Is 100KF an Isoform of Hemopexin? Immunochemical Characterization of the Vasoactive Plasma Factor 100KF

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Abstract. The human vasoactive plasma factor 100KF has been proposed to play a role in minimal change disease in relapse. Since preliminary data suggested similarity between 100KF and the human plasma glycoprotein hemopexin (Hx), this study was conducted to compare 100KF with purified Hx for sequence homology, immunostaining properties in Western and dot-blot assays, ability to affect glomerular ecto-ATPase and glomerular polyanions in vitro, as well as their glomerular permeability increasing effect following alternate perfusion into the rat kidney ex vivo. 100KF was purified from normal pooled plasma according to standard chromatographic techniques, and from the same batch Hx was prepared using affinity chromatography. A second batch of Hx was prepared directly from human serum according to a standard protocol. (For comparison, additional Hx samples obtained from other centers were also included in the study.) The results show: (1) 100% homology of 100KF with plasma Hx after internal sequence analysis; (2) positive staining of the eluate with both monoclonal and polyclonal anti-Hx IgG as well as anti-100KF IgG in dot-blot assays, and similar bands on Western blotting using the same antibodies; (3) affection of glomerular polyanions and glomerular ecto-ATPase after incubation of kidney tissue with either 100KF or Hx (1.5 respectively 1.0 mg/ml; 1.0 h, 37°C), as detected by computerized histochemical quantification; and (4) significant enhancement of urinary protein leakage after Hx perfusion followed by diluted rat serum into the rat kidney ex vivo (Hx: 210.65 ± 49.79 μg protein leakage per min versus heat-inactivated Hx control: 112.2 ± 49.18 μg per min [both n = 6]). From these data and from the observation that both Hx and 100KF activity can be inhibited by serine protease inhibitors but not by broad spectrum collagenase inhibitors, it is concluded that Hx may be closely related or identical to the active moiety of 100KF.

Circulating factors either in the form of plasma proteins or cytokines are supposed to play a role in the pathophysiology of proteinuria in minimal change disease (MCD) (1–3). For instance, in 1957 Tapie et al. (4) observed a relationship between the presence of lymphoid tumors and the occurrence of nephrotic syndrome in some patients; this observation was confirmed by other authors as well (5,6). Since removal of the tumor induced remission of the nephrotic syndrome, it was suggested that circulating factors might play a causal role in the mechanism of proteinuria.

Based on these observations and on various abnormalities of T cells in vitro from subjects with MCD, as well as on the recurrence of some nephrotic syndrome after transplantation, Shaloub suggested in 1974 (7) that abnormal production of T cell products may be toxic for glomerular filtration barrier, leading to albuminuria.

In the years that followed, a considerable number of articles dealing with lymphokines or lymphokine-like plasma factors were published, suggesting enhanced activity of these factors in patients with proteinuria (8–19). Unfortunately, the exact biochemical nature of most of the factors described is obscure.

Previously, we reported on a vasoactive plasma protease denoted as 100KF, present in the circulation of healthy subjects as well as in patients with MCD (20). This partly purified factor is able to affect glomerular extracellular matrix molecules, such as glomerular sialoglycoproteins or glomerular ecto-ATPase in vitro, as well as after perfusion into the rat kidney ex vivo (21,22). These alterations also occurred in association with increased glomerular permeability after either alternate kidney perfusion ex vivo or direct infusion of 100KF into the rat kidney in vivo (23).

Until now, the nature of this plasma constituent was uncertain, although preliminary molecular biologic studies suggested similarity with the plasma acute phase reactant hemopexin (Hx). Here, we describe further studies into the identity of 100KF using internal amino acid sequence analysis and immunoblotting with antibodies raised against 100KF as well as against human Hx.

The results suggest that 100KF is similar, if not identical, to plasma Hx or an isoform of Hx. The additional observation that besides 100KF, purified human Hx is also able to induce transient increased protein leakage after contact with the rat kidney ex vivo, supports this concept.
Materials and Methods

Preparation of 100KF from Normal Human Plasma

Isolation of 100KF was carried out by standard chromatographic methods as described previously (22). In brief, pooled normal human plasma from healthy donors was dialyzed against 0.01 M Tris-HCl buffer (0.045 M NaCl, pH 8.0) and run batchwise over a DE52 ion-exchange column (Pharmacia, Uppsala, Sweden), using subsequently 0.045, 0.080, and 1.000 M NaCl (in 0.01 M Tris-HCl buffer). Fractions were tested for their capacity to affect glomerular polyanion (GPA), i.e., 50-μl samples of desalted fractions (1.5 mg of protein per ml) were incubated for 60 min at 37°C with cryostat sections of normal rat kidneys. After washing, the sections were stained for GPA according to the colloidal iron method (see below). Concentrated and desalted fractions positive for GPA-affecting capacity (0.045 to 0.08 M) were dialyzed overnight to 100× volume of phosphate-buffered saline (PBS). The fractions were run over a Sephadex G150 SF column (Pharmacia), yielding three peaks that were assayed for their molecular weights using gradient polyacrylamide gel electrophoresis (PAGE; 4 to 30%; Isolab, Akron, OH), and again tested for GPA-affecting capacity. The positive fraction (estimated molecular weight, 70 to 100 kD) is referred to as 100KF. All isolation steps were carried out at 4°C using buffer solutions with pH 8.0.

Purification of Hx

Human plasma Hx was prepared using two different protocols. First by affinity chromatography using rabbit anti-hemopexin cross-linked to Sepharose (Pharmacia). Before the cross-linking, nonspecific antibody (i.e., anti-trypsin IgG) contaminating this commercially obtained anti-hemopexin antibody was removed using a Sepharose-transfer column (Sigma Chemical Co., St. Louis, MO). The affinity columns were carried out by the cyanogen bromide method according to standard procedures.

Thus, 100KF fractions were run over the anti-Hx affinity column and the bound proteins were eluted by 0.1 M citrate buffer (pH 2.5) and immediately neutralized with 1 M Tris/HCl to pH 8.0. After dialysis and concentration steps, the samples were tested on kidney tissues as described in the section, Histochemistry.

The second Hx isolation protocol was carried out according to Hrkal et al. (24) with minor modifications. In brief, the active fractions obtained from the DE52 ion exchange column as described above (see Preparation of 100KF from Normal Human Plasma) were run over a Blue Sepharose CL-6B column (Pharmacia) in 0.05M sodium phosphate buffer (flow rate 40.0 ml/h). After elution of the unbound proteins with 0.05 M phosphate (pH 7.1), a stepwise gradient was used consisting of 0.5 M sodium phosphate (pH 7.1), followed by elution with 1.5 M NaCl in 50 mM sodium phosphate (pH 7.1). The fractions eluted with 1.5 M NaCl (peak 3) consisted of highly purified Hx as shown by sodium dodecyl sulfate (SDS)-PAGE and Western blotting.

Internal Sequence Analysis of Protein

For partial sequence analysis, 100KF fractions were run over a polyacrylamide gel (4 to 30%) in the presence of SDS. The protein bands, showing positive staining in the Western blot with rabbit anti-100KF antibody (see below), have been cut out for further analysis (molecular weight approximately 100 kD). After trypsin digestion and preparative HPLC assay, the collected samples were tested for the amino acid sequence using the standard assay for Edman degradation (25). The highest peaks in the HPLC chromatogram derived from two individual 100KF fractions have been analyzed. The analysis revealed the sequence of 12 amino acids in both cases. The procedures for sequence analysis were carried out by Eurosequence (Groningen, The Netherlands).

Preparation of Rabbit Anti-100KF Antibodies

100KF fraction (0.5 mg proteins) was emulsified in 2.0 ml of complete Freund’s adjuvant (CFA) for the initial immunization, or incomplete Freund’s adjuvant for booster injection. Female chinchilla rabbits, 3 mo of age, were immunized intradermally in the shaved back skin with 2 ml of 100KF in CFA divided over six skin sites. Booster injections were given intramuscularly every month. According to the antibody titer, sera were harvested approximately 3 to 6 mo after initial immunization and precipitated in 50% ammonium sulfate. Subsequently, the precipitates were resolved, dialyzed against PBS (pH 7.4), and tested for specificity by dot-blot analysis. Positive fractions (see below) were collected, and the IgG immunoglobulins were isolated using a protein A column (BioRad Laboratories, Hercules, CA). This IgG fraction was tested again for specificity using dot-blot analysis.

Immunoabsorption Studies

Immune complexes were prepared by mixing 100KF and anti-Hx Ig in a ratio of approximately 3:1; this ratio appeared to be antigen-antibody equilibrium in the present condition, as tested previously. A solution of 5.0 ml containing 100KF (4.9 mg/ml) and rabbit anti-Hx antibody (Dako) (1.7 mg/ml) in PBS, pH 7.4, was gently stirred for 60 min at 4°C. The solution was subsequently run over a protein A column (BioRad; flow rate, 30.0 ml/h) to eliminate 100KF–anti-Hx complexes. The unbound fraction was collected and tested (in a concentration of 2.5 mg/ml) for its potential enzymatic activity following incubation with kidney sections. PBS and the 100KF complex solution (2.0 mg/ml) (100KF-Cx) fractions served as negative and positive control, respectively. After incubation, sections were processed as described under Histochemistry, and histochemical quantification was carried out as described under Quantification of Histochemical Results.

Immunoblotting Assays

For dot-blot analyses, BioBlot-Nitrocellulose membranes (Costar, Cambridge, Canada) and Bio-Dot apparatus (BioRad Laboratories) were used. 100KF as well as Hx samples (diluted in 0.02 M Tris/HCl, 0.5 M NaCl, pH 7.5; TBS) were applied on the membrane in a range of 0.05 to 25 μg/well. After an overnight blocking step (TBS supplemented with 1% bovine serum albumin) and several wash steps with TBS, mouse monoclonal anti-Hx antibody (26) (100 μl, 1:50 diluted), rabbit anti-100KF antibody (100 μl, 1:150 diluted), as well as the rabbit anti-Hx antibody (obtained from Dako A/S, Glostrup, Denmark) (100 μl, 1:2000 diluted) were applied. Also, unrelated rabbit antibody of the same isotype (i.e., anti-human trypsin IgG) was used to check for binding specificity (100 μl, 1:50). After washing thoroughly (0.05% Tween 20 in TBS), either peroxidase-conjugated rabbit-anti-mouse antibody (1:250; Dako) or peroxidase-conjugated goat-anti-rabbit antibody (1:500; Dako) was used as second step, and visualized by 4-chloro-1-naphthol (Aldrich-Chemical Company, Milwaukee, WI) according to standard methods.

Western Blotting

Hx, prepared according to Hrkal et al. (24) as well as after elution from the anti-Hx Sepharose column, or 100KF fraction was run over a gradient polyacrylamide gel (PAGE 4 to 30%; Isolab) in the presence of 0.1% SDS for 2 h at 150 V. [For comparison of staining pattern, two additional Hx samples kindly provided by Dr. Ann Smith...
(University of Missouri) and from the group of Dr. Muller-Eberhard (Cornell University New York) were also run on PAGE. Subsequently, the gel was blotted to BioBlot-nitrocellulose membrane (Costar, Cambridge, Canada) for 6 h at 100 V. After overnight blocking steps (TBS containing 1% bovine serum albumin; pH 7.5), the membrane was washed (TBS) and incubated with either polyclonal anti-100KF antibody (1:100) or the monoclonal anti-Hx antibody (1:100) for 1 h at 20°C. As a second step, either peroxidase-conjugated goat-anti-rabbit antibody (1:250; Dako) or peroxidase-conjugated goat-anti-mouse antibody (1:250; Dako) was used, followed by visualization with 4-chloro-1-naphthol (Aldrich-Chemical). As molecular weight determinant, a prestained marker (BioRad Laboratories) was also applied on the PAGE gel. For protein detection, blots were also stained using Ponceau S (Pharmacia).

**Histochemistry**

**Demonstration of GPA.** The presence of GPA was demonstrated by the colloidal iron method as described previously (27). Cryostat sections of normal rat kidneys (4 μm), fixed with alcohol/aceton (1:1, 30 min, −20°C), were incubated with 50 μl of either Hx solution (1.0 mg of protein per ml), 100KF (1.5 mg/ml), or PBS for 1 h at 37°C. After washing, the sections were allowed to incubate with acidified colloidal iron solution for 3 h at 20°C. The absorbed iron is subsequently visualized by conversion to ferric ferrocyanide using Perls’ solution (1% HCl and 1% K3Fe(CN)6, 20 min, 20°C). Blue reaction product represents polymers containing predominantly sialoglycoproteins.

**Demonstration of Glomerular ecto-ATPase.** Cryostat sections (4 μm) were fixed in aceton for 10 min at −20°C and followed by preincubation with test solutions as described in the previous section, i.e., rat kidney sections were incubated with either Hx (1.0 mg/ml), 100KF (1.5 mg/ml), or PBS for 1 h at 37°C. After this treatment, the sections were washed and processed for the histologic demonstration for ecto-ATPase according to standard methods (22), using a monoclonal mouse-anti-ATPase antibody (20 min, 1:150 diluted) and subsequently peroxidase-conjugated goat-anti-mouse antibody (30 min, 1:50 diluted, Pierce, Rockford, IL). Reaction products were visualized by 3-amin-9-ethyl-carbazole. All incubation steps of the staining procedures were carried out at 20°C.

**Quantification of Histochemical Results.** After incubation with the test solutions, glomerular reaction product of the kidney sections was quantitatively scored using computerized image analysis as described previously (28). Glomeruli were screened using a digitizing tablet (mm 1812, Summagraphics; Pro-Technology, Melbourne, Victoria, Australia), and the staining intensity of the reaction product as well as the area of the glomerulus were measured. Glomerular stain-to-tissue ratio, Australia), and the staining intensity of the reaction product as descre described previously (27). Cryostat sections of normal rat kidneys (4 μm), fixed with alcohol/aceton (1:1, 30 min, −20°C), were incubated with 50 μl of either Hx solution (1.0 mg of protein per ml), 100KF (1.5 mg/ml), or PBS for 1 h at 37°C. After washing, the sections were allowed to incubate with acidified colloidal iron solution for 3 h at 20°C. The absorbed iron is subsequently visualized by conversion to ferric ferrocyanide using Perls’ solution (1% HCl and 1% K3Fe(CN)6, 20 min, 20°C). Blue reaction product represents polymers containing predominantly sialoglycoproteins.

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**Statistics Analysis**

Statistical evaluation of data was carried out using either the paired Wilcoxon test (two-tailed) or the Mann–Whitney U test (paired; two-tailed) as indicated in the legends of Figures 5 and 6. P ≤ 0.05 was considered significant.

**Results**

**Amino Acid Sequence Determination**

After internal sequence analyses of the samples tested, the amino acid sequences SGAQATWTLPW and ALPQPQN-VTSL were determined. Comparison of these sequences in the protein library (Swiss-prot) revealed full homology of both fragments with the human plasma glycoprotein Hx. In Figure 1, the sequence of mature plasma Hx is shown. Regions of homology with the two partial 100KF sequences were underlined.

**Figure 1.** Amino acid sequence of human plasma hemopexin (Hx). The standard one-letter code for amino acids is used. Homologous amino acids as determined in 100KF fraction are underlined.
**Dot-Blot Analysis**

As depicted in Figure 2A, affinity-purified Hx (columns B, D, and F) showed a dose-dependent staining with the monoclonal anti-Hx antibody (column B; detection limit: 0.5 μg), polyclonal anti-100KF antibody (column D; detection limit: 0.05 μg), as well as the commercial polyclonal anti-Hx antibody (column F; detection limit ≤0.05 μg).

The staining of these antibodies for 100KF is shown in columns A, C, and E. 100KF is recognized by both polyclonal antibodies used (columns C and E). Our anti-100KF antibody (column C) stains 100KF with an optimal concentration at 5 μg (detection limit: 0.05 μg), whereas the commercially obtained polyclonal anti-Hx antibody (column E) shows an optimal concentration at 5 μg (detection limit: ≤0.05 μg). 100KF is also recognized by the monoclonal anti-Hx (column A), although to a lesser extent (detection limit: 0.5 to 5 μg). As can be seen in Figure 2B, 100KF (1) and Hx (2) show negative staining with anti-human trypsin antibodies. Identical results were obtained using Hx prepared according to Hrkal et al. (24) instead of Hx eluted from the Sepharose-anti-Hx column.

None of the control dot-blots, whereby human serum albumin, bovine serum albumin, or human IgG (all from Boehringer Ingelheim, Heidelberg, Germany), used as nonrelevant plasma proteins, showed positive staining up to 250 μg (not shown here).

**Western Blot Analysis**

The electrophoretic pattern of both Hx preparations as well as 100KF was detected by monoclonal anti-Hx antibody and polyclonal anti-100KF IgG (Figure 3). The left panel of Figure 3A shows several bands after staining for protein using Ponceau S, whereas in the right panel the fractions are stained with polyclonal anti-100KF antibodies. The 100KF sample (lane 2) and our Hx sample (lane 3) show bands at approximately 70 kD (arrow), whereas the Hx sample from Muller-Eberhard (26) shows an additional band of approximately 127 kD (lane 4, right panel). Figure 3B shows protein staining of two of our Hx preparations (lanes 2 and 3, left panel), as well as a control Hx sample, whereas in the middle panel staining with monoclonal antibody can be seen. Also here bands of approximately 70 kD are present in all of the Hx preparations tested, whereas again a 127-kD band fragment is seen in the control Hx sample (and to a lesser extent also in some of our original samples eluted from the Sepharose anti-Hx column [results not shown]). The right panel shows a Western blot of our Hx preparations (lane 2 and 3) stained with polyclonal anti-100KF IgG.

Although individual staining patterns as such may vary to a certain extent, in these Western blots the predominant bands reflect an identical molecular mass (i.e., approximately 70 kD) for both Hx and 100KF (Figure 3B, middle and right panel, versus Figure 3A, right panel, lane 2).

**Histochemistry**

The histochemical stainability for GPA as well as for glomerular ecto-ATPase following contact with 100KF, Hx, or PBS is demonstrated in Figures 4 and 5. As can be seen from the histochemical staining depicted in Figure 4, in vitro incubation with either 100KF (Figure 4, B and E) or Hx (Figure 4, C and F) resulted in a clear reduction of stainability for both GPA (the top set of micrographs) as well as glomerular ecto-ATPase (the bottom set), compared to sections treated with PBS (Figure 4, A and D).

Quantification of the reaction product following staining for GPA after incubation of kidney sections with either Hx, 100KF, or PBS is shown in Figure 5A. It can be seen that the mean amount of reaction product was reduced significantly after incubation with either 100KF (hatched columns) or Hx (solid column) compared to incubation with PBS (open col-
In Figure 5B, data of the stainability for glomerular ecto-ATPase were summarized. It is shown that the expression of this glomerular ectoenzyme was significantly reduced following incubation with 100KF (hatched columns) or Hx (closed column) compared to PBS (open column). (100KF: 15.30 ± 6.5 versus control: 38.66 ± 4.2, P ≤ 0.01; Hx: 13.65 ± 12.5 versus control: 38.66 ± 4.2, P ≤ 0.01).

Immunoabsorption

Figure 6 shows stainability for glomerular ecto-ATPase after incubation of kidney sections with PBS (open column), unbound fraction from the protein A column (solid column), and a fraction consisting of 100KF-anti-Hx complexes (hatched column). It can be seen that although the expression of glomerular ecto-ATPase is significantly decreased after incubation with 100KF, compared to PBS (P ≤ 0.01), no significant alteration is seen after contact with the unbound fraction (solid column). Unbound fractions obtained from experiments with 100KF-anti-Hx complexes prepared with antigen excess showed similar activity compared to 100KF alone (results not shown).

Urinary Protein Leakage

During kidney perfusion with diluted rat serum, perfusion with Hx, or heat-inactivated Hx, there was no significant difference in the volumes of the urine collected (mean urinary volume ± SD for Hx: 104.5 ± 7.4 μl/min versus 98.6 ± 6.5 μl/min for HI-Hx; NS). As can be seen from Figure 7, following alternate kidney perfusion with Hx and diluted rat serum (solid column), a significant increase of protein can be detected in the urine samples (210.65 ± 49.79 μg/min) compared to animals perfused with the control factor (open column), i.e., heat-inactivated Hx (112.26 ± 49.18 μg/min; P ≤ 0.01).

Discussion

The aim of the present study was to investigate the hypothesis that the human plasma factor 100KF is similar or identical to plasma Hx, or an isoform of this molecule. Therefore, we have performed sequence studies comparing amino acid sequences in 100KF (AQATWTELPW and ALPQPQNVTSLL), using the Swiss-prot library (Figure 1). In contrast to various enzymes containing so-called Hx-like domains, e.g., various metalloproteinases and extracellular matrix molecules like vitronectin (30,31), only in plasma Hx can a full homology be shown for the tested sequences of 100KF. Therefore, we feel that 100KF should be associated with plasma Hx rather than with collagenases, gelatinases, metalloproteinases, and the like.

Comparison of 100KF with isolated plasma Hx in blotting assays reveals striking similarities between 100KF and Hx as well (Figures 2 and 3). Thus, in dot-blot assays both 100KF and Hx are recognized by monoclonal and polyclonal anti-Hx antibodies in a similar way. Because nonrelevant antibody of the same isotype did not stain 100KF or Hx in this assay (Figure 2B), it is unlikely that staining by the relevant anti-
bodies used is due to nonspecific stickiness of IgG to the antigen \textit{per se}. These findings could be confirmed by Western blotting showing the recognition of different Hx preparations as well as 100KF using both monoclonal and polyclonal anti-Hx antibodies. As can be deduced from the Western blots, the estimated molecular weight of Hx equals that of 100KF, whereas 70 kD and higher may fit well within the range of molecular weights described in the literature. Thus, probably due to the extent of glycosylation or formation of dimers (32,33), molecular weight estimations of Hx may vary from 55 kD to more than 80 kD (26). Whether the 127-kD band occurring in some of our preparations as well as in those of others may represent a dimeric form of Hx remains to be settled.

Whatever the outcome may be, it is clear that our active 100KF moiety is recognized by monoclonal and polyclonal anti-Hx antibodies, whereas various Hx preparations are recognized by anti-100KF antibody in a similar pattern suggesting a very close relationship between these substances.

A functional similarity between 100KF and Hx deals with the potential of both fractions to affect glomerular extracellular matrix molecules (Figures 4 and 5). It has been shown that 100KF is able to cause glomerular alterations after incubation with human or rat kidney tissue, \textit{i.e.}, affection of glomerular ecto-ATPase and glomerular sialoglycoproteins (22), as well as glomerular heparan sulfate proteoglycans. Because this activity of 100KF can be inhibited by heat inactivation or by preincubation of 100KF with serine protease inhibitors (for instance phenylmethylsulfonyl fluoride), but not by broad-spectrum metalloproteinase inhibitors, it was suggested that a serine protease is involved as active moiety of 100KF.

To exclude the possibility that 100KF contains a moiety different from Hx, which might be responsible for the enzymatic activity, absorption studies with 100KF-anti-Hx complexes precipitated with protein A were done. As shown in Figure 6, the unbound fraction was completely inactive compared to native 100KF complexes, supporting the notion that the protease activity observed is associated with Hx.

If 100KF is closely related or identical to Hx, the question arises whether plasma Hx \textit{per se}, or after stimulation, is able to...
show enzymatic activity. Until now, protease activity in native human plasma Hx has not been documented.

However, the behavior of plasma Hx in particular tissue microenvironments is unknown, and it is conceivable therefore that under certain pathologic conditions, “activation” of Hx may occur. Indeed, in healthy subjects, various plasma serine proteases, for instance complement components or coagulation factors, are present in the circulation in a nonactive (i.e., zymogenic) form. Such zymogens can be activated after certain stimuli (for instance factor XII becomes factor XIIa, i.e., activated Hageman factor). Even isolation procedures as such may be able to stimulate certain zymogens to their activated form.

With respect to the putative enzymatic nature of plasma Hx, one report has been published in which Hx is associated with hyaluronidase via a complete sequence homology (34). This observation awaits further confirmation.

Figure 7 shows that after perfusion of Hx into the rat kidney followed by diluted plasma, increased leakage of plasma proteins occurs, as is the case after 100KF infusion. This also points to similar functional activity of both fractions upon the glomerular filtration barrier (23). Indeed, in this model glomerular alterations observed after either Hx or 100KF perfusion were identical, i.e., loss of GPA as well as glomerular...
References

Acknowledgments

This study was supported by the Dutch Kidney Foundation (Grant C94-1422). We are grateful to Dr. Ann Smith (Department of Molecular Biology and Biochemistry, University of Missouri), who supplied hemopexin fractions for comparison.

References


