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Glycosylation of Glomerular Basement Membrane in Type 1 (Insulin-Dependent) Diabetic Children

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Summary. Immunoelectrophoresis of glomerular basement membrane antigens in the urine of 20 Type 1 (insulin-dependent) diabetic and 10 healthy children was performed. In 10 of the diabetic children, there was altered α-1-mobility, while the other diabetic and normal children showed α-2-mobility. After incubation with glucose, glomerular basement membrane antigens in the urine of healthy children showed α-1-mobility. Isolated human kidney glomerular basement membrane split products obtained by proteolytic degradation (papain, trypsin, chymotrypsin) were also investigated by immunoelectrophoresis. A difference was observed in the immunoelectrophoretic pattern of native and glycosylated glomerular basement membrane split products. A distinct increase of thiobarbituric acid assay positive glomerular basement membrane structures after incubation with glucose provides suggestive evidence for the occurrence of non-enzymatic glycosylation of glomerular basement membrane proteins. Glycosylated glomerular basement membrane proteins may contribute to both functional and morphological changes in diabetic glomerulosclerosis.

Key words: Non-enzymatic glycosylation, glomerular basement membrane, immunoelectrophoresis, Type 1 diabetes mellitus.

Morphological changes of the glomerular basement membrane (GBM) have been described in diabetes mellitus [1]. These changes include thickening of the basal lamina, moth-eaten vacuolated areas and accumulation of electron dense granules. Genetic [2, 3], immunological [4] and metabolic disturbances have been discussed [5] in the pathogenesis of diabetic nephropathy.

An analogous mechanism similar to the non-enzymatic glycosylation of haemoglobin and other serum proteins [6–9] found in diabetic individuals with attendant hyperglycaemia, may theoretically affect the GBM glycoproteins [10, 11]. Recently, a method has been described for the immunoelectrophoretic characterization of human GBM split products in urine [12]. Using this method, we have investigated the excretion of GBM antigens in the urine from diabetic and healthy children. In addition, we have investigated the possibility that nonenzymatic glycosylation is involved in the altered immunoelectrophoretic behaviour of GBM proteins in diabetes. We have also examined the glycosylation of isolated human kidney GBM structures in vitro.

Subjects and Methods

Twenty children with Type 1 (insulin-dependent) diabetes mellitus were investigated at a summer camp. Clinical details are shown in Table 1. None of the diabetic patients had proteinuria or evidence of retinal vascular complications on fundoscopy.

For comparison we used urine samples obtained from 10 healthy children (five female, five male), mean age 12.4 years (range 8–14 years). Twenty-four hour urine samples were collected, using merthiolate as a preservative (1 g merthiolate/1000 ml urine). Urine samples were dialysed and concentrated 100-fold by an Amicon stirred Coll 2000 (Amicon, Lexington, USA) using a membrane permeable for molecule weights below 20,000 daltons. Protein concentration was adjusted to 1 mg/ml. This preparation was used for the immunoelectrophoresis and the glycosylation. Immunoelectrophoresis was performed with rabbit-anti-human GBM serum.

At 2 week intervals, rabbits were injected intracutanously with 10 mg dry GBM-preparation mixed with aluminium hydroxide and complete Freund's adjuvant (Difco, Detroit, USA). Two months later, the animals were bled by cardiac puncture. Absorption experiments were performed according to McPhaul and Dixon [13]. The antiserum (1 ml) was incubated with 10 mg of isolated human GBM for 24 h at 37 °C and the reactivity was assessed by subsequent immunofluorescence. No positive reaction could be detected on frozen 5 µ kidney sections. When the antiserum was absorbed with GBM, no precipitation lines were found on immunoelectrophoresis. There was no reaction with fibronectin before or after absorption with normal human plasma. No reactivity was found on immunofluorescence with Bowman's capsule, mesangial matrix or muscle basement membrane, but was found with lung basement membrane and placenta basement membrane [14]. As reactivity against human blood leucocytes was excluded, the probability that the antiserum would react with cell membranes is low.

Electrophoresis was performed using an LKB machine (LKB, Brommer, Sweden) and was allowed to run for 45 min, washed and then stained with amidoblack [12]. GBM antigens from the urine of 10 healthy children were incubated with a solution containing 18 g/

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Table 1. Clinical details of the diabetic children

	Total (n = 20)	α-1-mobility of urinary GBM antigen (n = 10)	α-2-mobility of urinary GMB antigen (n = 10)	p
Sex	11 males 9 females	5 males, 5 females	6 males, 4 females	
Age (years)	12.0 (8–16)	12.5 (8–16)	11.3 (8–13)	NS
Duration of disease (years)	5.6 (1–10)	5 (2–10)	6.1 (1–10)	NS
Glycosuria (g/24 h)	46.9 (17–110)	56.8 (25–110)	37.1 (17–60)	NS

Data given as mean with range in parentheses; NS = not significant

Table 2. Enzymatic degradation of the glomerular basement membrane

Enzyme (0.01 mg)	Buffer (mmol/l)	Inhibition
Papain[a]	1/15 phosphate 0.005 cystein 0.001 EDTA (pH 7.3)	0.001 ml 0.2 mmol/l sublimate
Chymotrypsin[b]	1/15 phosphate (pH 7.3)	0.001 ml trasylol
Trypsin[b]	1/15 phosphate (pH 7.3)	0.001 ml trasylol

Substrate concentration 1 mg/ml, incubation period 6 h at 37 °C.
[a] Sigma: Saint Louis, USA, [b] Serva: Heidelberg, FRG

Table 3. Estimation of ketoamine-linked glucose by the thiobarbituric acid assay of native and glycosylated glomerular basement membrane (GBM) prepared by digestion by various proteolytic enzymes

	Papain	Chymotrypsin	Trypsin
	(nmol 5-hydroxymethyl-furfural/mg protein)		
Native GBM	62.5 ± 5.8	47.5 ± 4.9	55.0 ± 5.3
Glycosylated GBM	875.0 ± 78.1[a]	1000.0 ± 97.9[a]	1225.0 ± 113.4[a]

Results expressed as mean ± SEM of four experiments;
[a] $p < 0.01$

100 ml glucose and merthiolate (1 : 10,000) as conservating agent for 10 days at 37 °C in the dark. Dialysis against distilled water was performed in the cold overnight (4 °C) and immunoelectrophoresis performed. GBM were prepared according to the principle of Krakower and Greenspon [15] as described previously [14] using kidneys from non-diabetic individuals obtained at surgery or within a few hours after death at necropsy. Native and non-enzymatically glycosylated GBM were degraded by proteases as indicated in Table 2 and 10 mg of GBM were used for each experiment. The protein concentration of the split products was adjusted to 1 mg/ml and used in that concentration for immunoelectrophoresis. Non-enzymatic glycosylation was also performed with 60 mg of native isolated human kidney GBM by the same procedure. Glycosylation of GBM proteins was estimated by the thiobarbituric acid assay of Flückiger and Winterhalter [16] as modified by McFarland et al. [17]. Results are expressed as nmol 5-hydroxymethylfurfural/mg protein.

The unpaired t-test was used for statistical analysis.

Results

Ten of the diabetic children showed α-1-mobility of the urinary excreted GBM antigens on immunoelectrophoresis. All the healthy children demonstrated α-2-mobility, which is usually found in normal individuals over the age of 5 years [14]. The remaining ten diabetic children also had α-2-mobility. We did not observe any evidence of intermediate lines or double lines in either the diabetic or healthy children. Figure 1 gives examples of the observed immunoelectrophoretic patterns. The diabetic children with α-1- and α-2-mobility showed no significant differences in their age or duration of disease (Table 1). There was a non-significant trend towards higher 24 h glycosuria in the patients with α-1-mobility (p = 0.06).

After incubation with glucose to produce non-enzymatic glycosylation, the urinary GBM antigens from the healthy children showed a change in immunoelectrophoretic mobility to the α-1-region. The GBM split products resulting from digestion with several proteases formed two precipitation lines, differing from each other in their electrophoretic mobility. After glycosylation, the GBM split products resulting from proteolytic digestion showed differences in immunoelectrophoretic

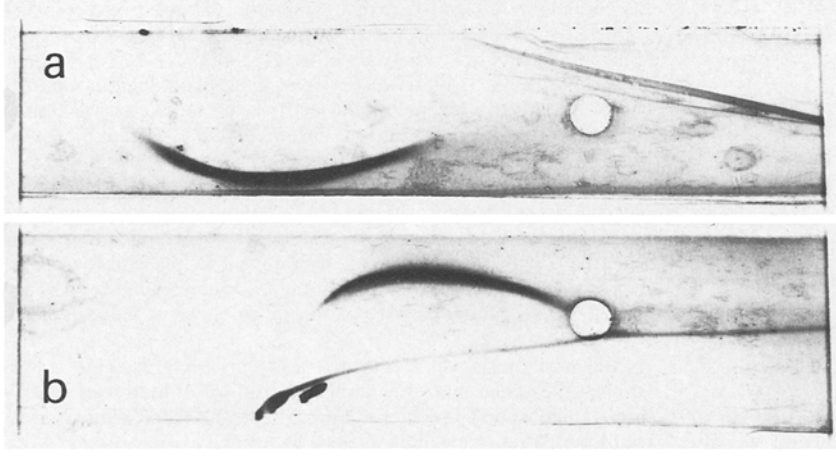

Fig. 1 a and b. Immunoelectrophoretic behaviour of glomerular basement membrane antigen excreted in urine. **a** α-1-mobility which was observed in 10 diabetic children. **b** α-2-mobility which was observed in 10 of the diabetic and all the healthy children

behaviour, with a shift to the α-1-region. Incubation of native GBM with glucose caused a significant increase of thiobarbituric acid positive material compared with the native GBM split products ($p < 0.01$; Table 3).

Discussion

We have demonstrated altered immunoelectrophoretic behaviour of urinary GBM proteins from healthy children after incubation with glucose, resulting in α-1-mobility which was also observed in a number of diabetic children. A change in immunoelectrophoretic mobility was also observed by GBM split products isolated from human kidneys following non-enzymatic glycosylation. The significant increase of thiobarbituric acid assay positive GBM split products after incubation with glucose indicated that non-enzymatic glycosylation of GBM protein had occurred.

Glucose, bound to GBM protein probably by a ketoamine linkage, may be incriminated in pathological alterations of diabetic GBM. Amino acids, e.g. lysine residues, could serve as acceptors for glucose molecules.

We conclude from our study that hyperglycaemia in diabetes might cause non-enzymatic glycosylation of GBM proteins which are excreted in the urine. Whether this increased basement membrane glycosylation contributes causally to the morphological changes and functional disturbances in diabetic nephropathy requires further investigation.

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