A METHOD FOR ASSESSING THE MOLECULAR SIEVING PROPERTIES OF RENAL BASEMENT MEMBRANES IN VITRO

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1. Introduction

Biochemical investigations of renal basement membranes have intensified in recent years following the recognition that glomerular basement membrane undergoes alteration in renal disease [1]. The glomerular membrane is believed to act as a macromolecular sieve [2] allowing an ultrafiltrate of blood to enter the lumen of the nephron as the initial stage in urine formation. This view of the function of basement membranes has been challenged by the proposal that the slit-pore membranes, which lie between the podocytes of the epithelial cells, exclude macromolecules (for discussion see ref. [3]). The controversy illustrates a fundamental problem in studying basement membranes which is to relate function to structure and composition.

In the kidney, the basement membranes form tubular layers around both the glomerular capillaries and the tubular nephrons. These structures are so small that indirect methods such as morphological [4] and urinary clearance [5] procedures have had to be used to assess the molecular exclusion properties of the filtration apparatus. Microperfusion methods have been used with some success but as it is impossible to measure total flux across the membranes in such experiments authors have resorted to mathematical models to assist in characterising the behaviour of the system [6,7]. All of these procedures are conducted in vivo, which is of advantage in studying the physiology of the filtration process, but they offer no advantage in biochemical studies since it is impossible to use perturbation methods to study the structure–function relationships of the basement membrane.

Here we report a simple and rapid method for measuring the molecular exclusion properties of isolated basement membrane in vitro. Filter pads are prepared from isolated basement membranes and are used for conventional studies of molecular rejection characteristics. Chemically modified basement membranes can be used so that the consequences of altering the composition and structure of the basement membranes can be assessed. This simple yet novel approach results from the development of a new method for isolating basement membranes in intact segments using detergent extraction [8]. Previous methods for basement membrane isolation have employed ultrasonication [9] which, although effective as a purification procedure, fragments the membranes, rendering them unsuitable for the formation of filter pads.

2. Methods

Basement membranes were isolated by the detergent method referred to above [8]. Kidney cortex was obtained from rabbits (New Zealand white, 2–2.5 kg) and after coarse mincing it was washed through a sieve (150 μm mesh) with buffered saline (0.15 NaCl containing 0.01 M Tris–HCl buffer, pH 7.4). Tissue fragments passing through the sieve were harvested by centrifugation and suspended in 0.5% *N*-lauroyl sarcosine (detergent/protein ratio 2:1). After 10 min the basement membranes were recovered by centrifugation and again treated with detergent followed by incubation in DNAase I (0.01% in 0.15 M NaCl) at room temperature for 30 min to remove nucleic acid, the basement mem-

branes were then washed four times in buffered saline. The final isolate was essentially devoid of cellular contaminants and on gross chemical analysis was found to be very similar to basement membrane isolated by sonication [8]. Morphological examination showed large segments of tubular basement membrane, glomerular basement membrane and some contaminant collagen fibre.

Basement membranes suspended in buffered saline were used to prepare the filter pads. The apparatus used was a conventional two-part filter assembly consisting of a funnel, fitted with a sinter (grade 1) ground flush with the surface, and a flanged glass cylinder (i.d. 40 mm) which could be clamped to the funnel so as to trap a filter membrane between the edges of the cylinder and the funnel. To prepare basement membrane filter pads, the filtration apparatus was assembled on a Buchner flask with a cellulose acetate filter disc (0.22 μm exclusion size) resting on a hardened filter paper (No. 1 grade) and both secured by the flanges of the filter. Basement membrane (3 mg protein) in 50 ml buffered saline was filtered through the apparatus by vacuum to compact the basement membranes on the filter surface. When approximately two-thirds of the suspension had been filtered, the flux through the filter had diminished to 0.5–1.0 ml/min. The excess suspension was withdrawn from the filter surface and replaced by buffered saline which was allowed to continue filtering under suction for a further 30 min to finally compact the basement membrane layer. The buffered saline was then replaced by solutions of protein and filtrates from these solutions were collected over timed periods; protein concentrations were measured by determining A_{280nm}-values of the solutions. During protein filtration the overstanding solution was stirred using a glass paddle 3 cm wide rotating at 150 rev./min. All solutions to be filtered were passed through a membrane filter (0.22 μm exclusion) before use. The rejection of proteins was taken as the fraction of the protein that did not pass through the filter, i.e.,

$$\frac{C_O - C_F}{C_O}$$

where C_O is the protein concentration of the overstanding solution and C_F that of the filtrate.

3. Results and discussion

In initial experiments it was necessary to gain some knowledge of the behaviour of the filter pads and to establish whether leakage of bulk solution occurred. Since large macromolecules do not pass through the glomerular basement membrane [5] passage of IgG through the filter pads was taken to indicate leakage of bulk solution. Tests with this protein showed a rejection of 0.98 ± 0.02, indicating that leakage accounted for only 2% of the total flux of solvent through the membrane. Concentration polarisation, resulting from the accumulation of an unstirred layer of rejected macromolecules at the filter surface, affects the behaviour of filtration membranes [10] and experiments were conducted to study the factors which influenced polarisation. As expected, stirring rate, stirrer position, and solute (macromolecule) concentration influenced the total flux through the membrane and the rejection values observed; both less efficient stirring and increased solute concentration resulted in a reduced total flux of solvent and increased rejection of solute. These results were similar to the effects observed with synthetic ultrafiltration membranes [10] and indicated that the basement membrane pads were indeed acting as molecular ultrafilters. In comparing the filtration properties of the filter pads towards different macromolecules, the solutes were dissolved in buffered saline at equal weight concentrations rather than at equimolar concentrations. Under the latter condition the solute occupies progressively more space in the solution as its molecular weight increases, a consequence of this was that for larger molecules a denser unstirred layer of solute accumulated at the membrane face and polarisation effects were more marked. Using equal weight concentrations of solute reduced but did not eliminate the more marked concentration polarisation effects which occurred when high molecular weight solutes were used.

When the rejections of proteins of different molecular sizes were measured it was found that rejection increased with molecular size (fig.1). Lysozyme behaved exceptionally in showing a lower rejection than was found for cytochrome *c*, a slightly smaller molecule, indicating that parameters other than molecular size influence the filtration of macromolecules through basement membrane. It is apparent

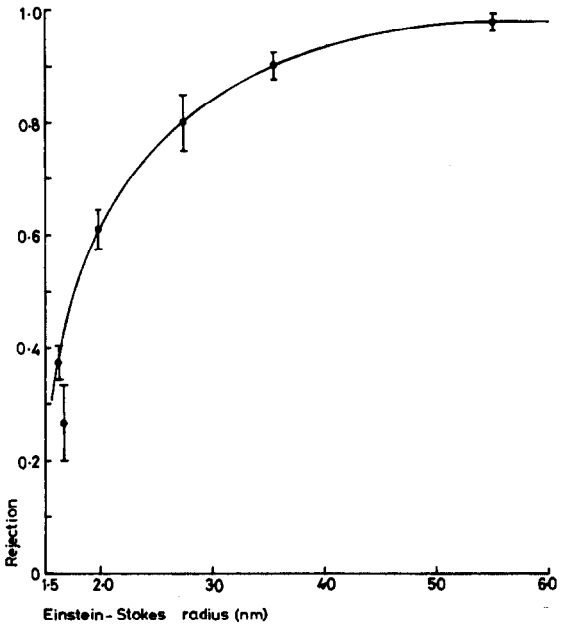

Fig.1. The rejection of proteins of differing sizes by basement membrane filters. Basement membrane filters were prepared by packing isolated basement membranes onto a cellulose acetate membrane filter and protein solutions (at a concentration of 0.5 mg/ml) were then filtered through the membrane pads by suction. The proteins filtered were, from left to right, cytochrome *c*, egg lysozyme, horse myoglobin, ovalbumin, bovine serum albumin and bovine immunoglobulin G. The concentrations of proteins in the overstanding solutions (C_O) and in the filtrates (C_F) were determined and the rejection values were calculated as

$$\frac{C_O - C_F}{C_O}.$$

Flux of buffered saline through the pads was 0.47 $\mu l.min^{-1} mm^{-2}$ at 750 mm Hg. All experiments were conducted at 20°C. The bars show the standard deviation of the results. The values of the Einstein-Stokes radii were calculated from published diffusion coefficients (D^0_{20w}) for the proteins [11–13] assumming that no aggregation had occurred under the conditions of the experiment. The radii indicate the relative sizes of the proteins in solution rather than their absolute dimensions.

from the results shown in fig.1 that the basement membrane pads exhibit molecular sieving characteristics which reflect those observed for glomerular filtration in vivo [5]. However it is pertinent to note that serum albumin exhibited a rejection of 0.9 in these experiments whereas it is almost wholly rejected by the glomerular membrane in vivo. It should be stressed that the rejection values obtained here were not absolute, since they were influenced by the conditions employed in the experiment. At higher protein concentrations rejection values were increased, but smaller proteins always showed lower rejections than larger proteins at the same concentration, admixture of proteins also increased the rejection of individual proteins. To compare these model filters with glomerular filtration in vivo, whole rabbit serum was filtered and the filtrate was found to contain less than 0.4% of the total serum protein. Examination of the proteins in the serum filtrate by gel chromatography and by electrophoresis on polyacrylamide gels showed that all the plasma proteins were present and there appeared to be no discrimination for proteins of lower molecular weight. Thus the protein that did pass through the membrane probably passed through leakage channels. The more effective retention of whole plasma protein as compared with albumin alone was probably due to concentration polarization resulting from the high concentration of the mixture of proteins in plasma and it is possible that polarization may influence the sieving properties of basement membrane in vivo. These results demonstrate that isolated basement membrane acts as a molecular sieve, effectively rejecting molecules of the size of albumin and above. Thus there seems no reason to suppose that the slit-pore membranes are involved in glomerular filtration.

The model system employed here differs in several important aspects from the situation pertaining in vivo. The membrane pads are comprised of multiple layers of basement membrane (fig.2), which may well influence the behaviour of the filters, and filtration was carried out with a high pressure differential across the membranes. However, it should be noted that the filtration pads were 20–30 membranes thick, as judged from electron micrographs, and the overall pressure difference was 750 mm Hg, giving a pressure drop across each membrane of between 37.5 mm and 25 mm Hg. This differential is similar to that reported in the glomerulus [2]. A further difference is that the bulk of the filter pad consists of tubular basement membrane rather than glomerular basement membrane and the properties of the two membranes may differ. However, the observation that the filter pads exhibit filtration characteristics which are similar to those

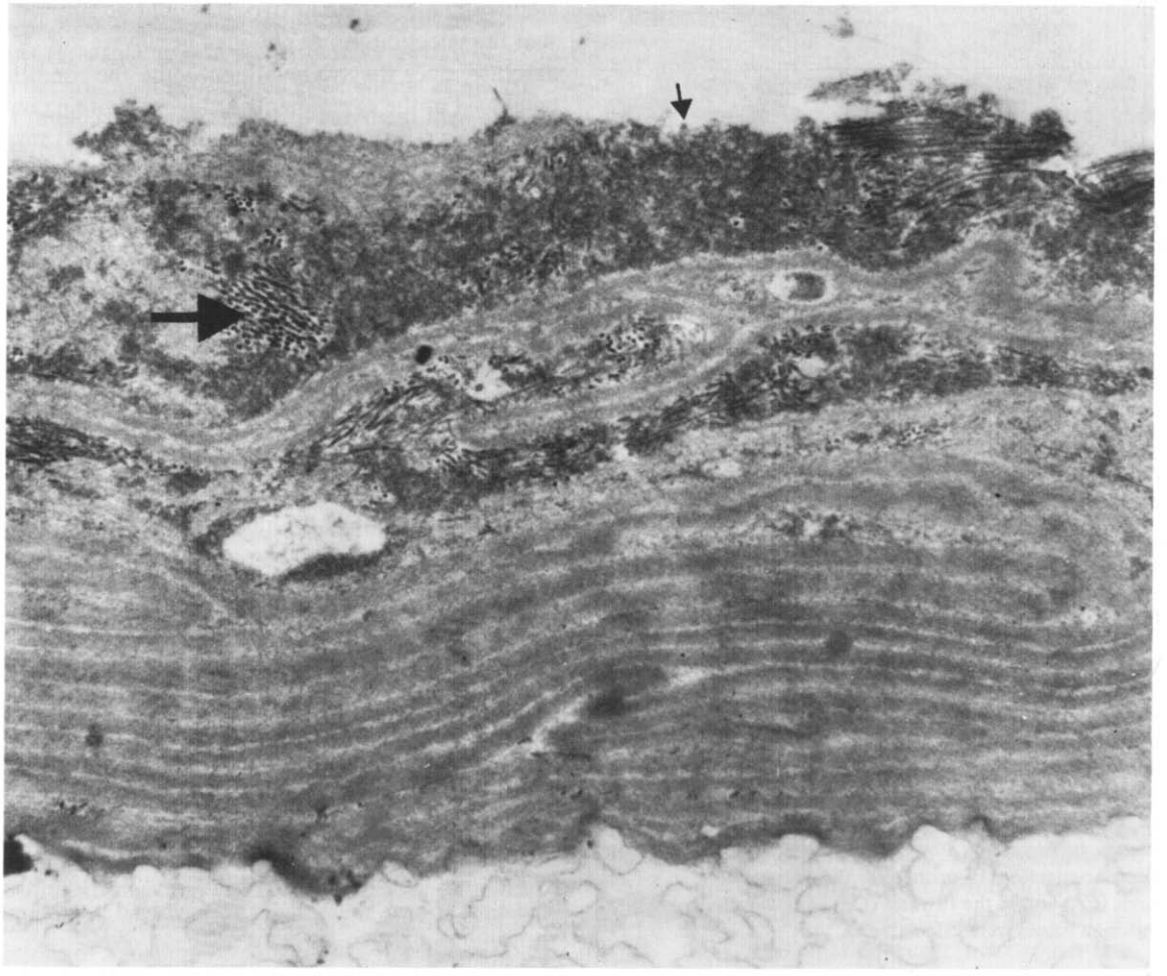

Fig.2. Electron micrograph of a basement membrane filter. Filter pads were fixed with a glutaraldehyde (2% in buffered saline), stained with osmium tetroxide before embedding and with lead citrate and uranyl acetate after sectioning. It can be seen that the basement membranes compact in layers on the surface of the cellulose acetate support (at the bottom of the photograph). Some collagen, an impurity in the preparation, is trapped between the membrane layers (thick arrow). Protein (IgG) which has been undergoing filtration is seen at the surface of the membrane gathered in irregularities on the surface of the membrane pad (thin arrow). No channels containing protein penetrate through the depth of the pad. Magnification : × 18 000.

reported for glomerular filtration in vivo, and that they are able to retain large macromolecules such as IgG, indicates that this model system may prove to be useful in studying the filtration characteristics of isolated basement membrane and may permit an examination of alterations in the filtration properties of the membranes consequent upon treatment with various agents such as proteases. It is anticipated that this approach will reveal information relating to the changes in glomerular filtration characteristics which are observed in renal disease.

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