Rapid communication

Reduced number of anionic sites is associated with glomerular basement membrane thickening in the diabetic BB-rat

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Summary. Glomerular basement membranes of diabetic and age- and sex-matched non-diabetic BB rats were studied morphometrically and ultrastructurally using a quantitative histochemical technique employing the cationic dye cuprolinic blue. Six months of diabetes resulted in a significant reduction in the density of anionic sites associated with increased thickness of the glomerular basement membrane. These findings

suggest that loss of anionic sites may be an important mechanism in the genesis of glomerular basement membrane dysfunction in diabetes.

Key words: Glomerular basement membrane, anionic sites, cuprolinic blue, BB-rat.

Thickening of glomerular basement membrane (GBM) and associated altered filtration properties of macromolecules is a characteristic feature of diabetic nephropathy [1, 2]. A combination of metabolic and haemodynamic changes is thought to be responsible for these abnormalities.

Biochemical alterations of GBM subsequent to increased non-enzymatic glycation of structural proteins and the formation of advanced glycosylation end products have been invoked in the genesis of an altered size selective barrier [2, 3]. The prevention of basement membrane thickening in hyperglycaemic and galactosaemic animals following treatment with aldose reductase inhibitors would suggest that an augmented polyol pathway activity may be of pathogenetic significance in the production of this lesion [4, 5]. An additional basement membrane abnormality that has been associated with diabetes is the decreased production of heparan sulphate proteoglycans (HSPG) [1, 2]. HSPG is the major proteoglycan in basement membranes, and is located at regular intervals along its surface [1]. The presence of proteoglycans in GBM is essential for the integrity of the glomerular charge selective barrier due to their properties of electrostatic repulsion and steric hindrance [2]. It has been suggested that decreased synthesis of proteoglycans in diabetes [1, 2, 6], may lead to GBM thickening either by increased trapping of plasma

proteins or secondarily by an increase in the synthesis of other GBM proteins [1, 2].

Proteoglycans can be visualized by cationic dyes that bind electrostatically with the polyanionic glycosaminoglycan chains of proteoglycans, or by immunocytochemical localization using core protein specific antibodies [2, 7]. The specificity and sensitivity of these staining techniques can be substantially enhanced by using phthalocyanin dyes and the critical electrolyte concentration method [7, 8].

In the present communication, we report the results of quantitative ultrastructural localization of proteoglycans in GBM of the diabetic BB-rat using the phthalocyanin dye cuprolinic blue.

Materials and methods

Animals

Six male diabetic and six age- and sex-matched non-diabetic BB-rats were used in this study. The animals were maintained in air filtered metabolic cages and fed rat chow (Wayne Lab Blox, Chicago, Ill, USA) and water ad libitum. Diabetic animals were treated with small daily doses (0.5–3.0 U/day) of protamine zinc insulin (Connaught Laboratories, Toronto, Ontario, Canada) to prevent ketosis. They were monitored daily with respect to urine volume, sugar and ketones. Blood glucose levels, HbA_{1c} levels and body weights were monitored regularly as previously described [5]. The rats were killed after 6 months of diabetes.

Fig. 1a and b. Electronmicrographs of the glomerular basement membrane of a non-diabetic rat (**a**) and of a diabetic rat (**b**) following exposure to the cationic dye cuprolinic blue. Note a thicker basement membrane and reduced number of anionic sites (some of which are shown with arrows) in **b**. Magnification 38,200 for both micrographs. P = Podocyte, E = Endothelial cell

Tissue collection

The animals were anaesthetized with Na-pentobarbital (50 mg/kg of body weight) and decapitated. Tissue blocks from the renal cortex were fixed in 0.02 mol/l acetate-buffered (pH 5.6) 2.5% glutaraldehyde, containing 0.2% cuprolinic blue (British Drug House Ltd., Poole, UK) and 0.2 mol/l magnesium chloride for adjustment of the critical electrolyte concentration [7, 8].

After overnight fixation at 4°C, the specimens were washed three times for 10 min in the same fixative containing no cuprolinic blue. Tissues were washed for a further three times in 1% aqueous Na-tungstate for 10 min, dehydrated in graded concentrations of alcohol, and embedded in Epon following standard procedures. The 30 and 50% alcohol concentrations contained 1% Na-tungstate to enhance the electron-density of the staining product [7, 8]. Separate kidney blocks were treated in the same fixative containing no dye and served as negative controls. Toluidine blue stained semi-thin sections were used for lightmicroscopic tissue orientation. Ultra-thin sections were stained with uranyl acetate and examined electronmicroscopically.

Morphometry

Electronmicrographs with a total magnification of 44,000 times from a minimum of two glomeruli per kidney were used for the measurement of GBM thickness by the orthogonal intercept method [9], GBM area, and the number of anionic sites expressed as $\#/\mu\text{m}^2$.

The morphometric analysis was carried out on a Hewlett Packard 9874A digitizer interfaced with a Hewlett Packard 9875A computer (Hewlett Packard, Fort Collins Colo., USA) and by an investigator unaware of the source of the samples.

Statistical analysis

The data are expressed as mean $\pm$ SEM and were analysed by students t-test and linear regression analysis by the least square method.

Results

Diabetic animals showed hyperglycaemia, elevated HbA₁ and decreased body weight gain compared to control animals (Table 1), as well as polyuria (> 30 ml/day) accompanied by glucosuria (> 110 mmol/l) and occasionally ketonuria. Control animals in contrast showed a 24 h urine output of < 5 ml and no glucosuria or ketonuria (data not shown).

GBMs of diabetic BB-rats showed increased thickness compared to non-diabetic control animals (Table 1, Fig. 1). Renal tissue blocks fixed with the solution containing cuprolinic blue demonstrated electron-dense filamentous stainings of the GBM corresponding to the anionic sites. When the staining products were cut on end they attained a dot-like appearance. Within the GBM, cuprolinic blue positive sites were distributed periodically along both the inner and outer surfaces. The staining sites were most numerous in the lamina rara externa followed by the lamina rara interna, while in the lamina densa they appeared to be distributed in a random fashion. No staining product was seen in tissues fixed in solutions containing no cuprolinic blue. Quantitation of the staining sites revealed a significant ($p < 0.001$) reduction in the density of anionic sites in diabetic rats compared to control rats (Table 1, Fig. 1).

Linear regression analysis of GBM thickness, with the density of anionic sites as the independent variable, showed a significant negative relationship ($r = -0.83$, $F = 21.428$, $p < 0.001$).

Discussion

The present study has demonstrated that GBM thickening in the diabetic kidney is associated with loss of anionic sites, identified by the cationic dye cuprolinic blue.

Table 1. Clinical and morphometric data in control and diabetic BB-rats

	Body weight (g)	Blood glucose (mmol/l)	HbA ₁ (%)	Basement membrane thickness (μm)	Density of anionic sites ($\#/\mu\text{m}^2$)
Control rats (n=6)	539.0 $\pm$ 21.6	6.1 $\pm$ 0.9	5.2 $\pm$ 0.4	0.326 $\pm$ 0.02	191.0 $\pm$ 6.9
Diabetic BB-rats (n=6)	409.7 $\pm$ 23.6 ^a	22.3 $\pm$ 1.3 ^b	11.2 $\pm$ 0.5 ^b	0.431 $\pm$ 0.06 ^a	129.8 $\pm$ 6.9 ^b

Data expressed as mean $\pm$ SEM; ^a $p < 0.01$ compared to control; ^b $p < 0.001$ compared to control

This technique identifies anionic sites as filamentous staining products that are due to the collapse of the glycosaminoglycan chain on the protein core of proteoglycans [7, 8]. Since Mg-ions in concentrations used in this study displace carboxylic groups but not sulphate groups, the cuproinic blue positive structures represent anionic sites of sulphated glycosaminoglycans. The staining sites are electron dense and discrete. Hence, this technique lends itself as a more reliable tool for the examination of anionic sites than the previously used ruthenium red technique [7, 8]. As cuproinic blue stains only the sulphated glycosaminoglycans, the loss of anionic sites could conceivably be due to either diminished sulphation or diminished production of glycosaminoglycans or by both. This notion is consistent with decreased incorporation of ^{35}S -sulphate into GBM [6, 10].

The loss of anionic sites in diabetic GBM may have an adverse effect on the glomerular charge selective barrier and may consequently alter the permeability properties of the GBM [1, 2]. Although it is believed that activation of the polyol pathway and/or non enzymatic glycation of structural proteins may underlie the characteristic basement membrane thickening in diabetes, it is not known whether or how these mechanisms relate to the loss of anionic sites. The highly significant relationship between GBM thickening and the loss of anionic sites may suggest that these two abnormalities are interrelated and that the dysfunctions of the size and charge selective barriers occur with equal pace in diabetes.

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