BOVINE RENAL GLOMERULAR BASEMENT MEMBRANE. ASSESSMENT OF PROTEOLYSIS DURING ISOLATION

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Summary. Existing methodology was modified to permit the rapid isolation of glomerular basement membrane at 0-3° with the use of specific inhibitors to continuously bathe the tissue during homogenization and fractionations. Membrane was prepared at 0-3° with inhibitors and at 0-3° and 27° without inhibitors. Analysis of these preparations by sodium dodecyl sulfate electrophoresis revealed identical polypeptide compositions except that the relative amount of the 55,000 dalton component observed at 0-3° increased with the inclusion of diisopropylfluorophosphate and decreased at 27° without inhibitors. These results show that some enzymatic degradation occurs during isolation in the absence of proteolytic inhibitors but suggest that the multiple polypeptide components are not produced during isolation.

Introduction

The glomerular basement membrane is considered to function as the sole or coarse filter which restricts the passage of proteins into the glomerular ultrafiltrate during purification of blood in the kidney (for reviews see refs 1, 2). It consists of a heterogeneous group of polypeptides ranging in molecular weight from 30,000 to greater than 700,000, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (3, 4). One of these polypeptides was recently isolated and shown to consist of both carbohydrate units of the whole membrane and low amounts of the amino acids characteristic of collagen (5). The presence of components more closely related to collagen have been identified in material solubilized on limited digestion of the membrane with pepsin (6, 7). The relationship of these components to those identified by SDS gel electrophoresis remains undefined.

Recently Grant et al. (8) concluded from biosynthetic studies that the

*Abbreviations used: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid: ε-ACA, ε-amino-caproic acid; NEM, N-ethylmaleimide; DFP, diisopropylfluorophosphate; GBM, glomerular basement membrane.

heterogeneous group of polypeptides, revealed by SDS gel electrophoresis, are not direct gene products nor do they arise from the assembly of lower molecular weight subunits, but possibly are generated artifactually by limited proteolysis during membrane isolation. Degradation of membrane by tissue-associated proteases is possible particularly since isolation procedures have not included rigorous steps to prevent or minimize proteolysis. This question of proteolysis is of utmost concern to those investigators involved in determining membrane structure, chemical alteration induced by disease, and nature of the interaction of vascular basement membrane with platelets on tissue injury. Therefore, the present report concerns an assessment as to whether the various polypeptide components are generated artifactually during isolation. This was accomplished by comparing the polypeptide composition of membrane prepared with proteolytic inhibitors at 0-3° and without inhibitors at 0-3° and 27°.

Materials and Methods

Materials. Bovine kidneys were obtained from a local abattoir and frozen with powdered dry ice within ten minutes after death. All electrophoretic chemicals were obtained from Bio-Rad. DFP, NEM, E-ACA, and EDTA were obtained from Sigma. All other chemicals were reagent grade or highest purity available.

Preparation of membrane. The isolation procedure was based on a combination of the method of Krakower and Greenspon (9) as modified by Spiro (10) and the method of Daniels and Chu (7). The cortex from 3-4 kidneys was shaved into ice-cold 0.85% NaCl ± proteolytic inhibitors, passed thrice through an automatic meat grinder, diluted to a total volume of 4ℓ with ice-cold 0.85% NaCl ± inhibitors, and mechanically homogenized (Brinkman Polytron PT10, setting 4) for two minutes with constant vigorous stirring. Glomeruli were separated from the homogenate by sieving techniques (10). The glomeruli were resuspended in ice-cold 1 M NaCl ± inhibitors in a 125 ml stainless steel beaker kept on ice. The suspension was sonicated for approximately two minutes at power setting 6 (Branson Sonifier, ½ inch probe) during which the temperature was monitored and maintained below 8°C. This suspension was then passed through a cold 200-mesh stainless steel sieve to remove DNA, and any tissue fragment contamination, which fortuitously are resistant to sonication. The membrane was then sedimented by centrifugation at 4° by applying 521 x g for 1 min. The sediment was washed 3X with ice-cold 1.0 M NaCl ± inhibitors by resuspending sedimenting at 521 x g for 10 min, and discarding supernatant. Finally, the NaCl was washed out using the same conditions above only using ice-cold distilled water. This final product was lyophilized to dryness and stored at -20°C.

Analyses. SDS polyacrylamide gel electrophoresis was performed as described previously by Hudson and Spiro (3) on 9 cm 5% and 7½% gels containing 0.1% sodium dodecyl sulfate. Staining and destaining was performed according to the method of Burgess and Jendrisak (11). The solubility of GBM was determined

as previously described (3) except protein was determined by the method of Geiger and Bessman (12).

Results and Discussion

Proteolysis during membrane isolation can be minimized or prevented by using a rapid procedure, low temperature, and specific enzyme inhibitors. Mammalian collagenases (13) and other metal-requiring proteases can be inhibited by EDTA. All the serine-proteases, such as plasmin, thrombin and elastase, can be inhibited by DFP (14) and the sulfhydryl-proteases can be inhibited by NEM. Also, the conversion of blood plasminogen to active plasmin that can occur in kidney tissue by the action of urokinase (15) and cytokinase (16) can be inhibited by using ε-ACA.

In order to isolate membrane under these conditions, the procedure recently devised by Daniels and Chu (7) was used with modification for the rapid isolation of glomeruli. Only cortical tissue was used, temperature was maintained at 0-3°, and solutions of proteolytic inhibitors were used to continuously bathe the tissue during homogenization and separation of released glomeruli from other tissue elements. The membrane was then isolated from glomeruli essentially as described by Spiro (10) except the temperature was maintained below 8°C, solutions of inhibitors were used during sonication and washing, and sonicated glomeruli were passed through a 200-mesh sieve to remove undisrupted glomeruli and contaminating tissue fragments and DNA.

In order to determine the effect of using these inhibitors and low temperature on GBM polypeptide composition, it was necessary to first examine the effect of sonication time and centrifugal force. These factors have been shown to affect the amino acid composition (17) and lipid content (18) of human GBM. The electrophoretic patterns of membrane prepared at 0-3° using increasing sonication time and increasing centrifugal force are shown in Fig. 1. There are essentially no differences in the electrophoretic patterns obtained for membrane prepared with sonication times of 2,4, and 8 minutes (gels A, B, and C, respectively) or with centrifugal forces of 521, 4680, and 13,023 x g (gels

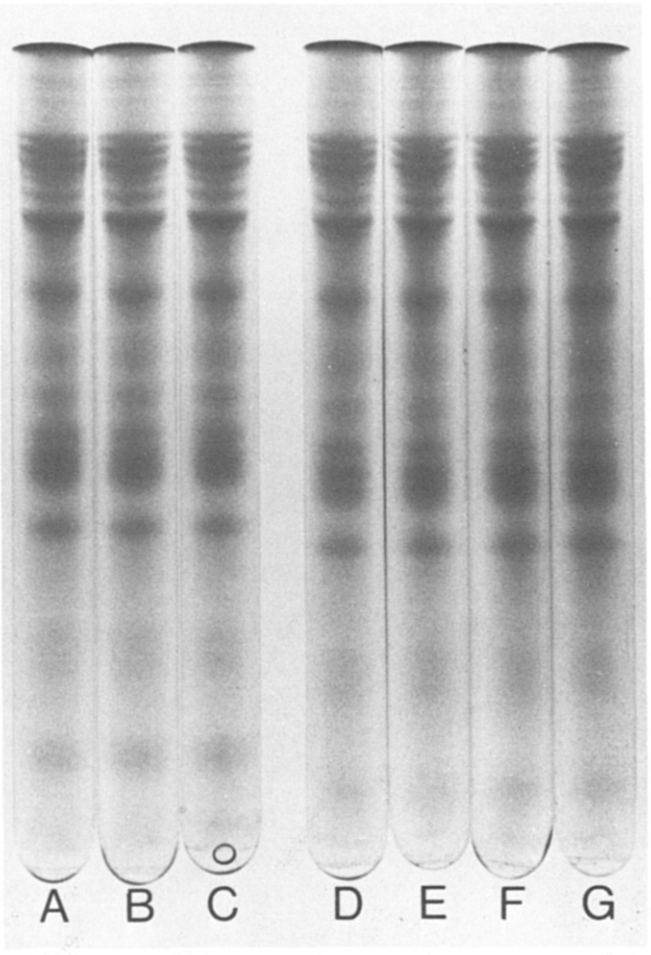

Figure 1. Effect of sonication time and centrifugal force on GBM polypeptide composition as determined by SDS gel electrophoresis. Gels A, B, and C are for membrane sonicated at 2,4, and 8 minutes, respectively, and centrifuged at 521 x g. Gels D, E, F, are for membrane centrifuged at 521 x g, 4680 x g, 13,023 x g, respectively, and sonicated for 2 minutes. Gel G is for membrane left in supernatant solution after centrifugation at 521 x g (gel D) which was recovered by further centrifuging at 13,023 x g. Electrophoresis was performed with 5% acrylamide gels.

D, E, F, respectively). The identical nature of these preparations is further substantiated by gel G which represents the membrane left in the supernatant solution from membrane centrifuged at 521 x g (gel D) but then centrifuged at 13,023 x g. Hence, any differences observed in the pattern for membrane pre-

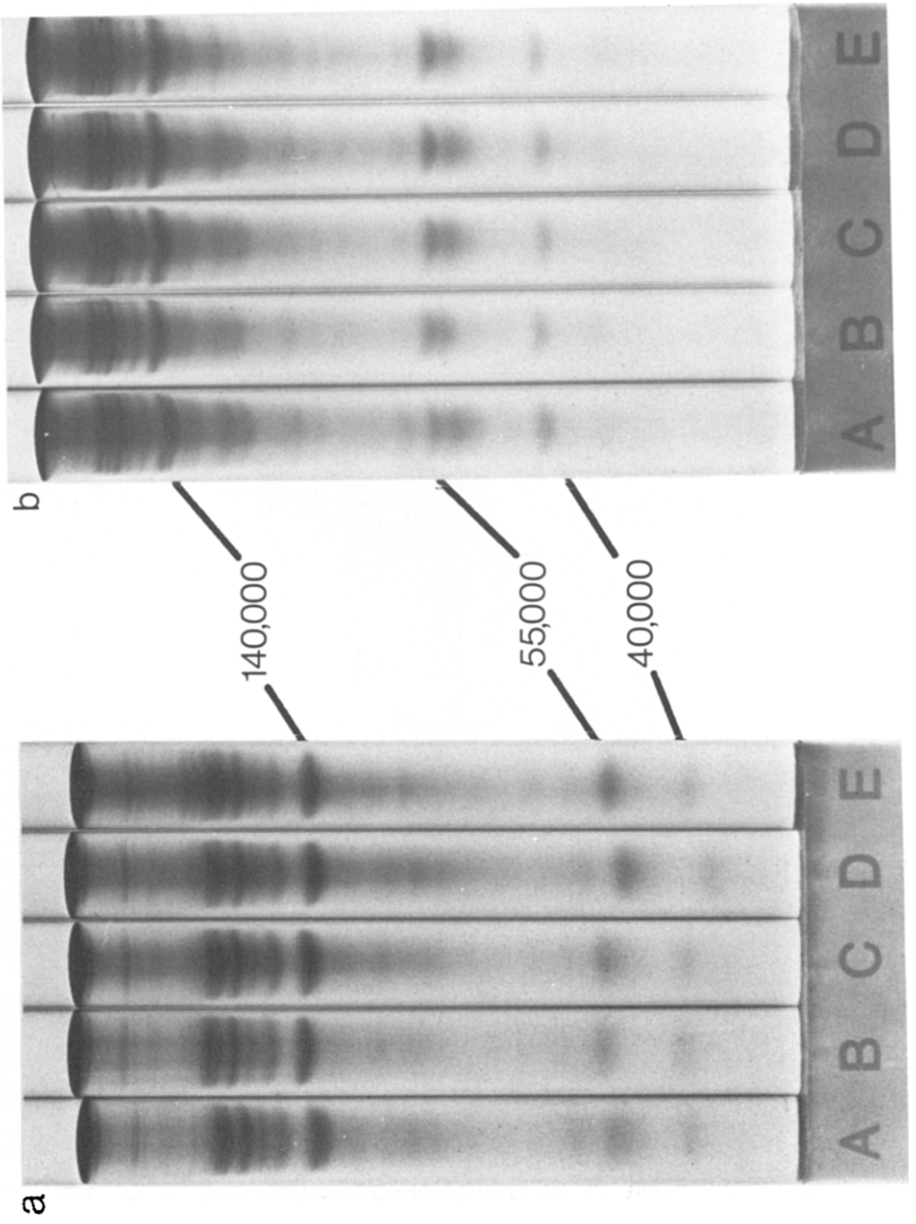

Figure 2. Effect of temperature and use of specific proteolytic enzyme inhibitors on GBM polypeptide composition as determined by SDS gel electrophoresis. Electrophoresis was performed with 5% (Fig. 2a) and 7½ (Fig. 2b) acrylamide gels. A, GBM isolated in eight hours at 27° without inhibitors; B, GBM isolated in three hours at 0-3° with no inhibitors; C, GBM isolated in three hours at 0-3° in presence of 20 mM EDTA-25 mM ε-ACA- 1 mM DFP; D, same as in C except

pared with inhibitors or at an elevated temperature can be interpreted confidently as due to proteolysis.

The electrophoretic patterns are shown in Fig. 2a for membrane prepared under identical conditions except the nature and concentration of inhibitor and temperature were varied. Patterns for membrane prepared at 0-3° with or without inhibitors (EDTA, ε-ACA, NEM, DFP) are virtually identical except that the relative amount of the 55,000 molecular weight polypeptide is increased with the use of DFP (compare gel B to C-E). ε-ACA or EDTA had no effect (gel not shown). Moreover, the pattern for membrane isolated without these inhibitors at room temperature (27° in which the tissue homogenate was allowed to set for an additional 4 hours was also identical to the membrane that was isolated at 0-3° except that the relative distribution of the polypeptides in the 40,000-60,000 molecular weight range was altered (compare gel A and B). These differences were further verified using 7½% acrylamide gels to improve resolution in the 40,000-60,000 molecular weight range (Fig. 2b). The single polypeptide (55,000) observed with the 5% gel was resolved into two components of molecular weight 51,000 and 55,000. As the conditions vary from that which permits maximum proteolysis to that of minimum proteolysis (gel A ⟶ E), the relative intensity of the 55,000 polypeptide increases.

These results show that the 55,000 dalton polypeptide is degraded by a protease during membrane isolation at 0-3° and to a further extent at 27° if DFP is not included. Degradation may also occur in that portion of membrane, 31% by weight for each preparation used in this study, which is insoluble in the SDS buffer and in that portion which is soluble but does not penetrate the 5% acrylamide gel. Thus, future structural studies should be performed on membrane that is isolated under conditions, such as those reported here, which prevent or minimize proteolysis.

These studies further suggest that the various polypeptides observed by SDS gel electrophoresis do not arise by proteolytic cleavage of a high molecular weight component during membrane isolation. It is more likely that these

are products of limited proteolysis *in vivo* as previously suggested (19) and represent integral membrane components. Multiple polypeptide components have recently been reported for a phylogenetically distant membrane prepared under identical conditions given here (20), suggesting that this structural feature may be characteristic of basement membranes.

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