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Reversibility of established diabetic glomerulopathy by anti-TGF-β antibodies in *db/db* mice

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Abstract

Treatment with a neutralizing anti-transforming growth factor-β (TGF-β) antibody can prevent the development of diabetic nephropathy in the *db/db* mouse, a model of type 2 diabetes. However, it is unknown whether anti-TGF-β therapy can reverse the histological lesions of diabetic glomerulopathy once they are established. Diabetic *db/db* mice and their non-diabetic *db/m* littermates were allowed to grow until 16 weeks of age, by which time the *db/db* mice had developed glomerular basement membrane (GBM) thickening and mesangial matrix expansion. The mice were then treated with an irrelevant control IgG or a panselective, neutralizing anti-TGF-β antibody for eight more weeks. Compared with control *db/m* mice, the *db/db* mice treated with IgG had developed increased GBM width (16.64 ± 0.80 nm vs. 21.55 ± 0.78 nm, $P < 0.05$) and increased mesangial matrix fraction ($4.01 \pm 0.81\%$ of total glomerular area vs. $9.55 \pm 1.04\%$, $P < 0.05$). However, the *db/db* mice treated with anti-TGF-β antibody showed amelioration of GBM thickening (18.40 ± 0.72 nm, $P < 0.05$ vs. *db/db*-IgG) and mesangial matrix accumulation ($6.32 \pm 1.79\%$, $P < 0.05$ vs. *db/db*-IgG). Our results demonstrate that inhibiting renal TGF-β activity can partially reverse the GBM thickening and mesangial matrix expansion in this mouse model of type 2 diabetes. Anti-TGF-β regimens would be useful in the treatment of diabetic nephropathy.

Keywords: Glomerular basement membrane thickening; Mesangial matrix expansion; Type 2 diabetes; Reversal

The pathologic lesions of diabetic nephropathy are well described [1]. They include cellular hypertrophy, glomerular basement membrane (GBM) and tubular basement membrane (TBM) thickening, tubulointerstitial fibrosis, and mesangial expansion with extracellular matrix, also known as glomerulosclerosis. These lesions, once established, were thought to be irreversible [2]. But the extracellular matrix does undergo remodeling and its quantity is determined by the rates of production and degradation [3]. In diabetic kidney disease, production of extracellular matrix outstrips degradation, and matrix proteins accumulate in the glomerular and tubulointerstitial compartments to manifest as glomerulosclerosis and tubulointerstitial fibrosis.

The pathogenesis of this extracellular matrix buildup has been convincingly linked to a metabolic cause, namely excessive levels of transforming growth factor-β (TGF-β) in the diabetic kidney [4]. TGF-β not only stimulates the synthesis of extracellular matrix but also inhibits the breakdown of matrix proteins, hence its reputation as a potent profibrotic cytokine. Furthermore, TGF-β is stimulated by high glucose [5], Amadori-modified proteins [6], advanced glycation endproducts [7], and angiotensin II [8], which are all metabolic features of diabetes. In addition, the intrarenal concentration of TGF-β is markedly increased in diabetes, both in animal models [9] and in humans [10]. To prove that increased TGF-β mediates the mesangial sclerosis of diabetic nephropathy, anti-TGF-β antibody therapy has been used to neutralize the renal TGF-β system and thereby prevent glomerular matrix overexpression in the short-term [11] and mesangial matrix expansion in the long-term [12].

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In these animal experiments, anti-TGF-β therapy was started concurrently with the onset of diabetes, demonstrating the efficacy of TGF-β blockade in preventing diabetic kidney disease. However, prevention of diabetic nephropathy is not an opportunity that is often afforded to the physician. More often than not, especially in type 2 diabetes, a patient has been hyperglycemic for many years before coming to the attention of a doctor, and by that time diabetic renal disease may have been established. Then the focus shifts from prevention to treatment of disease, with the hopes of arresting or even reversing the progressive course of diabetic nephropathy. One recent study gives hope that the natural history of diabetic renal disease can improve with optimal treatment of diabetes [13]. Eight type 1 diabetic patients with biopsy-proven diabetic nephropathy at baseline had pancreatic allografts that functioned well for many years. After 10 years of true euglycemia, renal function was preserved and, more important, the GBM thickness and index of mesangial expansion reverted to normal. Thus, it appears that aggressive and prolonged glycemic control can reverse the pathological lesions of diabetic nephropathy.

Given the above study and our encouraging results with anti-TGF-β therapy in preventive trials, we investigated whether the same neutralizing anti-TGF-β antibody would reverse the structural abnormalities of established diabetic kidney disease in an experimental model of type 2 diabetes, the obese *db*/*db* mouse [14]. This mouse model has a defective hypothalamic receptor for leptin, an adipocyte-derived hormone that normally decreases food intake, increases energy expenditure, and causes weight loss [15]. Because the *db*/*db* mouse is resistant to the effects of leptin, it becomes obese and develops insulin-resistance. By eight weeks of age, the *db*/*db* mouse is frankly hyperglycemic and has full-blown type 2 diabetes [12]. In another eight weeks (i.e., 16 weeks of age), the mice will have developed diabetic kidney disease with histologic lesions that are reminiscent of diabetic nephropathy in humans [16]. At that time, treatment with the anti-TGF-β antibody was begun to see if inhibiting the renal TGF-β system could reverse GBM thickening and mesangial matrix expansion.

Materials and methods

Experimental animals. Female diabetic *db*/*db* mice, with a C57BL/6J genetic background, and their non-diabetic counterparts, *db*/*m* mice, were purchased from Jackson Laboratories (Bar Harbor, ME). Blood glucose measurements with a glucometer confirmed that by eight weeks of age, the *db*/*m* mice were normoglycemic and the *db*/*db* mice were hyperglycemic. After eight more weeks of diabetes, six of the *db*/*db* mice were sacrificed to check whether the histological lesions of diabetic nephropathy had developed by 16 weeks of age. At that point, the *db*/*m* mice and the remaining *db*/*db* mice were treated with either a panselective, neutralizing mouse anti-TGF-β antibody (anti-T) [17] or an isotype-matched, irrelevant mouse antibody (IgG) that served as the control for anti-T. The antibodies were injected intraperitoneally three times per week for an additional eight weeks, until the mice were 24 weeks of age. This anti-T regimen has been shown to suppress the glomerular expression of TGF-β without any systemic ill effects [12]. In the control arm, 8 non-diabetic *db*/*m* mice and 10 diabetic *db*/*db* mice received IgG. In the treatment arm, 10 non-diabetic *db*/*m* mice and 10 diabetic *db*/*db* mice received anti-T. At the end of the experiment, the mice were euthanized and the left kidney was removed and immediately frozen in liquid nitrogen.

Mesangial matrix fraction. Portions of the renal cortex were fixed in 10% neutral buffered formalin and then embedded in paraffin. Sections were cut at 5 μm and stained with periodic acid–Schiff (PAS). The coded sections were read by an observer unaware of the experimental protocol. For each mouse, 20 glomeruli were selected at random. Using the computer program, Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD), the area of the PAS-positive material in the mesangium was factored by the glomerular tuft area to arrive at the fraction of mesangial matrix.

GBM thickness. For ultrastructural evaluation, renal cortical tissue was fixed in 3% glutaraldehyde, post-fixed in osmium tetroxide, and stained with uranyl acetate and lead citrate. The specimen was thin-sectioned and examined under a transmission electron microscope. Electron micrographic pictures were taken at the 10,000× magnification for each animal. Using the Image-Pro Plus 3.0 program, the thickness of the glomerular basement membrane was measured at random locations and averaged.

Statistical analysis. Data are presented as means ± SEM. Comparisons between two groups were made with the unpaired Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Results and discussion

Glycemic control

At eight weeks of age, the *db*/*m* mice were all normoglycemic and the *db*/*db* mice were all hyperglycemic (data not shown). By 24 weeks of age, at the end of the study, the average plasma glucose in the non-diabetic *db*/*m* group treated with control IgG was 6.94 ± 0.56 mmol/L. In the diabetic *db*/*db* group treated with IgG, plasma glucose was 35.28 ± 3.17 mmol/L and in the diabetic *db*/*db* mice treated with anti-T, plasma glucose was 42.50 ± 2.22 mmol/L. This tendency for anti-T treatment to modestly raise the blood glucose concentration in diabetic mice was previously encountered [12].

GBM thickening

Diabetes caused thickening of the GBM. The GBM width increased significantly from 16.64 ± 0.80 nm in the non-diabetic *db*/*m* mice to 22.61 ± 0.99 nm in the 16-week-old diabetic *db*/*db* mice (Fig. 1). After diabetic GBM thickening had occurred, eight further weeks of treatment with irrelevant IgG had no effect (GBM width: 21.55 ± 0.78 nm), but treatment with the neutralizing anti-T antibody partly reversed the GBM thickening to 18.40 ± 0.72 nm, $P < 0.05$ vs. 24-week *db*/*db*-IgG (Fig. 1). Of note, anti-T antibody did not change the GBM thickness in non-diabetic *db*/*m* mice (data not shown).

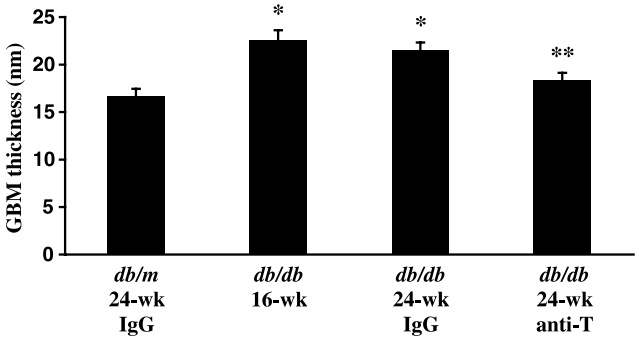

Fig. 1. Eight-week-old diabetic *db/db* mice were allowed to remain hyperglycemic until 16 weeks of age. At that time, selected *db/db* mice ($n = 6$) were sacrificed and the glomerular basement membrane (GBM) thicknesses were measured. The remaining 16-week-old *db/db* mice were then treated with either irrelevant IgG ($n = 10$) or neutralizing anti-TGF-β antibody (anti-T, $n = 10$) until 24 weeks of age. As the control, 16-week-old non-diabetic *db/m* mice were concurrently treated with IgG ($n = 8$) for eight weeks until 24 weeks of age. All mice were then sacrificed and their GBM thicknesses were measured. $^{*}P < 0.05$ vs. *db/m*, $^{**}P < 0.05$ vs. *db/db* 16-week or *db/db* 24-week, IgG.

Mesangial expansion

In the control *db/m* mice given IgG, the mesangial matrix fraction of the total glomerular tuft area was $4.01 \pm 0.81\%$ (Fig. 2). This percentage was slightly but not significantly decreased by anti-T treatment in the *db/m* mice ($3.86 \pm 1.23\%$). With diabetes, however, the percent mesangial matrix was significantly increased to $10.16 \pm 0.66\%$ in the *db/db* mice at 16 weeks and $9.55 \pm 1.04\%$ in the *db/db* mice at 24 weeks (Fig. 2). Eight weeks of treatment with control IgG did not affect the extent of mesangial matrix expansion in diabetes, but eight weeks of therapy with the neutralizing anti-TGF-β antibody significantly reduced the mesangial matrix fraction to $6.32 \pm 1.79\%$ (Fig. 2), approaching the non-diabetic norm of $4.01 \pm 0.81\%$.

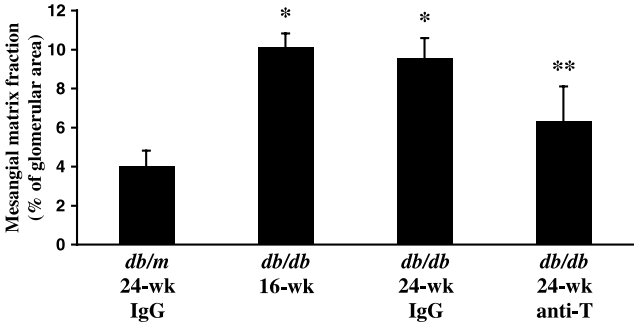

Fig. 2. Eight-week-old diabetic *db/db* mice were allowed to remain hyperglycemic until 16 weeks of age. At that time, selected *db/db* mice ($n = 6$) were sacrificed and the mesangial matrix fractions were calculated. The remaining 16-week-old *db/db* mice were then treated with either irrelevant IgG ($n = 10$) or neutralizing anti-TGF-β antibody (anti-T, $n = 10$) until 24 weeks of age. As the control, 16-week-old non-diabetic *db/m* mice were concurrently treated with IgG ($n = 8$) for eight weeks until 24 weeks of age. All mice were then sacrificed and their mesangial matrix fractions were measured. $^{*}P < 0.05$ vs. *db/m*, $^{**}P < 0.05$ vs. *db/db* 16-week or *db/db* 24-week, IgG.

The impact of these numbers can be appreciated visually by examining the representative light micrographs from each of the four animal groups. A micrograph of the renal cortex from a non-diabetic *db/m* mouse at 24 weeks shows a normal glomerulus with patent capillary loops (Fig. 3A). This contrasts with the glomerulus from a *db/db* mouse at 16 weeks of age, after about eight weeks of untreated diabetes. It shows hypertrophy and exuberant mesangial matrix expansion with relative closure of the capillaries (Fig. 3B). These diabetic changes persist in the *db/db* mice that lived to 24 weeks and were treated with irrelevant control IgG (Fig. 3C). On the other hand, therapy with the anti-TGF-β antibody for eight weeks partially reversed the mesangial matrix accumulation that had been established by 16 weeks of age. The glomerulus contains less PAS-positive matrix material and the capillary loops are more widely open (Fig. 3D).

Importance of TGF-β in diabetic nephropathy

The intrarenal TGF-β system plays a crucial part in the development of pathologic lesions in diabetic nephropathy. Two of the characteristic changes in diabetic kidney disease are glomerular basement membrane thickening and expansion of the mesangium with matrix. These structural lesions have their functional consequences and manifest as worsening proteinuria and progressive renal dysfunction in diabetes [18,19]. The underlying pathogenesis of GBM thickening and mesangial matrix expansion may be mediated in large part by the TGF-β system. After all, TGF-β expression is stimulated by nearly every aspect of the diabetic state in most kidney cell types and TGF-β overactivity drives the development of glomerulosclerosis [4]. As proof of this concept, when TGF-β activity in the *db/db* mouse was intercepted by systemic treatment with an anti-TGF-β antibody, renal function was preserved, matrix overexpression was normalized, and diffuse diabetic glomerulosclerosis was prevented [12]. Despite the continued hyperglycemia in the diabetic mice, therapy aimed at the TGF-β system alone was sufficient to protect the kidneys from diabetic damage.

Prevention vs. treatment of disease

However, that particular study was designed as a prevention trial in that anti-TGF-β treatment was started concurrently with the onset of type 2 diabetes [12]. Therefore, we designed the current experiment to be more of a therapeutic trial and commenced anti-TGF-β antibody therapy long after the onset of diabetes and after the establishment of diabetic glomerulopathy, evidenced by GBM thickening and mesangial matrix expansion. Even at this late stage, antagonizing the

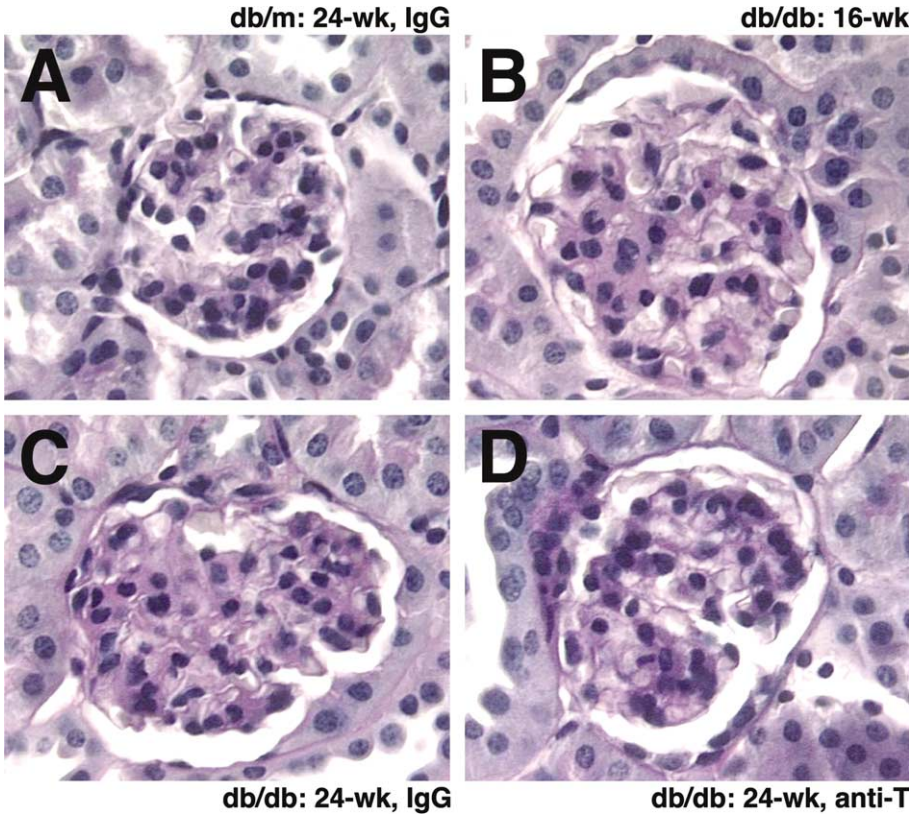

Fig. 3. Paraffin-embedded sections of the renal cortex were stained with periodic acid–Schiff (PAS). Representative light micrographs (magnification: 400×) from each of the mouse groups are shown. (A) Normal glomerulus from a non-diabetic *db*/*m* mouse at 24 weeks. (B) Glomerulus from an untreated *db*/*db* mouse at 16 weeks of age, showing hypertrophy and mesangial matrix expansion. (C) Glomerulus from a *db*/*db* mouse treated with an eight-week course of IgG from 16 to 24 weeks of age, displaying persistence of diabetic changes. (D) Glomerulus from a *db*/*db* mouse treated with eight weeks of anti-TGF-β antibody until 24 weeks of age, depicting partial reversal of mesangial matrix expansion.

intrarenal TGF-β system was able to partially reverse the histologic lesions of diabetic glomerular disease. It should be noted that anti-TGF-β therapy may not improve the course of diabetic proteinuria as our prevention study found that anti-TGF-β antibodies did not confer this type of renoprotection [12]. Nevertheless, it is tempting to speculate that reversal of the structural abnormalities would slow or halt the decline in renal function due to diabetes.

Success with glycemic control in reversing diabetic nephropathy

Before TGF-β, other metabolic abnormalities had been targeted in the hopes of reversing the glomerular lesions of diabetic renal disease. Hyperglycemia was an obvious choice and pancreatic transplantation allowed investigative subjects to achieve long-term normoglycemia. In one of the early studies, pancreas-after-kidney transplantation prevented the progression of modest mesangial expansion and GBM thickening in renal allografts of type 1 diabetic patients [20]. Subsequent studies have looked at simultaneous pancreas–kidney transplants [21–23]. Compared with a kidney transplant alone, simultaneous pancreatic-kidney transplantation was able to prevent the increased GBM thickness and increased mesangial volume that can develop in diabetic patients after receiving a donor kidney [21,22,24]. Likewise, the mesangial matrix fraction and GBM widening in renal allografts of type 1 diabetic patients have been attenuated by intensive insulin regimens [25]. Moreover, an interesting case report described how both cadaveric kidneys from a donor with proven diabetic nephropathy showed almost complete resolution of diffuse glomerulosclerosis and GBM thickening after being transplanted into non-diabetic recipients [26].

As for whether euglycemia can reverse the diabetic damage in native kidneys, animal work has shown that pancreatic transplantation can both prevent [27] and reverse [28] the mesangial expansion in alloxan-induced diabetic rats. In type 1 diabetic humans, pancreatic transplantation did not improve GBM width or mesangial fractional volume at five years [29] but did normalize these parameters at 10 years [13]. The decade-long time frame may mean that it takes normoglycemia as long to reverse the lesions of diabetic nephropathy as it takes diabetes to effect pathologic changes in the kidney. All of the above studies demonstrate that

excellent glycemic control can effectively reverse the microscopic features of diabetic nephropathy.

Potential non-specific effects of anti-TGF-β antibody

Compared to pancreatic transplantation, anti-TGF-β therapy performed nearly as well at regressing diabetic glomerulopathy, despite the fact that anti-TGF-β antibodies slightly worsened glycemic control. Therefore, the mechanism of anti-T treatment does not involve improvement of glucose metabolism, but we speculate that the combination of optimal glycemic control and anti-TGF-β antibodies would result in a more complete reversal of GBM thickening and mesangial expansion in the *db*/*db* mouse.

If not a glucose metabolic effect, perhaps anti-T therapy had a hemodynamic effect that contributed to the reversal of diabetic renal pathology. Anti-TGF-β antibodies can lower the blood pressure by a moderate amount [30]. In as much as blood pressure control with anti-hypertensive medication may cause regression of mesangial glomerulosclerosis [31,32], the mechanism of anti-T could be partly related to its anti-hypertensive effect. The majority of studies, however, have found that anti-hypertensive drugs ameliorate or prevent but do not reverse the histologic changes of diabetic nephropathy [33–38]. Furthermore, the *db*/*db* mouse may not develop clinically significant hypertension that could be improved by anti-T, because target organ damage in the form of cardiac hypertrophy was not observed in our previous study at 16 weeks [12]. It seems less likely, then, that a blood pressure-lowering effect explains the reversibility findings in the present study. In the end, anti-TGF-β antibodies probably reversed GBM thickening and mesangial matrix expansion by virtue of its specific antagonism of TGF-β overactivity, blocking a major profibrotic pathway in the diabetic kidney. Regardless of the exact mechanism, anti-TGF-β therapy represents an important and novel advance in the management of diabetic nephropathy.

Clinical considerations of anti-TGF-β therapy

In terms of applicability, the therapeutic trial matches the practice of medicine more closely than the preventive trial. With diabetic nephropathy, the physician is often faced with the challenge of treating a patient late in the natural history of the disease, particularly in type 2 diabetes. By the time that microalbuminuria, the earliest clinical marker of incipient nephropathy, is detected, the true extent of diabetic renal disease may already be quite advanced as indicated by the presence of glomerular hypertrophy, GBM thickening, and even mesangial matrix expansion [39,40]. To favorably modify the disease course, the optimal treatment program entails a multidisciplinary approach that tries to achieve tight glycemic control [41,42], to regulate the blood pressure to 130/80 mm Hg [43], to preferably use ACE inhibitors [44] or angiotensin receptor blockers [45,46], to treat hyperlipidemia [47], to quit smoking [48], and to moderately restrict dietary protein [49]. Unfortunately, even if all these measures are maximized, they may not prevent the progression of diabetic nephropathy to end-stage renal disease [50]. Perhaps this serves to emphasize that a multifactorial disease like diabetic nephropathy requires a multipronged treatment strategy. In the fight against diabetic kidney disease, inhibition of the renal TGF-β system can be an effective and complementary addition to the therapeutic armamentarium.

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