Clogging of the Glomerular Basement Membrane

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ABSTRACT The negative charges of the sulfated glycosaminoglycans (GAGs) of the glomerular basement membrane (GBM) were differentially neutralized by perfusion with high molarity buffers in order to determine whether or not these charges protect the GBM from being clogged by circulating plasma macromolecules. Progressive elimination of the negative charges resulted in clogging of the GBM by perfused native ferritin (NF) and bovine serum albumin as evidenced ultrastructurally by the increase in accumulation of NF in the GBM. In addition, the permeability of the GBM to $^{125}$I-insulin, a macromolecule which is normally freely permeable, and the glomerular filtration rate (as determined by $[^{3}H]$inulin clearance) were markedly reduced after the GBM had been clogged with NF in the presence of high molarity buffer, thereby indicating that clogging severely reduces the ability of the GBM to act as a selective filter. These findings are consistent with the idea that the sulfated GAGs of the GBM serve as anticlogging agents.

MATERIALS AND METHODS

Materials

Male Charles River CD rats weighing 250–300 g were used. Materials were purchased as follows: horse spleen ferritin (cadmium free) from Calbiochem-Behring Corp., San Diego, CA; bovine pancreatic insulin from Sigma Chemical Co., St. Louis, MO; $[^{3}H]$inulin (1.2 Ci/mmol) and Na$^{125}$I from Amersham Corp., Arlington Heights, IL; ruthenium red from Ventron Corp., Danvers, MA; and Ilford K-5 emulsion from Polysciences, Inc., Warrington, PA. Cationized ferritin was prepared as previously described (8). Insulin was iodinated by the lactoperoxidase-glucose oxidase method of Hubbard and Cohn (7) and the free iodine removed by passing the iodinated product through a Sephadex G-10 column. The efficiency of iodination was 45–50%.

Perfusion with Buffers of Various Molarities and NF

Rats were anesthetized with ether and the left kidney was exposed and isolated from the systemic circulation as previously described (12). Subsequently, each rat was heparinized (100 U/100 g body weight), the aorta and ureter were both cannulated (the former with an 18-gauge cannula and the latter with heparinized PE-10 polyethylene tubing), a snip incision was made in the right atrium (for egress of the perfusate), and the kidney was perfused for 1–2 min with oxygenated Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4 at 120 mm Hg to wash out the blood. Kidneys were then perfused separately for 5 min with KRB of varying molarities (0.15, 0.50, 1.00, 1.50, 2.00, and 2.50 M), which was adjusted by the addition of a suitable amount of NaCl, containing 7.5% dialyzed bovine serum albumin (BSA), NF (10 mg/ml), insulin (10 mg/ml, to maintain normal glomerular blood flow), and 25 mM Na-acetate, at a pressure of 120 mm Hg and a flow rate of 5–7 ml/min. While a constant flow rate of the perfusate was maintained, the kidneys were fixed in situ by a subcapsular injection of Karnovsky’s aldehyde fixative (12.5% glutaraldehyde and 10.0% paraformaldehyde in 0.15 M cacodylate buffer, pH 7.4) at the hilum, while simultaneously the fixative was dripping over the kidney surface, and processed for electron microscopy as previously described (8). For each buffer treatment, thin sections were prepared (from six tissue blocks derived from the kidneys of two animals) and 12 areas of the GBM (in normal...
section) were photographed in a systematic random manner and printed at a final magnification of × 100,000. The total number of ferritin particles in the laminae rarae interna (LRI) and externa (LRE) and lamina densa (LD) were counted and expressed as the number of NF particles/μm² of LRI, LRE, and LD (12).

**Perfusion with Iodinated Insulin and Light Microscopic Autoradiography**

Kidneys were exposed and perfused separately with each individual molarity buffer for 2 min as detailed above. Subsequently, the kidneys were perfused for an additional 10 min with the same molarity buffer (0.15–2.5 M) containing 10 μCi/ml of ¹²⁵I-insulin, and fixed and processed for microscopy as described above. Light microscope autoradiograms were prepared as previously described (16). For each buffer treatment, 20 glomeruli taken from seven tissue samples of the superficial kidney cortex of two animals were photographed under a light microscope (at or near their greatest diameter) in a systematic random manner and printed at a final magnification of × 1,500. The total number of autoradiographic grains over the capillary lumina, urinary space, and glomerular capillary wall (GCW) (including the podocytes and their processes, the GBM, and the axial regions of the glomerulus) were counted and the percent of the total grains over each compartment was determined. In addition, the percent area and the grain density (GD) of each compartment were also determined, the former by the point-counting method as described by Weibel (17) and the latter by dividing the percent grains by the percent area values (% grains/% area). The permeability of the GCW to ¹²⁵I-insulin was established by determining the ratio of the GD of the capillary lumina to that of the urinary space (grain density ratio [GDR]), with an increase in the GDR indicating a decrease in ¹²⁵I-insulin permeability.

**Determination of the GFR**

Kidneys were perfused separately with buffers with various molarities (0.15–2.5 M) for 2 min (containing BSA, NF, ismelin, and Na-acetate) and subsequently with the same buffers containing 0.1 μCi/ml [³H]inulin for 10 min. Urine was collected (via the catheterized ureter) during the entire period of perfusion with [³H]inulin. GFR was determined by [³H]inulin clearance (1). For each buffer treatment, GFR determinations were made on six animals.

**RESULTS**

**Morphology**

Perfusion of kidneys with high molarity buffers did not disrupt the structural integrity of the GCW as evidenced by the fact that the foot-process slit-diaphragm complex and the endothelium remained firmly attached to the GBM (Fig. 1). In addition, such perfusion did not destroy the anionic sites of the GBM but served only to neutralize the charge of their constit-

![Figure 1](image-url)

**FIGURE 1** Portions of the glomerular capillary wall from kidneys in which the negative charge of the glomerular basement membrane (GBM) has been differentially neutralized by perfusion with (A) 0.15 M, (B) 1.0 M, (C) 1.5 M, and (D) 2.0 M KRB buffer and the GBM subsequently clogged by perfusion with the same molarity buffers containing native ferritin (NF). With 0.15 M KRB, few NF particles enter the GBM and localize in the lamina rara interna (LRI) and inner portion of the lamina densa (LD). With 1.0–1.5 M KRB, NF enters the GBM in increased numbers and penetrates deeper: LD and lamina rara externa (LRE). Finally, with 2.0 M KRB, maximal accumulation of NF occurs and is restricted to the LRI and inner LD. CL, Capillary lumina; En, endothelium; B, glomerular basement membrane; fp, foot process; US, urinary space. x 80,000.
uent polyanionic macromolecules since subsequent staining with ruthenium red and binding studies with cationized ferritin (CF) (probes previously shown to be specific for the GBM anionic sites (8)) utilizing buffers at physiological molarity revealed no changes in either their number or pattern of distribution within the GBM (Fig. 2).

Clogging of the GBM with NF

The negative charges of the GBM's polyanionic macromolecules were differentially neutralized with buffers of various molarities and changes in the accumulation of protein, in this case NF, were determined in order to assess whether these charges protect the GBM from being clogged by comparable plasma macromolecules. With no neutralization of the anionic charge (0.15 M KRB) few NF particles entered the GBM, with most being confined to the LRI and inner portion of the LD (Fig. 1A and Table I). Partial neutralization of the anionic charge (0.50-1.5 M KRB) resulted in a two- to sevenfold increase in the number of ferritin particles entering the GBM and in an enhanced penetration of the NF to deeper layers of the GBM, the latter most likely due to the partial abolition of the GBM's charge barrier properties (Figs. 1B and C, and Table I). Total neutralization of the anionic charge (2.0-2.5 M KRB) led to maximal accumulation of NF in the GBM with the overwhelming majority of the particles being restricted to the LRI and few reaching the LD or LRE, in spite of the fact that the charge barrier of the GBM had been totally eliminated (Fig. 1D and Table I). Since BSA is present in the perfusate, and since it is itself a protein macromolecule, it too, most likely, participates in the clogging process although it cannot be directly visualized electron microscopically. These results are consistent with the idea that removal of the GBM's anionic charge renders it susceptible to being clogged by circulating plasma macromolecules.

Permeability Experiments with Iodinated Insulin

To determine whether or not clogging of the GBM interferes with its ability to act as a selective filter, the GBM was differentially clogged with NF and BSA, and changes in the permeability of 125I-insulin across the GCW were determined. With no clogging of the GBM (0.15 M KRB) the GDR was near unity, indicating an equal concentration of 125I-insulin in the CL and US and thus free passage of the tracer across the GCW (Fig. 3A, Table II). Moderate clogging (0.50-1.50 M KRB) resulted in only a slight increase in the GDR and a small decrease in 125I-insulin permeability, suggesting that this level of clogging does not reduce the porosity of the GBM sufficiently to dramatically alter the flow of 125I-insulin (Figs. 3B and C, and Table II). Extensive clogging (2.0-2.5 M KRB) resulted in a dramatic increase in the GDR (threefold), indicating a marked reduction in the permeability of 125I-insulin across the GCW (Figs. 3D and E, and Table II). In addition, the percent of the grains as well as the G of the GCW increased progressively with higher degrees of clogging, consistent with the idea that since insulin is a protein it, too, participates in the clogging process. This is dramatically illustrated in glomeruli perfused with 2.5 M KRB in which the autoradiographic grains (which represent 125I-insulin localization) faithfully decorate the contours of the GCW (Fig. 4). Thus, clogging of the GBM materially alters its ability to act as a selective filter.

GFR after Differential GBM Clogging

Increasing levels of GBM clogging resulted in a decrease in the GFR (which was greatest after clogging in the presence of 2.0-2.5 M KRB) as gauged by [3H]inulin clearance. The GFR of kidneys perfused with 0.15 M KRB in either the presence or absence of NF and BSA was the same, indicating that with 0.15 M KRB no clogging of the GBM occurs. Kidneys perfused with 2.50 M KRB without NF and BSA (clogging proteins) showed no reduction in the GFR, thereby establishing that it is not the high molarity of the KRB alone that is the cause of the reduced GFR but rather the combination of high molarity

![Figure 2. Portions of the glomerular capillary wall (Cap) from kidneys that were perfused with: (A) 0.15 M KRB containing CF, (B) 0.15 M KRB containing CF followed by 2.5 M KRB, and (C) the same as in B followed by 0.15 M KRB containing CF. Perfusion with 0.15 M KRB containing CF results in a normal pattern of binding of the CF to the anionic sites. This binding is abolished as a result of perfusion with 2.5 M KRB, which neutralizes the charge of the anionic sites, and is subsequently restored after perfusion with CF in 0.15 M KRB. This clearly indicates that high molarity buffers do not destroy the integrity of the anionic sites but serve only to neutralize their negative charge. × 50,000.](image-url)
Effect of Different Molarity Buffers on the Distribution of Ferritin Molecules/μm² in the GBM *

| Buffer molarity | Lamina rara interna mean ± SD (%) | Lamina densa mean ± SD (%) | Lamina rara externa mean ± SD (%) |
|-----------------|-----------------------------------|----------------------------|----------------------------------|
| 0.15            | 115 ± 35 (77)                     | 34 ± 18 (23)              | -                                |
| 0.50            | 124 ± 28 (63)                     | 72 ± 28 (36)              | 2 ± 1 (1)                        |
| 1.00            | 140 ± 25 (55)                     | 105 ± 33 (40)             | 12 ± 6 (5)                       |
| 1.50            | 269 ± 47 (32)                     | 514 ± 115 (61)            | 53 ± 13 (7)                      |
| 2.00            | 609 ± 98 (73)                     | 221 ± 50 (27)             | -                                |
| 2.50            | 1465 ± 196 (90)                   | 177 ± 55 (10)             | -                                |

* An average of 0.5 μm² of GBM area was utilized for ferritin counts which was derived from 12 capillaries from each of two animals.
† Percent of the total native ferritin particles in the GBM.

DISCUSSION

The data obtained in the present investigation clearly establish that the negative charge of the polyanionic macromolecules of the GBM, most importantly HS-PG, protects the GBM from being clogged by circulating plasma macromolecules. This conclusion is substantiated by the finding that neutralization of the intrinsic negative charge of the GBM by perfusion with buffer and protein (Table III). Finally, the GFR values obtained at physiological buffer molarity are in agreement with those previously obtained by deMello and Maack (4) using an isolated perfused rat kidney system. Thus, the GFR data corroborate the findings with 125I-insulin that clogging of the GBM impairs its ability to act as a selective filter.

FIGURE 3 Glomerular profiles from kidneys in which the negative charge of the glomerular basement membrane (GBM) has been differentially neutralized by perfusion with (A) 0.15 M, (B) 1.0 M, (C) 1.5 M, and (D) 2.0 M KRB, the GBM clogged with native ferritin (NF), and the kidneys subsequently perfused with 125I-insulin. With no clogging of the GBM (A), insulin freely permeates the glomerular capillary wall (GCW), resulting in an equal concentration of 125I-insulin in the urinary space (US) and capillary lumina (CL): equal number of autoradiographic grains in the CL and US. Increasing degrees of clogging (B–D) results in a marked reduction in the permeability of 125I-insulin across the GCW: fewer grains in the US. X 500.
high molarity buffer renders the GBM readily cloggable by NF, a molecule whose biophysical properties (charge and shape) are similar to those of plasma albumin, and that such clogging results in a marked decrease in the permeability, as well as to a reduction in the GFR.

Previously, HS-PG and the other GAGs (chondroitin sulfate and hyaluronic acid) of the GBM, the latter being present in only minute quantities, were shown to participate in imparting to the GBM its properties as permeability barrier to plasma macromolecules since removal of the GAGs either singly or in combination resulted in a significant increase in the permeability of the GBM to both NF (12) and iodinated BSA (5). The present findings extend the role of GAGs, most importantly the sulfated ones, by indicating that not only do they confer upon the GBM its ability to selectively exclude macromolecules on the basis of their charge (2, 15) and size (3, 15) but, in addition, render it resistant to being clogged by these very same macromolecules. Given the fact that the components of the GBM turn over at a very slow rate (14) and that the renewal time of the GBM is therefore very long, a mechanism to resist

| TABLE II |
|---|
| Changes in the Permeability of the GCW to $^{125}$I-Insulin after Various Degrees of Clogging |

| Buffer concentration | Grain percent | Grain Density | GDR | GFR |
|---|---|---|---|---|
| | CL | US | GCW | CL | US | GCW | CL/US |
| M | 0.15 | 34.83 | 27.23 | 1.00 | 0.80 | 1.51 | 1.25 |
| | 0.50 | 26.77 | 38.16 | 35.07 | 0.79 | 0.79 | 1.95 | 1.00 |
| | 1.00 | 29.95 | 28.55 | 41.50 | 0.83 | 0.68 | 1.94 | 1.22 |
| | 1.50 | 26.10 | 26.23 | 47.67 | 0.83 | 0.57 | 2.09 | 1.46 |
| | 2.00 | 35.45 | 10.49 | 53.56 | 0.85 | 0.28 | 2.67 | 3.04 |
| | 2.50 | 35.04 | 10.95 | 54.01 | 0.91 | 0.27 | 2.55 | 3.37 |

CL, capillary lumen; US, urinary space; GCW, glomerular capillary wall; GDR, grain density ratio.

| TABLE III |
|---|
| [H]Insulin Clearance after Various Degrees of Clogging |

| Buffer molarity | $^{3}$H-Insulin/ml/min.g | GFR/ml/min.g of kidney weight |
|---|---|---|
| M | 0.15‡ | $0.189 ± 0.029$ |
| | 0.15 | $0.181 ± 0.013$ |
| | 0.50 | $0.182 ± 0.007$ |
| | 1.00 | $0.094 ± 0.024$ |
| | 1.50 | $0.066 ± 0.005$ |
| | 2.00 | $0.099 ± 0.002$ |
| | 2.50‡ | $0.009 ± 0.002$ |
| | 2.50‡ | $0.185 ± 0.022$ |

* Data represent mean ± SD. Six animals per experimental variable were utilized.
‡ Perfusates devoid of proteins.

**FIGURE 4** Profile of a glomerulus from a kidney in which the negative charge of the glomerular basement has been totally neutralized by perfusion with 2.5 M KRB, the GBM clogged with native ferritin (NF), and the kidney subsequently perfused with $^{125}$I-insulin. Few autoradiographic grains are seen in the urinary space (US), indicating a marked reduction in $^{125}$I-insulin permeability across the glomerular capillary wall (GCW). In addition, the contours of the GCW are decorated with grains (arrows), suggesting that the $^{125}$I-insulin itself participates in the clogging process. CL, Capillary lumen. × 1,250.
sulfated GAGs protects the GBM from being clogged due to the fact that the sulfate groups remain hydrated and prevent hydrophobic interactions between the GAGs and the macromolecules in the solution being filtered (in this case NF, BSA, and insulin) resulting in a normal glomerular filtration rate (GFR) (solid arrow). By contrast, neutralization of the anionic charge of the GBM-GAGs by perfusion with high molarity (2.5 M) KRB (in which the molarity is adjusted by the addition of NaCl) results in an abolition of the charge barrier of the GBM and a deactivation of the SO₄²⁻ groups of the GAGs (by formation of the sodium salts of the GAGs) leading to an increase in the entry and accumulation of macromolecules in the GBM (clogging) and a reduction in the permeability of the GBM to freely filterable molecules such as insulin as well as in the GFR (broken arrow).

clogging would seem to be absolutely essential if the GBM is to continue to function as an effective filter.

This then raises the question as to the mechanism whereby the sulfated polyanionic macromolecules of the GBM impart to the GBM its clogging resistivity. By analogy to data obtained in artificial membrane systems, the answer most likely resides in the properties of the sulfate group itself: these include (a) its hydrophilic nature, which maintains the GAGs of the GBM wet at all times, thereby blocking hydrophobic interactions between the GAGs and the macromolecules in the solution being filtered; (b) its strong negative charge, which generates an electric field within the GBM capable of repelling negatively charged plasma macromolecules and salt; and (c) its extremely stable negative charge, since the sulfate group represents the conjugate base of a strong acid (sulfuric acid), and therefore resists being deactivated by low pH or heavy metals.

On the basis of the data obtained previously (5) and in the present study, the following model for the role of GAGs in GBM function seems plausible (Fig. 5). Under physiological conditions, the negative charge and gel-forming properties of the GAGs of the GBM confer on the GBM its permeselectivity barrier properties, while the sulfate groups of the GAGs protect the GBM from being clogged by means of the mechanism detailed above. Neutralization of the negative charge of the GBM-GAGs, such as by perfusion with high molarity buffer, abolishes the charge barrier properties of the GBM, thereby enabling plasma macromolecules to readily enter the GBM and interact with the deactivated sulfate groups of the GBM-GAGs, resulting in a reduction in the porosity of the GBM and in its ability to serve as an effective ultrafilter.

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