Ultrastructural and Functional Analyses of Nephropathy in Calmodulin-Induced Diabetic Transgenic Mice

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ABSTRACT

Background: Previous animal models of diabetic nephropathy have used diabetic animals for which the underlying defect was either uncertain or the diabetes was induced by potentially specific toxins. In this report, we describe the renal abnormalities in a transgenic mouse model that develops early-onset diabetes due to overexpression of calmodulin in pancreatic beta cells.

Methods: Renal tissues were collected from normal and transgenic mice at 112, 182, and 300 days. These were prepared for light microscopic observation, stained with polyethyleneimine (for anionic sites), or rendered acellular by detergent extraction prior to observation by transmission and scanning electron microscopy. Morphometric analysis of glomerular basement membrane thickness was carried out by the "orthogonal intercept" method. Twelve-hour urine samples of fed and fasting mice were collected for urine volume and glucose and protein analyses. Blood glucose, blood urea nitrogen, serum insulin, and creatinine were determined in 60–90-day-old and 255-day-old mice by established methods.

Results: Morphometric analyses revealed age-related and transgene-related increases in glomerular basement membrane thickness. A 22% increase in transgenic diabetics over controls was seen at 112 days of age that developed to increases of 43% and 37% at 182 and 300 days of age, respectively. Mesangial matrix area was also increased markedly in transgenic mice. Surprisingly, even in the oldest diabetic mice, there was no reduction in anionic sites. Moreover, despite an eightfold increase in urine volume, these mice did not become significantly proteinuric.

Conclusions: These results indicate that proteinuria of diabetes may be delayed or prevented by maintenance of a normal complement of glomerular basement membrane anionic sites. They also demonstrate that transgenic mice can provide a valuable model for discriminating between different aspects of diabetic nephropathy. Anat. Rec. 247:9–19 © 1997 Wiley-Liss, Inc.

Key words: diabetic nephropathy, glomerular basement membrane, mesangial matrix, anionic sites, diabetic transgenic mice

OVE26 mice are a transgenic diabetic line maintained continuously since 1988 that overexpress calmodulin in pancreatic beta cells (Epstein et al., 1989, 1992). Histology of the pancreas reveals that the calmodulin transgene produces degeneration of pancreatic beta cells (Epstein et al., 1989, 1992). These mice present many characteristics that may make them a particularly valuable, reproducible, and well-defined model of diabetes and diabetic complications. The specificity of the transgene and the insulin promoter allows certainty as to the primary defect in these diabetic mice. Induction of diabetes requires no administration of a beta cell toxin with the incumbent variability and possibility of nonspecificity. Symptoms of diabetes are rapid in onset. Mice demonstrate elevated blood glucose levels within the first days of life and have blood glucose values over 450 mg/dl by 30–55 days of age (Epstein et al., 1989). At this age, they have serum insulin levels that are 42% of controls. Probably due to their lasting residual insulin secretion, these mice can survive at least a year without exogenous insulin or

Received March 22, 1996; accepted July 30, 1996.
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Contract grant sponsor North Dakota Lions Foundation; Contract grant number DK44519.

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other treatments. Thus, these mice require no more care than do any other strain of normal laboratory mice. Husbandry is straightforward because males are usually fertile up to at least 6 months and females are fertile for several months without insulin therapy. In addition, transgenic mice of this line are easily distinguishable from nontransgenic siblings by 2 weeks of age due to the development of obvious cataracts. This occurs due to the coinjection and cointegration of the cataract-inducing GR19 gene (Overbeek et al., 1985; Epstein et al., 1989) with the diabeticogenic calmodulin transgene. Diabetic transgenic mice have been used extensively for the study of the etiology of diabetes (Lipes and Eisenbarth, 1990). However, their advantages have not been employed for the study of diabetic complications.

Diabetic nephropathy is one of the most serious complications of diabetes in humans. Nephropathy is a leading cause of death among diabetics and one-third of American dialysis patients are diabetic (Teutsch et al., 1989). The specific causes of diabetic nephropathy and proteinuria have not been established. It is clear, however, that elevated blood glucose is one important parameter in its development. Nevertheless, it cannot be the only factor because different patients with similar blood glucose levels often have completely different renal outcomes. Characteristics of diabetic nephropathy include both morphological and functional abnormalities. Major morphological abnormalities include increased mesangial matrix (MM) volume, thickened glomerular basement membranes (GBM), and reduced anionic sites in these and other renal extracellular matrices. Functional characteristics include alterations in glomerular filtration rate and proteinuria. The OVE26 model of diabetes presents an alternative model for investigation of diabetic nephropathy particularly because of the rapid onset of hyperglycemia and the fact that no expression of the calmodulin transgene could be detected in the kidney. In this report, we describe several features of kidney pathology and selected parameters of function in the diabetic transgenic line that includes both similar and contrasting characteristics of human diabetic nephropathy.

MATERIALS AND METHODS

The initial production and characterization of the diabetic OVE26 line was previously described (Epstein et al., 1989, 1992). OVE26 mice were maintained by mating transgenic males to ICR outbred females and were identified by the presence of cataracts. Control mice were nontransgenic animals from the same strain. The mice were maintained on a 12-hr light–dark cycle. Animals received continuous access to Purina rodent lab chow and water. All animal treatment was in accordance with NIH Guide for the Care and Use of Laboratory Animals.

Control and transgenic 112-, 182-, 225-, and 300-day-old mice were killed by cervical dislocation, after which kidneys were removed and placed in normal saline on ice. Three to six mice in each group were killed. Renal cortical tissues were prepared for microscopic examination as previously described (Carlson and Kenney, 1980a,b). Briefly, renal capsules were incised and removed to expose the underlying cortex. Kidneys were then longitudinally divided and sliced to 2 mm in thickness with a razor blade. The medullary portion of each section was removed and discarded, and the remaining cortex was minced and divided into three aliquots. The first aliquot was used for light microscopic studies, and the second was fixed immediately for electron microscopy and served as a control for acellular preparations. The cellular components in the third aliquot were solubilized by sequential detergent extraction (Carlson et al., 1978). This procedure resulted in translucent acellular tissue blocks that subsequently were prepared for observation by transmission (TEM) and scanning (SEM) electron microscopy.

Microscopic Technique

Aliquot 1 was placed in 10% neutral buffered formalin for 24 hr at room temperature, dehydrated, and embedded in paraffin for conventional light microscopy. Sections were stained with periodic-acid Schiff (PAS) and counterstained with hematoxylin. These were observed and photographed in an Olympus BH-2 brightfield light microscope under oil immersion with an original magnification of 473 diameters.

Aliquots 2 and 3 were fixed for 2–4 hr in Karnovsky’s (1965) fixative at 4°C, pH 7.4. They were then rinsed with 0.2 M sodium cacodylate buffer, postfixed in 1% OsO₄ at 4°C (90 min), rinsed with distilled water, and stained en bloc with 1% tannic acid (60 min) followed by 0.5% aqueous uranyl acetate at 4°C (90 min). These were dehydrated through graded ethanol (and propylene oxide for TEM). Aliquot 2 was prepared for TEM, and aliquot 3 was prepared for both TEM and SEM.

Tissue for TEM was embedded in Epon/Araldite (Carlson and Hinds, 1981) and cured for 48 hr at 60°C. Epoxy blocks were thin sectioned with a diamond knife on a Dupont-Sorval MT2-B ultramicrotome. Sections were mounted on 200-μm mesh naked copper grids and stained with lead citrate (Venable and Coggeshall, 1965) and uranyl acetate (4% in absolute ethanol). Sections were observed and photographed at 2,000–15,000 diameters in a JEOL 100S TEM.

Each sample to be examined by SEM was immersed in Freon 22 (cooled by liquid N₂) for 10 sec and then in liquid N₂ for 20 sec. During immersion in liquid N₂, each tissue block was cleaved with a chilled single-edged razor blade and returned to absolute ethanol. Following drying by Peldri II (Ted Pella) sublimation, samples were mounted on aluminum specimen stubs with colloidal silver and coated with a thin layer of gold-palladium (150 Å) in a Hummer VI sputter coater. Whole and fractured acellular glomeruli were observed and photographed in an Hitachi S-800 field emission SEM at original magnifications of 1,000–25,000 diameters.

Anionic Site Staining

Renal tissues from control and transgenic diabetic mice were prepared for staining with polyethyleneimine (PEI) by a modification of the method of Schurer et al. (1978). Freshly isolated tissue blocks (1–2 mm³) were washed in 0.1 M sodium cacodylate/HCl buffer, pH 7.4 (osmolality adjusted to 300 mOsm with sucrose) and then immersed for 30 min in a 0.5% solution of PEI (1,800 mol. wt.; Polysciences) in 0.9% NaCl, pH 7.3. These blocks were washed twice in cacodylate buffer.
and immersed for 1 hr in 2% phosphotungstic acid in cacodylate buffer, pH 7.3. These tissues were postfixed in 1% OsO₄ (90 min) at 4°C and rinsed in buffer before dehydration and final processing for TEM observation as described above.

**GBM Thickness**

The procedure adopted for morphometry was a modification of the harmonic mean method summarized by Dische (1992) and adapted from Jensen et al. (1979) and Hirose et al. (1982).

Transmission electron micrographs were taken of random peripheral GBMs in acellular preparations of 112-, 182-, and 300-day-old control or diabetic kidneys. Two to four glomeruli were studied for each of the three to six animals in each group. For TEM calibration, a standard cross-grating grid (2,160 lines/mm) was photographed under the same conditions as were the GBMs (×15,000 original magnification). All prints were made at a constant enlarger setting to produce a final print magnification of 40,000 diameters.

A clear plastic sampling line grid consisting of 63 squares (3 cm × 3 cm) was superimposed on 20 micrographs obtained from three to six randomly selected epoxi blocks. Peripheral GBM was marked wherever it was intersected by grid lines (mesangial regions were excluded). A transparent measuring ruler (adapted from Hirose et al., 1982) was laid over electron micrographs at each point, where they were intersected by grid lines, and the perpendicular distances (“orthogonal intercepts”) across GBMs were recorded as “classes” as indicated by the measuring ruler. Approximately 350 measurements were made for each acellular glomerulus. Harmonic mean apparent thicknesses were calculated (Hirose et al., 1982) and then multiplied by 4/3π (Jensen et al., 1979) to remove an expected right-sided skew (Gundersen and Osterby, 1972) and to provide true GBM thicknesses.

**Measurement of Serum and Urine Parameters**

Urine was collected in Nalgene metabolic chambers from 11 a.m. to 11 p.m. During this period, urine was centrifuged and refrigerated approximately every 3 hr to minimize contamination. To obtain fasting urine samples, mice were fasted for 2 hr prior to entering the metabolic chamber and during the time spent in the chamber. Fed and fasting mice had continuous access to water. Urine and serum glucose values were determined spectrophotometrically with a hexokinase assay (Sigma, HK-20). Urine protein was measured with bovine serum albumin as a standard using a Coomassie dye binding protein assay kit from BioRad. Blood samples were obtained from tribromoethanol anesthetized mice (160 mg/kg, i.p.). Serum was prepared by a serum separator apparatus (Becton Dickson). Determinations of serum insulin levels were made by double antibody radioimmunoassay by using a coated tube insulin radioimmunoassay kit (Diagnostic Products) and rat insulin standards (Novo Riolabs). Blood urea nitrogen (BUN) and serum creatinine were measured by an automated method (Cobas Fara, Roche Diagnostic Systems) according to Tiffany et al. (1972) and Larson (1972), respectively.

**Statistical Analyses**

Differences between values obtained from same-age and same-treatment control and transgenic mice were evaluated by Student’s t test.

**RESULTS**

**Light Microscopic Studies of Renal Glomeruli**

Light microscopy (LM) of renal tissues from control and diabetic mice show that as early as 112 days of age there was a significant increase in PAS-positive material in centrolobar regions of glomeruli (Fig. 1A & 1B). Tubular BMs and GBMs also stained positively at this time, but alterations in transgenics were not seen. When kidneys from control 225-day-old mice were observed, an age-related increase in PAS positivity was noted in mesangial regions (compare Fig. 1A with 1C), but even more striking was the major increase in mesangial matrix-related PAS-positive materials in 225-day-old transgenic diabetic mice (Fig. 1C & 1D). In many of the glomeruli, masses of PAS-positive material reminiscent of nodular glomerulosclerosis were present. As a consequence of this early development of glomerular lesions, several electron microscopic experiments and a morphometric analyses of this material were done.

**Electron Microscopic Investigations of Acellular Renal Glomeruli**

In an effort to examine carefully the morphology and disposition of extracellular matrix components of glomeruli (including the MM) from control and transgenic diabetic mice, renal tissues were collected and rendered acellular by sequential detergent extraction. This method yielded TEM and SEM preparations that clearly demonstrated the histoearchitecture of GBMs and MM in control animals and showed their increased thickness and spatial expansion in transgenics. Acellular GBMs in 182-day-old control mice showed typical histoearchitectural arrangements by TEM (Fig. 2A). Peripheral GBMs were thin and formed loops that extended outward from axial regions. MM material was minimal and was limited to centrolobar regions. SEM observations confirmed the TEM analyses and showed minimal MM material, with large patent loops of relatively smooth and regular peripheral GBM (Fig. 2B). In contrast, TEM of 182-day-old transgenic diabetic mice showed heavy depositions of MM material that extended onto the internal (endothelial/mesangial) surfaces of peripheral GBM loops (Fig. 2C). Consequently, GBMs were significantly thicker and irregular. Likewise, by SEM, acellular GBMs from transgenic mice showed increased MM material, which formed fenestrated plates in axial regions and extended onto peripheral GBM loops as a non-uniform layer of extracellular matrix (Fig. 2D). GBMs appeared significantly thicker than did their control counterparts (compare Fig. 2B with 2D).

In an attempt to document the progression of diabetic glomerulopathy in the transgenic mice, electron microscopic studies similar to those done in 182-day-old mice (Fig. 2) were carried out at 300 days of age (Fig. 3). At this time, TEM of control mice showed an age-related thickening of GBMs and an increase in MM material (compare Fig. 2A with 3A). In addition, small
numbers of thin collagen fibrils were located within the MM. SEM also showed that GBMs from control 300-day-old mice were slightly thicker than those from 182-day-old mice (compare Fig. 2B with 3B). MM, however, did not expand significantly in these animals. At 300 days of age, TEM of acellular GBMs in transgenic diabetic mice showed thick, densely irregular peripheral GBM loops and major MM changes including heavy deposition of striated collagen fibrils (Fig. 3C). Likewise, SEM showed fibrillar components within the MM and masses of material that appeared to reduce the caliber of capillary loops and could form the core of glomerulosclerotic nodules (Fig. 3D).

**GBM Thickness**

Because both TEM and SEM examinations of acellular glomeruli suggested age- and diabetes-related thickening of GBMs, a morphometrical analysis of GBM thickness was done. Measurements were made of two to four glomeruli from three to six control or transgenic diabetic mice at 112, 182, and 300 days of age.

Figure 4 shows the results of our studies and indicates that an age-related thickening occurs in control animals where GBMs increase from 77 nm at 112 days of age to 104 nm at 182 days to 141 nm at 300 days. The rate of increase in thickness is much greater in diabetics, however, where GBMs range from 95 nm at 112 days of age to 148 nm at 182 days to 193 nm at 300 days. Together with the qualitative morphological studies described above, these data suggested that significant glomerulopathy was present in the transgenic diabetic mice, the characteristics of which were similar to some of those seen in chronic stages of human diabetic renal disease. Accordingly, serum studies and urinalysis were done to determine some possible func-
Fig. 2.
tional renal deficits that may accompany the structural changes.

**Serum Analyses**

Serum values for glucose, insulin, creatinine, and BUN were obtained in diabetic and normal mice at two age ranges, 60-90 days of age and 225-255 days of age (Table 1). As expected, blood glucose values were elevated significantly above those of control mice for both old and young mice. Serum insulin was reduced relative to control in the diabetic mice at both age ranges. The fact that blood glucose was over four times normal whereas insulin was only reduced by 50% indicated marked insulin resistance in the transgenic mice. Serum creatinine of diabetic mice between 60 and 90 days of age was about 50% higher than normal. This indication of possible renal failure was reinforced by the BUN values that were also approximately twice that of normal. Older diabetic mice also demonstrated elevated BUN and creatinine values. The elevation in these parameters was almost identical to the elevation seen in the younger diabetic mice. Because these mice were more severely diabetic than were the younger transgenics and the older mice had been diabetic for a longer period of time, we would have anticipated a worsening of these values in the older animals; however, this was not seen. The changes in BUN and creatinine were not likely due to body weight differences, which typically were small (data not shown).

**Urinalysis**

Urinalysis was performed on 190-220 day-old diabetic and normal mice (Fig. 5). Initial measurements were made on free-fed mice. Under these conditions, the diabetic mice had almost a tenfold increase in urine volume (Fig. 5A). The enhancement of urine volume appeared to be due to the very high urine glucose levels of the diabetic mice because fasting eliminated most of the elevated urine volume and most of the glycosuria (Fig. 5B). Despite the enormous increase in urine volume and the possible signs of renal failure, these diabetic mice showed only small changes in proteinuria (Fig. 5C). Under fasting conditions, proteinuria of the diabetic was actually lower than the proteinuria of the controls. Most rodent models of diabetes report 400-800% increases in proteinuria (Larson, 1972; Tochino, 1984; Velasquez et al., 1990; Cohen et al., 1995). Thus, despite more than 200 days of diabetes and elevated serum creatinine and BUN, these animals did not clearly exhibit a crucial characteristic of diabetic nephropathy, elevated proteinuria.

The reason for the different levels of proteinuria during fed and fasting states was unclear. Part of this may have been due to incomplete sample collection because the fed/fasting difference was less when normalized to urine creatinine (data not shown).

**Anionic Site Investigations**

PEI is a positively charged molecule that when coupled with phosphotungstic acid is identified in TEM by small electron-dense dots that provide a rough approximation of the location and density of anionic sites in extracellular matrices. In an effort to provide a possible explanation for the surprising lack of proteinuria in late stages of nephropathy in the transgenic diabetic animals, we carried out a TEM study of PEI-positive GBM- and MM-related anionic sites in 300-day-old control and transgenic diabetic mice. The results (Fig. 6) indicated that PEI-positive anionic sites were present in these extracellular matrices in approximately equal numbers in control and transgenic diabetic mice, where intersite distances averaged 70.5 nm and 72.8 nm, respectively.

**DISCUSSION**

A number of transgenic mouse lines have been developed that lead to overt insulin-dependent diabetes mellitus (IDDM). These lines have been used mainly to investigate possible cellular and molecular mechanisms that may initiate the cascade of events leading to the diabetic state (for review, see Lipes and Eisenbarth, 1990). The OVE26 line used in the current study carries a calmodulin minigene regulated by the rat insulin II promoter. This regulation leads to a major overexpression of calmodulin specific to pancreatic beta cells and to subsequent reductions of serum and pancreatic insulin (Epstein et al., 1989). Because this model shows high serum levels of glucose at an early age, does not require exogenous insulin for survival, and does not require injection of potentially nonspecific toxins, it appeared to be an excellent candidate for examining morphological and functional features of diabetic nephropathy.

Our initial observations showed diffuse increased PAS positivity in mesangial regions in transgenic mice as young as 112 days of age. Subsequent PAS studies have shown that mesangial increases are progressive, and at 225 days of age heavy diffuse accumulations were seen. Nodules indicative of the diabetic lesions described by Kimmelsteil and Wilson (1936) were present but small in 225-day-old transgenic mice. An age-related increase in PAS-positive material was evident in control animals (compare Fig. 1A with 1C), but nodular glomerulosclerosis was never seen in these animals. Preliminary data also indicated a trend toward increased kidney weight and glomerular size in older diabetic animals as compared with controls (data not shown). These data strongly suggested that renal involvement in our transgenic mice was substantial.

The age- and diabetes-related increase in glomerular extracellular matrix identified by LM led us to initiate several electron microscopic experiments. Because we had previously extensively used the sequential detergent extraction method (Carlson et al., 1978) for generating isolated GBM and MM starting material for electron microscopic studies in normal (Carlson and Hinds, 1981, 1983; Carlson and Chatterjee, 1983; Carlson and Kenney, 1980a,b) and diabetics (Carlson and Surerus, 1986; Marion and Carlson, 1989), similar techniques were employed in the current study. As in all acellular preparations, GBMs were necessarily de-
fined as detergent-insoluble, electron-dense laminae densae (Carlson et al., 1978) and could not be compared directly with the trilaminar basement membranes seen in conventional cellular preparations (Hay, 1991). It was quite likely, however, that at least portions of electronlucent laminae rarae were present in these preparations because pelleted acellular basement membranes remained separated from each other by "clear spaces" that may be morphological equivalents of laminae rarae seen in cellular control specimens (Carlson and Kenney, 1980a,b).

TEM studies of acellular preparations of normal mouse kidneys showed that at 182 days of age GBMs appeared thin, regular, and ultrastructurally indistinguishable from the laminae densae of GBMs in cellular preparations (Like et al., 1972). Histoarchitecturally, acellular mouse GBMs closely resembled those of the human (Carlson and Kenney, 1982) and could be divided into peripheral portions surrounding capillary lumens and mesangial portions that lined the axial stalk. MM was clearly identifiable at this time and appeared as light condensations of basement membrane-like substances. Similar MM materials have been identified in centrolobular regions of glomeruli in acellular preparations from normal rabbits, rats, rhesus monkeys, and humans (Carlson and Kenney, 1982). SEM studies of these preparations showed strikingly the three-dimensional disposition of MM and, as in other species (Carlson and Hinds, 1983), exhibited irregular ridges and struts extending onto internal surfaces of peripheral GBMs.

When acellular GBMs and MM from normal 182- and 300-day-old animals were examined at the same magnification (compare Fig. 2A & 2B with 3A & 3B), they were histoarchitecturally identical but showed expected age-related increases in thickness and density. In addition, MM in normal 300-day-old mice exhibited striated collagen fibrils, which have been similarly identified in a number of other species (Carlson and Hinds, 1981).

Morphological changes in acellular glomeruli were highly exaggerated in diabetic animals (compare Figs. 2C & 2D with 3C & 3D). Transgenic 300-day-old animals showed major increases in MM matrix when compared with their 182-day-old counterparts, but the differences were even greater when they were compared with glomeruli from control animals. At 300 days of age, MM in diabetic mice was concentrated in nodules comprised of "nests" of striated collagen fibrils and other basement membrane-like materials that, like their human diabetic equivalents (Carlson and Sures, 1986), were located in centrolobular regions of glomeruli. Mouse MM nodules were not as compact as those seen in human diabetic end-stage glomerulopathy but closely resembled those reported in acellular diabetic rhesus monkey kidneys (Marion and Carlson, 1989).

Because our morphological studies of acellular glomeruli strongly suggested age- and diabetes-related thickening in mice peripheral GBMs, a morphometric analysis was done. To our knowledge, careful thickness studies of GBMs in normal and diabetic mice have not been done, although some investigators have reported general increases in GBM width in several strains of spontaneously diabetic mice (Orci et al., 1970; Like et al., 1972; Wehner et al., 1972). Our measurements of

<table>
<thead>
<tr>
<th>Mice</th>
<th>Blood glucose (mg/dl)</th>
<th>Serum insulin (μU/ml)</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>60–90 day db</td>
<td>657 ± 38.9 (4)***</td>
<td>20.9 ± 6.3 (4)</td>
<td>49.8 ± 10.8 (6)*</td>
<td>0.7 ± 0.2 (6)*</td>
</tr>
<tr>
<td>60–90 day con</td>
<td>170 ± 7.1 (6)</td>
<td>38.3 ± 9.9 (6)</td>
<td>28.4 ± 1.3 (10)</td>
<td>0.5 ± 0.0 (10)</td>
</tr>
<tr>
<td>255 day db</td>
<td>803 ± 30.2 (4)***</td>
<td>21.0 ± 2.0 (4)*</td>
<td>41.0 ± 8.1 (3)</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>225 day con</td>
<td>177 ± 12.9 (4)</td>
<td>51.4 ± 15.0 (4)</td>
<td>25.3 ± 0.9 (3)</td>
<td>0.4 ± 0.3</td>
</tr>
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1Values are mean ± S.E. Numbers in parentheses indicate the sample size. All statistical comparisons are between diabetic (db) and control (con) animals at the same age and of the same parameter.

*P < 0.05 by one-tailed Student's t test.

**P < 0.01 by two-tailed Student's t test.

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**TABLE 1. Serum analyses of control and diabetic mice**
acellular GBMs showed a clear increase in GBM thickness with age, with 300-day-old control animals averaging 81.5% more than those at 112 days. By comparison, diabetic mice showed a 103.5% increase over the same time period, but, more importantly, transgenics of all ages showed GBMs that averaged 34.3% thicker than did controls, with a range of 22.5-42.9%.

The foregoing ultrastructural and morphometric data provided convincing evidence that our diabetic OVE26 mouse line exhibited a number of the characteristics of established diabetic nephropathy in human subjects. In fact, GBM thickening and widening of the glomerular mesangium (primarily due to expansion of MM) are constant findings in all human subjects with diabetic nephropathy (Mauer et al., 1981). The most characteristic functional pathology of diabetic nephropathy is proteinuria. In the current investigation, diabetic mice were tested for proteinuria at an age (200 days) when the morphological changes observed were well established. However, diabetic proteinuria was mild or absent regardless of whether the mice were fed or fasted. Most investigators have reported elevated severe proteinuria in rodent models of hypoinsulinemic diabetes (Valesquez et al., 1990). NOD mice showed significant proteinuria shortly after onset of diabetes (Tochino, 1984), but conflicting reports have indicated that BB Wistar rats may (Brown et al., 1983) or may not (Cohn et al., 1987) demonstrate proteinuria despite prolonged diabetes. Interestingly, both of these latter studies showed that the only morphological pathology observed in the glomerulus of the BB rat was GBM thickening, without the increased MM seen in our transgenic mice.

The lack of clearly elevated proteinuria in our transgenic diabetic mice, despite many of the morphological characteristics of late-stage diabetic nephropathy, was indeed enigmatic. In an effort to shed some light on this apparent paradox, a TEM analysis of GBM anionic sites was undertaken. No significant differences in PEI positivity could be shown in 300-day-old control and transgenic mice (Fig. 6). GBMs from 112-, 225-, and 182-day-old mice were also examined and likewise showed no significant anionic site differences in normal and transgensics (data not shown).

We have previously demonstrated that, by preincubating renal tissues in heparitinase (an enzyme specific for heparan sulfate residues), PEI staining was virtually abolished (Rada and Carlson, 1991). Similar experiments were carried out as controls for PEI experiments in the current study with the same results (data not shown), and therefore the relationship of PEI-positive anionic sites to heparan sulfate residues was positively established. This result was important because a number of investigations have shown that reduced

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Fig. 5. Urinalysis of 225-250-day-old control and diabetic mice. A shows urine volume; B shows urine glucose; and C shows urine protein. Detailed procedures are described in Materials and Methods. Nine to 10 mice were used for each experiment. Vertical bars indicated the S.E.M. Asterisk indicates that the diabetic mouse is different from the control mouse under the same feeding conditions, P < 0.025 by Student's t test. Two asterisks indicate that the diabetic mouse is different from the control mouse under the same feeding conditions, P < 0.00002 by Student's t test.
heparan sulfate proteoglycan in GBMs is associated with increased permeability (Kanwar et al., 1980; Rosenzweig and Kanwar, 1982) including significant proteinuria (Kanwar, 1984). Moreover, several TEM studies have shown a loss of heparan sulfate-rich GBM anionic sites in animal models of diabetes with known proteinuria (Chakrabarti et al., 1989; Moriya et al., 1993; Sugimoto et al., 1994).

Therefore, the presence of normal complements of anionic sites in GBMs of OVE26 diabetic transgenic mice may be functionally related to the absence of an otherwise expected proteinuria. From this hypothesis, it may be inferred that the proteinuria of diabetic nephropathy possibly may be delayed or prevented by maintenance of normal distributions and densities of GBM anionic sites.

We conclude that, because the OVE26 transgenic mouse described in the current study expresses a number of pathological features of diabetic nephropathy without an expected proteinuria or loss of anionic sites, it may provide a valuable model for future studies aimed at elucidating and discriminating between structural and functional aspects of diabetic nephropathy.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Jason Graff. Thanks are extended to Julie Horn who typed the manuscript. The work was supported in part by a grant from the North Dakota Lions Foundation (E.C.C.), by PHS grant DK44519 (P.N.E.), and by the Grand Forks Human Nutrition Research Center of the Agricultural Research Service, USDA.

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