Determination of the Bifunctional Properties of High Molecular Weight Kininogen by Studies with Monoclonal Antibodies Directed to Each of Its Chains*

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In normal human plasma two forms of kininogen exist, low molecular weight kininogen (LMWK) and high molecular weight kininogen (HMWK). When these proteins are cleaved they are found to have a common heavy chain and bradykinin, but each has a unique light chain. Monoclonal antibodies to the heavy and light chains of HMWK have been developed, and the effects of each on the function of this protein are defined. Initial studies showed that an antibody, C11C1, completely neutralized the coagulant activity of plasma HMWK whereas another antibody, 2B5, did not. On a competitive enzyme-linked immunosorbent assay (CELISA) the C11C1 antibody was consumed by kininogen antigen in normal plasma but not by kininogen antigen in HMWK-deficient plasma. On immunoblot, the C11C1 antibody recognized one kininogen protein in normal plasma and did not recognize any kininogen antigen in HMWK-deficient plasma. These combined studies indicated that the C11C1 antibody was directed to an epitope on the unique 46-kDa light chain of HMWK. In contrast, the 2B5 antibody on a CELISA was consumed by kininogen antigen in both normal plasma and HMWK-deficient plasma but not by total kininogen-deficient plasma. On immunoblot, the 2B5 antibody recognized both kininogens in normal plasma but only LMWK in HMWK-deficient plasma. These combined studies indicated that the 2B5 antibody was directed to the common 64-kDa heavy chain of the plasma kininogens. Utilizing direct binding studies or competition kinetic experiments, the 2B5 and C11C1 antibodies bound with high affinity (1.71 and 0.77 nM, respectively) to their antigenic determinants on the HMWK molecule. The 2B5 antibody did neutralize the ability of HMWK to inhibit platelet calpain. These studies with monoclonal antibodies directed to each of the HMWK chains indicate that HMWK is a bifunctional molecule that can serve as a cofactor for serine zymogen activation and an inhibitor of cysteine proteases.

Recent studies indicate that high molecular weight kininogen (HMWK) is a bifunctional molecule. Kallikrein cleaves HMWK to release the nonpeptide bradykinin and expose a 56-kDa light chain on the carboxyl-terminal portion of the molecule that has the ability to shorten the coagulant activity of plasma (1) and to act as a cofactor in the activation of the serine zymogens factor XII, prekallikrein, and factor XI (2) by promoting the binding of the latter two proteins to activating surfaces (3). In addition, HMWK contains a 64-kDa domain on the NH2-terminal portion of the molecule which has the ability to inhibit cysteine proteases (4–6), including platelet calpain (7). The heavy chain of HMWK is identical in amino acid structure to the heavy chain of low molecular weight kininogen (LMWK), since the syntheses of the plasma kininogens are controlled by one gene (8, 9). The difference between HMWK and LMWK is differential splicing to produce two forms of mRNA which are translated to yield a 56-kDa light chain in HMWK and a 4-kDa light chain in LMWK (8, 9). To further probe the structure-function relationship of HMWK in complex systems such as plasma or cells, we sought to produce monoclonal antibodies to different regions of this protein. This report characterizes two monoclonal antibodies which recognize epitopes on each functional site of HMWKs.

MATERIALS AND METHODS

The 2B5 and C11C1 antibodies were each found to be IgG1 subtype, light chain. Purified C11C1 antibody neutralized the coagulant activity of plasma HMWK as a function of concentration, and at high concentration the inhibition was complete (data not shown). The purified 2B5 antibody at similar concentrations did not inhibit HMWK coagulant activity. These findings indicated that the C11C1 antibody was directed to a region at or near the light chain of HMWK, since that portion of the molecule is involved with coagulant activity. Since the 2B5 antibody did not inhibit clotting, it

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1 The abbreviations used are: HMWK, high molecular weight kininogen; LMWK, low molecular weight kininogen; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; CELISA, competitive enzyme-linked immunosorbent assay; ELISA, enzyme-linked immunosorbent assay.

2 Portions of this manuscript (including "Materials and Methods") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry; 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-1480, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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must be directed to the heavy chain of HMWK or to an epitope on the light chain of HMWK not involved with coagulant activity. Immunochemical studies were performed to determine more definitively which chain of HMWK these antibodies were against.

**Immunochemical Studies with the 2B5 Antibody**—On a CELISA, the 2B5 antibody was consumed by kininogen antigen present in both normal plasma and the variant HMWK-deficient plasma, Fitzgerald plasma (data not shown). Little consumption of the 2B5 antibody occurred with dilutions of total kininogen-deficient (Williams) plasma. Since Fitzgerald plasma does not contain HMWK but does contain 0.98 µM LMWK, the 2B5 antibody appeared to be directed to the heavy chain of the plasma kininogens. This assessment was further evaluated by immunoblot studies on these proteins in plasma. In preliminary studies, it was found that the 2B5 antibody was unable to recognize kininogen antigen that had been treated with SDS even after preparation of blots on nitrocellulose paper. An immunoblot was performed on protein that was electrophoresed on 5.5% alkaline PAGE in the absence of deagents. As shown in Fig. 1, purified HMWK when stained with Coomassie Blue migrated mostly as a single band on this system. Purified HMWK on immunoblot was also mostly a single band with a smear of higher molecular weight aggregates (35) at the top of the gel. The immunoblot of kininogen antigen in normal plasma resulted in two bands: one of lower amount with an identical migration to purified HMWK and another at a larger amount that migrated further into the gel. The immunoblot of HMWK-deficient plasma (Fitzgerald plasma) was a single band similar to the lower but larger band in normal plasma and below the bands of purified HMWK. This band represents LMWK since this protein is the only form of kininogen in Fitzgerald plasma. These experiments indicated that the 2B5 antibody was directed toward LMWK and the heavy chain of HMWK, the kininogen antigen common in both normal and Fitzgerald plasmas.

The specificity of the 2B5 antibody for the heavy chain of the kininogens was further determined on affinity chromatography experiments with purified proteins. Kallikrein-cleared HMWK was applied to an affinity column which had purified 2B5 antibody attached. This preparation of kallikrein-cleared HMWK after reduction and alkylation on 8% SDS-PAGE consisted of three bands at 64, 56, and 46 kDa (data not shown). When applied to the affinity column, the unbound protein, which contained all the coagulant activity, consisted of two bands at 56 and 46 kDa (Fig. 2), representing the light chain of HMWK and its proteolytic derivative. Alternatively, protein bound to the column and eluted with potassium thiocyanate was a single band at 64 kDa (Fig. 2), representing the heavy chain of HMWK. This study confirmed that the 2B5 antibody recognized only the heavy chain of HMWK and was directed to an epitope on this chain not altered by reduction and alkylation.

**Immunochemical Studies with the C11C1 Antibody**—On a CELISA, the C11C1 antibody was consumed by kininogen antigen in normal plasma but not by kininogen antigen in HMWK-deficient (Fitzgerald) plasma (data not shown). Since Fitzgerald plasma does not contain HMWK and the C11C1 antibody neutralized the coagulant activity of normal plasma, this antibody appeared to be directed to the light chain of HMWK, which consists of three bands at 64, 56, and 46 kDa (Fig. 2), representing the light chain of HMWK and its proteolytic derivative. Alternatively, protein bound to the column and eluted with potassium thiocyanate was a single band at 64 kDa (Fig. 2), representing the heavy chain of HMWK. This study confirmed that the 2B5 antibody recognized only the heavy chain of HMWK and was directed to an epitope on this chain not altered by reduction and alkylation.
chain of HMWK. This assessment was further evaluated by immunoblot. Like the 2B5 antibody, the C11C1 antibody was unable to recognize kininogen antigen that had been treated with SDS. In Fig. 3 purified HMWK stained with Coomassie Blue or on immunoblot using the C11C1 antibody was mostly a single band on alkaline PAGE. Immunoblot of normal human plasma with the C11C1 antibody resulted in the detection of a single band of protein with a migration similar to purified HMWK (Fig. 3). No kininogen antigen was detected on immunoblot using the C11C1 antibody on HMWK-deficient (Fitzgerald) plasma (Fig. 3). These plasma studies confirmed that the C11C1 antibody was directed to an epitope on the light chain of HMWK.

Further characterization of the specificity of the C11C1 antibody was determined by affinity purification of partially purified HMWK. A resuspended 45% ammonium sulfate precipitate of an eluate of a normal plasma-loaded QAE-gel was applied to a C11C1 affinity column (Fig. 4A). In this experiment the plasma was not pretreated with diisopropyl fluorophosphate. After washing the column the eluted protein, which hadHMWK coagulant activity, consisted of three major bands at 120, 64-56, and 46 kDa on reduced SDS-PAGE, consistent with both intact and cleaved HMWK (Fig. 4A). Since the middle band on this gel was wide and might be interpreted as only the 64-kDa heavy chain of HMWK, further immunoblot studies were performed to determine whether the band seen contained the 56-kDa light chain of HMWK. The protein eluted from the C11C1 affinity column was run on 8% SDS-PAGE and electroblotted onto nitrocellulose paper. An immunoblot using a polyclonal anti-light chain of HMWK antiserum from an 8% SDS-PAGE stained with Coomassie Blue R-250, as described in the legend to Fig. 1, of a 45% ammonium sulfate precipitate of an eluate of a normal plasma-loaded QAE-gel (AS) and a potassium thiocyanate eluate of the protein bound to a C11C1 affinity column (BOUND HMWK). The numbers to the left of the gels represent mass standards in kilodaltons. B is an immunoblot of the protein eluted with potassium thiocyanate (HMWK) using polyclonal anti-human light chain of HMWK antiserum from an 8% SDS-PAGE. The numbers to the right of B represent mass standards in kilodaltons.

Affinity Constants of the Antibodies—The affinity of each of the monoclonal antibodies to bind to their antigenic determinants on HMWK was determined. An $^{125}$I-2B5 antibody was used to determine the affinity constant of this antibody for the heavy chain of HMWK by direct binding experiments (Fig. 5). In these studies, nonspecific binding in the presence of a 50-fold molar excess unlabeled antibody was less than 4%, and binding of the $^{125}$I-2B5 antibody to HMWK was saturable when 9.4 nM $^{125}$I-2B5 was added. As indicated by the slope of the plot of bound/free versus bound, the $^{125}$I-2B5 antibody bound to the heavy chain of HMWK with an apparent $K_d$ of 1.7 nM (Fig. 5). This finding was confirmed by analysis of a competition inhibition experiment with normal plasma on the CELISA using the 2B5 antibody. After determining the IC50 for total kininogen antigen by the 2B5 antibody (33), the calculated apparent dissociation constant (34) of the 2B5 antibody for HMWK was 1.9 nM.

The affinity constant of the C11C1 antibody binding to the light chain of HMWK was determined by coagulant assay (Fig. 6). Increasing concentrations of purified C11C1 antibody from 84 to 168 nM inhibited the coagulant activity of normal plasma. On a secondary plot of the slope of the reciprocal plot versus the antibody concentration (Fig. 6, inset) the mean apparent $K_i$ of the C11C1 antibody to inhibit plasma HMWK coagulant activity was 0.77 nM (12 to 0.3 nM, using ± S.D. of
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FIG. 5. 2B5 affinity constant for the heavy chain of HMWK. 

\[ \frac{B}{F} = \frac{K_d}{K_d} \]  

Mean values to calculate \( K_d \). Since the variation of the apparent \( K_d \) was large using a coagulant assay, the affinity constant was also calculated from competition inhibition experiments with normal plasma on the CELISA. After determining the \( IC_{50} \) for plasma HMWK antigen by the C11C1 antibody (33), the mean apparent dissociation constant from two experiments (34) with the C11C1 antibody was 2.1 nM.

Influence of the Monoclonal Antibodies on the Functional Activities of HMWK—Studies were performed to determine how each of the monoclonal antibodies altered the functional activities of HMWK. Initial investigations sought to determine whether both chains of HMWK can contribute to the rate and extent of generation of plasma HMWK procoagulant activity. The extent and rate of plasma HMWK procoagulant activity in the absence or presence of each monoclonal antibody was evaluated when the plasma was incubated for variable times with a negatively charged artificial surface (Fig. 7). The maximal amount of HMWK procoagulant activity in normal plasma was detected when the plasma was incubated for 9 min with the surface. Preincubation of the plasma with the C11C1 antibody limited the extent of HMWK activity measured but not its rate. Preincubation of the plasma with the 2B5 antibody neither inhibited the extent nor the rate of generation of HMWK procoagulant activity. These findings indicated that the heavy chain of HMWK did not influence the procoagulant activity of HMWK.

Further studies were performed to determine whether either of the monoclonal antibodies to HMWK influenced the ability of HMWK to inhibit the proteolytic activity of platelet calpain. Using purified HMWK at a predetermined concentration that inhibited 65% of the activity of purified platelet calpain, a preincubation of the HMWK with a 20-fold molar excess of purified murine IgG, a light chain antibody or C11C1 antibody did not alter HMWK's ability to inhibit platelet calpain (data not shown). However, when HMWK was preincubated with a 20-fold molar excess of 2B5 antibody (10 μM), the 2B5-treated HMWK produced less (47% ± 5.6 (mean ± S.E.)) inhibition of platelet calpain activity (Fig. 8).
The ability of the 2B5 antibody to neutralize the inhibitory activity of HMWK on platelet calpain was studied in a concentration-dependent manner (Fig. 8). When the HMWK was preincubated with a 50-100-fold molar excess of purified 2B5 antibody (25 and 50 μM, respectively), these 2B5-treated aliquots of HMWK produced 31% ± 5.1 and <1%, respectively, inhibition of platelet calpain activity. This concentration-dependent reversal of the inhibition of platelet calpain by HMWK suggested that this antibody may be directed to a site on the heavy chain of HMWK near the portion of the HMWK molecule involved in the inhibition of platelet calpain.

**DISCUSSION**

Using two different monoclonal antibodies, each directed to a different chain of HMWK, we are able to alter HMWK's procoagulant activity or inhibitory activity on platelet calpain. These findings provide further evidence for the bifunctional nature of HMWK. The ability of normal plasma but not HMWK-deficient plasma to consume the C11C1 antibody on a CELISA and the ability of the C11C1 antibody to recognize only one of the two forms of plasma kininogen in normal plasma but none in HMWK-deficient plasma (Fig. 3) indicates that the C11C1 antibody is directed to the light chain of HMWK, the unique antigenic portion of HMWK that distinguishes it from LMWK. The evidence that the C11C1 antibody neutralized the procoagulant activity of HMWK (Figs. 6 and 7) and recognized both the 56-kDa intermediate light chain and its 46-kDa proteolytic derivative (Fig. 4) indicates that the epitope recognized is in the latter polypeptide.

Since the expression of HMWK procoagulant activity relies on two properties of the 46-kDa light chain, its ability to bind to activating surfaces (3) and its ability to complex with the contact zymogens, prekallikrein and factor XI (36-38), further studies should allow definition of the amino acid sequence defining this epitope.

The absence of inhibition of coagulant activity by the 2B5 antibody (Fig. 7) indicated that the 2B5 antibody may be directed toward the light chain of HMWK. However, since HMWK has a 10-kDa N-terminal peptide on its 56-kDa light chain which does not contribute to the light chain's procoagulant activity, the 2B5 antibody could have also been directed to this portion of the light chain of HMWK. Immunochromatography studies indicate that the 2B5 antibody is directed to the heavy chain of HMWK. The finding that the 2B5 antibody on a CELISA was consumed by kininogen antigen in both normal and HMWK-deficient plasma indicates that it must be directed to the kininogen antigenic determinant present in both of these plasmas, i.e. the heavy chain of HMWK or LMWK. Immunoblot on plasmas and affinity chromatography on purified cleaved HMWK confirm that the 2B5 antibody is directed to the heavy chain of the kininogen molecules since it only recognizes the 64-kDa heavy chain of HMWK in plasmas or purified alkylated and reduced kinogen (Figs. 1 and 2). The 2B5 antibody did neutralize the ability of HMWK to inhibit platelet calpain, the functional activity of the heavy chain of HMWK (Fig. 8). Further studies are needed to determine if the Fab derivative of the 2B5 antibody inhibits HMWK's ability to neutralize platelet calpain in order to define more precisely the epitope on the heavy chain of HMWK that this antibody is directed toward.

The functional properties of the light and heavy chains of HMWK may not be entirely separate. Although the studies with the 2B5 monoclonal antibody indicate that the heavy chain of HMWK contributes nothing to the extent and rate of generation of plasma HMWK procoagulant activity (Fig. 7), the light chain of HMWK may contribute to the inhibition of platelet calpain. In previous studies we showed that purified 56-kDa light chain alone could not inhibit platelet calpain (7). However, HMWK, either as a purified protein or in plasma, was a 3-6-fold better inhibitor of platelet calpain than purified heavy chain of HMWK or plasma LMWK (7). Alternatively, the interaction of HMWK with platelet calpain may contribute to the procoagulant activity of HMWK. In other studies we have shown that when calpain in platelet cytosol is inhibited by a molar excess of purified HMWK, some alteration in the purified HMWK allows it to develop increased procoagulant activity (15). This finding suggests that in the process of inhibiting platelet calpain, HMWK becomes an activated cofactor for serine zymogen activation. Co-localization of the cofactor activity of HMWK for serine zymogen activation and inhibitory activity of HMWK on cysteine protease activity may occur in vivo on cell membranes since HMWK is contained within platelet granules (11, 15) and HMWK binds to the surface of platelets (39) by two sites on its molecule, one on the heavy chain and another on its light chain (40). In conclusion these studies using two novel monoclonal antibodies to each chain of HMWK extend the notion that this protein is a bifunctional molecule for modulation of plasma serine proteases and inhibition of tissue cysteine proteases. These two monoclonal antibodies will be useful probes to further map the functional domains of each chain of the HMWK molecule.

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Immunohematology: Competition inhibition assays using normal or variant deficient plasma and purified monoclonal antibodies were performed as previously reported 

Production of Monoclonal Antibodies: Monoclonal antibodies to HMWK were produced by modifications of the procedure of Kohler and Milstein (27). The inoculation procedure for production of each of the monoclonal antibodies differed. Four male BALB/c nude mice, 6 weeks old (Tangle University) Skin and Cancer Hospital) were immunized intraperitoneally with 100 µl of sterile HMWK/mouse in complete Freund's adjuvant (week 0) and then again intraperitoneally with 200 microliters of protein/mouse on week one. On week 2 a third 100 µg injection in 6,4 ml incomplete Freund's adjuvant was administered intraperitoneally. Cells from the hardiest clone were grown in culture and the strongest positive clones were injected intraperitoneally with 200 µg/mouse on week one. Antigens differed. Producing hybridomas and selecting clones were made identical to those of Dreu. A modified procedure of Macek et al (28) was used. The survival of the clones was followed in culture and the supernatants were directly added to cuvette wells linked with purified HMWK.

Another four male or female BALB/c AnSkh mice, 8-10 weeks old were immunized subcutaneously with 50 µg HMWK/mouse in complete Freund's adjuvant (week 0) and then again subcutaneously with 50 µg HMWK/mouse in incomplete Freund's adjuvant at week 5. At week 11, 50 µg/mouse in 0.15 M sodium chloride were intraperitoneally injected. Four days later, two mice were selected at spleen donors and their spleenocytes were fused with Sp2/0-Ag14 myeloma cells. Supernatants and purified monoclonal antibodies were produced by limiting dilution (29). Cells from the best clones were grown in culture and the supernatants were harvested. This culture supernatant was designated DCS1.

Affinity Chromatography: Approximately 14 µg of purified DCS1 antibody was bound to 5 ml of Affi-Gel 10 according to the manufacturer's recommendations in 0.1 M HAc-C1, 0.1 M NaCl pH 7.4. Bound protein was eluted by 3 M potassium thiocyanate in the same buffer.

Determination of affinity constants: The affinity constants of each of the purified monoclonal antibodies for their antigenic determinants on HMWK were approached by two techniques. Purified 285 antibody was radiolabeled with 125I using iodogen by a modification (30) of the technique of Frazer and Speck (31). The affinity constant of 125I-285 binding to HMWK was determined by direct binding experiments. Two hundred µl of purified HMWK at an initial concentration of 2 molar was linked to individual Immunomax cuvette wells at 4°C for 20-30 min. Cells from the hardiest clone were grown in culture and the supernatants were directly added to cuvette wells linked with purified HMWK. The affinity constant of 125I-285 binding to HMWK was determined from the slope of a plot of bound/free versus the amount bound according to the analysis of Songkram (32).

Another four male or female BALB/c AnSkh mice, 8-10 weeks old were injected intraperitoneally with 200 µg/mouse on week one. After washing the cultures with PBS-Tween, 1-285 in concentrations from 0.031 to 25 µg/mouse were added to individual Immunomax cuvette wells in triplicate in the absence or presence of a 50-fold molar excess of unlabeled 285 antibody. After incubation for 2 h at 37°C, each well was washed twice with PBS-Tween and then treated with a 1/50 dilution of peroxidase conjugated (PO-285). The peroxidase binding determined from the amount of 125I-285 bound in the presence of a 50-fold molar excess of unlabeled 285 from the bound and free was determined from the slope of a plot of bound/free versus the amount bound according to the analysis of Songkram (32).

The apparent affinity constant of the 285 antibody to bind to HMWK was determined from the slope of a plot of bound/free versus the amount bound according to the analysis of Songkram (32). The apparent affinity constant of the 285 antibody to bind to HMWK was determined from the slope of a plot of bound/free versus the amount bound according to the analysis of Songkram (32).