Prevention of Diabetic Glomerulopathy in Streptozotocin Diabetic Rats by Insulin Treatment

The Mesangial Regions

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Summary. A quantitative morphological study of the mesangial regions has been performed on the kidneys of two groups of insulin treated diabetic rats 6 months after the induction of diabetes. In one group reasonably good control of plasma glucose levels (182 ± 20 (SD) mg/100 ml at 0800 h; 95 ± 35 mg/100 ml at 2300 h) was achieved. This group showed no mesangial changes when compared to a non-diabetic control group. In the second diabetic group poor control was intended (plasma glucose 452 ± 41 mg/100 ml and 555 ± 86 mg/100 ml respectively). The following differences were noted when this group was compared to the non-diabetic controls and to the rats in which the blood glucose was well-controlled: 1. Increase in total mesangial volume per glomerulus by 42% and 38% ($2p = 0.025$ and 0.037); 2. Increase in the total amount of basement membrane-like material (BMLM) per glomerulus by 30 and 27% ($2p = 0.030$ and 0.046); 3. Increase in the total mesangial cell volume per glomerulus by 46 and 43% ($2p = 0.033$ and 0.048); 4. Increase in volume of electron dense material in the BMLM by about 200% compared to both groups ($2p = 0.001$ and 0.0003). The study has shown that the mesangial regions also are involved in the diabetic glomerulopathy of experimental diabetes. The morphological changes including increased amounts of basement membrane material are prevented by proper glycaemic control.

Key words: Mesangial region, streptozotocin diabetes, glucose control in rats, diabetic glomerulopathy, stereology, insulin treatment.

It has previously been shown that kidneys and glomeruli become enlarged over a 6 months period in rats with constant high plasma glucose levels [27].

Glomerular basement membranes (GBM) were also significantly thicker than in non-diabetic control rats [28]. There is also evidence of mesangial changes in experimental diabetes in the rat [11, 13, 19, 34, 35], similar to those found in humans [21, 23, 24, 35]. The mesangial changes due to experimental diabetes are described as an increase of the mesangium, with increased amounts of basement membrane-like material (BMLM). The mesangial regions also become strongly reactive to periodic acid-Schiff (PAS) and with the immunofluorescent technique a positive reaction to immunoglobulin and complement is seen.

Consequently, the experimental diabetic rat was considered an acceptable model for studying the prevention of diabetic mesangial changes using morphometric techniques. The cationic stain Alcian Blue [31], which reveals the surface coat of the glomerular cells [2], is also bound to the mesangial regions of diabetic and non-diabetic rats [25] and this electron microscopic histochemical technique was also used in the present study.

Material and Method

Detailed description of the animals, their treatment and the tissue sampling procedure has been given previously [27, 28]. In summary, streptozotocin diabetes was induced by administering 90 mg/kg intravenously. Three groups of female Wistar rats were used. In one group of 8 streptozotocin diabetic rats good diabetic control was planned. These animals constitute the 'well-controlled' diabetic group. In another group of rats poor control was intended and this group of rats is referred to as the 'poorly-controlled' group. These degrees of control were achieved by administering different amounts of insulin to the two diabetic groups [26], 7.7 ± 2.2 U/kg/day and 1.5 ± 0.2 U/kg respectively. The daily insulin dose was governed by plasma glucose measurements. A special non-commercial, very long-acting Ultralente preparation (NOVO) [32] was administered once a day. The two diabetic groups were compared with a non-diabetic control group, age and weight matched with the diabetic rats at the start of the experiment.

Fig. 1. Low magnification electron micrograph of part of a mesangial region, illustrating the localization of the electron dense (ED) material in the basement membrane-like material (BMLM). The mesangial cell is placed in the centre. The cell membrane (arrowheads) can be followed around the cell. Outside the cell, in the BMLM, the ED (arrows) can be seen. The full line is the border between the mesangial BMLM and the basement membrane of the capillary (BM). The dotted line is the border between the mesangial region and a capillary (CAP) lined by endothelial cells (EN). N = nucleus of the mesangial cell. C = cytoplasm of the mesangial cell. FP = foot processes of the epithelial cells. US = urinary space

Six months after the onset of diabetes the left kidney was fixed by retrograde perfusion via the aorta. The kidney was perfused first with 1% glutaraldehyde in a modified Tyrode solution [17, 28] and then continued for 10 minutes with the addition of 0.5 g/100 ml of the copperphthalocyanine, Alcian Blue (8 GX), pH 7.1 [31]. Four control experiments were performed without the addition of Alcian Blue and four experiments performed with Alcian Blue solution at pH 2.5. Tissue was processed for electron microscopy and the mesangial regions were studied in 3 glomeruli from each animal.

In the present study the mesangial regions have been defined as the areas delineated towards the basement membrane of the capillary at the place where the endothelial and epithelial side of the basement membrane material start to run parallel. Towards the capillary it is bordered by the endothelial cells and towards the urinary space by the foot processes of the epithelial cells (Fig. 1). Thus the region consists of mesangial cells and BMLM.

Perfusion of the kidneys with an Alcian Blue containing medium results in the appearance of electron dense, black, areas in the mesangial regions. These are always located in the BMLM very close to the mesangial cells and sparing the most peripheral part of the BMLM towards the urinary space (Fig. 2). If the perfusions are performed without the addition of Alcian Blue the BMLM is seen as an even gray area. In the perfusions at low pH the electron dense areas appear gray.

With point counting [7] the fractional areas of the total mesangium, basement membrane-like material (BMLM), electron dense material due to the Alcian Blue staining, mesangial cell fraction and number of nuclear profiles per glomerulus (= total tuft area

omitting Bowman's space) were measured. The electron dense material is here and in the further estimations of the absolute volumes considered part of the BMLM.

About 1100 points were counted over each mesangial region. All measurements were performed blindly in random sequence by the same technician. A line marking the border between mesangial BMLM and the basement membrane of the capillary and between the endothelial cells and the mesangium was drawn by the author, not knowing the source of the material.

From previous investigations [27] the mean glomerular volume in each animal was known, making it possible to estimate the absolute volumes of the different structures.

Student's t-test for independent samples has been used to compare the groups. Results are presented as mean $\pm$ SD.

Results

The glycaemic control of the two groups of diabetic animals during the experiment is shown in Table 1. Mean plasma glucose concentration at 0800 h was 182 ± 20 mg/100 ml in the 'well-controlled' diabetic rats just before the insulin injection, falling to normal values after 3 hours and remaining at normal values for the next 12 hours. Urine glucose excretion was 0.19 ± 0.10 g/day in the 'well-controlled' diabetic rats. On 62% of all days no glucose was found in the

Fig. 2 A and B. High magnification electron micrographs showing part of mesangial regions. The basement membrane-like material (BMLM) in **A** contains little electron dense material (ED) whereas a large proportion of the BMLM in **B** contains ED. The ED (arrows) is localized close to the mesangial cells and sparing the most peripheral part of the BMLM towards the epithelial cells. Between the ED and the cytoplasm of the mesangial cell (C) the cell membrane can be seen (arrow-heads). The full line is the border between the mesangial BMLM and the basement membrane of the capillary (BM). The dotted line is the border between the mesangial region and a capillary. EN = endothelial cell. N = mesangial cell nucleus

urine. The 'poorly-controlled' rats had constant high plasma glucose levels and a constant high glucose excretion.

The results from the measurements of the mesangial regions are presented in Table 2. A statistically significant increase in the absolute volumes of all the parameters studied was found in the 'poorly-controlled' group compared to both other groups. The relative areas of the total mesangium, BMLM and mesangial cells of the glomeruli were identical in the three groups. ($2p = 0.10$, 0.53 and 0.11 respectively). In contrast, the relative areas of the electron dense material in the BMLM showed significantly different results. The electron dense material constituted 4.1% of the BMLM in the non-diabetic controls, 3.9% in the rats in good control, but 9.9% in the diabetic rats in poor control ($2p = 0.0013$ and 0.0003). The number of nuclear profiles per $100 \mu^2$ mesangial area was the same in the three groups.

Discussion

In these studies the experimentally diabetic rat was chosen because it develops kidney changes with some

similarities to those found in humans [21, 23, 24, 35]. However, the diabetic rat does not develop typical Kimmelstiel-Wilson changes. This discrepancy could be due to the shorter duration of diabetes in the rat. On the other hand there are striking similarities, such as increase in kidney size [22, 27, 29], in glomerular volume [9, 27, 38], in glomerular basement membrane thickness [4, 12, 28, 36], increase of mesangium [10, 14, 19] and basement membrane-like material [1, 35, 37].

An increase in mesangial regions in the diabetic rat has been described before in both light microscopic [10, 14, 19] and electron microscopic studies [1, 35]. However, no detailed quantitation of the mesangial regions has been reported. The quantitative studies presented here confirm the impression found in other studies that diabetic rats develop mesangial changes, such as increase in mesangial areas and increase in BMLM. An increase in total mesangial cell volume was also found in the present study, but without any differences in nuclear densities between the groups. The mesangial regions have thus increased their content of both BMLM and cellular mass. Nothing can be concluded about the

number of cells since the nuclear size is unknown. In the same rats GBMT has been measured and was found to be significantly thicker in the rats in poor control compared to each of the two other groups [28].

The studies have thus shown that streptozotocin diabetic rats develop enlarged mesangial regions with increased BMLM and cellular mass, furthermore, they have shown that these changes can be prevented by proper control of plasma glucose.

After conventional tissue preparation for electron microscopy the BMLM is described as consisting of a peripheral part, ultrastructurally similar to the basement membrane of the capillary and a more central part, close to the mesangial cell which appears to be less compact [8].

In tracer studies particles from the blood stream will be located in these less compact regions in the mesangium and can later be seen within the mesan-

gial cells, indicating that the mesangial regions play a role in the handling of macromolecules [6, 8, 16].

In diabetic rats this same part of the BMLM has been described as containing electron dense areas [1, 35]. Their localization is identical to that of the electron dense material in the present studies, but the electron density is not as pronounced and as well-defined as in preparations stained with Alcian Blue.

Alcian Blue is a copperphthalocyanine, molecular weight 1342 with 2–4 positively charged groups. It most likely binds electrostatically to negatively charged sites in the tissues [31]. Alcian Blue is not visible in itself on the electron micrographs, but when Alcian Blue molecules bound in the tissues are exposed to osmium this complex becomes visible and adds to the electron density in the tissue.

Alcian Blue is used in electron microscopy to enhance the electron density of the surface coat of cells [2]. In glomeruli it binds most strongly to the epithelial cells at neutral pH [2, 5]. In the present studies Alcian Blue is also bound to the epithelial cell coat and the staining complex appears as a uniform dense layer, Fig. 2. No striking differences in the pattern of binding to the cell membranes in diabetic and non-diabetic animals has been observed, but this has not been quantitated.

Alcian Blue has also been used to study the distribution of anionic sites in the basement membrane where Alcian Blue reveals densely stained fibrils [5]. In the present study fibrils are also seen in the mesangial regions in the BMLM under the epithelial cells and close to the mesangial cells.

The nature of the electron dense areas in the BMLM can only be speculated upon. The electron dense material could be abnormal BMLM synthesized by the mesangial cell and to which the Alcian Blue binds in greater amounts than to normal BMLM. It has been shown before [3] that the composition of the basement membranes in diabetics is

Table 1. Blood and urine glucose concentrations and body weight in normal and diabetic rats during the 6 months experiment

	Non-diabetic control rats	'Well-controlled' diabetic rats	'Poorly-controlled' diabetic rats
n	8	8	8
Plasma glucose 0800 h (mg/100 ml)	87 ± 9 ^b	182 ± 20 ^a	452 ± 41 ^b
1100 h	–	120 ± 47 ^c	338 ± 71 ^c
2300 h	–	95 ± 35 ^c	555 ± 86 ^c
Urine glucose (g)	–	0.19 ± 0.10 ^d	10 ± 2 ^d
Body weight at termination of the experiment (g)	310 ± 28	320 ± 27	222 ± 28
Insulin (U/kg/day)	–	7.7 ± 2.2	1.5 ± 0.2

^a In each animal 123 measurements were made. The individual mean values were used to calculate the mean of the group

^b Based on 12 measurements in each animal

^c Based on 2 measurements in each animal

^d Based on 32 measurements in each animal. Values are given as mean ± SD

Table 2. Results from the measurements of the mesangial regions in normal and diabetic rats

	Non-diabetic control rats		'Well-controlled' diabetic rats		'Poorly-controlled' diabetic rats
Mesangium (μ^3) × 10 ³	87.4 ± 17.4	NS	89.7 ± 19.9	*	124.0 ± 38.8 *
BMLM (μ^3) × 10 ³	23.8 ± 4.6	NS	24.5 ± 4.4	*	31.0 ± 7.0 *
Mesangial cell volume (μ^3) × 10 ³	63.6 ± 13.3	NS	65.2 ± 16.5	*	93.0 ± 33.7 *
Electron dense material (μ^3) × 10 ³	0.98 ± 0.33	NS	0.97 ± 0.46	●	3.08 ± 1.19 ●
Number of nuclear profiles per 100 μ^2 mesangial area	1.83 ± 0.30	NS	1.84 ± 0.41	NS	1.56 ± 0.50 NS
Number of animals	8		8		8
Number of glomeruli per animal	3		3		3

The values in the table are given as absolute amounts per mean glomerulus per animal. Values are given as mean ± SD. * and ● between the columns refer to comparison between the two columns.

* and ● after the last column refer to comparison between the first and the last column. * 2P < 0.05; ● 2P < 0.01

abnormal. The basement membrane preparations on which the analyses are made, however, include the mesangial BMLM where the present studies have revealed a structural abnormality. Whether these biochemical and histochemical changes are a manifestation of the same abnormality is not known.

Alcian Blue could also be bound to large molecules for unknown reasons trapped in the BMLM as it is described with the immunofluorescent technique [13, 19].

The Alcian Blue staining could thus be an expression of the phenomenon seen with the immunofluorescent technique but with more precise information about the localization of the abnormal material.

In the studies presented here as in the mentioned studies with the immunofluorescent technique, the abnormalities in the mesangial regions due to diabetes can be influenced by the control state [12, 15, 20].

The mesangial cell has phagocytotic properties and the mesangium is important in clearing the BMLM from deposited aggregated proteins and macromolecules [6, 8, 16, 18]. A disturbance in these functions or an increased demand for processing of macromolecules, could lead to accumulations of substances in the BMLM. It has recently been shown that the mesangial cells in diabetic rats contain more actomyosin filaments than those in normal rats [30], which could be interpreted as an increased demand for processing macromolecules present in the BMLM in the diabetic state.

In conclusion it can be said that the streptozotocin diabetic rat in poor blood glucose control develops mesangial changes, well-known ones such as increase in the mesangium and BMLM, as well as ultrastructural changes revealed by the Alcian Blue technique. If the diabetic rats are kept in good blood glucose control no mesangial changes develop.

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Received: November 16, 1978,
and in revised form: May 4, 1979

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