Deficiency of plasminogen activator inhibitor 2 in plasma of patients with hereditary angioedema with normal C1 inhibitor levels

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Background: Hereditary angioedema with normal C1 inhibitor levels (HAE-N) is associated with a Factor XII mutation in 30% of subjects; however, the role of this mutation in the pathogenesis of angioedema is unclear.
Objective: We sought evidence of abnormalities in the pathways of bradykinin formation and bradykinin degradation in the plasma of patients with HAE-N both with and without the mutation.
Methods: Bradykinin was added to plasma, and its rate of degradation was measured by using ELISA. Plasma autoactivation was assessed by using a chromogenic assay of kallikrein formation. Plasminogen activator inhibitors (PAIs) 1 and 2 were also measured by means of ELISA.
Results: PAI-1 levels varied from 0.1 to 4.5 ng/mL (mean, 2.4 ng/mL) in 23 control subjects, from 0.0 to 2 ng/mL (mean, 0.54 ng/mL) in patients with HAE-N with a Factor XII mutation (12 samples), and from 0.0 to 3.7 ng/mL (mean, 1.03 ng/mL) in patients with HAE-N without a Factor XII mutation (11 samples). PAI-2 levels varied from 25 to 87 ng/mL (mean, 53.8 ng/mL) in control subjects and were 0 to 25 ng/mL (mean, 4.3 ng/mL) in patients with HAE-N with or without the Factor XII mutation. Autoactivation at a 1:2 dilution was abnormally high in 8 of 17 patients with HAE-N (4 in each subcategory) and could be corrected by supplemental C1 inhibitor in 4 of them. Bradykinin degradation was markedly abnormal in 1 of 23 patients with HAE-N and normal in the remaining 22 patients.
Conclusions: Bradykinin degradation was normal in all but 1 of 23 patients with HAE-N studied. By contrast, there was a marked abnormality in PAI-2 levels in patients with HAE-N that is not seen in patients with C1 inhibitor deficiency. PAI-1 levels varied considerably, but a statistically significant difference was not seen. A link between excessive fibrinolysis and bradykinin generation that is estrogen dependent is suggested. (J Allergy Clin Immunol 2016;137:1822-9.)

Key words: *Hereditary angioedema, C1 inhibitor, bradykinin, plasminogen activator inhibitor*

Hereditary angioedema with normal C1 inhibitor levels (HAE-N; previously known as type III HAE)[1,2] is associated with a mutation in Factor XII in approximately 30% of patients and is strikingly estrogen dependent.[3,4] However, the relationship of this mutation to the pathogenesis of angioedema is uncertain; initial evidence to suggest that the mutated Factor XII is abnormally active[5] was refuted soon thereafter.[6] What is currently known is that the likely mediator of the swelling is bradykinin based on (1) the striking similarity of the clinical presentation to that of type I and II hereditary angioedema (HAE)[7] and (2) successful control of acute symptoms by blocking the bradykinin receptor[8] or inhibiting plasma kallikrein.[9] Curiously, infusion of C1 inhibitor (C1-INH) can also be effective acutely, suggesting that supranormal C1-INH levels can be therapeutic.[4]

We have considered the possibility that the underlying abnormality of this form of HAE is a deficiency of some other plasma inhibitor, the consequence of which might be enhanced activation of the plasma bradykinin-forming cascade when compared with normal values. Here we report that the main abnormality in patients with HAE-N is a deficiency of plasminogen activator inhibitor (PAI) 2 with diminished PAI-1 levels while all other parameters of activation or inhibition of the plasma bradykinin-forming cascade are normal. There was no distinction based on the presence or absence of the Factor XII mutation.

METHODS

Patients and sample collection

The diagnosis of HAE was confirmed by a clinical presentation of recurrent angioedema in the absence of urticaria, plus low C1-INH and/or function levels[10] by using a commercial assay performed at the site of origin of the samples. Patients with HAE-N had a clear family history of HAE, normal C1-INH protein and function levels, and in most instances striking estrogen dependence. Citrated plasma from 23 patients with HAE-N, 23 patients with type I HAE, and 23 healthy control subjects was separated by means of centrifugation of freshly collected blood at 2000 rpm for 10 minutes at 4°C. All samples were immediately placed in aliquots and stored at −80°C. Samples were handled similarly at all participating sites and shipped overnight on dry ice. The protocol was approved by the ethics committees and data protection agencies at all participating sites.

ELISA to measure C1-INH protein levels in plasma

Immulon 2HB plates (Thermo Scientific, Waltham, Mass) were coated with 5 μg/mL polyclonal antibody to C1-INH. After blocking with 1% BSA in

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Abbreviations used
BCIP/NBT: 5-Bromo-4-chloroindolyl phosphate/nitroblue tetrazolium
C1-INH: C1 inhibitor
HAE: Hereditary angioedema
HAE-N: Hereditary angioedema with normal C1 inhibitor levels
HK: High molecular weight kininnogen
PAI: Plasminogen activator inhibitor

PBS, samples and standards were added and incubated at room temperature for 1 hour. Bound C1-INH was probed with alkaline phosphatase–conjugated mAb to C1-INH, followed by color development with 5-bromo-4-chloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

Functional assay for C1-INH based on inhibition of kallikrein and Factor XIIa

Immulon 2HB plates were coated with 5 μg/mL avidin in coating buffer (100 μL) overnight at 4°C. Plates were washed 3 times with PBS-Tween (200 μL of each). Subsequently, 200 μL of 1% BSA in PBS was added to block the unused sites. The plates were incubated at 37°C for 1 hour and washed 3 times with PBS-Tween (200 μL of each). Samples or standards were added to the plates along with biotinylated protein (25 μL of standards or samples, 25 μL of biotinylated Factor XII or biotinylated kallikrein [1 μg/mL], and 50 μL of binding buffer), mixed, and incubated at 37°C for 1 hour. After incubation, plates were washed 3 times with PBS-Tween (200 μL of each). A polyclonal antibody to C1-INH was added, followed by incubation at room temperature for 1 hour. The wells were washed with PBS-Tween 3 times. Alkaline phosphatase–conjugated secondary antibody was added, and the samples were incubated at room temperature for 1 hour, followed by color development with the phosphatase substrate BCIP/NBT. The OD at 450 nm was read, and calculations were performed by using the standard curve.[11]

Functional assay for α_2-macroglobulin based on kallikrein inhibition

The functional assay for α_2-macroglobulin was developed in the same way as for C1-INH by using biotinylated kallikrein. After incubation, antibodies to α_2-macroglobulin were used for detection, followed by color development, as described above.

Bradykinin degradation in plasma

For bradykinin degradation studies, samples were prepared by incubating a known amount of bradykinin (300 ng) with each plasma (patients with HAE-N and healthy control subjects), and aliquots were collected at the indicated time. The proteins in the aliquots were precipitated with ice-cold ethanol and centrifuged for 1 hour at 10,000 rpm in a microcentrifuge at 4°C, and the supernatant containing free bradykinin was collected. The supernatant was then evaporated with a centrifugal concentrator to dryness and resuspended in EIA buffer. Bradykinin enzyme immunoassay was performed with an assay kit from Peninsula Laboratories (San Carlos, Calif), according to the manufacturer's recommendations. The concentrations of bradykinin present in samples were calculated by using a standard curve and are expressed as picograms per milliliter.

Prekallikrein activation assay

Activation of prekallikrein to form kallikrein on incubation of plasma in room temperature without any added activators was considered to represent "spontaneous" activation.[12] Kallikrein activity assays were performed, as described previously.[13]

Measurement of protein levels of plasma inhibitors

Protein levels of β_2-glycoprotein I, α_2-antiplasmin, activated protein C inhibitor, and inter-α-trypsin inhibitor in patients' samples were compared with control values by means of Western blotting, ELISA, or both.

SDS-PAGE and Western blotting

SDS-PAGE was performed with the buffer system of Laemmli.[14] Gradient gels (4-20%) were used for separation of proteins. After electrophoresis, the proteins were transferred to nitrocellulose membranes overnight. The membranes were then incubated with blocking buffer (1% BSA in PBS) for 1 hour after blocking and probed with mAbs for an additional 1 hour. Bound probes were visualized by incubating the membranes with alkaline phosphatase–conjugated secondary antibodies, followed by color development in BCIP/NBT.

ELISA to measure PAI-1 and PAI-2 levels in plasma

ELISA kits for the measurement of PAI-1 and PAI-2 levels were obtained from Cloud-Clone (Houston, Tex). The assays were initially performed, as suggested by the manufacturer, with a plasma dilution of 1:100. However, the results from 1:100 and 1:50 dilutions of plasma from patients with HAE-N were all less than the level of detection. Therefore we repeated the assays by using plasma dilutions of 1:10 and 1:5, and the values obtained were used to calculate nanograms per milliliter. Values from normal plasma and plasma from patients with type I/II HAE exceeded the standard curve at dilutions of less than 1:50.

Statistical analysis

The primary outcomes of interest were PAI-1 and PAI-2 levels among the 4 groups. Associations between PAI-1 and PAI-2 levels by disease group were evaluated by using linear regression models. Model assumptions were checked graphically. Pairwise comparisons between groups were evaluated by using contrast statements with a Tukey adjustment to control for multiple comparisons. All analyses were conducted in SAS software (version 9.3; SAS Institute, Cary, NC).

RESULTS

Quantitation of functional C1-INH

We first considered the possibility that patients' C1-INH levels could be normal based on inhibition of activated C1 yet abnormal if assayed based on inhibition of kinin-forming enzymes. The results for 17 patients who were assayed and compared with 17 healthy control subjects are shown in Fig 1, *A* and *B*. Some patients with HAE-N had inhibitory levels of 60% to 75%, and this was evident whether we assayed inhibition of kallikrein or activated Factor XII. This might represent secondary depletion of C1-INH in some subjects. Although functional C1-INH levels of 40% of normal or less are typical of symptomatic patients with type I or II HAE, C1-INH levels determined by means of inhibition of Factor XIIa or kallikrein were significantly decreased in patients with HAE-N irrespective of whether the Factor XII mutation was present or absent.

Assay of other possible inhibitors of kinin-forming enzymes

We next developed a functional assay for α_2-macroglobulin, as described in the Methods section; applied it to plasma of patients with HAE-N; and compared it with plasma of healthy control subjects. The results are shown in Fig 1, *C*. Similar to C1-INH, functional α_2-macroglobulin in patients' plasma seemed