Nonproteinuric Diabetes-Associated Nephropathy in the Cohen Rat Model of Type 2 Diabetes

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The Cohen diabetic rat is an experimental model reminiscent of human type 2 diabetes. The aim of this study was to characterize the development of end-organ damage in this model. Cohen diabetic sensitive (CDs) and Cohen diabetic resistant (CDr) rats were fed regular diet or a diabetogenic diet. Glucose tolerance, renal function, and renal and retinal histology were studied at set intervals. CDs fed diabetogenic diet were the only strain that expressed the diabetic metabolic phenotype. In this strain, urinary protein excretion did not increase with the development of diabetes, but plasma urea and creatinine levels increased and creatinine clearance decreased. Light microscopy revealed in CDs enlarged glomeruli with increased mesangial matrix and thickening of the glomerular capillary wall; electron microscopy demonstrated thickened basement membrane and mesangial abundance. There was increased staining for type IV collagen in glomeruli and interstitium of CDs. The retinas of diabetic CDs demonstrated pathology consistent with nonproliferative diabetic retinopathy. The histological findings in the kidneys, the absence of proteinuria, the impairment in glomerular filtration, and the development of retinopathy in CDs are consistent with diabetes-associated nephropathy that is similar to a nonalbuminuric type of nephropathy associated with type 2 diabetes in humans. *Diabetes* 54:1487–1496, 2005

The Cohen diabetic rat is an experimental rodent model reminiscent of human type 2 diabetes (1,2). The model consists of two strains: the Cohen diabetic sensitive (CDs) rat, which invariably develops diabetes when fed a diabetogenic diet, and the Cohen diabetic resistant (CDr) rat, which remains normoglycemic even when fed a similar diet (1). The original colony was established over 30 years ago (2). We recently rebred animals from the original colony and characterized the metabolic phenotype of the new colony (1). We subsequently set forth to investigate whether target organ damage related to diabetes develops in this model. Because nephropathy is among the most common and morbid complications of diabetes, our focus was on the kidney.

Earlier studies on the renal phenotype of this model placed much emphasis on histopathology, but paid little or no attention to kidney function (3–13). Preliminary studies by our group indicated that in the CDs strain, an unusual type of nephropathy evolves with no significant proteinuria but with progressive impairment of kidney function. In the current study, we present the results of our investigation on the development of end-organ damage in animals from the new Cohen diabetic rat colony. We provide histopathological evidence of the diabetes-related renal and retinal lesions and characterize functional aspects of the diabetic renal injury in this model. We demonstrate that the CDs is an experimental model of type 2 diabetes that develops a nonproteinuric type of diabetic nephropathy.

RESEARCH DESIGN AND METHODS

The animals that were used in this study were male CDs and CDr rats that had been recently re-inbred in the Israeli Rat Genome Center in Ashkelon, Israel (1).

Animals were fed either regular rat diet or a diabetogenic diet that induces diabetes in CDs but not in CDr (1). Regular diet consists of a mixture of ground whole wheat, ground alfalfa, bran, skimmed milk powder, and salts, resulting in 21% protein, 60% carbohydrates, 5% fat, and 0.45% NaCl content (Koffolk) and is provided along with tap water ad libitum. The diabetogenic diet consists of a custom-prepared mixture of 18% casein, 72% sucrose, 4.5% butter, 0.5% corn oil, 5% salt no. II USP, distilled water, and fat soluble vitamins and was provided with distilled water ad libitum. Of note, this diet must be copper poor for the animals to develop the diabetic phenotype (1).

Shortly after weaning, animals were fed either regular or diabetogenic diet. Four groups were thus studied: CDs rats fed regular diet or diabetogenic diet and CDr rats fed regular diet or diabetogenic diet. An oral glucose tolerance test (OGTT) was performed at monthly intervals to monitor the development of the metabolic phenotype. Renal function was determined at 1, 3, and 6 months after initiation of the diabetogenic diet. Upon termination of the studies, animals were anesthetized, blood was obtained from the bifurcation

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OGTT, oral glucose tolerance test; PAS, para-amino-salicylate.

of the aorta, and the animals were killed by exsanguination. The kidneys and eyes were then retrieved.

OGTT. Animals were fasted overnight. In the morning hours, animals were placed in individual cages. Blood was obtained from the tip of the tail of the fully awake, unanesthetized animal. Whole-blood glucose levels were determined using a glucose reagent strip and a standard automated glucometer (Elite; Bayer). After the fasting blood glucose level was determined, the animal was loaded by gavage with a solution composed of 3.5 g glucose/kg body wt dissolved in distilled water. Blood glucose levels were measured 15, 30, 60, 120, and 180 min later.

Plasma insulin levels. Insulin levels were measured during the OGTT at fasting (time 0), 30 min (estimated glucose peak), and 180 min (glucose nadir) after glucose loading. Plasma insulin levels were assayed by enzyme-linked immunosorbent assay using an ultrasensitive rat insulin assay (Mercodia, Uppsala, Sweden) that requires only 5 μl plasma per sample.

HbA_{1c}. HbA_{1c} levels were measured at 1, 3, and 6 months of diabetogenic diet. Since the assay required a relatively large blood volume, it was performed only in animals that were killed. Different animals were thus used at each of the time points. Levels were assayed using a kit from Roche Diagnostics on a Cobas Integra system.

Renal function. Renal function was determined by measuring urinary protein excretion, plasma urea levels, and creatinine clearance.

Protein excretion. Urine was collected for 24 h in metabolic cages at monthly intervals. Urinary protein excretion was measured by the microprotein-PR method (Sigma Diagnostics). To determine the albumin fraction, we measured total protein concentration in a select number of samples using the pirogallol red molibdate complex and albumin concentration with bromocresol green.

Plasma urea. Plasma urea was determined in blood samples obtained upon termination of experiments at 1, 3, and 6 months.

Creatinine clearance. Plasma creatinine was determined in blood samples obtained upon termination of the experiments. Urinary creatinine was determined in 24 urine collections in metabolic cages at 1, 3, and 6 months. Creatinine levels in plasma and urine were measured using the standard Jaffe reaction. Creatinine clearance was calculated using the standard clearance formula. Data were normalized for body weight and, when possible, for kidney weight.

Blood pressure. Blood pressure was measured at ambient temperature (26–28°C) in awake animals by the tail-cuff method using the IITC-31 computerized blood pressure device (IITC Life Science, Woodland Hills, CA), as previously described (14). Blood pressure values were derived from at least three replicate measurements.

Histology

Kidneys. Kidneys were removed after 3 and 6 months of diabetogenic diet and studied by light microscopy, electron microscopy, and immunocytochemistry.

For light microscopy, kidneys were fixed in 4% formaldehyde and histology was studied in thin sections stained with hematoxylin and eosin or para-amino-salicylate (PAS). For electron microscopy, kidney sections were fixed in Karnovsky's solution.

Kidney sections were also stained by immuncytochemistry for type IV collagen. Paraffin sections (4 μm) were deparaffinized in xylene, hydrated in gradual ethanol concentrations, and reacted for 1 h at room temperature with a monoclonal mouse anti–human collagen type IV antibody (Zymed, South San Francisco, CA), which has cross-reactivity to the rat. This was followed by incubation with an appropriate biotinylated second antibody for 30 min and with biotin avidin complex–peroxidase for 30 min (Vectastain ABC kit; Vector, Burlingame, CA). The reaction was developed with 3,3′-diaminobenzidine as a substrate. The intensity of the staining was evaluated under light microscopy in 50 glomeruli within each kidney in a semiquantitative manner (0 to +2).

Retina. A series of pilot studies led us to study the retinas of CDs and CDr rats after 6 and 12 months of feeding with diabetogenic diet. The animals were killed and the eyes removed and fixed with a formaldehyde-gluteraldehyde mixture. Blocks were sectioned and stained with hematoxylin and eosin. Serial sections of retinas, starting from the posterior pole of the retina and continuing up to the ora serrata, were examined by light microscopy. The retinal thickness and architecture were compared in CDs and CDr.

Statistics. Data are presented as means ± SE. Between-group analyses were performed using the unpaired Student's *t* test (two-tailed) and ANOVA, as applicable.

RESULTS

Animal survival and body weight. CDs and CDr fed regular diet and CDr fed diabetogenic diet survived the entire 6-month study period and appeared in good health throughout. In contrast, CDs fed diabetogenic diet looked unwell after 1 month and became gradually emaciated; all survived 1 and 3 months, but only 8 of 10 rats survived the full 6 months of the study.

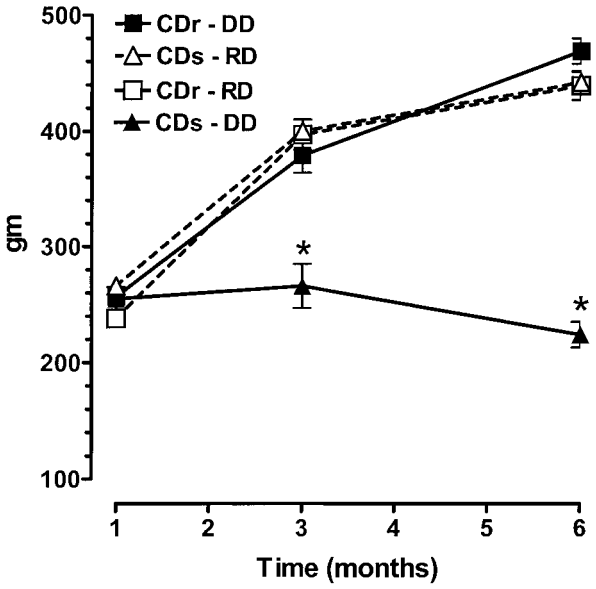

FIG. 1. Body weight of CDs and CDr rats after 1, 3, and 6 months of feeding with regular diet (RD) or diabetogenic diet (DD). Data are presented as means ± SE. *P < 0.01 vs. all other groups.

Body weights of all groups of animals at the different time points are shown in Fig. 1. One month after initiation of diabetogenic diet, there was no difference in body weight between CDs and CDr. CDr fed regular diet or diabetogenic diet and CDs fed regular diet continued to grow thereafter, with no difference in weight between the groups at 3 and 6 months. In contrast, CDs fed diabetogenic diet failed to grow, with their body weight remaining unchanged over the entire study period.

Metabolic phenotype

OGTT. The glucose levels during the OGTT performed after 1, 3, and 6 months of regular diet or diabetogenic diet are shown in Fig. 2. The pattern demonstrated by CDr fed regular diet or diabetogenic diet and of CDs fed regular diet was within a "normal" nondiabetic range. The glucose response was similar between the three groups and without any apparent change over the 6-month study period. In contrast, CDs fed diabetogenic diet already exhibited a diabetic pattern 1 month after initiation of the diet. The area under the curve and glucose levels at 120 and 180 min gradually increased at 3 and 6 months. These findings are consistent with those previously reported in this model of diabetes (1).

Plasma insulin. Plasma insulin levels after overnight fasting and following glucose loading during the OGTT are shown in Fig. 3. One month into the study, basal plasma insulin levels were not different in CDs and CDr. At 3 months, basal insulin rose in CDs and CDr fed regular diet or diabetogenic diet. In contrast, in CDs fed diabetogenic diet, basal insulin levels were very low. The insulin response to the oral glucose load in CDs fed diabetogenic diet for 1 month was similar to that observed in CDr fed diabetogenic diet; at 3 and 6 months, the insulin response was flat. With glucose levels being high during the OGTT and insulin levels being low, the glucose-to-insulin ratio

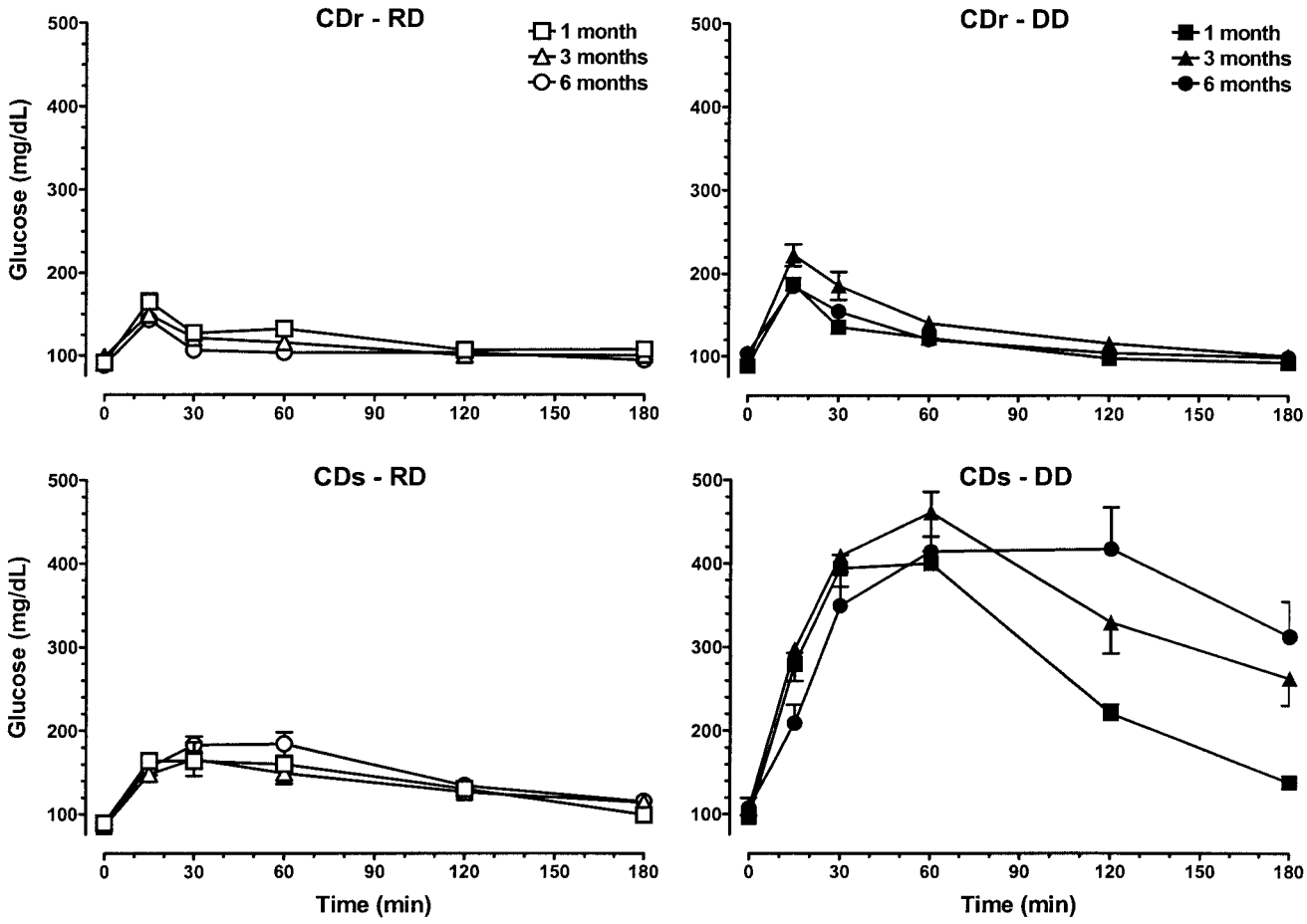

FIG. 2. Results of OGTT in CDr and CDs rats fed for 1, 3, and 6 months with either regular diet (RD) or diabetogenic diet (DD).

was the highest in CDs fed diabetogenic diet compared with all other groups (data not shown). These data indicate an inappropriately low insulin secretion rate in CDs that developed diabetes.

HbA$_{1c}$. In animals fed diabetogenic diet, HbA_{1c} levels were significantly elevated in CDs compared with CDr at 1, 3, and 6 months after initiation of diabetogenic diet, as shown in Fig. 4. In animals fed regular diet, HbA_{1c} levels were 3.91 ± 0.08% (n = 6) in CDs and 4.04 ± 0.04% (n = 7) in CDr at 3 months (P = NS); at 6 months, levels remained similar at 4.09 ± 0.04% (n = 5) in CDs and 4.08 ± 0.01% (n = 5) in CDr (P = NS). These values were not different from those observed in CDr fed diabetogenic diet at 1, 3, and 6 months.

Protein excretion. Total protein excretion by CDs and CDr fed diabetogenic diet for 1, 2, 3, and 6 months is shown in Fig. 5. After 1 month on diabetogenic diet, at which time glucose intolerance had developed in CDs but not in CDr, protein excretion averaged a low 13 ± 1 mg/24 h in CDs (n = 35) and 21 ± 3 mg/24 h in CDr (n = 18) (P = 0.007). Nearly half of the amount of protein excreted was albumin: 6 ± 1 mg/24 h in CDs (n = 16) and 6 ± 1 mg/24 h (n = 11) in CDr. After 2 months on diabetogenic diet, CDs rats expressing the diabetic phenotype continued to excrete a low amount of protein, averaging 12 ± 1 mg/24 h (n = 6). In contrast, protein excretion in CDr, which continued to express a normal OGTT, began to rise, averaging 53 ± 7 mg/24 h (n = 6). After 3 months of feeding with diabetogenic diet, protein excretion in CDs stayed at 12 ± 1 mg/24 h (n = 20) while in CDr rose further to 62 ± 8 mg/24 h (n = 17). Nearly half of the protein excretion consisted of albumin: 7 ± 1 mg/24 h (n = 8) in CDs and 38 ± 10 mg/24 h (n = 10) in CDr. After 6 months of diabetogenic diet, protein excretion in CDs continued to be low at 9 ± 2 mg/24 h (n = 8), 6 ± 2 mg/24 h (n = 5) of which consisted of albumin; in CDr, protein excretion remained elevated at 35 ± 1 mg/24 h (n = 10).

In a control group of animals fed regular diet for 6 months, CDs excreted 7 ± 1 mg/24 h protein (n = 9), which was not different from the amount excreted by the group fed diabetogenic diet. Thus, neither diabetogenic diet nor the development of diabetes in CDs affected the amount of protein excretion. Similarly in CDr, animals fed regular diet for 6 months excreted 30 ± 5 mg/24 h protein (n = 8), which was not different from the amount excreted by CDr fed diabetogenic diet.

Glomerular filtration. Glomerular function was measured after 1, 3, and 6 months of feeding with diabetogenic diet by measuring plasma urea and plasma and urinary creatinine levels and by calculating creatinine clearance.

Plasma urea. One month after initiation of diabetogenic diet, plasma urea levels were higher in CDs compared with CDr (Table 1). At 3 and 6 months, plasma urea increased steadily in CDs and tripled, whereas levels in CDr increased over the same period of time only ~1.5-fold,

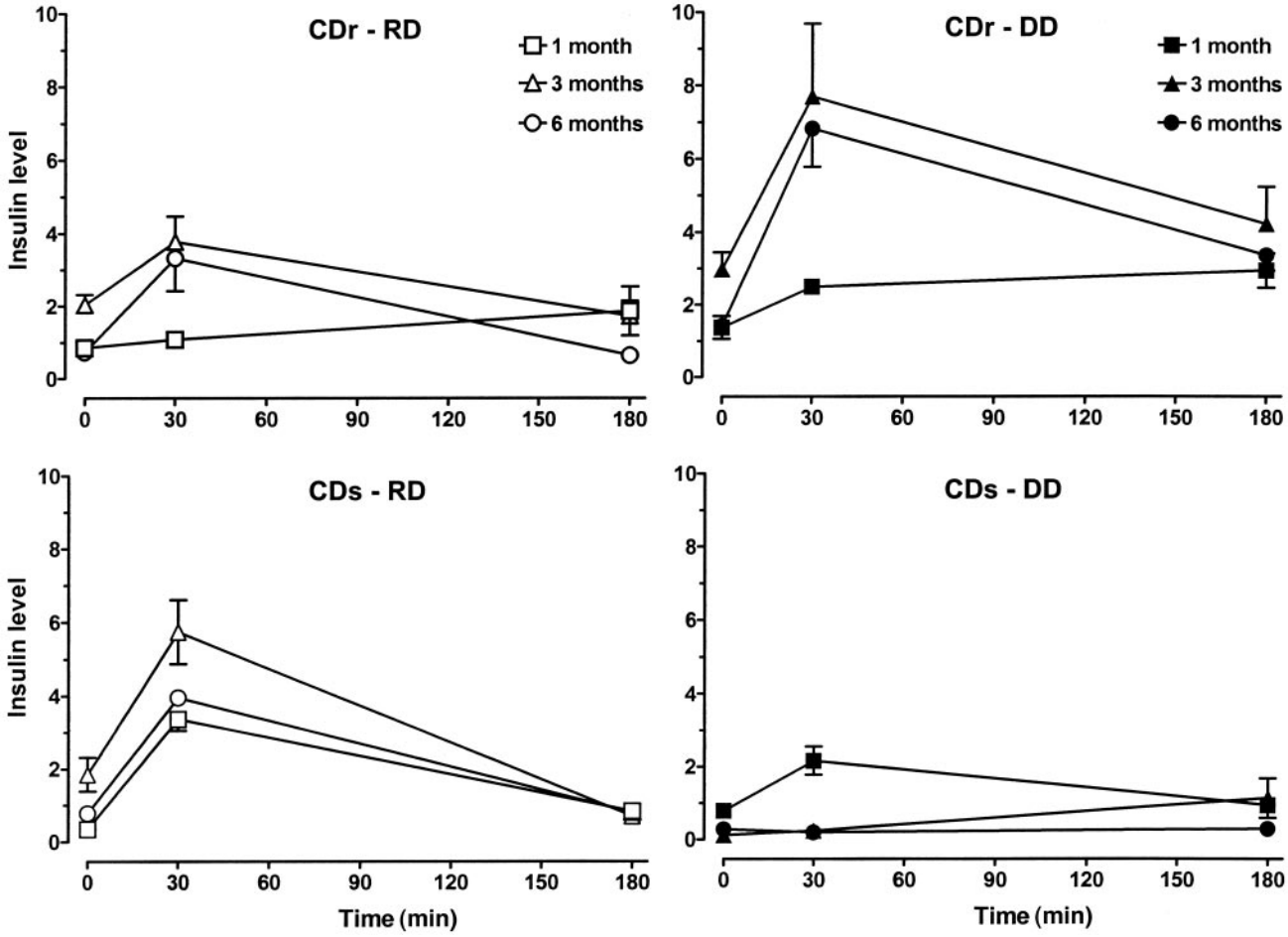

FIG. 3. Plasma insulin levels (units/ml) after overnight fasting (t = 0, 30, and 180 min into the OGTT) in CDr and CDs rats aged 1, 3, and 6 months.

maintaining a statistically significant difference between the groups.

Plasma creatinine. After 1 month of feeding diabetogenic diet and in parallel to the differences in plasma urea levels, plasma creatinine levels were higher in CDs than in CDr (Table 1); this difference in plasma creatinine levels dissipated thereafter and was no longer apparent at 3 and 6 months. Of note, though, is that the body weight of CDs fed diabetogenic diet did not rise further, whereas the weight of CDs fed regular diet and those of CDr provided either diet continued to rise. The failure of serum creatinine to rise in CDs fed diabetogenic diet may thus be accounted for by the relative loss of muscle mass in the course of the development of diabetes in this strain over 3 and 6 months, as evidenced by the lower urinary creatinine excretion rate by CDs than CDr at 3 and 6 months (data not shown).

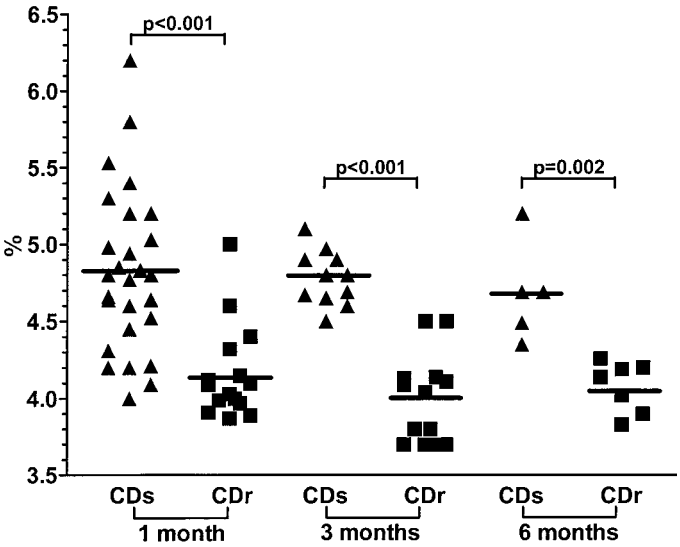

FIG. 4. HbA_{1c} levels 1, 3, and 6 months after initiation of diabetogenic diet in CDs and CDr. Group comparison was by unpaired Student's t test.

Creatinine clearance. Creatinine clearance after 1 month of diabetogenic diet was already significantly lower in CDs than in CDr (Fig. 6 and Table 2). At 3 and 6 months, creatinine clearance steadily declined in CDs but remained largely unchanged in CDr. The differences in creatinine clearance between the groups remained highly significant when normalized for kidney weight. When normalized for body weight, the differences were not significant at 1 and 3 months but became significant at 6 months, with the most likely reason being that CDs did not gain weight as did CDr over the study period (Fig. 4).

Urine flow, urine osmolality, and fractional sodium excretion. To rule out "prerenal azotemia" as a cause of the reduction in creatinine clearance in CDs, we measured urine volume, osmolality, and fractional sodium excretion

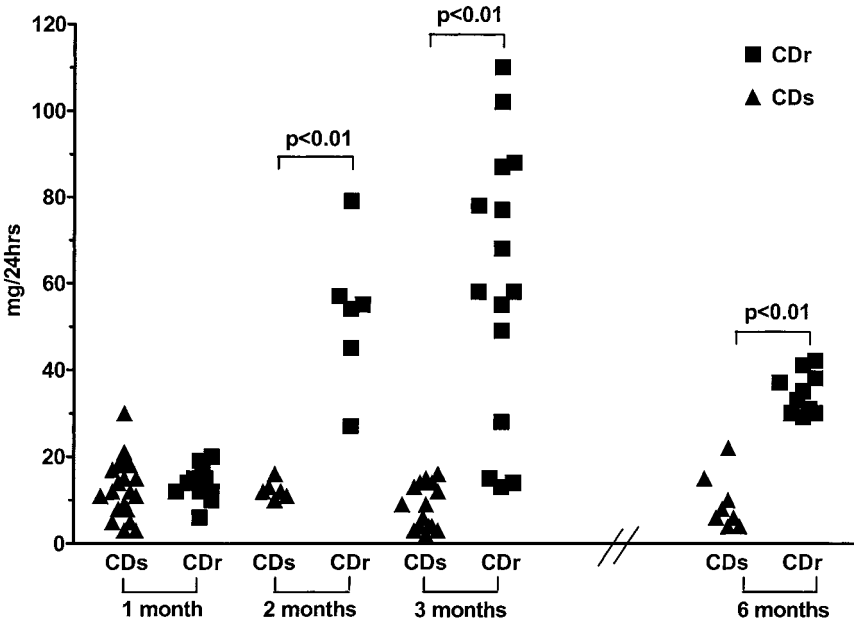

FIG. 5. Urinary protein excretion in CDs and CDr fed diabetogenic diet at 1, 2, 3, and 6 months after initiation of diabetogenic diet. Between-group comparison by Student's unpaired *t* test.

in CDs and CDr after 6 months of diabetogenic diet. The 24-h urine volume in CDs was more than twice that found in CDr (24 ± 4 vs. 11 ± 1 ml/24h, respectively; $P = 0.002$). Urine osmolality in CDs was half of that found in CDr (802 ± 94 vs. 1,174 ± 123 mOsm/l; $P = 0.0002$). Fractional sodium excretion was not different in CDs and CDr (0.25 ± 0.05 vs. 0.25 ± 0.01, respectively; P = NS). These findings are not consistent with prerenal azotemia.

Blood pressure. In view of the observed decline of creatinine clearance in CDs, we measured systolic arterial pressure after 1, 3, and 6 months of diabetogenic diet. The data are shown in Table 3. Blood pressure was higher at all time points in CDr than in CDs by ~10–15 mmHg.

Renal histology

Light microscopy. After 3 months on the diabetogenic diet, the kidneys of CDr (as viewed by thin slices of kidney sections stained with hematoxylin and eosin and PAS) appeared normal (Fig. 7). In contrast, the glomeruli in CDs appeared abnormal with mesangial matrix expansion narrowing the lumen of the capillary loops. The glomerular changes in CDs were diffuse and generalized, consistent with grade 3, using the grading system previously described by Rosenmann and Cohen (12). The interstitium and the blood vessels appeared normal.

Electron microscopy. The glomeruli of CDr appeared normal (Fig. 8). In contrast, CDs exhibited increased mesangial matrix protruding into and narrowing the capillary lumen, thickening of the basement membrane, and flattening of the foot processes.

Immunohistochemistry. Immunocytochemical staining for type IV collagen in CDs revealed increased staining in the glomeruli and to a lesser degree in the interstititum (Fig. 9). The average total score in the glomeruli of CDs was 75 ± 7 (three animals, 50 glomeruli per animal), which was twice that found in CDr (28 ± 10; three animals, 50 glomeruli per animal) ($P = 0.018$).

Retinal pathology. After 6 months, the retinas of CDs provided diabetogenic diet ($n = 6$) or regular diet ($n = 6$) were entirely normal, with complete preservation of all retinal layers. At 12 months, however, severe retinal atrophy was found in the diabetic CDs fed diabetogenic diet ($n = 4$), with almost complete atrophy of the retinal photoreceptors and only remnants of ganglion cells. Severe atrophy was also found in the inner and outer nuclear layer cells. The retinal blood vessels presented normal architecture. There were no signs of new blood vessel formation consistent with proliferative diabetic retinopathy. The retinas of CDr fed diabetogenic diet ($n = 3$) were entirely normal (Fig. 10). Severe retinal atrophy thus developed in the CDs rat between 6 and 12 months after initiation of the diabetogenic diet and the appearance of diabetes.

DISCUSSION

The major finding in the current set of studies was that the CDs rat develops a diabetes-associated nephropathy that is expressed by a reduction in glomerular filtration rate but without the development of proteinuria or hypertension and in the presence of histopathological changes in the kidney that are consistent with diabetic nephropathy. This

TABLE 1
Plasma urea and creatinine levels in CDs and CDr after feeding with diabetogenic diet

	Urea (mg/dl)			Creatinine (mg/dl)		
	1 month	3 months	6 months	1 month	3 months	6 months
CDs	40 ± 2 (25)	62 ± 9 (16)	151 ± 26 (5)	0.47 ± 0.01 (25)	0.52 ± 0.02 (14)	0.56 ± 0.02 (6)
CDr	31 ± 1 (15)	38 ± 4 (16)	49 ± 3 (8)	0.43 ± 0.01 (15)	0.50 ± 0.02 (16)	0.57 ± 0.01 (8)
CDs vs. CDr (*P*)	<0.01	<0.01	<0.001	0.019	NS	NS

Data are means ± SE (n). Between-group comparisons were made using Student's unpaired t test.

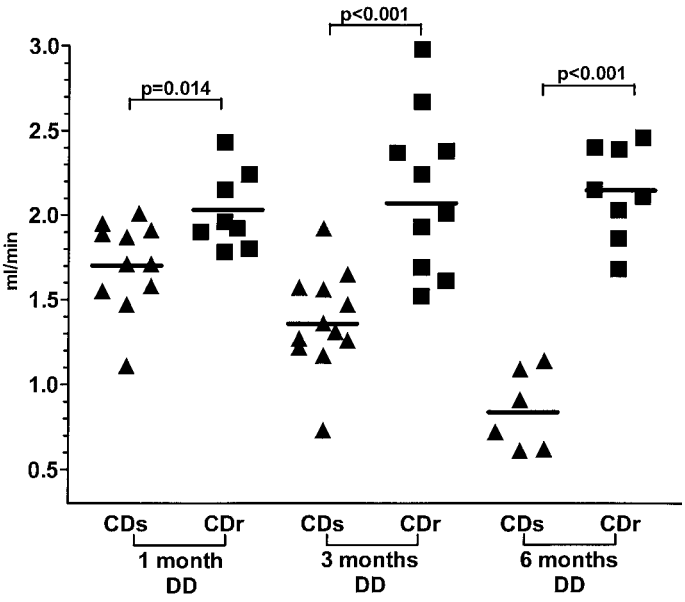

FIG. 6. Creatinine clearance in CDs and CDr fed diabetogenic diet (DD) for 1, 3, and 6 months with diabetogenic diet. Between-group comparisons by Student's unpaired *t* test.

type of nephropathy in the rat is reminiscent of a recently reported nonalbuminuric type of nephropathy that is associated with type 2 diabetes in humans (15).

The development of a nephropathy in the diabetic CDs strain is unquestionable. Yet, what evidence suggests that this renal lesion is consistent with diabetic nephropathy? Our evidence consists of classic histopathological changes, increased deposition of type IV collagen, and the appearance of retinopathy in CDs but not in CDr.

In terms of kidney histopathology, diabetic nephropathy typically presents with extracellular matrix accumulation, mesangial expansion, and an increase in glomerular basement membrane width. The light and electron microscopy findings in our study in CDs provided diabetogenic diet consist of such mesangial expansion and widening of the glomerular basement membrane and are entirely consistent with those previously reported in CDs (8–11,16–18). Are these histopathological findings in the kidneys of CDs specific enough to make the unequivocal diagnosis of diabetic nephropathy? There are no histopathological features that are unique to diabetic nephropathy that can provide a conclusive answer to that question. Therefore, we sought additional evidence that would support the specificity of our diagnosis of diabetic nephropathy in CDs and found a relative abundance of type IV collagen in the kidneys of CDs fed diabetogenic diet. Increased renal deposition of type IV collagen deposition has been associated with diabetic nephropathy in a large number of studies as early as a decade ago (19) and more recently as well (20). Our immunocytochemical findings of increased type IV collagen staining, more in the glomeruli but also in the interstitium of CDs but less in CDr, are thus also consistent with diabetic nephropathy.

The appearance of retinopathy in CDs rats fed diabetogenic diet and the absence of such retinopathy in the nondiabetic CDr strain can be taken as further circumstantial evidence for the presence of diabetic nephropathy in our model. The type of retinopathy we found in CDs is,

TABLE 2
Creatinine clearance in CDs and CDr after feeding with diabetogenic diet

	Creatinine clearance (ml/min)									
	1 month			3 months			6 months			
	Total	Per 100 g body wt	Per gram kidney wt	Total	Per 100 g body wt	Per gram kidney wt	Total	Per 100 g body wt	Per gram kidney wt
CDs	1.70 ± 0.08 (11)	0.72 ± 0.04 (11)	0.96 ± 0.06 (11)	1.37 ± 0.09 (12)	0.53 ± 0.04 (11)	0.60 ± 0.04 (8)	0.85 ± 0.10 (6)	0.38 ± 0.03 (6)	0.29 ± 0.04 (6)
CDr	2.02 ± 0.08 (8)	0.80 ± 0.04 (8)	1.16 ± 0.06 (8)	2.05 ± 0.14 (12)	0.62 ± 0.07 (10)	0.93 ± 0.09 (10)	2.14 ± 0.10 (8)	0.45 ± 0.01 (8)	0.74 ± 0.03 (8)
CDs vs. CDr (*P*)	0.014	NS	0.029	<0.001	NS	<0.001	<0.001	0.039	<0.001

Data are means ± SE (*n*). Between-group comparisons were made using Student's unpaired *t* test.

TABLE 3
Systolic arterial pressure in CDs and CDr after feeding with diabetogenic or regular diet

	1 month	3 months	6 months
CDs (mmHg)	122 ± 1 (4)	135 ± 3 (6)	130 ± 4 (8)
CDr (mmHg)	136 ± 2 (3)	144 ± 3 (6)	143 ± 2 (10)
CDs vs. CDr (*P*)	0.002	0.049	0.005

Data are means ± SE (*n*). Between-group comparison was made using Student's unpaired *t* test.

however, unlike that classically described in humans, which is typically characterized by, among other findings, vascular changes including the appearance of microaneurysms. Yet, this retinopathy is similar to that which has been described in other experimental models of diabetes (21–26) and is entirely consistent with earlier reports in the CDs strain (17,27,28). Of note, though, is that we were able to detect the retinopathy only between 6 and 12 months after initiation of the diabetogenic diet and the appearance of diabetes, while diabetic nephropathy developed earlier. We cannot rule out, however, that early diabetic retinopathy, undetectable by our investigative tools, was already present before 6 months of diabetogenic diet. Further investigations using more sensitive tools to detect the early appearance of retinopathy are required to resolve this issue.

Based on the evidence discussed above, our working assumption is that CDs develop a diabetes-associated type of nephropathy. Other experimental models of diabetes in rats and mice have previously been shown to develop various degrees of diabetic nephropathy. The hallmark of the diabetic phenotype in these other models, however, has been the appearance of proteinuria. As a result, the functional expression of diabetic nephropathy has usually been described as a leak of protein, with early microalbuminuria followed by macroproteinuria and a subsequent decline in glomerular filtration rate. The most striking and unusual finding in the current study was that the diabetic CDs strain developed a nephropathy that was not associated with proteinuria, yet exhibited a decline in creatinine clearance, which presumably reflects a decline in glomerular filtration within 1 month of the development of the diabetic metabolic syndrome. We did not detect an early hyperfiltration phase that has been reported to occur in early phases of diabetic nephropathy. It is possible that our study design did not allow us to determine whether a short-lived hyperfiltration phase occurred during the development of diabetes in CDs between initiation of diabetogenic diet and before our first measurement of kidney function at 1 month. Such a possibility, however, is unlikely, since the full metabolic diabetic state developed in CDs only after ~4 weeks of diabetogenic diet, at which time we already detected a decline in glomerular filtration.

The contrasting CDr strain, which remained normoglycemic despite the diabetogenic diet, developed significant proteinuria but without a reduction in glomerular filtration or the development of histopathological changes in the kidneys or the retinal pathology found in CDs. Because proteinuria developed in CDr with either regular or diabetogenic diet and because these animals did not develop diabetes with either diet, the appearance of proteinuria in CDr is obviously unrelated to the diabetogenic diet or diabetes. Could the proteinuria be related to the 13–14 mmHg higher blood pressure found in CDr than in CDs? This study does not allow us to provide a definitive answer to that question. The issue of proteinuria in CDr thus remains open and certainly deserves to be investigated in a separate study.

Have previous studies in the Cohen diabetic model reported similar findings with respect to proteinuria? Interestingly enough, in earlier studies on the phenotype of this model, little emphasis was placed on kidney function (3–13). No data were provided with respect to the level of protein excretion or kidney function in all but one study in which varying levels of proteinuria were reported in both CDs and CDr strains, ranging from low to abnor-

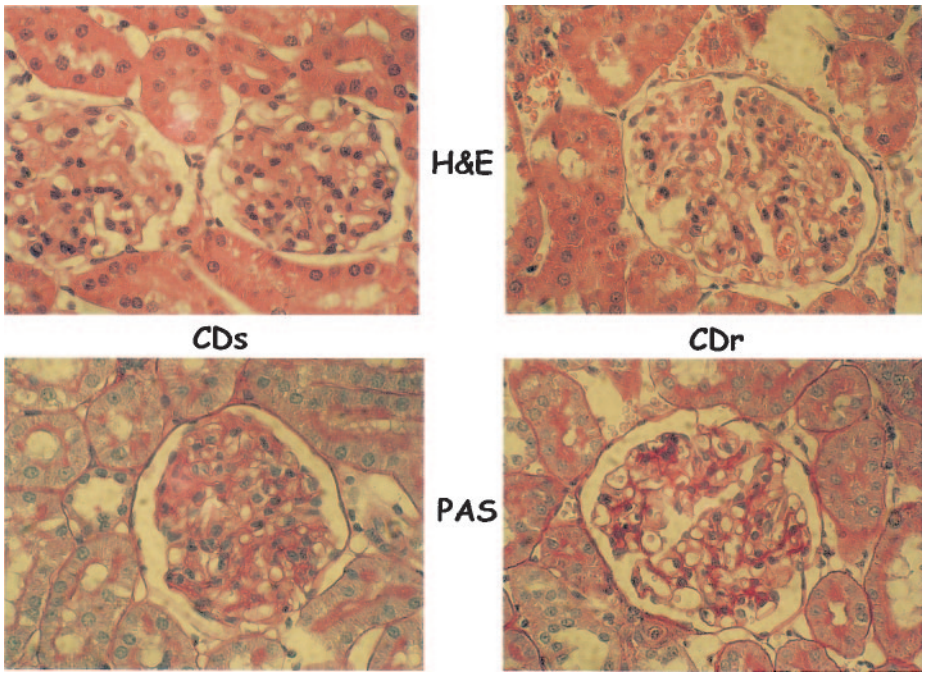

FIG. 7. Hematoxylin and eosin (H&E) and PAS stains of 2- to 3-μm sections of kidneys (×400) from CDs and CDr fed diabetogenic diet for 3 months.

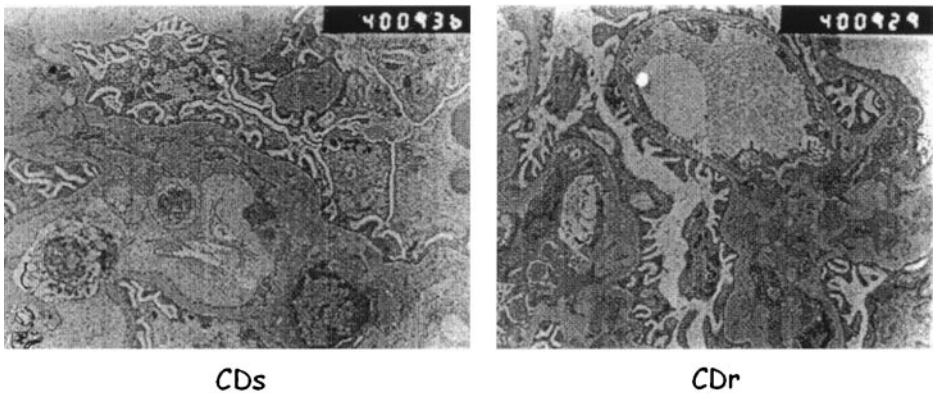

FIG. 8. Electron micrographs (×4,000) of representative kidney sections of CDs and CDr fed diabetogenic diet for 3 months.

mally high (7). In that particular study, however, no information was available as to the level of genetic homogeneity of the CDs strain, raising the possibility of cross-strain contamination affecting the level of protein excretion.

Why is there no proteinuria in the diabetic CDs strain that does develop nephropathy? Our current data suggest that the absence of proteinuria in the diabetic CDs is genetically determined. In favor of this conclusion are the observations that CDs fed diabetogenic diet (which became diabetic) or regular diet (which did not become diabetic) did not develop proteinuria, whereas paradoxically the CDr strain that was fed either diet (both groups not developing diabetes) excreted an abnormally high amount of protein in the urine. Thus, the dietary manipulation that induces the diabetic phenotype in CDs but not in CDr cannot be linked to the presence or absence of proteinuria. Proteinuria must, therefore, have been determined by the differing genetic background of the two strains. At this time, it is unclear which factor in the CDs genetic background prevents the development of proteinuria in the face of diabetic nephropathy. However, is proteinuria a sine qua non of the renal diabetic lesion? The pathophysiology of proteinuria in diabetes remains incompletely understood. The classic modeling claims increased porosity of the glomerular basement membrane in diabetes and a relative decrease in anionic charge, which together contribute to protein leakage. Such modeling does not appear to apply in the diabetic CDs rat. The reduction in glomerular filtration, on the other hand, is typically accounted for by a decrease in filtration surface caused by extracellular matrix expansion and obliteration of the capillary lumen, leading to a glomerulosclerosis type of lesion (16,29). In CDs, the abnormal morphology of the glomeruli that was found in the current study is consistent with the progressive decline in glomerular filtration. Judging from this model, therefore, protein leakage does not appear to be a prerequisite for the decline in glomerular filtration, and our results suggest that the two pathologies may be unlinked.

What is the importance of our findings with respect to our understanding of diabetes and end-organ damage in humans? Proteinuria is generally considered a hallmark of the clinical diagnosis of diabetic nephropathy, a prognostic marker for the development of renal failure in diabetic patients, and an indication for initiation of treatment designed to slow down progression of renal disease. The absence of proteinuria is usually considered to be a reassuring sign by clinicians, suggesting the absence of nephropathy in diabetic patients. The development of renal failure is attributed to other causes in these patients, including atherosclerosis and hypertension. And yet, during the past decade, several reports have emerged suggesting that a reduced glomerular filtration rate can also occur in normoalbuminuric patients with long-standing type 1 and type 2 diabetes (15,30–33). The Third National Health and Nutrition Survey (NHANES III) indicates further that the finding of nonalbuminuric renal insufficiency in diabetes is not uncommon (34). The major question in this regard is (as has been in our current study of the rodent model) whether the nephropathy causing the decline in glomerular filtration in the absence of proteinuria in these individuals is consistent with diabetes (i.e., diabetic nephropathy), or whether other renal pathologies are involved that are not related to diabetes. No histopathological data are available in most of these patients because in the absence

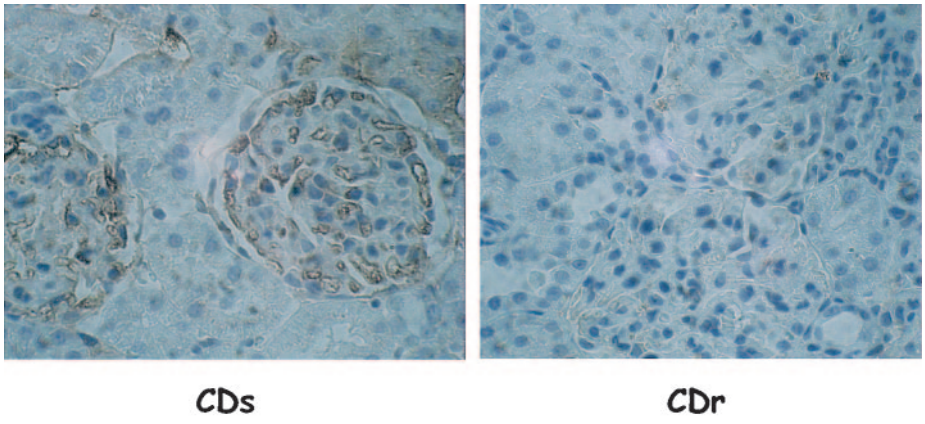

FIG. 9. Immunocytochemical staining for type IV collagen (in brown) in sections of kidneys of CDs and CDr fed diabetogenic diet for 3 months.

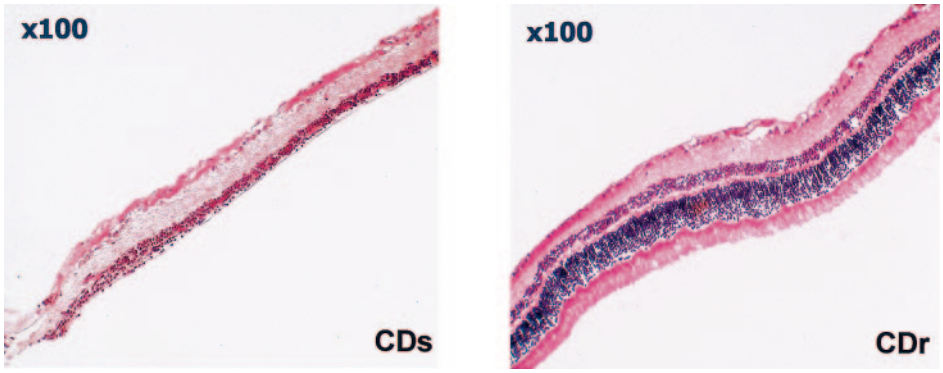

FIG. 10. Photomicrographs of hematoxylin and eosin–stained sections of the retinas of CDs and CDr (×100) after 12 months of diabetogenic diet.

of proteinuria, most clinicians refrain from performing a renal biopsy. There are some data, nonetheless, that indicate that a significant proportion of normoalbuminuric long-standing diabetic patients have well-established diabetic nephropathy lesions (33,35,36). In the current investigation of the renal phenotype of a rodent model of type 2 diabetes, we provide strong evidence that a decline in glomerular filtration can occur in the absence of proteinuria and in the presence of a renal histopathological lesion that is consistent with diabetic nephropathy. These findings imply that the impaired glomerular filtration and normoalbuminuria observed in diabetic patients may indeed be due to diabetic nephropathy per se and not to another type of renal lesion. Can one extrapolate such findings from the rodent model to humans? Is it possible that diabetic nephropathy develops in humans silently and without early warning signs such as microalbuminuria? Judging from studies in humans that claim that a significant percentage of patients with type 2 diabetes may progress to renal insufficiency while remaining normoalbuminuric (15,30,31,33,36), as well as the additional experimental evidence currently provided in our study, that may indeed be the case. The practical implication already raised by others, yet not sufficiently appreciated by diabetologists, nephrologists, or general practitioners, is that diabetic patients with normoalbuminuria are not safe or immune from diabetic nephropathy, which may develop insidiously and progress silently and unannounced to renal failure.

Several questions remain unanswered. Are there early signs, other than microalbuminuria, that can predict the onset of the nonproteinuric type of diabetic nephropathy? Is the traditional treatment designed to prevent progression of diabetic renal disease also effective in the nonproteinuric modality of the disease? Is the course of the disease similar to that of the proteinuric moiety? The Cohen diabetic rat is the experimental model that may allow us to provide at least some answers to these questions.

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REFERENCES

1. Weksler-Zangen S, Yagil C, Zangen DH, Ornoy A, Jacob HJ, Yagil Y: The newly inbred Cohen diabetic rat: a nonobese normolipidemic genetic model of diet-induced type 2 diabetes expressing sex differences. *Diabetes* 50:2521–2529, 2001
2. Cohen AM: Development of the model. In *The Cohen Diabetic Rat*. Cohen AM, Rosenmann E, Eds. Basel, Karger, 1990, p. 1–9
3. Cohen AJ, McGill PD, Rossetti L, Guberski DL, Like AA: Glomerulopathy in spontaneously diabetic rat: impact of glycemic control. *Diabetes* 36:944–951, 1987
4. Cohen AM, Rosenmann E: Diffuse intercapillary glomerulosclerosis in sucrose-fed rats. *Diabetologia* 7:25–28, 1971
5. Cohen AM, Rosenmann E: Effect of the estrogen antagonist, tamoxifen, on development of glomerulosclerosis in the Cohen diabetic rat. *Diabetes* 34:634–638, 1985
6. Cohen AM, Rosenmann E, Rosenthal T: The Cohen diabetic (non-insulin-dependent) hypertensive rat model: description of the model and pathologic findings. *Am J Hypertens* 6:989–995, 1993
7. Cohen AM, Wald H, Popovtzer M, Rosenmann E: Effect of myo-inositol supplementation on the development of renal pathological changes in the Cohen diabetic (type 2) rat. *Diabetologia* 38:899–905, 1995
8. Cohen AM, Rosenmann E: Male sex hormone and nephropathy in Cohen diabetic rat. (Genetically selected sucrose fed). *Diabete Metab* 10:199–205, 1984
9. Cohen AM, Rosenmann E: Acarbose treatment and diabetic nephropathy in the Cohen diabetic rat. *Horm Metab Res* 22:511–515, 1990
10. Rosenmann E, Teitelbaum A, Cohen AM: Nephropathy in sucrose-fed rats: electron and light microscopic studies. *Diabetes* 20:803–810, 1971
11. Rosenmann E, Yanko L, Cohen AM: Female sex hormone and nephropathy in Cohen diabetic rat (genetically selected sucrose-fed). *Horm Metab Res* 16:11–16, 1984
12. Rosenmann E, Cohen AM: The pathology of the Cohen Diabetic rat. In *The Cohen Diabetic Rat*. Cohen AM, Rosenmann E, Eds. Basel, Karger, 1990, p. 54–75
13. Rosenmann E, Teitelbaum A, Cohen AM: Nephropathy in sucrose fed rats: electron and light microscopic studies. *Diabetes* 20:803–810, 1971
14. Yagil C, Katni G, Rubattu S, Stolpe C, Kreutz R, Lindpaintner K, Ganten D, Ben-Ishay D, Yagil Y: Development, genotype, and phenotype of a new colony of the Sabra hypertension prone (SBH/y) and resistant (SBN/y) rat model of slat sensitivity and resistance. *J Hypertens* 14:1175–1182, 1996
15. MacIsaac RJ, Tsalamandrisc C, Panagiotopoulos S, Smith TJ, McNeil KJ, Jerums G: Nonalbuminuric renal insufficiency in type 2 diabetes. *Diabetes Care* 27:195–200, 2004
16. Raptis AE, Viberti G: Pathogenesis of diabetic nephropathy. *Exp Clin Endocrinol Diabetes* 109 (Suppl. 2):424–437, 2001
17. Yanko L, Rosenmann E, Cohen AM: A correlative study on the occurrence of retinopathy and nephropathy in sucrose-fed diabetic rats. *Acta Diabetol Lat* 12:52–65, 1975
18. Rosenmann E, Yanko L, Cohen AM: Comparative study of the pathology of sucrose-induced diabetes in the rat and adult-onset diabetes in man. *Isr J Med Sci* 11:753–761, 1975
19. Ziyadeh FN: Renal tubular basement membrane and collagen type IV in diabetes mellitus. *Kidney Int* 43:114–120, 1993
20. Abe H, Matsubara T, Iehara N, Nagai K, Takahashi T, Arai H, Kita T, Doi T: Type IV collagen is transcriptionally regulated by Smad1 under advanced glycation end product (AGE) stimulation. *J Biol Chem* 279:14201–14206, 2004
21. Sima AA, Garcia-Salinas R, Basu PK: The BB Wistar rat: an experimental model for the study of diabetic retinopathy. *Metabolism* 32:136–140, 1983

22. Papachristodoulou D, Heath H, Kang SS: The development of retinopathy in sucrose-fed and streptozotocin-diabetic rats. *Diabetologia* 12:367–374, 1976
23. Hausler HR, Sibay TM, Stachowska B: Observations of retinal microaneurysms in a metahypophyseal diabetic Chinese hamster. *Am J Ophthalmol* 56:242–244, 1963
24. Hausler HR, Stachowska B, Sibay TM: Observation of retinopathy in Metahypophyseal Diabetic Chinese Hamsters. *Invest Ophthalmol* 31:378–383, 1963
25. Bloodworth JM, Engerman RL Jr., Anderson PJ: Microangiopathy in the experimentally diabetic animal. *Adv Metab Disord* 2 (Suppl. 2):245–250, 1973
26. Engerman RL, Bloodworth JM, Jr.: Experimental diabetic retinopathy in dogs. *Arch Ophthalmol* 73:205–210, 1965
27. Cohen AM, Yanko L, Rosenmann E: Control of blood glucose levels and development of diabetic microangiopathy: effect of glibornuride. *Isr J Med Sci* 19:311–318, 1983
28. Yanko L, Michaelson I, Cohen A: The retinopathy of sucrose fed rats: genetics and diet as factors in development of diabetes mellitus. *Isr J Med Sci* 8:990–991, 1972
29. Steffes MW, Osterby R, Chavers B, Mauer SM: Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. *Diabetes* 38:1077–1081, 1989
30. Lane PH, Steffes MW, Mauer SM: Glomerular structure in IDDM women with low glomerular filtration rate and normal urinary albumin excretion. *Diabetes* 41:581–586, 1992
31. Caramori ML, Fioretto P, Mauer M: Low glomerular filtration rate in normoalbuminuric type 1 diabetic patients: an indicator of more advanced glomerular lesions. *Diabetes* 52:1036–1040, 2003
32. Tsalamandris C, Allen TJ, Gilbert RE, Sinha A, Panagiotopoulos S, Cooper ME, Jerums G: Progressive decline in renal function in diabetic patients with and without albuminuria. *Diabetes* 43:649–655, 1994
33. Caramori ML, Fioretto P, Mauer M: The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? *Diabetes* 49:1399–1408, 2000
34. Garg AX, Kiberd BA, Clark WF, Haynes RB, Clase CM: Albuminuria and renal insufficiency prevalence guides population screening: results from the NHANES III. *Kidney Int* 61:2165–2175, 2002
35. Fioretto P, Steffes MW, Mauer M: Glomerular structure in nonproteinuric IDDM patients with various levels of albuminuria. *Diabetes* 43:1358–1364, 1994
36. Chavers BM, Bilous RW, Ellis EN, Steffes MW, Mauer SM: Glomerular lesions and urinary albumin excretion in type I diabetes without overt proteinuria. *N Engl J Med* 320:966–970, 1989