

Markers of autoreactivity, coagulation and angiogenesis in patients with nonallergic asthma

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Abstract

Background: Patients with nonallergic asthma frequently show autoreactivity as do subjects with chronic urticaria (CU). Activation of the coagulation cascade and hyper-expression of vascular endothelial growth factor (VEGF) were recently found in CU, and there is sparse evidence that the same may occur in asthma.

Objective: To investigate autoreactivity, activation of the coagulation cascade, and expression of VEGF in patients with nonallergic asthma.

Methods: Twenty-one adults with nonallergic asthma underwent autologous plasma skin test (APST) and the measurement of plasma levels of the prothrombin fragment F1+2, D-dimer, VEGF, and the inflammatory marker C-reactive protein (CRP). Twenty-one healthy sex- and age-matched subjects served as normal controls.

Results: The APST scored positive in 19 of 21 (90%) patients vs 0 controls. Mean fragment F1+2 plasma levels were significantly higher in patients with asthma (267 ± 243 pM) than in controls (150 ± 51 pM; $P = 0.0001$). Similarly, plasma levels of both D-dimer and VEGF were significantly higher in patients than in controls (D-dimer: 2364 ± 1467 vs 1301 ± 525 pM; $P = 0.0001$; VEGF: 1721 ± 2566 vs 76 ± 375 fM; $P = 0.0001$). A trend toward increased levels of F1+2, D-dimer, VEGF, and CRP was found in patients with a more severe disease according to GINA classification.

Conclusion: Nonallergic asthma is characterized by autoreactivity as well as increased coagulation and angiogenesis markers, which are known to enhance vascular permeability. The presence of circulating vasoactive factors may be relevant to understand the disease pathophysiology and to detect novel therapeutic strategies in nonallergic asthma.

It is generally agreed that a persistent airway inflammation, characterized by the participation of both chemical mediators and cells, plays a pivotal role in the pathophysiology of asthma (1). Because atopy and allergen exposure account for only a variable proportion of the asthmatic population as a trigger for developing asthma, it is thought that other factors play an essential role in the development of the disease (2, 3). Inflammation of respiratory airways is present in both allergic and nonallergic asthmatics, and what differentiates the two groups is the triggering event, which in the latter is often undefined. Mastcells and eosinophils play a central patho-

genic role in asthma. Mechanisms underlying recruitment and activation of eosinophils appear to be similar in allergic and nonallergic conditions (4), and increased inflammatory cells populations, including eosinophils, are seen in the airways on lavage and biopsy (5). Despite the absence of a detectable specific IgE synthesis, in nonatopic asthma there is an elevated expression of cytokines and chemokines (including IL-13 and IL-5) that does not significantly differ from that observed in atopic asthma (6). The involvement of autoimmune mechanisms has been hypothesized in patients with nonatopic asthma following the detection of circulating

autoantibodies specific for a 55-kDa epithelial antigen (7, 8), but those preliminary observations have not been further substantiated.

Chronic urticaria (CU), a skin disorder characterized by the recurrent eruption of transitory itchy wheals, sometimes associated with angioedema, for more than 6 weeks is another disorder whose pathogenesis has been considered obscure for a long time. However, during the last decade it has become clear that CU is frequently characterized by autoreactivity as the intradermal injection of autologous serum causes a wheal-and-flare reaction in 30–60% of patients (9–12); moreover, a proportion of autoreactive patients appear to have a real autoimmune disorder, as serum IgG autoantibodies to IgE or to the high affinity IgE receptor, FcεRI, can be detected in 25–40% of patients (9–15). The autoreactive nature of CU has been recently confirmed by the finding that the intradermal injection of autologous plasma induces a wheal-and-flare reaction even more frequently than autologous serum in patients with CU (16). Studies carried out by us have also shown that CU is characterized by an activation of the coagulation cascade by the extrinsic pathway (17, 18) and by an increase in plasma vascular endothelial growth factor (VEGF) (19). All these mechanisms seem to parallel disease activity in patients with urticaria (16–19). Experimental data indicate that thrombin induces edema through an increase in vascular permeability (20, 21), an effect that is much reduced by antihistamines and abolished if mast cells granules are depleted (21); moreover, thrombin triggers mast cell degranulation (22) and may activate protease-activated receptor-1 (PAR-1) on mast cells (23). In some mast cell populations, the response to thrombin is equipotent with FcεRI-mediated activation (24). Further, recent studies demonstrated that eosinophils are an important source of tissue factor (TF), the key initiator of the coagulation cascade (25). In keeping with this last observation, we found that both the activation of the coagulation cascade and VEGF hyper-expression are associated with eosinophils (26).

During the last years, several clinical and experimental data providing a link between these two apparently distant conditions, namely CU and nonallergic asthma, have appeared in the medical literature. First, it was found that patients with nonallergic asthma frequently score positive on the autologous serum skin test (ASST), suggesting the presence of circulating histamine-releasing factors in this disease (27), a finding that confirms pioneering observations by a Swedish doctor who carried out experiments with the so-called autoserum test as long as 50 years ago (28). This observation was further analyzed by other researchers who showed that in patients with asthma, a positive ASST is associated with an increase in bronchial hyper-responsiveness (29). Moreover, bronchial hyper-responsiveness, which is a hallmark of asthma, is a frequent finding in patients with CU (30). Finally, recent reports suggest an activation of the extrinsic coagulation pathway in acute asthma (31). In light of these observations and of our recent findings in CU, we looked for the presence of autoreactivity (by evaluation of skin reactivity to autologous plasma), activation of the coagulation cascade (by measuring plasma levels of two markers of coagulation activation, i.e., the prothrombin fragment

Table 1 Demographic features of the study population and asthma severity

Patient no.	Sex	Age	APST	GINA score
1	F	17	Pos	MP
2	F	41	Pos	MP
3	F	16	Pos	MP
4	F	39	Pos	LP*
5	F	46	Pos	LP
6	F	22	Pos	MP
7	F	18	Pos	LP
8	F	63	Pos	MP
9	F	46	Pos	MP
10	F	51	Neg	LP*
11	F	70	Pos	LP
12	F	44	Pos	LP
13	F	22	Pos	LP*
14	F	36	Pos	MP
15	M	53	Pos	LP*
16	F	65	Neg	MP*
17	F	54	Pos	MP
18	F	42	Pos	MP
19	F	48	Pos	I
20	F	75	Pos	LP
21	F	43	Pos	LP

GINA score: I, intermittent; P, persistent; L, slight; M, moderate.

APST, autologous plasma skin test.

*Presence of aspirin-exacerbated asthma.

F1+2 and the fibrin fragment D-dimer), and angiogenesis involvement (by measuring plasma VEGF levels) in patients with nonallergic asthma.

Patients and methods

Patients

This cross-sectional study was carried out on 21 adult patients (M/F 1/20; mean age 40.6 years [SD 17.6 years], range 16–70) with nonallergic asthma. Nonallergic asthma was diagnosed if the following criteria were fulfilled:

- Clinical history of repeated asthma episodes responding to short acting beta agonists.
- Negative skin-prick tests with a large series of commercial (Allergopharma, Reinbek, Germany) extracts of both seasonal and perennial airborne allergens including pollens (grass, mugwort, ragweed, pellitory, plantain, birch, olive, and cypress), molds (*Alternaria tenuis*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Candida albicans*, *Penicillium notatum*, and *Tricophyton mentagrophytes*), house dust mites, and both cat and dog dander.
- Bronchial hyper-responsiveness, as demonstrated by positive metacholine provocation.

Asthma severity was graded following the Global Initiative for Asthma (GINA) guidelines.

The disease duration ranged between 1 and 25 years. Five of the 21 (24%) patients had a history of aspirin-exacerbated

asthma. No patient had a present or past history of CU, and no patient had a history of recent viral or bacterial respiratory infections.

Twenty-one healthy subjects (M/F 1/20; mean age 42.0 years, range 18–68) served as control group.

The study was approved by the local review board, and all of the subjects gave their informed consent before participating in the study.

Autologous plasma skin test

All patients underwent the intradermal injection of 0.05 ml of sterile, autologous plasma anticoagulated with Na citrate (autologous plasma skin test, APST) that was carried out and read as previously described (16). We performed the APST rather than the ASST because in previous studies of patients with urticaria we found that APST scores more frequently positive, and this prompted us to start studying the coagulation cascade in that skin disorder (16–18). No patient was taking antihistamines when APST was performed, whereas they continued their usual anti-asthma medication. A skin-prick test with histamine 10 mg/ml and an intradermal test with 0.05 ml of saline were used as positive and negative controls, respectively. The APST was regarded as positive if a clear-cut wheal-and-flare reaction was present both at 15 and 30 min in the total absence of any reactivity to saline.

Plasma measurements

Prothrombin fragment F1+2, a marker of thrombin generation, was measured in Na citrate–anticoagulated plasmas by sandwich ELISA (Enzygnost F1+2; Behring Diagnostics GmbH, Frankfurt, Germany). Intra-assay and inter-assay coefficients of variation were 5% and 8%, respectively.

D-dimer, a marker of fibrin generation and degradation, was measured in Na citrate–anticoagulated plasmas by ELISA (Zymutest D-dimer; Hyphen BioMed, Neuville sur Oise, France) according to the manufacturer's instructions. The intra- and inter-assay CVs were 10% and 15%, respectively.

Plasma VEGF concentration was measured by a sandwich enzyme immunoassay (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Intra-assay and inter-assay coefficients of variation were 5% and 7%, respectively.

C-reactive protein (CRP) plasma concentration was measured using a sandwich enzyme immunoassay (Zymutest CRP, Hyphen BioMed, Neuville-sur-Oise, France). Intra- and inter-assay variability was lower than 11%.

Statistics

Data were analyzed using the SPSS PC statistical package, version 17.00 (SPSS Inc., Chicago, IL, USA). As the plasma measurements were positively skewed, they were log-transformed before analysis. The F1+2, D-dimer, and VEGF results are given as antilog values of mean values (geometric mean) with standard deviation (SD). Student's *t*-test for

unpaired data was used to assess the statistical significance of the differences between the patients and normal controls. Correlations were assessed by means of least-squares linear regression. Proportions were compared with the chi-square test with Yates' correction. Probability (*P*) values < 0.05 were considered statistically significant.

Results

The autologous plasma skin test scored positive in 19/21 (90%) patients with nonallergic asthma (Table 1) and scored negative in all control subjects ($P = 0.0001$). An example of positive APST in patients with asthma is shown in Fig. 1. Interestingly, the only two APST-negative patients with asthma had a history of aspirin-exacerbated asthma, although the association did not reach statistical significance. In all patients and controls, the intradermal injection of saline did not induce any skin reaction. Mean fragment F1+2 plasma levels were significantly higher in patients with asthma (267 ± 243 pM) than in controls (150 ± 51 pM; $P = 0.0001$) (Fig. 2). Similarly, plasma levels of both D-dimer and VEGF were significantly higher in patients than in controls (D-dimer: 2364 ± 1467 vs 1301 ± 525 pM; $P = 0.0001$; VEGF: 1721 ± 2566 vs 76 ± 375 fM; and $P = 0.0001$) (Figs 3 and 4). In patients with asthma, CRP plasma levels (0.74 ± 2.57 mg/ml) were slightly higher than those of normal controls (0.45 ± 0.47) without reaching statistical significance.

A significant correlation was found between F1+2 and D-dimer plasma levels in patients with nonallergic asthma ($r = 0.427$, $P = 0.05$). F1+2 also correlated with CRP ($r = 0.572$, $P = 0.007$), whereas no other correlation was observed between coagulation markers and VEGF or CRP. A trend toward increased levels of F1+2, D-dimer, VEGF, and CRP was found in patients with a more severe disease according to the GINA classification: patients ($n = 10$) with



Figure 1 Positive autologous plasma skin test in a patient with nonallergic asthma. P, plasma; S, serum; C, negative control (saline); H, SPT with histamine 10 mg/ml.

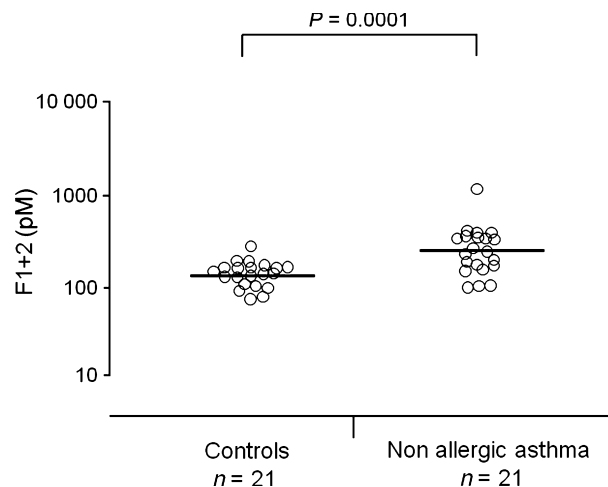


Figure 2 Plasma prothrombin fragment F1+2 levels in 21 healthy controls and 21 patients with nonallergic asthma. The results are expressed as pM, and the horizontal lines represent geometric means. F1+2 levels are higher in patients with nonallergic asthma than in the normal controls.

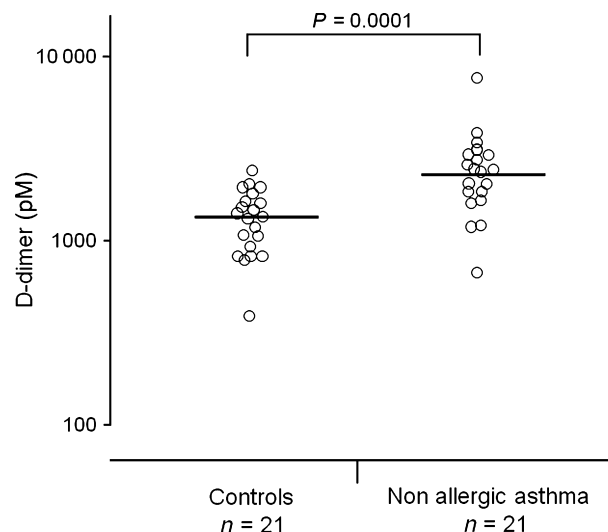


Figure 3 Plasma D-dimer levels in 21 healthy controls and 21 patients with nonallergic asthma. The results are expressed as pM, and the horizontal lines represent geometric means. D-dimer levels are higher in patients with nonallergic asthma than in normal controls.

moderate persistent asthma showed higher levels than patients ($n = 10$) with mild persistent asthma, although a statistical significance was not reached (F1+2: 390 ± 326 vs 256 ± 116 pM; D-dimer: 2890 ± 1911 vs 2500 ± 992 pM; VEGF: 3374 ± 2807 vs 2169 ± 2400 fM; and CRP: 1.05 ± 2.79 vs 0.63 ± 2.53 μ g/ml).

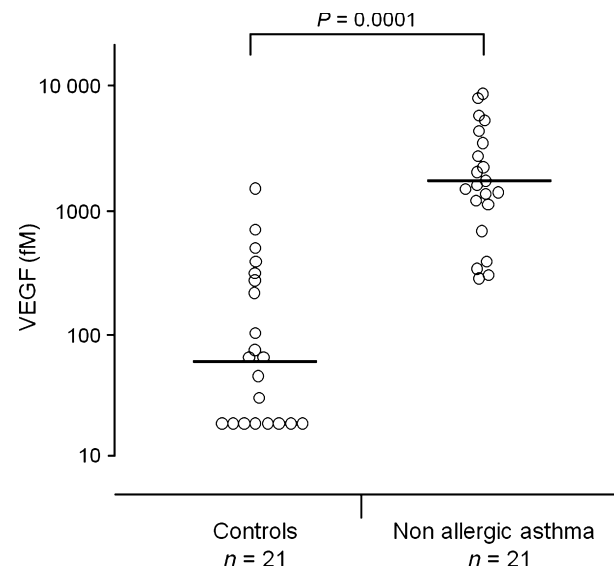


Figure 4 Plasma vascular endothelial growth factor (VEGF) levels in patients and controls. Plasma levels of VEGF in 21 healthy controls and 21 patients with nonallergic asthma. The results are expressed as fM, and the horizontal lines represent geometric means. Vascular endothelial growth factor levels are higher in patients with nonallergic asthma than in normal controls.

Discussion

Both allergic and nonallergic asthma are essentially inflammatory diseases. In this study, we were able to detect circulating vasoactive factors in most patients with nonallergic asthma as shown by the wheal-and-flare reaction upon intradermal injection of autologous plasma. This finding confirms and strengthens previous observations based on skin testing using autologous serum instead of autologous plasma (27). In light of our recent observations in CU, a disease that is also characterized by autoreactivity (16), we studied both coagulation markers and VEGF in patients with nonallergic asthma (17–19). The findings in patients with asthma were strikingly similar to those in patients with CU, with significant thrombin generation, activation of the coagulation cascade, and increase in plasma VEGF. So far, we did not perform immunohistochemical studies on patients with asthma yet; however, based on our previous observations in patients with CU, eosinophils are a possible source of both TF and VEGF (32). The activation of the coagulation has been recently observed in both allergic and nonallergic asthma (31, 33, 34). Interestingly, in animal experimental models, coagulation factor VII deficiency is associated with a reduced asthmatic response (35). Furthermore, ample evidence exists on the close link between inflammation and coagulation (36, 37). Proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α) induce the expression of TF, the main initiator of blood coagulation. Tissue factor activates the coagulation cascade generating thrombin, which leads to the formation of fibrin clot from fibrinogen. At the cellular level, the coagulant

mediators act on protease-activated receptors (PAR) inducing the expression of proinflammatory cytokines (37, 38). In turn, the two systems – coagulation and inflammation – activate each other, thus amplifying the response. Because asthma is regarded as a chronic inflammatory disorder, our data showing a correlation between the coagulation marker F1 + 2 and the inflammatory marker CRP is not surprising and is in accordance with previous observations in other inflammatory diseases (39).

In view of the present observations, nonallergic asthma enters a (hitherto restricted) group of diseases characterized by 'autoreactivity', including CU, the multiple drug allergy syndrome, and multiple NSAID intolerance (40–43). It is possible that in all these conditions the activation of the coagulation cascade as well as VEGF play a pathogenic role, maybe as amplification systems; in effect, there are indirect data suggesting a relevant role for coagulation cascade in the

clinical expression of asthma (34, 35). Although such diseases appear to be distinct from an etiological and pathogenic point of view, they are, nonetheless, all characterized by a state of chronic reactivity of different organs that is triggered both by exogenous and endogenous factors. Specifically, in the case of asthma, the activation of the coagulation cascade might be secondary to bronchial mucosa inflammation. In conclusion, nonallergic asthma is characterized by autoreactivity as well as increased coagulation and angiogenesis markers, which are known to enhance vascular permeability. The presence of circulating vasoactive factors may be relevant to understand the disease pathophysiology and to detect novel therapeutic strategies in nonallergic asthma.

Conflict of interest

The authors have no conflicts of interest to declare.

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