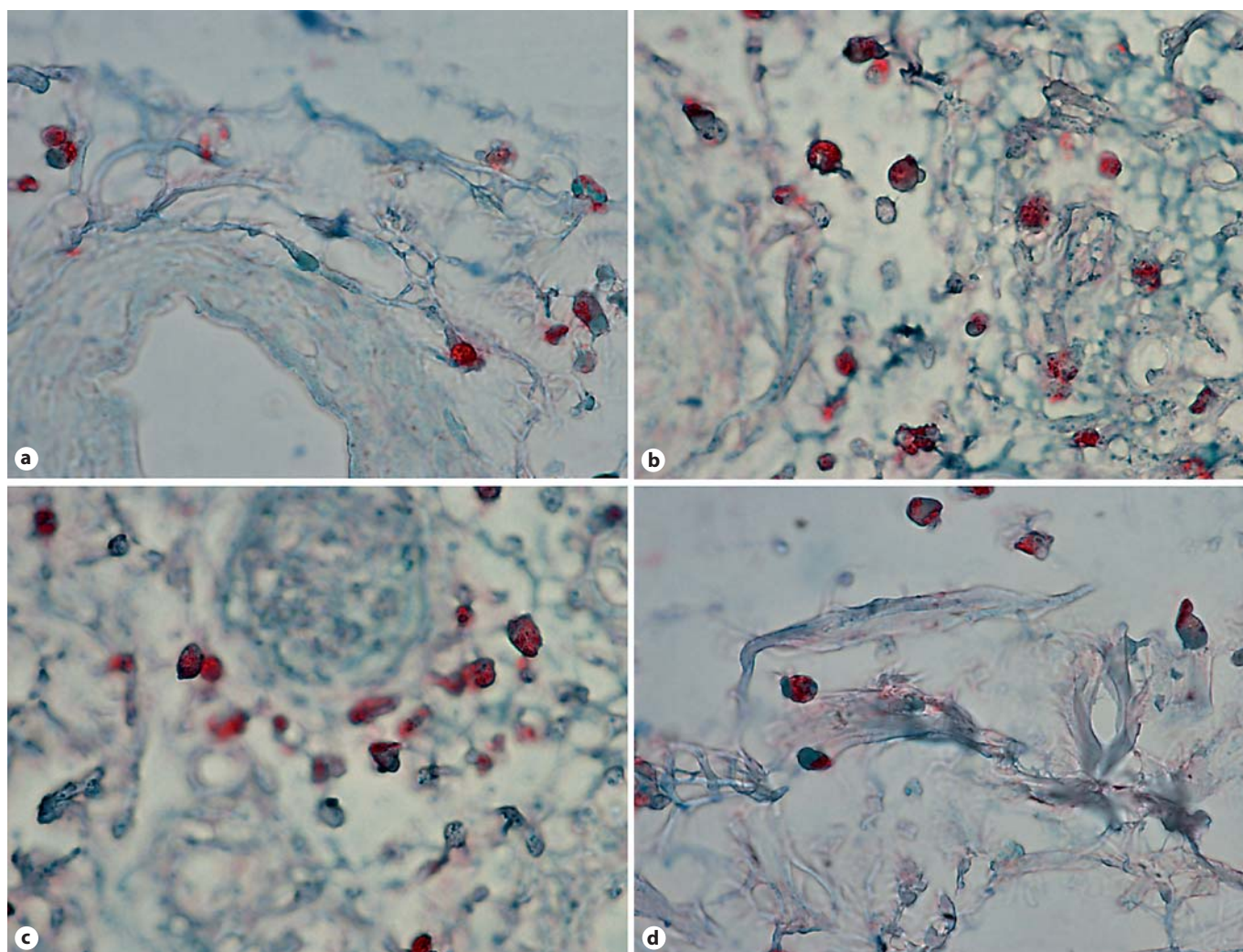
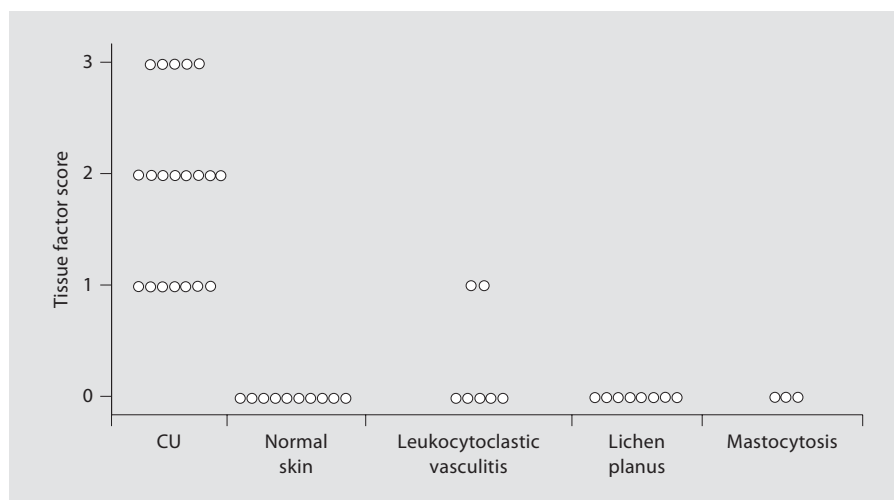


Fig. 3. Immunohistochemical double staining for TF (red) and ECP (blue) in 4 patients with the highest TF reactivity scores. TF co-localizes with ECP, indicating that the TF-positive cells are eosinophils. $\times 1,000$.

nuclear cells [11]; eosinophil infiltration may be relevant, sparse or totally absent [11]. Sabroe et al. [12] observed that eosinophil accumulation occurs early in the evolution of a wheal in patients with CU, namely in less than 4 h, but eosinophil number increases after 12 h. Our study did not aim to evaluate the kinetics of eosinophil infiltration, but incidentally, a high number of eosinophils was found in a biopsy taken about 12 h from the onset of the wheal (fig. 1a). Such a pattern resembles that of so-called late-phase urticaria, which is considered as a distinct clinical subset characterized by a predominant eosinophilic infiltrate [13]. The co-localization experiments, showing that the main infiltrating cells of CU lesions express both TF and ECP, support the involvement of eosinophils. Moreover, tissue-infiltrating macrophages do not express ECP, which can be detected only in circulating monocytes [14]. The present findings along with previous data suggest that eosinophils expressing TF are the cell type that triggers the activation of the TF pathway of coagulation in patients with CU [7–9]. Future studies on RNA expression could be useful to confirm our immunohistochemical results. The lack of TF expression we found in skin disorders characterized by non-eosinophil-

ic infiltrates (that is, leukocytoclastic vasculitis, lichen planus and mastocytosis) further supports this view. Although our data are not enough to clarify whether this mechanism is the cause of CU or simply an epiphenomenon, some considerations should be taken into account. The activation of the TF pathway of coagulation results in the generation of thrombin which, in experimental models, has been shown to induce oedema through an increase in vascular permeability [15, 16] by a direct effect on endothelial cells [17] and indirectly by a thrombin-related release of inflammatory mediators. Most effects of thrombin are probably histamine mediated (and hence mast cell mediated) as they have been reported to be reduced by anti-histamines and mast cell granule depletion in animal models [16]. Furthermore, thrombin triggers mast cell degranulation [18] and may activate protease-activated receptor-1 on mast cells [19].

The identification of eosinophils as relevant actors in the pathogenesis of CU potentially paves the way for new therapeutic strategies for this often distressing disorder. Moreover, other eosinophil-driven skin diseases should be the matter for future investigation.

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