Brief Definitive Report

DETECTION OF ACTIVE KALLIKREIN IN INDUCED BLISTER FLUIDS OF HEREDITARY ANGIOEDEMA PATIENTS*

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Hereditary angioedema (HAE) is a disease characterized clinically by recurrent episodes of swelling in the tissues of the extremities, face, abdomen, and respiratory tract, and biochemically by a deficiency of C1 inhibitor function, usually in association with an absence of C1 inhibitor protein (1–5). In studies of HAE, Landerman et al. (6) demonstrated the deficiency of a serum inhibitor of kallikrein, and postulated that unregulated kallikrein activity in tissue interstitial fluids was the etiologic agent in HAE. Subsequent studies revealed that C1 inhibitor is the deficient serum inhibitory protein in HAE and also established it as a major inactivator of kallikrein (4, 7). In addition to kallikrein inactivation, further investigations of C1 inhibitor showed it also could inactivate activated Hageman factor, factor XIa, plasmin, $C\overline{1r}$, and $C\overline{1s}$ (8–13). Thus C1 inhibitor is multifunctional and acts as a primary regulatory protein in both the Hageman-factor-dependent pathways (contact system) and in the classical pathway of complement.

As yet, the etiologic role(s) for either or both of these pathways in the pathogenesis of HAE is not clearly established. Studies by Donaldson and Evans (13), Austen and Sheffer (14), and Pitts et al. (15) showed that plasmas from HAE patients often contained $C\overline{1}$, the activated first component of complement, and low levels of C4 and C2, indicative of the unregulated cleavage of these substrate proteins by the $C\overline{1s}$ subunit of $C\overline{1}$. In vitro experiments showed that mixtures of $C\overline{1s}$, C4, C2, and plasmin produced a kinin-like activity (C2 kinin) similar to the kinin activity isolated from plasmas of patients who experience acute HAE attacks (16, 17). This C2-kinin was postulated to be the mediator of HAE.

Because C1 inhibitor is a major regulatory protein in the Hageman-factor-dependent pathways and because plasmin was required for the in vitro generation of C2-kinin, one anticipates activation of the contact system in HAE. To assess activation of the Hageman-factor-dependent pathways in the interstitial fluids from the sites of traumatic swelling, we have induced suction blisters on these sites in patients with HAE and in normal volunteers. We found that the blister fluid obtained from HAE patients contained large amounts of active kallikrein that was inhibitable by purified C1 inhibitor or by antibodies specific for plasma prekallikrein. These observations suggest that trauma initiates activation of the Hageman-factor-dependent pathways

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in the tissues of HAE patients and produces kallikrein activity. Furthermore, this kallikrein activity persists and is unregulated in these damaged tissues.

Materials and Methods

Subjects and Study Protocol. The patients with HAE who volunteered for the study were patients receiving their medical care at the Clinical Research Center (CRC) of Scripps Clinic and Research Foundation, La Jolla, Calif. The normal volunteers were selected from the employees of this institution. The age, sex, medical treatment, clinical symptoms, and plasma C1 inhibitor levels at the time of the study are shown in Table I. The study protocol was approved by the Human Research Committee of the Scripps Clinic and Research Foundation and consisted of admission to the CRC, solicitation of informed consent, precipitation of localized swelling on the forearm by traumatic vibration for 10 min with an inverted vortex Genie mixer (Scientific Products Div., American Hospital Supply Corp., McGaw Park, Ill.) and immediate induction of a suction blister. The suction blisters were induced on the vibrated forearm with the apparatus described by Kiistala (18) and modified by Kaplan et al. (19) (John Markle Specialty Parts, San Diego, Calif.). The temperature in the apparatus was maintained between 48 and 51°C for 30–60 min with a suction of 200 mm mercury. The blisters were then aspirated, and the fluids (~0.2–0.5 ml) stored in plastic tubes at −70°C until analysis.

Studies of Blister Fluids. The total protein concentration of each blister fluid was estimated with the Folin reagent by comparison with bovine albumin standard solutions. The presence of the following proteins was qualitatively ascertained by double immunodiffusion against monospecific antisera in 1% agarose (Seakem; Marine Colloids, Inc., Rockland, Maine) gels that contained 0.01 M veronal-buffered saline, pH 8.6, and 0.02 M EDTA: Hageman factor, prekallikrein, high molecular weight kininogen (HMWK), plasminogen, C1s, C1 inhibitor, α_2-macroglobulin, α_1-anti-trypsin, anti-thrombin III, inter α-trypsin inhibitor, α_2-anti-plasmin, and anti-chymotrypsin. Sodium dodecyl sulfate (SDS) polyacrylamide (9%) slab gels were prepared according to Laemmli (20) and used to detect enzymes capable of cleaving ^{125}I-Hageman factor, ^{125}I-prekallikrein ^{125}I-HMWK, and ^{125}I-plasminogen. 5 μl of each blister fluid was incubated with of each radiolabeled protein at 37°C for 45 min. After electrophoresis, the distribution of radioactivity in the gel was determined by autoradiography of the dried Coomassie-blue-R250-stained gel. Inhibition of the cleavage of HMWK by the blister fluid was accomplished by preincubation of the diluted blister fluid (1/20) with either purified C1 inhibitor (90 μg/ml final concentration) or with goat antibodies (1 mg/ml final concentration) to prekallikrein isolated from a prekallikrein-Sepharose affinity column. The ability of the blister fluids to cleave HMWK and release bradykinin from 5 μg purified HMWK was measured with the estrus rat uterus in a 3-ml bath that contained de Jalon's solution. The latent period between the time of addition and the onset of contraction was employed to assess the activity. The quantity of kinin present was estimated from a standard curve generated with synthetic bradykinin (Sandoz Pharmaceuticals, Hanover, N. J). A final concentration of 1×10^{-3} M 1,10-phenanthroline and SQ 20881 (E. R. Squibb and Sons, New York), an inhibitor of angiotensin-converting enzyme, was added to the blister fluids before incubation with HMWK to prevent inactivation of the generated bradykinin. The Hageman factor, prekallikrein, HMWK, and plasminogen employed in these studies were purified from normal human plasma and were homogeneous as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), immunoelectrophoresis, and double immunodiffusion (21–23). The purified Hageman factor and prekallikrein reconstituted the functional clotting activities of plasmas that were genetically deficient in these proteins. All proteins were radiolabeled with ^{125}I by the chloramine T method (24). Kallikrein was prepared from prekallikrein by incubation with trace amounts of activated Hageman factor.

Results

We obtained six blister fluids from five patients with HAE and eight blister fluids from eight normal volunteers. The blister fluids were assayed immunochemically for

TABLE I

Characteristics of Subjects Who Volunteered for Induction of Suction Blisters

Subject	Diagnosis	Age	Sex	Medications	Symptoms	C1 inhibitor level
		yr				
1 C.W.	HAE	50	F	None	16 attacks/mo	28
2 P.M.	HAE	27	F	Stanozolol	3 attacks/mo	46
3 V.C.	HAE	13	F	Stanozolol	1 attack/mo	27
4 S.M.	HAE	51	F	Danocrine	None	54
5a J.L.	HAE	60	M	Danocrine	daily swelling	72
5b J.L.	HAE	60	M	None for 6 d	daily swelling	58

HAE indicates established diagnosis of HAE. All HAE patients exhibited clinical symptoms of HAE and deficiency of C1 inhibitor function and protein. Patient J.L. has no family history of angioedema. The normal subjects studied included five males and three females ranging in age from 28 to 40 yr. Plasma levels of C1 inhibitor were determined by radial immunodiffusion and are given as μg/ml with a normal range being 145–227 μg/ml. Plasma from all normal individuals contained apparently normal concentrations of C1 inhibitor as determined by double diffusion immunoprecipitation techniques.

Hageman factor, prekallikrein, HMWK, plasminogen, C1s, C1 inhibitor, α_2-macroglobulin, α_1-anti-trypsin, and α_1-anti-chymotrypsin. All blister fluids analyzed contained detectable amounts of all of the proteins except C1 inhibitor. The blister fluids from the patients with HAE exhibited qualitatively less HMWK and C1 inhibitor than those obtained from the normal volunteers.

Because the patients with HAE had low levels of C1 inhibitor in their plasmas (Table I), we questioned whether the blister fluids might contain active Hageman factor and/or kallikrein, two of the proteases inactivated by C1 inhibitor. Studies of the HAE blister fluids showed that all six fluids from the five patients contained kallikrein-like activity capable of cleaving radioiodinated HMWK (^{125}I-HMWK), whereas only two of eight blister fluids obtained from normal volunteers contained any detectable amount of this activity (Fig. 1). The HAE blister fluids contained more ^{125}I-HMWK-cleaving activity than the two normal blister fluids that contained this activity. None of the blister fluids from either the HAE patients or the normal volunteers contained enzymes that cleaved radioiodinated Hageman factor, prekallikrein, or plasminogen under the conditions of the experiment. The kallikrein-like activity in HAE blister fluids cleaved ^{125}I-HMWK into fragments similar to the fragments of ^{125}I-HMWK produced by incubation with purified human plasma kallikrein (Figs. 1 and 2). The HMWK cleaving activity in HAE blister fluids and purified human kallikrein were both completely inhibited by preincubation with excess purified C1 inhibitor (Fig. 2). Preincubation of the HAE blister fluids and isolated human kallikrein with immunopurified goat antibodies specific for human prekallikrein also produced marked inhibition of the cleavage of ^{125}I-HMWK (Fig. 2). Preincubation of HAE blister fluids and kallikrein with goat antibodies specific for C1s (2 mg/ml final concentration) did not inhibit the cleavage of ^{125}I-HMWK (data not shown).

Because the enzymatic digestion of HMWK by kallikrein produces bradykinin, we studied the abilities of HAE blister fluids to generate smooth-muscle-contracting activity during incubation with purified HMWK. Four HAE blister fluids generated kinin activity as assayed on rat uterus, whereas none of three normal blister fluids produced kinin (Table II). Comparison of the kinin activity produced by the HAE blister fluids with the kinin activity produced by purified kallikrein and purified HMWK indicates that four HAE blister fluids contained large amounts of kallikrein-like activity (Table II). The smooth-muscle-contracting substance released from

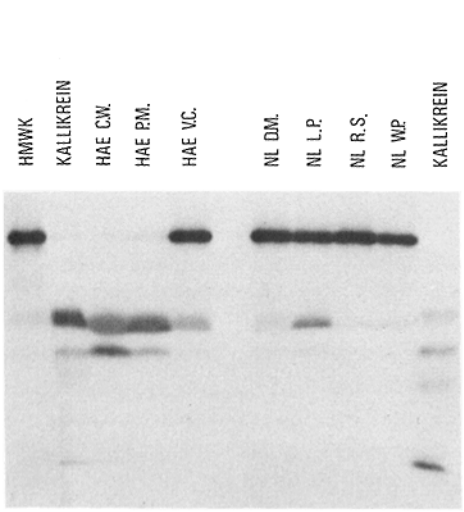

FIG. 1.

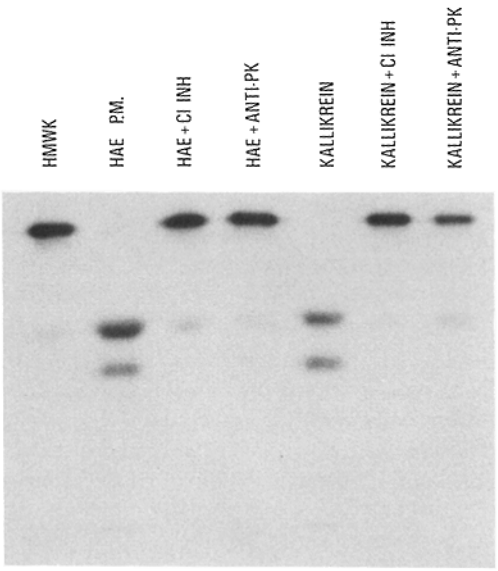

FIG. 2.

FIG. 1. Representative autoradiogram of SDS-PAGE demonstrating cleavage of ^{125}I-HMWK by blister fluids obtained from patients with HAE. Kallikrein preparation utilized in lane on right side of SDS-PAGE was different from preparation utilized on left. NL, normal control.

FIG. 2. Autoradiogram of SDS-PAGE demonstrating cleavage of ^{125}I-HMWK by blister fluid and purified kallikrein and inhibition of cleavage by purified C1 inhibitor or anti-prekallikrein antibodies. C1 INH, C1 inhibitor; ANTI-PK, anti-prekallikrein.

TABLE II

Determinations of Kinin-releasing Activities in Blister Fluids

Subject	Kinin released
	ng
HAE patients	
1 C.W.	21.9
3 V.C.	6.4
4 S.M.	5.2
5a J.L.	26.0
5b J.L.	0
Kallikrein (650 ng)	38.4

The quantity of kinin was obtained by comparison of the contraction of the estrus rat uterus with that obtained using synthetic bradykinin. The blister fluids from three normal subjects contained no detectable amounts of released kinin.

HMWK by HAE blister fluids or by kallikrein was abrogated by preincubation with chymotrypsin (10 μg). Both of the normal blister fluids that had cleaved small amounts of ^{125}I-HMWK were included in the three normal blister fluids studied with the kinin-generating assay and neither generated detectable smooth-muscle-contracting activity.

Discussion

The primary observation of these studies is that the suction blister fluids obtained from patients with HAE contained large amounts of active kallikrein, whereas similar blister fluids obtained from normal individuals did not. The kallikrein activity present

in HAE blister fluids appeared similar to plasma kallikrein in that it cleaved HMWK, released kinin from purified HMWK, and was inhibited by antibodies specific for plasma prekallikrein. The kallikrein activity was also abolished by incubation with C1 inhibitor, but not by antibodies specific to C1s. The concentrations of kallikrein activity in some blister fluids approached the concentration of total activatable kallikrein in human plasma (50 μg/ml), although the estimates of kallikrein activity in HAE blister fluids varied from 10 to 44 μg/ml. These observations suggest activation of the Hageman-factor-dependent pathways with generation of plasma kallikrein during the induction of blisters. However, the activation of a tissue kallikrein that bears antigenic cross-reactivities with plasma kallikrein has not been excluded. The persistence of kallikrein activity in blister fluids appears to result from the lack of C1 inhibitor in the blister fluid. The decreased levels of C1 inhibitor in the blister fluids from HAE patients probably reflects the low plasma levels of this protein. One HAE blister fluid (5b J.L.) contained only small amounts of kallikrein activity and did not release kinin from HMWK. This result was unexpected because another blister fluid obtained from this patient during Danocrine treatment did contain large amounts of kallikrein. The reasons for these differences are not clear.

The presence of kallikrein in HAE blister fluids indicates that activation within the Hageman-factor-dependent pathways can occur in the tissues of these patients. These studies do not distinguish among the putative inflammatory mediators that may be responsible for the pathophysiological features of HAE. However, they do suggest that the Hageman-factor-dependent pathways or, possibly, a tissue kallikrein may play a role in this disease, either by production of bradykinin from HMWK, by activation of plasmin *in situ*, or by some other yet undefined mechanism. Further studies of blister fluids obtained from patients with HAE may provide new insight into the pathogenesis of HAE.

Summary

Six suction-induced blister fluids obtained from five patients with hereditary angioedema (HAE) contained active kallikrein, whereas only two blister fluids obtained from eight normal volunteers contained small amounts of this activity. Kallikrein was present in large amounts of HAE blister fluids as assessed by its ability to liberate smooth-muscle-contracting activity from purified high molecular weight kininogen. It was inhibited by purified antibodies specific for plasma prekallikrein and also by purified C1 inhibitor, but not by antibodies specific for C1s. These observations suggest that activation of the Hageman-factor-dependent pathways occurs in the tissues of HAE patients, and once generated, active kallikrein persists in these tissues.

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