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HUMAN PLASMA KALLIKREIN PROCESSING: PROTEOLYSIS AS AN ALTERNATIVE CONTROL

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SUMMARY

Human plasma kallikrein (HuPK) was detected in normal non-activated and dilution-activated plasma by immunoblotting, using polyclonal antibodies. In non-activated plasma, the predominatly detected protein corresponds to prokallikrein (Mr 80,000-90,000). Activated plasma, besides kallikrein, contains larger proteins (Mr > 130,000) that possibly represent complexes between kallikrein and proteinase inhibitors. Plasma also contains species (Mr 43,000) which corresponds to kallikrein heavy chain. In activated plasma, monoclonal antibodies against kallikrein heavy chain detected, besides these same bands described above, two additional bands (Mr 30,000 and 20,000) possibly correspondent to fragments of kallikrein heavy chain.

INTRODUCTION

Human plasma kallikrein is a serine proteinase present in plasma as prokallikrein, a glycoprotein zymogen synthesized by liver as a single polypeptide chain with 619 aminoacid residues. The activation of prokallikrein to kallikrein requires the cleavage of the peptide bond Arg^{371}-Ile^{372} in the single chain, forming a molecule with a heavy chain (371 residues) and a light chain (248 residues) held together by dissulfide bridges (1).

Prokallikrein circulates in plasma complexed with high molecular weight kininogen as a bimolecular complex 1:1 (2), being the heavy chain of prokallikrein bound to the light chain of high molecular weight kininogen (3).

Prokallikrein is activated in blood by activated factor XII (4). Kallikrein itself activates factor XII at the early phase of intrinsic blood coagulation (5), and hydrolyzes high molecular weight kininogen liberating the hypotensive peptide bradykinin (6). Plasma kallikrein also participates in some reactions of fibrinolysis (7) and complement systems (8), and promotes the aggregation of platelets (9) and leucocytes (10).

Plasma kallikrein can be found in two different molecular weight species (Mr 88,000 and 85,000) (11). The NH_2-terminal heavy chain (43,000) is important for the specific activity of kallikrein and is similar to the heavy chain of factor XI (12); the COOH-terminal light chain (Mr 36,000-33,000) lodges the

catalytic site of kallikrein, and is similar to trypsin (1).

Kallikrein is inactivated either by plasma inhibitors such as C1-inhibitor, alpha 2-macroglobulin, antithrombin III (13) and protein C inhibitor (14), or by the liver, where it is recognized by its alpha-galactose residues (15).

In this report, we studied the proteolysis of human plasma kallikrein in activated human plasma, as an alternative control of plasma kallikrein metabolization.

MATERIAL AND METHODS

Human plasma kallikrein (HuPK) was isolated from Cohn's fraction IV (16) or fresh plasma (17). The materials were submitted to SBTI-Sepharose chromatography (0.02 M tris-HCl, 0.3 M NaCl, pH 8.0 buffer) and the adsorbed kallikrein was eluted by benzamidine 1.0 M. The protein concentration was calculated according to the mesurement of the extinction coefficient ($E_{280,1\%}$=10.6), determined by Nagase and Barrett (18). The heavy and light chains of plasma kallikrein were isolated by reduction with dithiothreitol, alkylation with iodoacetamide (19) and separated on SBTI-Sepharose.

Polyclonal antibodies against HuPK were raised in rabbits. The antiserum was purified on HuPK-Sepharose (0.05 M tris-HCl, 0.15 M NaCl, pH 8.0 buffer), and the bound antibodies were eluted with 0.2 M glycine-HCl, pH 2.0 buffer, and the pH of

the collected samples was kept at 8.0 by addition of 1.0 M tris. Antibody against rabbit IgG (second antibody) was raised in goats, and anti-serum was purified on anti-HuPK-Sepharose, by the same procedure.

Monoclonal antibodies (PK 9) against heavy chain of HuPK, kindly provided by Prof. Dr. Werner Muller-Esterl (University of Mainz-Germany), were precipitated from ascitic fluids with saturated ammonium sulfate solution, pH 7.0 (11:9, v/v). The solution was kept at 4°C during 60 minutes and centrifuged at 3,500 x g at 4°C for 30 minutes. The pellet was ressuspended in 50% ammonium sulfate solution and recentrifuged, under the same conditions. This pellet was ressuspended in 0.01 M tris-HCl, pH 7.5 buffer and dialyzed against the same buffer, overnight at room temperature. The antibodies were cromatographed on a column (100 ml) of DEAE-Sepharose CL 6B (0.01 M tris-HCl, pH 7.5 buffer), and eluted with a sodium chloride gradient from 0 to 0.25 M. The PK9 antibodies were eluted with 0.14 M sodium chloride.

Antibodies were labelled with iodine-125 using as oxidant reagents either Iodo-beads (Pierce) or Chloramine T (20). The specific activity of the polyclonal or monoclonal antibodies was 10-20 uCi/ug, and in each experiment 2 x 10^6 cpm (100 ng of protein) were used.

Human normal blood was collected directely into plastic tubes mixed with sodium citrate solution 3.8%, 9:1 (v/v). The

sample was centrifuged at 1,500 x g, during 15 minutes at 4°C, and the separeted plasma was aliquoted and kept frozen at -20°C.

For activation, plasma samples (2 ul) were thawed, diluted in water in a final volume of 200 ul, and incubated at 37°C during 15 minutes. The activation reaction was stopped by freezing, and the samples were submitted to electrophoresis. Control samples of non activated plasma (2 ul) were thawed and diluted directly in electrophoresis buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels from 5% to 15%, according to the method of Laemmli (21), with 3% stacking gels. Apparent molecular weights were determined by running standard proteins of known molecular weights (range 14,000 to 200,000) on the gel.

Immunoblotting was performed according to the modification described by Burnette et al. (22) or according to Kyhse-Andersen (23). The immunodetection of the antigen was achieved by a double antibody reaction: the nitrocellulose membranes were incubated with anti-HuPK antibody (20 ug/ml) and the complexes were developed with ^{125}I-second antibody (2 x 10^6 cpm per assay). Alternatively, the immunodetection of the antigens was achieved by reaction with the monoclonal antibody against heavy chain ^{125}I-PK9 (2 x 10^6 cpm per assay).

RESULTS

Human plasma kallikrein was purified by a described procedure (16, 17) and the two known forms, alpha and beta, were prepared; both preparations resulted in single bands on non reduced gel electrophoresis (Figure 1a).

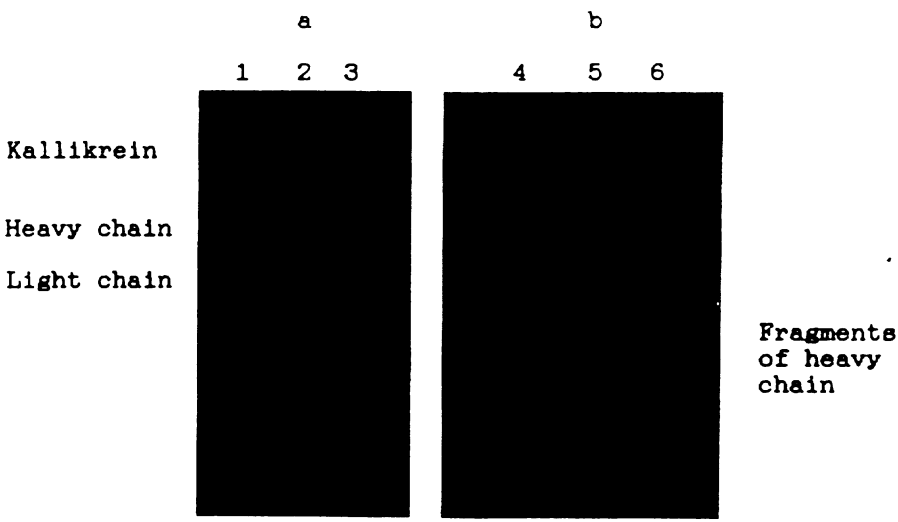

FIGURE 1: SDS-polyacrylamide electrophoresis of kallikrein (HuPK) forms.
a. Non reduced gel
1. alpha-HuPK (15 ug)
2. alpha-HuPK and beta-HuPK (20 ug)
3. beta-HuPK (10 ug)

b. Reduced gel
4. alpha-HuPK (15 ug)
5. alpha-HuPK and beta-HuPK (20 ug)
6. beta-HuPK (10 ug)

After reduction with DTT, alpha-HuPK presents a heavy chain (Mr 45,000) and a light chain (Mr 36,000 and 33,000); beta-HuPK has its heavy chain hydrolyzed into two bands (Mr 30,000 and 20,000), and a light chain (Mr 36,000 and 33,000).

On an immunoblotting using anti-HuPK and ^{125}I-second antibodies, in non-activated plasma (figure 2a) prokallikrein gives a strong band (Mr 80,000) and also weak (Mr > 150,000) bands. The 100-fold diluted plasma (figure 2b) presents the same prokallikrein-kallikrein band (Mr 80,000), and the high molecular bands (Mr > 130,000), that represent kallikrein-inhibitor complexes. The activated plasma also exhibits a band (Mr 43,000) that corresponds to the purified heavy-chain of the enzyme used as marker in the experiment (figure 2d); this band does not coincide with IgG fragments which may contaminate kallkrein preparations (figure 2c).

The same experiment indicated in figure 2 was performed with a monoclonal antibody against heavy chain (PK9). Diluted human plasma (1:100) was incubated with purified HuPK or its isolated heavy chain and the antigens were immunoprecipitated with ^{125}I-PK9 (figure 3).

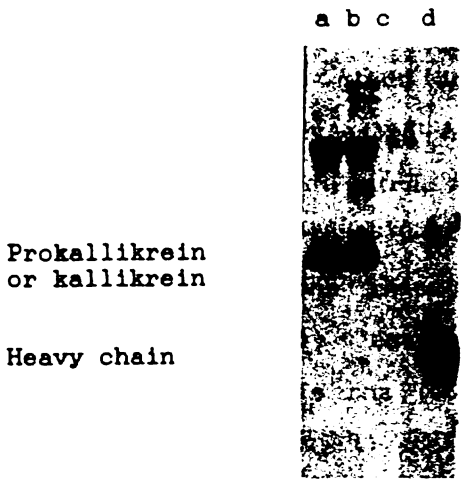

FIGURE 2: Activation of human normal plasma by dilution (1:100).

a. human plasma (2 ul)
b. human plasma (2 ul) diluted 1:100
c. human IgG (6.5 ug)
d. isolated heavy chain of HuPK (1.0 ug)
Immunoprecipitation: anti-HuPK
Development: ^{125}I-second antibodies

In activated plasma the monoclonal antibodies (PK9) identified kallikrein (Mr 80,000) and larger proteins (Mr > 130,000); in this case, the higher molecular weight complexes correspond to kallikrein-inhibitors complexes, and not to eventual complexes between free light chain of HuPK and inhibitors, because the monoclonal PK9 used to develop the blotting does not react with light chain (24).

The addition of isolated kallikrein heavy chain to activated plasma causes a strong enhancement of the weak protein band (Mr 43,000), showing that the band corresponds to heavy chain.

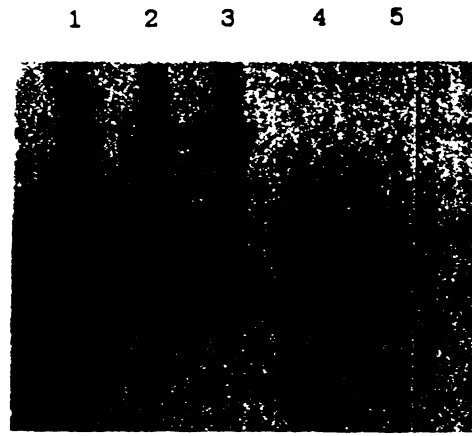

FIGURE 3: Diluted human plasma (1:100) incubated with purified kallikrein and kallikrein heavy chain.

1. human normal plasma (2 ul)
2. human normal plasma (2 ul) and HuPK (0.5 ug)
3. human normal plasma (2 ul) and isolated heavy chain (0.3 ug)
4. HuPK (0.5 ug)
5. isolated heavy chain (0.3 ug)

Development: ^{125}I-PK9

Plasma (2 ul) was diluted (1:100) and incubated with increasing quantities of purified HuPK, and the antigens were immunoprecipitated with ^{125}I-PK9 (figure 4). The increasing quantities of purified kallikrein added to activated plasma causes a strong enhancement of the kallikrein-inhibitors complexes bands (Mr > 130,000), as well as the heavy chain band (Mr 43,000), and also the appearance of smaller proteins bands (Mr 30,000 and 20,000). These results are the first indication of the hydrolysis of kallikrein heavy chain, in plasma.

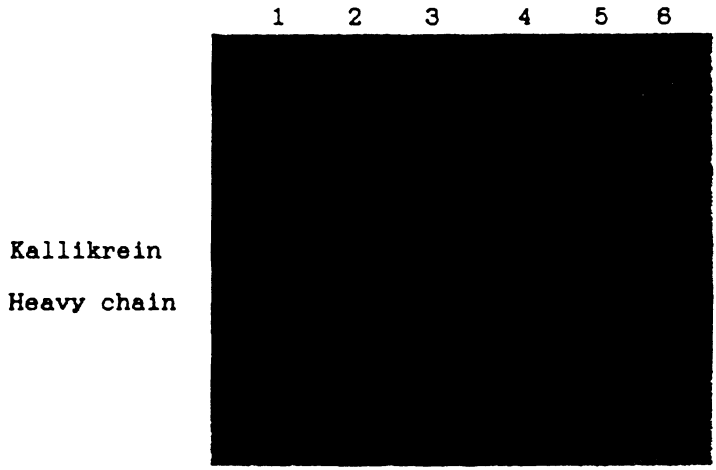

FIGURE 4: Diluted human plasma (1:100) incubated with different quantities of HuPK.

1. human plasma (2 ul)
2. human plasma (2 ul) and HuPK (0.5 ug)
3. human plasma (2 ul) and HuPK (1.0 ug)
4. human plasma (2 ul) and HuPK (2.5 ug)
5. HuPK (2.5 ug)
6. HC (0.2 ug)

Development: ^{125}I-PK9

DISCUSSION

Beta-HuPK derives from a clevage in the heavy chain of the alpha-HuPK, and one possibility for this modification is to occur to keep pure kallikrein solutions at room temperature. The beta form has the same activity on synthetic substrates, but

alpha-kallikrein cleaves high molecular weight kininogen (HMWKgn) releasing braykinin, faster than the beta form (25).

Contact of plasma with negatively charged surfaces, such as glass or kaolin, causes the activation of the intrinsic pathway of blood clotting, by bringing together kallikrein, kininogen and factor XII on the surface. However, dilution induces activation by decreasing the inhibitor concentration in plasma and thus promoting activation of the key clotting enzymes. In our experiments the activation was confirmed by the formation of complexes, equivalent to those formed by incubation of kallikrein and purified inhibitors (13).

Only in the activated plasma the protein band corresponding to the heavy chain (Mr 43,000) was detected by both polyclonal and monoclonal antibodies (26). The addition of large quantities of active kallikrein in activated plasma brings evidences that the transformation of alpha to beta-kallikrein proceeds in the plasma, as it was observed for purified samples of kallikrein. The larger protein bands (Mr > 130,000) probably correspond to complexes of kallikrein and plasma proteinase inhibitors. The polypeptide chains with Mr < 43,000 may indicate that kallikrein or its heavy chain is being processed in activated plasma, resulting in shorter fragments.

No corresponding free light chain is found in activated plasma containing free heavy chain. One possible explanation for this finding is an extensive hydrolysis of the light chain, and no immunnodetectable fragments would be available for reaction

with polyclonal antibodies. Or, alternatively, the formation of light chain-inhibitors complexes might occur but difficult to resolve from the HuPK-inibitor complexes.

Thus, the control of kallikrein activity, especially in the blood clotting cascade, might also be exerted by a proteolytic degradation of its molecule. The resulting free heavy chain might alter the surface binding ability of high molecular weight kininogen, and the whole activity of the contact phase would be lowered. Proteolysis of the heavy chain itself might also be taken as part of this control mechanism, that would not depend on inhibition and liver clearance alone.

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