Cleavage of Human High-Molecular Weight Kininogen by Purified Kallikreins and Upon Contact Activation of Plasma

By Sessa Reddigari and Allen P. Kaplan

To study the digestion pattern of human high-molecular weight (mol wt) kininogen (HMWK) in plasma during contact activation we have prepared monoclonal antibodies (MoAbs) to the light-chain (LC) and the heavy-chain moiety of HMWK. One MoAb from each set was purified, and neither MoAb inhibited the clotting activity of HMWK. In enzyme-linked immunoabsorbent assay and immunoblotting experiments neither antibody bound to kininogen-deficient plasma. Digestion of purified HMWK with plasma kallikrein yielded, on reduced sodium dodecyl sulfate gels, two LC forms, at 62 and 49 kd, respectively. Digestion of HMWK with tissue kallikrein (TK) yielded mainly the 62-kd form. In plasma kallikrein, the 62-kd species slowly shifted to 49 kd, and with TK, the 62-kd species accumulated with time. Anti-LC MoAb was also used as a probe in immunoblotting experiments to study the digestion pattern of HMWK in whole plasma activated with kaolin or dextran sulfate. In activated normal pooled plasma (NHP) and factor XI-deficient plasma, native HMWK (mol wt, 115 kd) was cleaved within five to ten minutes, and two LC forms at 62 and 49 kd were detected. In kaolin-activated prekallikrein (PK)-deficient plasma, the disappearance of the 115-kd form was relatively slow, and only the 62-kd form of LC was seen. HMWK was not cleaved when factor XII-deficient plasma was incubated with kaolin. LC-dependent coagulant activity paralleled the presence of LC bands seen in the immunoblots, and lower-mol wt fragments of LC were not identified. These data indicate that in activated NHP two forms of LC of HMWK (62 and 49 kd) are formed sequentially. Further, the LC-dependent coagulant activity remains detectable long enough to suggest that proteolytic inactivation of LC is too slow to be an important control mechanism.

Materials and Methods

Proteins. HMWK was prepared from fresh NHP as previously described. Its coagulant activity was 12 U/mg where 1 unit is defined as the coagulant activity obtained in 1 mL of pooled NHP. PK was purified from fresh NHP as described previously. It was activated to kallikrein by reaction with 1% (wt/wt) Hageman factor fragment as described previously. The specific activity of kallikrein was 1.6 U/µg with Chromozym PK (Boehringer Mannheim Biochemicals, Indianapolis). One unit of enzyme activity is defined as the amount of enzyme that causes a change in absorbance at 405 nm of 1.0/min.

Tissue kallikrein (human urinary) was a gift from Dr Harry Margolius (Medical University of South Carolina, Charleston), and its specific activity was 74.0 p-tosyl-L-angininemethylester (TAME) esterase U/mg/min. Protein concentrations were determined according to the method of Lowry et al. MoAbs. MoAbs to the HMWK LC and HC were prepared by the method of Lipschitz et al. Anti-LC MoAbs were produced by immunizing the mice with native HMWK. Anti-HC MoAbs were made by immunizing the mice with purified reduced and alkylated HC, which was a gift from Dr Werner Muller-Esterl (University of Munich). Ascites fluids were prepared by intraperitoneal injection of 2 to 3 x 10^6 cells of active subcloned hybridoma cells into pristane-treated BALB/c female mice (retired breeders).

Antibody isotyping was done by enzyme-linked immunosorbent assay (ELISA) techniques using a commercial isotyping kit (from Southern Biotechnology Associates, Birmingham, AL). Antibodies were purified by 40% ammonium sulfate precipitation followed by ion-exchange chromatography on DE-52 (Whatman, Maidstone, England) in 10 mmol/L Tri-HCl (pH 8.0) and eluted with a 0 to 0.3 mol/L NaCl gradient in the same buffer. Peak fractions containing anti-LC activity were pooled, concentrated to 3.0 mg/mL, and stored at -70°C.

Coagulation assays. The coagulant activity of HMWK in purified preparations or in plasmas congenitally deficient in factor XII, PK, or factor XI was determined as described previously. Briefly, 50 µL each of Williams plasma (deficient in both HMW and LMW kininogens), the sample, and kaolin-cephalin (rabbit brain cephalin, Sigma Chemical Co, St Louis) suspension (10 mg kaolin/mL) in
CLEAVAGE OF HIGH MOL WT KINIGIN

0.01 mol/L potassium phosphate containing 0.15 mol/L NaCl, pH 7.4 (PBS), was preincubated at 37°C for two minutes. Clotting at room temperature was initiated by the addition of 50 μL of 0.05 mol/L CaCl₂. The LC coagulant activity of test samples was measured against a daily standard curve obtained from serially diluted NHP. NHP was prepared by pooling plasmas from at least three people. All plasmas were quick frozen in aliquots after collection and stored at -70°C. Only freshly thawed samples were used in all assays. All contact factor-deficient plasmas were obtained from George King Biomedical, Inc (Overland Park, KS) and were thawed only once and used immediately.

Activation of plasma with kaolin and dextran sulfate. NHP or plasma deficient in factor XII, PK, or factor XI were activated with 2 mg/mL kaolin (stock 20 mg/mL in PBS) at 37°C with frequent agitation. Aliquots were withdrawn at indicated time points and either diluted 1:10 into Tris-buffered saline (TBS) and added to the coagulation assay mixture or added to SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and heat denatured immediately. Activation of plasma with dextran sulfate (mol wt, 500,000) was performed at 4°C as described by van der Graaf et al.27 at a final concentration of 12.5 μg/mL. Samples at various time points were taken for immunoblotting and determination of HMWK coagulant activity as described earlier.

ELISA. ELISAs were performed by standard procedures with 96-well microtiter plates (Immulon-2, Dynatech Laboratories, Inc, Alexandria, VA). Wells were coated with 20 or 40 ng of antigen in 0.05 mol/L sodium carbonate buffer (pH 9.6) by incubation at 4°C overnight. Blocking was for 30 minutes at 37°C with 3% bovine serum albumin (BSA) in PBS. Wells were then incubated with various dilutions of primary antibody in PBS containing 0.05% Tween 20 for two hours followed by a two-hour incubation with the secondary antibody (goat antimouse IgG (γ chain) conjugated to alkaline phosphatase, Jackson Immunoresearch Laboratories, Inc, West Grove, PA) at a dilution suggested by the manufacturer. After washing, p-nitrophenylphosphate (1 mg/mL) in 10% diethanola-mine-HCl buffer (pH 9.6) was added to the wells and the absorbance read at 405 nm on Dynatech ELISA reader.

Gel electrophoresis. SDS-PAGE under reducing (with β-mercaptoethanol) or nonreducing conditions was performed according to the method of Laemmli23 and stained with Coomassie brilliant Blue R-250 or used for immunoblotting. Gels were either 10% (purified proteins) or 7.5% (plasma) acrylamide.

Immunoblotting. Proteins were electroblotted from gels onto nitrocellulose sheets (0.2 μm, BA83, Schleicher and Schuell, Inc, Keene, NH) according to the method of Towbin et al.24 After transfer, the nitrocellulose membrane was treated with 3% BSA in PBS-Tween at room temperature for one hour to block the unbound sites. The membrane was then treated with a 1:1,000 dilution of primary antibody at room temperature for two hours, rinsed, and then treated with goat antimouse IgG (γ chain)-alkaline phosphatase conjugate in PBS-Tween at a dilution suggested by the manufacturer for two hours at room temperature. The final wash was done in PBS-Tween containing 0.5 mol/L NaCl for one hour at 37°C. The bands were developed in substrate mixture containing 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium in Tris-HCl buffer (pH 9.5).26

RESULTS

MoAbs to HMWK. We have produced a total of 22 anti-LC and six anti-HC MoAbs as described in Materials and Methods. One MoAb from each set was purified. Both antibodies were of IgG1 x subtype. These did not recognize any antigens in total kininogen-deficient plasma in ELISA experiments (Table 1) or in immunoblotting experiments. In

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HMWK</th>
<th>LC</th>
<th>HC</th>
<th>Williams Plasma</th>
<th>Normal Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-LC (571-28)</td>
<td>0.838†</td>
<td>1.6</td>
<td>0.06</td>
<td>-0.015</td>
<td>0.33</td>
</tr>
<tr>
<td>Anti-HC (11-2)</td>
<td>0.94</td>
<td>-0.03</td>
<td>1.6</td>
<td>-0.02</td>
<td>0.78</td>
</tr>
</tbody>
</table>

*Plasma was diluted 1:100 in bicarbonate coating buffer.
†Optical density, 405 nm by ELISA.

Fig 1, lane A, we see a major band of HMWK at mol wt 120 kd and a minor component at 103 kd. These are present in most normal plasma tested. Above are faint bands that are variable, perhaps representing traces of undissociated HMWK aggregates. None of these is seen in lane B in which kininogen-deficient plasma is tested. Also, neither antibody inhibited the coagulant activity of HMWK (data not shown).

Digestion of purified HMWK by plasma and tissue kallikrein. Purified HMWK was digested with either purified plasma kallikrein or tissue kallikrein and subjected to
Fig 2. SDS-PAGE (10%) of HMWK digestion with purified plasma kallikrein (A) and tissue kallikrein (B). HMWK was incubated with either kallikrein at a 100:2 (wt/wt) ratio at 37°C. Aliquots were withdrawn at indicated times, denatured in reducing SDS-PAGE sample buffer, and electrophoresed. The gel was stained with Coomassie blue R-250 and destained.

Fig 3. HMWK digestion with plasma kallikrein. HMWK was incubated with plasma kallikrein at 1% wt/wt of enzyme per substrate. Samples at indicated time points were electrophoresed under reducing and nonreducing conditions and immunoblotted with anti-LC MoAb. (A) Immunoblot. (B) Coomassie blue-stained gel.
CLEAVAGE OF HIGH MOL WT KININOGEN

SDS-PAGE. Figure 2 shows the time course of such digestion under reducing conditions. By five minutes virtually all the HMWK was digested and converted to an HC of 65 kd with an LC of 62 kd just beneath it. As the digestion proceeded, this LC was further digested to a smaller LC of 49 kd. Digestion of HMWK by tissue kallikrein was similar; complete cleavage of native HMWK was evident by five minutes except that conversion of the high-mol wt LC to the lower-mol wt form was only beginning by 30 minutes and, although not shown, continues to slowly progress over many hours.

Immunoblotting of HMWK after digestion with plasma kallikrein. A time course of digestion of a different batch of HMWK by plasma kallikrein was performed and subjected to SDS-PAGE under reducing and nonreducing conditions (Fig 3). The slab gel was then immunoblotted with anti-LC MoAb. As can be seen in Fig 3A, under nonreducing conditions the antibody recognized major bands of native
HMWK at 120 and 103 kd. A minor band is seen at about 116 kd. With increasing time of digestion the 120-kd material was converted to bands seen at 103 and 96 kd.

Upon reduction bands were seen, at zero time, at 116, 100, 85, 62, and 49 kd, thus indicating that the HMWK used in these experiments was partially cleaved, although these cleavage products were not that apparent in Coomassie blue–stained gels at zero time (Fig 3B). The 116-, 100-, and 85-kd bands were very quickly digested, and as digestion proceeded, the 62-kd band became more intense (Fig 3A, ten minutes) and then appeared to decrease in intensity (Fig 3B), whereas the band seen at 49 kd progressively increased (Figs 3A and 2A).

Contact activation and immunoblotting of plasma. NHP or plasmas deficient in PK, factor XI, or factor XII were activated with kaolin, and at indicated time points aliquots were electrophoresed under reducing conditions and immunoblotted with anti-LC MoAb. The results are shown in Fig 4. It is evident that when NHP is activated with kaolin the 116-kd form of HMWK and the minor form seen at 100 kd are cleaved within the first five minutes and the two forms of LC become visible at 62 and 49 kd. These two bands are seen essentially unchanged at the end of 30 minutes (Fig 4A). Very similar results were obtained with factor XI–deficient plasma (Fig 4B). In PK-deficient plasma, the rate of disappearance of native HMWK is slow relative to normal or factor XI–deficient plasma, and some uncleaved material can still be seen after two hours. The 62-kd form of LC is seen and remained prominent, and its conversion to the 49-kd form was minimal even after 90 minutes of incubation (Fig 4C). There was no apparent cleavage of HMWK upon activation of factor XII–deficient plasma (Fig 4D).

In a separate experiment NHP and factor XI–deficient plasmas were activated with dextran sulfate. Aliquots immediately before and 5, 10, and 30 minutes after the addition of dextran sulfate were immunoblotted. The results shown in Fig 5 indicate that, in NHP, HMWK was cleaved almost completely within five minutes and the two LC forms could be seen at 62 and 49 kd, respectively. At ten minutes, the 62-kd form was fainter, and the 49-kd form was more intense. By 30 minutes, all of the 62-kd forms were converted to the lower forms. In factor XI–deficient plasma the cleavage was minimal at five minutes, but after that it was similar to NHP.

Time course of depletion of HMWK coagulant activity. The time course of depletion of HMWK coagulant activity during the first 30 minutes of activation by kaolin was assessed in the same samples shown in the immunoblots of Fig 4. Aliquots of each sample were diluted, added to HMWK-deficient plasma, and clotted as described in Materials and Methods. Results are shown in Fig 6. Approximately 30% depletion of coagulant activity was seen in NHP by 30 minutes whereas only 18% depletion was seen in factor XI–deficient plasma. Depletion of coagulant activity in PK-deficient plasma appeared slower during the initial 20 minutes but was very close to the other two plasmas by 30 minutes.

When NHP was activated with dextran sulfate for 24 hours, its HMWK coagulant activity decreased by 60%, whereas in similarly treated factor XI–deficient plasma, the decrease was 40% (Table 2).

DISCUSSION

It is known that when purified HMWK is cleaved by purified plasma kallikrein, two initial cleavages result in bradykinin release and the formation of a two-chain form of HMWK in which one chain is susceptible to further proteolysis. Some reports indicate that reduction of kinin-free HMWK yields chains of equal size (65 kd) on SDS gel electrophoresis. Others find an LC of 56,000 distinguishable from the HC, which then undergoes further digestion.11,12,13 We considered the possibility that LCs of 62 to 65, 56, and 46 to 49 kd form sequentially and that conversion of the 62- to 65-kd form to the 56-kd form might be exceedingly rapid and therefore variable in different experiments in different laboratories. Immunoblotting, with an anti-LC monoclonal allows one to follow this pattern in gels without superimposed HC. Our data indicate only two forms of LC when we assess digestion of purified HMWK by kallikrein. The 62-kd band can be converted to the 49-kd band progressively,11 and the rate is proportional to the enzyme concentration. We also confirm the relative inability of tissue kallikrein to convert the 62-kd LC to 46 to 49 kd during the digestion times used in our experiments. Further slow con-
CLEAVAGE OF HIGH MOL WT KININOGEN

Fig 6. Coagulant activity of LC of HMWK in activated plasmas. Two hundred fifty microliters of NHP or deficient plasma were incubated with kaolin (final concentration, 2 mg/mL) at 37°C. Aliquots at indicated time points were diluted into PBS (1:10), and 60 μL samples (in triplicate) were immediately assayed for HMWK clotting activity as described in Materials and Methods. Data obtained are expressed as percent initial (zero time) activity. Normal plasma; Δ, factor XI–deficient plasma; ■, PK-deficient plasma.

Table 2. HMWK-Dependent Coagulant Activity in Dextran Sulfate-Activated Plasma

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Unactivated</th>
<th>24-h Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHP</td>
<td>1.4 (100%)</td>
<td>0.55 (39.3%)</td>
</tr>
<tr>
<td>Factor XI deficient</td>
<td>0.95 (100%)</td>
<td>0.58 (61%)</td>
</tr>
</tbody>
</table>

were observed, although almost all of the 62-kd forms were converted to 49-kd ones within ten minutes, and they remained stable (Fig 5). A 30% decrease in HMWK-dependent coagulant activity was seen during the 30-minute period in kaolin-activated NHP (Fig 6). Although there was no such decrease in dextran sulfate activated NHP in the same time period (data not shown), a 24-hour incubation with dextran sulfate resulted in a 60% loss of coagulant activity (Table 2).

Comparison of the rates of cleavage of NHP and plasma deficient in factor XII, PK, or factor XI revealed rate differences that are predictable on the basis of the known requirements for contact activation. Factor XII-deficient plasma did not become activated. PK-deficient plasma showed a marked diminution in the rate of activation as well as no conversion of the 62-kd LC to the 49-kd LC. These are each kallikrein-dependent processes; thus those cleavages seen are due to an interaction of factor XIIa or perhaps factor XIa with HMWK. Factor XI–deficient plasma did not differ from NHP with kaolin activation but was slower to activate with dextran sulfate; it is unclear whether this is true of the particular plasma or indicative of a subtle contribution of factor XIa to either the initiation of contact activation by Hageman factor or to direct HMWK cleavage.

The decrease in coagulant activity observed for NHP or factor XI–deficient plasma with prolonged incubation with either kaolin or dextran sulfate was relatively small, and the maximum, a 60% loss, occurred after a 24-hour incubation with dextran sulfate (Table 2). Thus although data shown in Table 2 and Fig 5 suggest that Factor XI can have a role in the metabolism of HMWK, considering that a level of HMWK of 15% is sufficient to normalize coagulation in kininogen-deficient plasma and any error inherent in our clotting assay would not change our data from a 70% residual activity to less than 15%, the data presented here indicate that proteolytic inactivation of the LC of HMWK by factor XIa or other enzymes is too slow to be an important control mechanism.

ACKNOWLEDGMENT

We gratefully acknowledge Rebecca Rowehl and Jackie Bortzner for their technical assistance and Dr Michael Silverberg for his computer programs, which were used for routine standard assays.

REFERENCES

kininogens are identical with $\alpha_2$-cysteine protease inhibitors. Evidence from immunological, enzymological, and sequence data.

FEBS Lett 182:310, 1985


Cleavage of human high-molecular weight kininogen by purified kallikreins and upon contact activation of plasma

S Reddigari and AP Kaplan