Originals

Sorbinil does not prevent galactose-induced glomerular capillary basement membrane thickening in the rat

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Summary. We investigated the role of the polyol pathway in the pathogenesis of glomerular basement membrane thickening in galactosaemic rats, an animal model that develops basement membrane lesions comparable to those of human diabetic subjects. Normotensive Wistar-Kyoto rats fed a 30% galactose diet for nine months developed significant glomerular basement membrane thickening by comparison with rats on a control test diet ($p = 0.008$). However, addition of an aldose reductase inhibitor, sorbinil (250 mg/kg diet), to the galactose diet did not prevent the increase in glomerular basement membrane thickness. Furthermore, by using a quantitative electron microscopic immunogold technique, we examined biochemical alterations in the composition of glomerular basement membranes in this animal model. The labelling density (comparable to relative concentration) of collagen type IV in thickened glomerular base-

ment membranes of galactosaemic animals was significantly increased by comparison to those of control rats ($p = 0.015$). However, there was no significant difference in labelling densities of laminin and heparan sulfate proteoglycan core protein of these animals. Thus, our results indicate that an increase in glomerular basement membrane thickness accompanied by an increase in the labelling density of collagen type IV occurs in the galactosaemic rats, but this thickening is not prevented by sorbinil at the dose used in this experiment. Our study raises the strong possibility that glomerular basement membrane thickening in galactosaemic rats may not be due to excessive polyol pathway activity.

Key words: Basement membrane, galactosaemia, aldose reductase inhibitors, glomerulus, microvessels.

The polyol pathway has been implicated in the development of various complications of diabetes such as cataract, retinopathy and neuropathy [1, 2]. Whether such a mechanism plays an important role in the pathogenesis of diabetic nephropathy remains controversial. The enzyme, aldose reductase has been immunocytochemically localized in glomerular podocytes [3] and cultured mesangial cells [4], and elevated concentrations of galactitol in the renal cortex of galactosaemic rats [5] and sorbitol in the renal papillae in diabetes [6] have been described. Although several investigators have shown that administration of aldose reductase inhibitors prevents renal hyperperfusion and increase in glomerular filtration rate in diabetic rats [7–9], one report failed to show such effect in these animals [10]. However, aldose reductase inhibition does not prevent glomerular basement membrane (GBM) thickening in diabetic rats [11–13].

To examine further the relationship of the polyol pathway to diabetic nephropathy, we fed rats a diet containing 30% by weight galactose for nine months. These animals

develop thickened retinal and cerebral capillary basement membranes comparable to those found in diabetic animals and humans [14–16]. Glomerular hyperperfusion has been reported in the galactosaemic rats [17], and GBM thickening has been described in long-term galactosaemic dogs [18]. Both retinal and cerebral capillary basement membrane thickening in galactosaemic rats are prevented by the simultaneous administration of aldose reductase inhibitors [14–16]. One group of investigators reported prevention of an important early lesion of diabetic retinopathy, capillary pericyte loss, by giving aldose reductase inhibitors to galactosaemic dogs [19]. However, this has not been confirmed by others [20]. In the present study, we examined the GBMs of galactosaemic rats by quantitative electron microscopy in the presence and absence of an aldose reductase inhibitor. Using a quantitative electron microscopic immunocytochemical technique, we investigated the relative concentrations of various macromolecular components of GBMs in these animals. In a previous study, we used this technique to demonstrate increases in

collagen type IV and laminin in retinal capillary basement membranes in galactosaemic rats, and the prevention of these changes in galactosaemic rats that were simultaneously fed an aldose reductase inhibitor [21].

Materials and methods

Animals

Fifteen normotensive Wistar-Kyoto female rats (Taconic Farms, Germantown, NY, USA) at six weeks of age (body weight about 100 g) were used in this study and maintained on different diets for nine months. The animals were divided into three groups (five animals in each group), and each group was given a different type of diet: a) the standard Ralston Purina (Purina Mills, Richmond, Ind., USA) test diet (carbohydrate, 61%; protein, 19%; fat, 10%; fiber, 3%; vitamins and minerals) (control animals), b) a diet in which the major carbohydrate source, dextrin was replaced by D-galactose to 30% by weight of the diet, and c) a diet of 30% galactose-plus-sorbinil (250 mg/kg diet; a gift from Dr. N. Hutson, Pfizer Central Research, Groton, Conn., USA). The food consumption of these rats was approximately 15 g per day per animal, and each animal in the sorbinil treated group received about 3 mg sorbinil per day.

Tissue fixation and processing

Animals were maintained on the dietary protocols for nine months, and then killed by barbiturate anaesthesia followed by bilateral pneumothorax. The kidneys from these animals were excised, and small pieces of cortical tissues from the upper poles of the right kidneys fixed in 3% formaldehyde and 0.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.4, for 2 h at 4°C, while the rest of the kidney tissues were frozen for other biochemical assays. Tissues were washed in 0.5 mol/l NH₄Cl in buffer for 1 h, dehydrated in increasing concentrations of methanol and embedded in LR White (Polysciences Inc., Warrington, Pa, USA). Polymerization was carried out in tightly capped gelatin capsules at 45°C for three days. One micron thick sections were cut, and random selection was done to find the centermost intact glomeruli in the block [22]. Ultrathin sections were then obtained, mounted on 200-mesh nickel grids, and stored in the desiccator until further use for immunogold staining.

Primary antibodies

The rabbit antiserum against mouse Engelbreth-Holm-Swarm (EHS) tumour collagen type IV (BioDesign, Kennenbunkport, Me, USA) has been characterized by RIAs as described elsewhere [23]. The specificity of the rabbit antiserum against mouse EHS tumour laminin (Polysciences) has been determined in our laboratory by immunoblotting. The rabbit antiserum against heparan sulfate proteoglycan (HSPG) extracted from EHS tumour (a gift from Dr. J. Hassell, Department of Ophthalmology, University of Pittsburgh, Pittsburgh, Pa, USA), was passed through a column of laminin bound to Sepharose CL-4B, and then adsorbed to a second column of proteoglycan and finally eluted [24]. This antibody was specific for the HSPG protein core, and did not react with either laminin or collagen type IV.

Electron microscopic immunocytochemistry

Ultrathin sections on grids were floated on drops of water for 5 min, and then transferred to drops of the blocking reagent, containing 1% bovine serum albumin (BSA) (Janssen Pharmaceutica,

Piscataway, NJ, USA) and 5% normal goat serum (NGS) (Vector Laboratories Inc., Burlingame, Calif., USA) in 20 mmol/l Tris buffered 0.9% NaCl solution (TBS), pH 7.4 for 30 min at room temperature. Grids were then incubated on drops of specific primary antibodies diluted in 20 mmol/l TBS containing 1% BSA and 1% NGS, for 2 h. The following dilutions of primary antibodies were used: collagen type IV (1:10), laminin (1:5) and HSPG core protein (1:15). After several washes with TBS, containing 0.1% BSA, grids were floated on drops of goat anti-rabbit IgG labelled with 10 or 15 nm gold particles (Janssen) diluted 1:20 in TBS, pH 8.2, containing 1% BSA, for 1 h. After washes with TBS and distilled water, sections were further stained with uranyl acetate and lead citrate, and examined in a Philips 300 transmission electron microscope.

Sections incubated with non-immune sera served as controls. All the tissues from the three different groups of animals were fixed and processed on the same day in identical ways. For each individual antibody, all the sections were immunostained on the same day in the same way. This was very important for our quantitative immunocytochemical study for comparison purposes. After several trials of different concentrations of primary and secondary antibodies, the optimal concentrations were worked out so that there was maximal specific staining on the basement membranes and minimal background staining.

Quantitative morphometry

For each antibody staining, 20 capillaries from each group of animals (four capillaries from each animal) were photographed at the same magnification of $\times 5,900$, and enlarged to the final magnification of approximately $\times 16,225$ during printing. From each animal, two blocks of kidney tissues were chosen at random, and in each block two capillaries in the centermost glomerulus were chosen. Only those capillaries, whose major axes did not differ in length by more than a factor of two, and with sharply delineated borders of basement membranes, were photographed in this study. As the GBM width is highly influenced by section angle, the sharpness of both basement membrane borders served as a criterion for perpendicularity to the plane of section [25]. The exact magnification of the photographs was calculated using electron micrographs of a diffraction grating replica (2,160 lines per mm; Polysciences). Quantitative morphometry was done on these prints by an observer, unaware of the groups of animals from which they were chosen. An Apple IIe microcomputer, equipped with BioQuant image analysis software (R & M Biometrics, Nashville, Tenn., USA) and a HiPad digitizer tablet (Houston Instruments, Division of Bausch and Lomb, Austin, Tex., USA) was used for quantitation.

By outlining the borders of basement membranes, basement membrane area (BMA) and basement membrane perimeter (BMP; taken by summing both the inner and outer perimeters of all basement membranes) were measured for each capillary. The beginning of the mesangium was determined on the criterion of disappearance of parallelism between the endothelial and epithelial cells [26], and the GBM interposed between the mesangium and epithelial cells was excluded from measurement. Thus, only the peripheral glomerular capillary basement membrane was included for quantitation. Next, the basement membrane length (BML) and the relative basement membrane thickness (BMT) were determined as follows:

$$\text{BML} = \text{BMP}/2$$

$$\text{BMT} = \text{BMA}/\text{BML}$$

The total number of individual gold particles distributed over the basement membranes for each antibody was counted. The labelling density for each antigen could then be expressed as the number of gold particles per unit area of basement membrane. Labelling densities for each antigen were then compared among these groups of animals. Occasionally, clusters of gold particles were found on the basement membranes, and these were excluded from counting.

Fig. 1 a–c. Immunogold labelling for collagen type IV in glomerular basement membranes in rats on **a** control diet, **b** galactose diet, and **c** galactose plus sorbinil diet. Antigenic sites are specifically labelled with 15 nm size gold particles. P = podocyte; E = endothelium; L = lumen of capillary. Bar, 0.5 μm

Statistical analysis

As four capillaries were chosen per animal, the individual measurements were averaged for each animal, and then these values were averaged for five animals in each experimental group to obtain the means and SD used for the final calculation of significance. The unpaired Student’s *t*-test was used to determine the significance of differences among the different groups of animals.

Results

Basement membrane thickness

Galactose-fed rats showed a significant increase in basement membrane thickness of glomerular capillaries (both peripheral and central) compared to rats that were on the control diet (*p* = 0.008; Table 1; Figs. 1–3). The thickening of the basement membranes was evenly distributed. In the galactose plus sorbinil-treated group, GBM thickness was not significantly different from that in the galactose-fed group (Table 1) (Figs. 1–3).

Labelling densities of basement membrane components

Sections stained with collagen type IV antibody showed specific localization of gold particles throughout the thickness of the glomerular basement membranes

(Fig. 1 a–c). The intensity of labelling in the mesangium was always much higher than that in the basement membranes. The labelling density for collagen type IV in the galactose-fed animals was significantly higher than that in the control group (*p* = 0.015) (Table 2). Capillaries in the sorbinil-treated animals did not show any significant difference in labelling density for collagen type IV in comparison with capillaries from the galactose-fed animals.

The localization of laminin in the GBMs was also very specific, and the labelling was found throughout the thickness of basement membranes (Figs. 2 a–c). The labelling density for laminin in galactose-fed animals was not significantly different from that in either the control animals or the sorbinil-treated animals (Table 2).

Table 1. Glomerular basement membrane thickness (GBMT) in three groups of animals, each group composed of five animals (mean ± SD in nm)

Diet	GBMT
Control	314.0 ± 22.62
Galactose	416.6 ± 61.63 ^b
Galactose + Sorbinil	389.4 ± 20.17 ^{a, c}

^a No significant difference from galactose value; ^b differs from control value (*p* = 0.008); ^c differs from control value (*p* < 0.001)

Fig. 2a–c. Immunogold labelling for laminin in glomerular basement membranes in rats on **a** control diet, **b** galactose diet, and **c** galactose plus sorbinil diet. Antigenic sites are labelled with 10 nm size gold particles. P = podocyte; E = endothelium; L = lumen of capillary. Bar, 0.5 μ m

The labelling for HSPG core protein in the GBMs was specific, but did not show any definite distribution (Figs. 3a–c). There was no significant difference in labelling densities for HSPG core protein in glomerular basement membranes among the three groups of animals (Table 2).

All of the animals fed galactose only developed cataracts at the time of killing; most of them had cataracts within four months on the diet. None of the animals on control and galactose-sorbinil diets had cataracts.

Table 2. Labelling densities for macromolecular antigens in glomerular capillary basement membranes in three groups of animals, each composed of five animals (Mean number of gold particles/ μ m² basement membrane area $\pm$ SD)

Diet	Collagen IV	Laminin	HSPG core protein
Control	7.59 $\pm$ 1.34	60.53 $\pm$ 10.84	7.79 $\pm$ 3.33
Galactose	14.21 $\pm$ 4.64 ^a	59.20 $\pm$ 11.91 ^b	5.40 $\pm$ 0.89 ^b
Galactose + Sorbinil	12.41 $\pm$ 0.53 ^{c,d}	42.31 $\pm$ 12.04 ^c	5.59 $\pm$ 2.03 ^e

^a Differs significantly from the control value ($p = 0.015$); ^b No significant difference from either control or sorbinil values; ^c Differs from the control value ($p < 0.001$); ^d No significant difference from the galactose value; ^e No significant difference from the control values. HSPG = Heparan sulfate proteoglycan

Discussion

Our results demonstrate that nine months of experimental galactosaemia in rats for nine months induces GBM thickening as it does in capillaries of the retina [14, 15] and the cerebral cortex [16]. Long-term galactosaemia has also been reported to produce similar GBM thickening in dogs, although other typical features of diabetic nephropathy such as mesangial expansion, glomerular obliteration and renal hypertrophy were absent in these animals [18]. In a relatively short-term study (seven weeks), it has been shown that galactose-fed rats develop functional abnormalities, including increases in urine volume, creatinine clearance, urinary albumin excretion and urinary N-acetyl glucosaminidase similar to those seen early in the course of diabetic nephropathy [27].

However, GBM thickening in galactosaemic rats could not be prevented by sorbinil at a dose that prevents thickening of capillary basement membranes in the retina [14, 15, 20] and the cerebral cortex [16]. Our study is the first demonstration, to our knowledge, that an aldose reductase inhibitor fails to prevent thickening of any microvascular basement membrane in experimental galactosaemia. Recent studies in rats with streptozotocin-induced diabetes have also shown that aldose reductase inhibitors fail to prevent glomerular structural changes including thickened GBMs [11–13]. While one study failed to show

Fig. 3a-c. Immunogold labelling for heparan sulfate proteoglycan (core protein) in glomerular basement membranes in rats on **a** control diet, **b** galactose diet, **c** galactose plus sorbinil diet. Antigenic sites are labelled with 15 nm size gold particles. P = podocyte; E = endothelium; L = lumen of capillary. Bar, 0.5 μ m

any effect of aldose reductase inhibitors on increased mesangial fractional volume in diabetic rats [12], another study demonstrated that these drugs could prevent mesangial expansion in diabetic rats on a high protein diet [11]. Recently, Mauer et al. reported that sorbinil can decrease GBM width in normal rats, although the mechanism is not known [8]. The disparity in effects of aldose reductase inhibitors in different tissues indicates that the polyol pathway may not be involved in the pathogenesis of GBM thickening in galactosaemic rats, even though it appears to have a role in the basement membrane thickening that occurs in the retinal and cerebral cortical capillaries of these animals. Experiments on aldose reductase inhibition and renal haemodynamics have shown mixed results. Administration of sorbinil or *myo*-inositol partially decreases the elevated glomerular filtration rate in diabetic rats [7–9, 28], whereas other investigators were unable to show any effect of aldose reductase inhibition [10] or *myo*-inositol supplementation [29] on hyperfiltration in diabetic rats.

Our immunogold experiments indicate that the labelling density of collagen type IV in the GBMs of galactosaemic rats is significantly increased ($p = 0.015$) by comparison with the control rats, although there was no change in the labelling density of laminin and HSPG core protein. We have described increases in labelling densities of collagen type IV and laminin in basement membranes

in the retinal capillaries of galactosaemic animals [21]. Our results are in agreement with the findings that increased levels of collagens occur in the renal glomeruli of rats in experimental diabetes [30, 31]. Kanwar and Farquhar [32] have shown that the array of negatively charged HSPG residues in the GBMs provide an effective filtration barrier, and their loss in glomeruli of diabetic animals [33] and human diabetic subjects [34] might be a major cause for the breakdown of this barrier. However, a different study has shown that there is an increased incorporation of $^{35}\text{SO}_4$ into proteoglycan of the basement membrane-producing murine EHS tumour grown at high glucose concentrations [35]. Cohen, et al. have demonstrated undersulphation in GBMs in experimental diabetes, that was independent of the polyol pathway activation [36]. Although we did not find any change in the labelling density of HSPG core protein in our experimental animals, our results do not exclude the possibility that there might be alterations in the degree of sulphation of the heparan sulphate moiety of the HSPG and possibly the number of anionic sites in the GBMs of galactosaemic rats without any change in the concentration of the HSPG core protein.

The immunogold technique used in our study does not permit determination of the absolute concentrations of various components of the GBMs, but it does allow us to compare the relative concentrations of individual compo-

nents among different groups of experimental animals. The labelling density of antigens in the post-embedding immunogold technique is independent of the thickness of the sections and penetration of antibodies as only those antigenic sites exposed at the surface of the sections react with the antibodies and are visualized as gold particles [37]. As some of the epitopes might be affected during fixation and processing, all tissue samples were fixed, processed and embedded identically, and also sections were stained immunocytochemically in the same way. By using such a standardized protocol, one can effectively compare the relative concentrations of a specific antigen in similar tissue sites [37, 38]. That the amount of gold labelling for antigens obtained with the immunogold technique correlates well with the concentrations of antigens estimated with biochemical [39, 40] or RIAs [41] supports the validity of the quantitative electron microscopic immunogold technique.

The precise biochemical mechanisms involved in thickening of the GBMs in diabetes and galactosaemia are not known. It is unclear whether the increase in collagen type IV in thickened basement membranes in capillaries of the kidneys, and the retina [21] in galactosaemic rats is due to increased synthesis or decreased degradation of the basement membrane macromolecules. Increased enzymatic [41] or non-enzymatic [42] glycation that results in post-translational modification of proteins may play an important role in the pathogenesis of GBM thickening in diabetes or galactosaemia. However, other results suggest a direct effect of hexose levels on the genome. Elevated levels of laminin B1 mRNA have been reported in the kidneys of streptozotocin-induced diabetic rats [43], and increased expression of collagen type IV and fibronectin [44] mRNA have been described in cultured endothelial cells exposed to high glucose concentrations.

In summary, our study demonstrates that an increase in thickness together with an increase in relative concentrations of collagen type IV takes place in the GBMs of galactosaemic rats, but treatment with an aldose reductase inhibitor does not prevent the GBM thickening. The polyol pathway, therefore, seems an unlikely biochemical cause for this lesion. The biochemical mechanism that is responsible for the pathogenesis of these changes in the kidneys of diabetic and galactosaemic animals remains to be determined.

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