Prevention of renal apoB retention is protective against diabetic nephropathy: role of TGF-β inhibition

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Abstract Animal studies demonstrate that hyperlipidemia and renal lipoprotein colocalize with biglycan, a renal proteoglycan. The purpose of this study was to determine whether prevention of renal apoB accumulation attenuates DN. Biglycan-deficient and biglycan wild-type Ldlr−/− mice were made diabetic via streptozotocin and fed a high cholesterol diet. As biglycan deficiency is associated with elevated transforming growth factor-β (TGF-β), in some experiments mice were injected with either the TGF-β-neutralizing antibody, 1D11, or with 13C4, an irrelevant control antibody. Biglycan deficiency had no significant effect on renal apoB accumulation, but led to modest attenuation of DN with 1D11, or with 13C4, an irrelevant control antibody. Biglycan deficiency had no significant effect on renal apoB accumulation, but led to modest attenuation of DN with ~30% reduction in albuminuria; however, biglycan deficiency caused a striking elevation in TGF-β. Use of 1D11 led to sustained suppression of TGF-β for approximately 8 weeks at a time. The 1D11 treatment caused decreased renal apoB accumulation, decreased albuminuria, decreased renal hypertrophy, and improved survival, compared with the 13C4 treatment. Thus, prevention of renal apoB accumulation is protective against development of DN. Furthermore, this study demonstrates that prevention of renal apoB accumulation is a mechanism by which TGF-β inhibition is nephroprotective.

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Diabetic nephropathy (DN) is the leading cause of end stage renal disease in the Western world. Dyslipidemia is highly prevalent in patients with nephropathy and can exacerbate progression of the disease (1, 2). Renal deposition of apoB and apoE accelerates the progression of glomerulosclerosis in many renal diseases (3). While it is unknown how lipid accumulates in the mesangium, we previously demonstrated that renal proteoglycans have a binding affinity for LDL in the physiological range (4). Proteoglycans are a class of matrix molecules composed of glycosaminoglycan (GAG) side chains attached to core proteins; proteoglycans are classified based on their core protein and GAG structure. Proteoglycans can bind lipoproteins via ionic interactions between negatively charged sulfate and carboxyl groups on their GAG chains and positively charged residues on apoB and apoE. Proteoglycan binding of LDL induces irreversible changes in the conformation of apoB inhibiting particle efflux and increasing susceptibility to modifications, such as oxidation. Oxidized lipoproteins are avidly taken up by macrophages leading to foam cell formation [for review see (5)].

Transforming growth factor-β (TGF-β) is known to be elevated in diabetes and critically involved in the initiation and progression of DN [for review see (6)]. The literature suggests that hyperlipidemia may contribute to elevated TGF-β levels (7), possibly due to increased angiotensin II or due to effects of hyperlipidemia worsening glomerular pathology, which would lead to increased TGF-β synthesis. TGF-β has been shown to mediate the accumulation of proteoglycans, such as biglycan, in the extracellular matrix (ECM) (8, 9). Furthermore, we have previously shown that TGF-β increases the size and LDL binding affinity of proteoglycans (10, 11). Biglycan is a small leucine-rich proteoglycan with two GAG side chains attached to its core protein. Increased circulatory biglycan is seen in a number of pro-inflammatory renal diseases, including transplant rejection and lupus nephritis (12). Renal biglycan has been

Abbreviations: DN, diabetic nephropathy; ECM, extracellular matrix; GAG, glycosaminoglycan; Ldlr−/−, LDL receptor-deficient; PAS, periodic acid Schiff; STZ, streptozotocin; TGF-β, transforming growth factor-β; UAE, urinary albumin excretion.

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shown to be elevated in diabetes and in fibrotic disease (13–16). Biglycan is well-known to be upregulated by TGF-β. However, the relationship between TGF-β and biglycan is complicated, as biglycan (and another related proteoglycan called decorin) have been proposed to be natural inhibitors of TGF-β, as they can bind TGF-β and sequester it in the ECM [for review see (17)]. It has been shown that the absence of biglycan in the ECM prevents TGF-β sequestration within the ECM, leading to overactive TGF-β signaling (18). Several studies have also reported increased TGF-β levels in biglycan- or decorin-deficient mice, providing further evidence that proteoglycans may act as natural inhibitors of TGF-β (19–22).

Previously, we reported that diabetes and hyperlipidemia led to continuous renal injury characterized by increased urine albumin excretion, elevated TGF-β, renal and glomerular hypertrophy, mesangial matrix expansion, and glomerular lipid and apoB accumulation (15, 16). Furthermore, lowering dietary cholesterol in these mice improved renal function by reversing renal injury (15). By immunohistochemistry, we demonstrated colocalization of renal apoB with biglycan (16). Thus, we proposed that renal apoB retention may be mediated by accumulation of renal biglycan (16). The goal of this study was to determine whether biglycan deficiency protected against DN. As biglycan deficiency causes increased TGF-β, which can confound findings, these studies were done in the presence or absence of TGF-β inhibition.

MATERIALS AND METHODS

Animals

Male biglycan-deficient and biglycan wild-type mice were crossed with LDL receptor-deficient (Ldlr−/−) mice (C57BL6 background) as previously described (19, 22). The studies were approved by the Institutional Animal Care and Use Committee in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Diabetes was induced via streptozotocin (STZ) injections as described; nondiabetic control groups received citrate injections (15, 16). In some experiments, mice were concurrently injected intraperitoneally with 2 mg/kg body weight TGF-β-neutralizing antibody, 1D11 (R&D Systems, Minneapolis, MN), or irrelevant control antibody, 13C4 (Genscript, Piscataway, NJ), on the first day of STZ injections. Injected with 1D11 or 13C4 were repeated every 8 weeks. All mice were fed a 0.12% g/kg cholesterol diet with 40% calories from fat (TD000242; Harlan Teklad, Madison, WI) (15, 16) for 26 weeks; time 0 is defined as the first day of diet and is 2 weeks after STZ or vehicle was first administered.

Metabolic and renal characterization

Animal studies were performed as previously described (15, 16). Briefly, glucose was measured at least weekly by tail vein prick using a commercial glucose meter. Mice received insulin via placement of subcutaneous pellets as needed (indicated by weight loss, body condition, or blood glucose over 500 mg/dl). Nonfasting blood samples were collected periodically to measure TGF-β and cholesterol, as previously described (15, 16). Mice were placed individually into metabolic cages every 4 weeks for collection of 24 h urine samples for measurement of albumin (Bethyl Laboratories, Montgomery, TX) and creatinine (R&D Systems). Blood pressure was measured by the tail cuff method in week 25 following a week of acclimation to the apparatus, as previously described (15, 16). After 26 weeks of diet and diabetes, mice were euthanized by lethal anesthesia and tissues were collected. Kidneys were decapitated, weighed, and divided. Portions of kidneys were snap-frozen in liquid nitrogen, fixed in 10% formalin, and then embedded in paraffin, fixed in methyl Carnoy, or frozen in OCT. Renal sections (4 μm thick) were stained with periodic acid Schiff (PAS), Mason’s trichrome, or oil red O, or immunostained for apoB (catalog number K23300R; Meridian Life Sciences, Memphis, TN), as previously described (16), and matrix accumulation was scored and glomerular cross-sectional area was measured by two blinded observers and scores averaged. Total renal protein was immunoblotted for apoB and proteoglycans as previously described (15, 16, 25) (the relative amount of apoB48 was very low, so only apoB100 is shown in the blots).

Renal TGF-β

Frozen kidney tissues were weighed and pulverized, then homogenized in a hypotonic buffer with protease inhibitors (24). The homogenate was centrifuged at 15,000 g at 4°C for 20 min and the supernatant was removed, aliquoted, and frozen at −80°C. Protein concentrations were determined by standard Bradford assay (Bio-Rad, Hercules, CA). The supernatant was used to measure renal TGF-β by ELISA (Promega, Madison, WI), which was normalized to total renal protein. Acid activation of homogenate was used to measure total TGF-β, while an aliquot of nonactivated homogenate was used to measure biologically active TGF-β according to the assay protocol.

LDL binding assay

Primary mesangial cells were isolated from 4- to 5-week-old nondiabetic biglycan-deficient and biglycan wild-type mice and used in an LDL binding assay in vitro, as previously described (25). In some experiments, cells were stimulated with TGF-β (2 ng/ml) and 1D11 or 13C4 (10 μg/ml) for 24 h, then washed prior to incubation with LDL. Cell protein was collected from parallel wells for analysis of biglycan by immunoblot.

Statistics

Data are presented as mean ± SEM unless otherwise specified or clear from the context. Most data analyses were based on a 2 × 2 factorial structure with diabetic status and genotype (see Table 1; Figs. 1, 2) or genotype and treatment (see Table 2; Figs. 3, 4) as the two factors. Males were not directly compared with females. If significant interactions between diabetic status and genotype (or genotype and treatment) were detected, pairwise comparisons within each genotype and diabetic status (or genotype and treatment) stratum were performed, adjusted for multiplicity using the Bonferroni method; otherwise, inferences were based on the presence or absence of significant main effects. For most quantitative outcomes collected longitudinally, test results for interactions and main effects are reported based on overall trajectories, trajectory slopes, or areas under trajectory curves, as estimated from linear mixed modeling. For quantitative outcomes analyzed at one time point, test results for interactions and main effects are based on between-animal ANOVA, which may have included log transformations to reduce nonnormality and/or weights to address different within-group variances. Survival was compared by log rank or Fisher’s exact test. Insulin pellet consumption was assessed for diabetic mice only using negative binomial or zero-inflated Poisson regression for number of pellets received. Data analyses were performed using version 9.5 of SAS or SigmaPlot 12.0. Statistical significance was defined by P < 0.05.
RESULTS

Metabolic characterization of biglycan deficiency

As previously reported, biglycan-deficient mice were smaller than biglycan wild-type mice (19). For male mice, weight gain was significantly decreased in diabetic compared with nondiabetic mice (P = 0.001); in biglycan-deficient compared with biglycan wild-type mice (P = 0.001). For female mice, there was a significant interaction between diabetes status and genotype (P = 0.001; Table 1). Biglycan deficiency had no impact on susceptibility to STZ-induced diabetes and all mice that received STZ had elevated glucose levels compared with control mice (P < 0.001 for diabetic vs. nondiabetic mice for both genders; Fig. 1A, B). Mice received insulin via placement of subcutaneous pellets as needed; nondiabetic mice did not require insulin. Biglycan-deficient male mice needed more insulin than biglycan wild-type mice (P = 0.035); however, there were no significant differences in insulin use between female biglycan-deficient or biglycan wild-type diabetic mice (Table 1). Per study protocol, mice that did not respond to insulin or had poor body condition were euthanized. There were no significant differences between groups in male mice, but female diabetic mice of both genotypes had decreased survival compared with female nondiabetic mice (P = 0.012); there was no significant effect of biglycan genotype on survival (Table 1). As previously described (15), plasma cholesterol increased over time while mice were fed the 0.12% cholesterol diet. There were no significant differences in cholesterol levels between groups in male mice. Female biglycan-deficient mice had significantly higher cholesterol levels than female biglycan wild-type mice at study end (P < 0.001), but there was no significant effect of diabetes (Table 1). In male mice, triglycerides did not differ significantly between groups; however, in female mice, there was a significant interaction between diabetes and genotype (P = 0.033; Table 1). Systolic blood pressure was increased in male diabetic biglycan wild-type mice compared with male nondiabetic biglycan wild-type mice (P = 0.026; Table 1; there was a significant interaction between biglycan genotype and diabetes status, P = 0.004). There were no significant differences between group blood pressures in female mice (Table 1).

Elevated TGF-β in biglycan deficiency

As previously described (19), biglycan-deficient mice (both males and females) had higher plasma TGF-β levels both at baseline and throughout the study; diabetes caused an increase in plasma TGF-β levels over time. Thus, diabetic biglycan-deficient mice had the highest plasma TGF-β levels over the study. For both male and female mice, there was a significant interaction between diabetes status and genotype (P < 0.001 for both males and females). For male mice, there were statistically significant differences between groups by pairwise comparisons (P = 0.026 for biglycan genotype in nondiabetic mice and P < 0.001 for other comparisons; Fig. 1C). For female mice, there were statistically significant differences between groups (P < 0.001 for each comparison; Fig. 1D). Renal TGF-β was measured after 26 weeks. Total renal TGF-β was increased by diabetes

| TABLE 1. Effect of biglycan deficiency on metabolic parameters |
|---------------------------------------------------------------|
|                                                                 |
| **Bgn<sup>-/-</sup> Control**                                   |
| **Bgn<sup>-/-</sup> DM**                                        |
| **Bgn<sup>-/-</sup> Control**                                   |
| **Bgn<sup>-/-</sup> DM**                                        |
|                                                                 |
| **Weight gain, male (g)**                                      |
| 5.9 ± 0.6                                                      | 5.0 ± 0.4                                                      |
| 6.2 ± 0.4                                                      | 6.4 ± 0.6                                                      |
| <0.001 for diabetes and genotype                               |
|                                                                 |
| **Weight gain, female (g)**                                    |
| 6.2 ± 0.4                                                      | 5.6 ± 0.5                                                      |
| 6.4 ± 0.6                                                      | 4.0 ± 0.5                                                      |
| <0.001 for diabetes and genotype                               |
|                                                                 |
| **Insulin use, male (mg/kg)**                                   |
| 0/13 (0%)                                                      | 4/26 (15%)                                                     |
| 0/10 (0%)                                                      | 25/26 (96%)                                                    |
| 0/12 (0%)                                                      | 10/12 (100%)                                                   |
| 1/10 (0%)                                                      | 16/24 (67%)                                                    |
| NS                                                             |
|                                                                 |
| **Insulin use, female (mg/kg)**                                 |
| 0/13 (92%)                                                     | 0/10 (0%)                                                      |
| 25/26 (96%)                                                    | 16/16 (100%)                                                   |
| 10/12 (100%)                                                   | 12/12 (100%)                                                   |
| 0/12 (0%)                                                      | 16/24 (67%)                                                    |
| NS                                                             |
|                                                                 |
| **Survival, male (N %)**                                       |
| 12/13 (92%)                                                    | 12/13 (100%)                                                   |
| 16/16 (100%)                                                   | 20/24 (83%)                                                    |
| 0.012                                                          |
|                                                                 |
| **Survival, female (N %)**                                     |
| 12/13 (92%)                                                    | 10/10 (100%)                                                   |
| 12/12 (100%)                                                   | 20/24 (83%)                                                    |
| 0.012                                                          |
|                                                                 |
| **Cholesterol, male (mg/dL)**                                  |
| 109 ± 21                                                       | 1,573 ± 137                                                   |
| 1,570 ± 163                                                    | 1,598 ± 113                                                    |
| NS                                                             |
|                                                                 |
| **Cholesterol, female (mg/dL)**                                |
| 963 ± 79                                                       | 1,073 ± 66                                                    |
| 1,426 ± 167                                                    | 1,595 ± 145                                                    |
| <0.001 for genotype                                           |
|                                                                 |
| **Triglycerides, male (mg/dL)**                                |
| 160 ± 21                                                       | 150 ± 43                                                      |
| 213 ± 53                                                       | 168 ± 30                                                      |
| NS                                                             |
|                                                                 |
| **Triglycerides, female (mg/dL)**                              |
| 102 ± 20                                                       | 178 ± 74                                                      |
| 79 ± 19                                                        | 495 ± 76                                                      |
| 0.033 for interaction between diabetes and genotype            |
|                                                                 |
| **sBP, male (mm Hg)**                                          |
| 109 ± 2                                                        | 118 ± 2                                                       |
| 116 ± 3                                                        | 111 ± 6                                                       |
| 0.004 for interaction between diabetes and genotype            |
|                                                                 |
| **sBP, female (mm Hg)**                                        |
| 111 ± 2                                                       | 108 ± 3                                                       |
| 109 ± 5                                                        | 104 ± 2                                                       |
| NS                                                             |
|                                                                 |
| **Total renal TGF-β, males (pg/mg)**                           |
| 119 ± 25                                                       | 230 ± 10.9                                                    |
| 160 ± 6.7                                                      | 690 ± 135.7                                                   |
| 0.006 for interaction                                        |
|                                                                 |
| **Total renal TGF-β, females (pg/mg)**                         |
| 113 ± 5.2                                                      | 19.2 ± 37.9                                                   |
| 176 ± 21.6                                                     | 677 ± 239.3                                                   |
| 0.02 for diabetes; 0.048 for genotype                          |
|                                                                 |
| **Bioactive renal TGF-β, males (pg/mg)**                       |
| 10 ± 1.0                                                       | 17 ± 0.8                                                      |
| 12 ± 1.0                                                       | 21 ± 1.9                                                      |
| <0.001 for diabetes                                           |
|                                                                 |
| **Bioactive renal TGF-β, females (pg/mg)**                     |
| 7 ± 0.3                                                       | 15 ± 0.8                                                      |
| 12 ± 2.4                                                       | 18 ± 5.5                                                      |
| NS (0.053 for diabetes)                                       |
|                                                                 |
| **Renal weight/body weight, males (mg/g)**                     |
| 3.7 ± 0.2                                                      | 5.9 ± 0.4                                                     |
| 3.9 ± 0.2                                                      | 4.8 ± 0.2                                                     |
| 0.016 for interaction between diabetes and genotype            |
|                                                                 |
| **Renal weight/body weight, females (mg/g)**                   |
| 3.7 ± 0.2                                                      | 6.3 ± 0.5                                                     |
| 4.2 ± 0.3                                                      | 6.2 ± 0.4                                                     |
| 0.002 for diabetes                                             |

Biglycan-deficient (Bgn<sup>-/-</sup>) and wild-type (Bgn<sup>+/+</sup>) Ldlr<sup>-/-</sup> mice were injected with vehicle (control) or STZ to cause diabetes (DM) and fed a 0.12% cholesterol diet for 26 weeks. Weight gain is defined as weight at week 26 − weight at day 0 (first day of diet, once hyperglycemia established) in grams. Insulin use is the number of mice per total enrolled mice per group that received insulin pellets; some mice may have received insulin more than once. Survival shows the number of mice alive at week 26 per number of mice enrolled (%). Cholesterol and triglycerides were measured in nonfasting samples at week 26. Systolic blood pressure (sBP) was measured in individual mice in week 25 using a tail cuff apparatus. Renal and bioactive TGF-β were measured in homogenized kidney samples from n = 4–6 per group. Data shown is mean ± SEM from N = 10–35 per group (number enrolled per group is shown in survival) unless otherwise specified.

<sup>a</sup>P < 0.05 for diabetes status within genotype.

<sup>b</sup>P < 0.05 for genotype within diabetes status.
Fig. 1. TGF-β and renal measures in biglycan deficiency. Biglycan wild-type (Bgn\textsuperscript{+/-}; solid symbols) and biglycan-deficient (Bgn\textsuperscript{-/-}; open symbols) Ldlr\textsuperscript{-/-} mice were injected with STZ to induce diabetes (DM; triangles) or vehicle (ctrl; squares) and then fed a 0.12% cholesterol diet for 26 weeks. A, B: Blood glucose was measured weekly. Mean ± SEM is shown for N = 10–35 per group. P < 0.001 for diabetic versus control groups. C, D: TGF-β was measured at the indicated times. Mean ± SEM is shown for N = 8–34 per group. *P = 0.024 for biglycan genotype effect in male nondiabetic mice and P < 0.001 for other pairwise comparisons. E, F: Mice were placed individually in metabolic cages every 4 weeks for the collection of 24 h urine samples for the determination of UAE/urinary creatinine excretion. Mean ± SEM is shown for N = 8–11 per group at each time point. *P < 0.001 for diabetic versus nondiabetic mice and for biglycan-deficient versus biglycan wild-type mice. G, H: Renal sections were stained with PAS and at least 20 glomeruli per mouse were scored using a semi-quantitative scale by two observers blinded to group. Mean ± SEM is shown for N = 7–16 per group. Panels A, C, E, and G, males; panels B, D, F, and H, females.
(P < 0.05 for both males and females) and by biglycan deficiency (P < 0.05 for both males and females) and there was a significant interaction between diabetes and biglycan genotype for males (P = 0.006; Table 1). Endogenously active renal TGF-β was significantly increased by biglycan deficiency (P = 0.016) and diabetes (P < 0.001) for male mice (Table 1), but was not significantly different across groups for female mice (Table 1; near-significant P = 0.053 for diabetes).

**Effect of biglycan deficiency on renal parameters**

All groups had a progressive increase in urinary albumin excretion (UAE) over time, with much higher levels in diabetic mice (P < 0.001 vs. nondiabetics). UAE in biglycan-deficient mice was significantly decreased compared with biglycan wild-type mice in both males and females, with UAE ∼30% lower than their biglycan wild-type counterparts over the time course (P < 0.001; Fig. 1E, F). Early DN is associated with renal hypertrophy and, in more advanced stages, renal atrophy is seen. Diabetes caused renal hypertrophy in both biglycan wild-type and biglycan-deficient mice (P ≤ 0.002 for both males and females); but in males, biglycan-deficient mice had significantly less renal hypertrophy than biglycan wild-type mice (P = 0.002; Table 1). In this murine model, we have previously demonstrated that diabetes causes increased mesangial matrix accumulation, as assessed by a semi-quantitative score (15). There was a tendency toward increased PAS score in diabetic compared with nondiabetic biglycan wild-type mice in both males and females, but this did not reach statistical significance. Biglycan deficiency may protect against mesangial matrix expansion, as biglycan-deficient diabetic mice did not demonstrate increased PAS score compared with biglycan-deficient nondiabetic mice in either gender; however, there were no statistically significant effects (Fig. 1G, H; supplemental Fig. S1). In male, but not female, mice, there was an increase in renal collagen staining in diabetic compared with nondiabetic biglycan wild-type mice in both males and females, but this did not reach statistical significance.

**Biglycan deficiency does not affect renal apoB retention**

Renal apoB accumulation was measured by Western blot and immunostaining. As expected (16), Western analysis demonstrated increased renal apoB content in diabetic mice compared with nondiabetic mice (P = 0.027), but there was no effect of biglycan deficiency (Fig. 2A, B). Immunostaining for apoB revealed a similar trend (supplemental Fig. S2A). Kidney sections were stained with oil red O, but there were no significant differences between groups (supplemental Fig. S2B). Cholesterol content was also measured in renal tissue homogenates and did not differ between groups (supplemental Fig. S2C). Primary mesangial cells were isolated from nondiabetic biglycan-deficient or biglycan wild-type mice and LDL binding was measured. There was no significant difference in LDL binding to biglycan wild-type or biglycan-deficient cells (Fig. 2C). As all proteoglycans can bind apoB, the renal content of other proteoglycans was measured by immunoblot to see whether another proteoglycan was upregulated in biglycan-deficient mice. No substantial differences in the renal content of perlecan,
decorin, versican, lumican, fibronectin, or fibromodulin were seen between biglycan-deficient or wild-type mice, or between control and diabetic mice (supplemental Fig. S3).

**Effect of TGF-β inhibition on metabolic parameters**

As both systemic and renal TGF-β were significantly increased in biglycan-deficient mice, the experiments were repeated using administration of TGF-β-neutralizing antibody, 1D11, or irrelevant control antibody, 13C4. To minimize animal use, only male diabetic mice were used. There was no significant effect of antibodies on susceptibility to STZ-induced diabetes. All mice gained weight during the study, but mice treated with 1D11 gained more weight than those treated with 13C4 ($P = 0.02$; Table 2). All mice in the study had elevated glucose levels throughout the study (Fig. 3A). There was no significant effect of genotype or either antibody on blood glucose levels. Mice received insulin via subcutaneous pellets as needed; biglycan-deficient mice needed more insulin than biglycan wild-type mice ($P = 0.002$), but there was no effect of the 1D11 on insulin use (Table 2). No mice that received 1D11 in either genotype died of any cause during the study. However, 15% of biglycan-deficient and 18% of biglycan wild-type mice that received 13C4 died or were euthanized prior to the end of the study (Table 2, $P = 0.011$ for antibody effect). Cholesterol increased over time in all groups (not shown), but did not significantly differ between groups (week 26 values shown in Table 2). After 26 weeks of diet, mice that received 1D11 had approximately double the triglyceride levels compared with those that received 13C4 ($P = 0.019$, Table 2) with no effect of genotype. There were no significant differences in systolic blood pressure between any of the groups (Table 2).

**Effectiveness of TGF-β inhibition**

Plasma TGF-β was measured at least weekly and injections of 1D11 (and 13C4) were repeated when TGF-β levels began to rise, which was every 8 weeks. As expected, use of the TGF-β-neutralizing antibody, 1D11, almost completely suppressed plasma TGF-β levels (Fig. 3B, the curves for 1D11-treated biglycan wild-type and biglycan-deficient mice overlap; $P < 0.001$ for 1D11 vs. 13C4 treatment within each genotype). In the 13C4-treated groups, there was an initial spike in TGF-β levels immediately after STZ injections, then a gradual drop over the next 7 days. TGF-β levels in 13C4-treated biglycan wild-type mice remained at approximately 1,000–1,500 pg/ml for the duration of the study. However, in 13C4-treated biglycan-deficient mice, TGF-β levels rose again after the first 5 weeks reaching levels of 10,000–15,000 pg/ml after 10 weeks, and remained significantly elevated over the rest of the study ($P < 0.001$ for 13C4-treated biglycan wild-type vs. biglycan-deficient groups; Fig. 3B). The timing of the increase in TGF-β levels in 13C4-treated biglycan-deficient mice is similar to the onset of albuminuria (Fig. 3C). The 13C4-treated biglycan-deficient mice had higher total and bioactive renal TGF-β content than 13C4-treated biglycan wild-type mice ($P < 0.05$ for each; Table 2); treatment with 1D11 lowered both total and bioactive renal TGF-β compared with treatment with 13C4 within each genotype ($P \leq 0.006$ for each).

**Effect of TGF-β inhibition on renal parameters**

There was a striking effect of 1D11 treatment to limit increases in UAE in both genotypes ($P < 0.001$; Fig. 3C). UAE in 1D11-treated mice remained at levels similar to those seen in nondiabetic mice in our prior studies (compare Fig. 3C with Fig. 1E, F) (15, 16). Furthermore, mice that received 1D11 had less renal hypertrophy compared with those that received 13C4 ($P < 0.001$; Table 2). However, there was no significant effect of 1D11 on PAS scores. Biglycan-deficient mice had slightly lower, but statistically significant, PAS scores than biglycan wild-type mice, but there was no significant effect of 1D11 on PAS scores. Biglycan-deficient mice had slightly lower, but statistically significant, PAS scores than biglycan wild-type mice, but there was no significant effect of 1D11 on PAS scores. Biglycan-deficient mice had slightly lower, but statistically significant, PAS scores than biglycan wild-type mice, but there was no significant effect of 1D11 on PAS scores (Fig. 3D; $P = 0.038$ for genotype; supplemental Fig. S4). There were no differences between groups in renal collagen content (supplemental Fig. S4) or renal macrophage accumulation (not shown).

**TABLE 2. Effect of biglycan genotype and TGF-β inhibition on metabolic parameters**

| Genotype/Inhibition | 1D11 | 13C4 |
|---------------------|------|------|
| **Weight gain (g)** | 5.6 ± 1.7 | 7.9 ± 1.3 |
| **Insulin use (number treated/total number)** | 7/17 (41%) | 4/19 (21%) |
| **Cholesterol (mg/dl)** | 123 ± 8 | 104 ± 9 |
| **Triglycerides (mg/dl)** | 1,429 ± 108 | 1,461 ± 58 |
| **Total renal TGF-β** | 263 ± 35 | 567 ± 130 |
| **Bioactive renal TGF-β** | 13 ± 1 | 5 ± 1 |
| **Renal weight/body weight (mg/g)** | 4.5 ± 0.2 | 3.5 ± 0.1 |

| Genotype/Inhibition | 1D11 | 13C4 |
|---------------------|------|------|
| **Survival, N (%)** | 14/17 (82.3%) | 19/19 (100%) |
| **sBP (mm Hg)** | 23/27 (85.2%) | 26/26 (100%) |
| **Total cholesterol (mg/dl)** | 1,192 ± 108 | 1,161 ± 58 |
| **Total triglycerides (mg/dl)** | 252 ± 56 | 517 ± 130 |
| **Total renal TGF-β** | 257 ± 29 | 542 ± 11 |
| **Bioactive renal TGF-β** | 13 ± 1 | 5 ± 1 |
| **Renal weight/body weight (mg/g)** | 4.5 ± 0.2 | 3.5 ± 0.1 |

Biglycan-deficient (Bgn<sup>−/−</sup>) and wild-type (Bgn<sup>+/+</sup>) Ldlr<sup>−/−</sup> mice were injected with STZ to cause diabetes and concurrently injected with TGF-β-neutralizing antibody, 1D11, or irrelevant control antibody, 13C4, at 2 mg/kg ip on the first day of STZ injections. Mice were fed a 12% cholesterol diet for 26 weeks. Weight gain is defined as weight at week 26 – weight at day 0 (first day of diet, once hyperglycemia established) in grams ($His P$ was derived from the time/antibody interaction term in a linear mixed model for weight). Insulin use is the number of mice per group that received insulin pellets; some mice may have received insulin more than once. Survival shows number of mice alive at week 26 per group of mice enrolled (%). Cholesterol levels were measured in nonfasting samples at week 26. Systolic blood pressure (sBP) was measured in individual mice in week 25 using a tail cuff apparatus. Renal and bioactive TGF-β were measured in homogenized kidney samples from 4–6 mice per group. Data shown is mean ± SEM from N = 17–27 per group (number enrolled per group is shown in survival) unless otherwise specified.

<sup>a</sup>P < 0.05 for diabetes status within genotype.

<sup>b</sup>P < 0.05 for genotype within diabetes status.
Effect of TGF-β inhibition on renal apoB accumulation

Treatment with 1D11 significantly decreased renal apoB content determined by Western blot within both genotypes (Fig. 4A, B; \( P = 0.002 \)). Immunostaining for apoB revealed a similar pattern (supplemental Fig. S5A). Kidney sections were stained with oil red O, but there were no significant differences between groups (supplemental Fig. S5B). Cholesterol content was also measured in renal tissue homogenates and did not differ between groups (supplemental Fig. S5C). Primary mesangial cells were isolated from non-diabetic biglycan wild-type or biglycan-deficient cells; cells were treated ex vivo with TGF-β or vehicle and with 13C4 or 1D11, then LDL binding was measured. In the absence of TGF-β stimulation, there were significant main effects for antibody \( (P = 0.029) \) and biglycan genotype \( (P = 0.013) \) on LDL binding; in the presence of TGF-β stimulation, there was less LDL binding in 1D11-treated compared with 13C4-treated cells \( (P < 0.0001; \text{Fig. 4C}) \). As expected, no biglycan was detectable in cells isolated from biglycan-deficient mice. Biglycan content was increased by TGF-β stimulation, which was inhibited by the presence of 1D11 in cells isolated from biglycan wild-type mice (Fig. 4D).
In summary, biglycan deficiency led to a modest decrease in UAE, but there was no significant effect on renal apoB accumulation. There was a striking elevation in TGF-β in biglycan-deficient mice. Although we did not find any compensatory increase in other proteoglycans in biglycan-deficient mice, the lack of biglycan did not significantly affect renal apoB accumulation implying that other proteoglycans (or, indeed, other mechanisms) contribute to renal apoB accumulation.
apoB retention. TGF-β leads to the elongation of GAG chains in many proteoglycans (4, 26–28). Proteoglycan-lipoprotein interactions are ionic in nature, with binding occurring between negatively charged GAG chains and positive residues on apoB or apoE. Thus, the elevated TGF-β in biglycan-deficient mice may have led to elongated GAG chains on other proteoglycan species, which could then bind LDL, even in the absence of biglycan. This is supported by the finding that TGF-β inhibition prevented renal apoB accumulation and decreased LDL binding to primary mesangial cells ex vivo. When biglycan-deficient mice were treated with the TGF-β-neutralizing antibody, 1D11, there was a suppression of UAE to levels seen in nondiabetic mice (compare Fig. 3C with Fig. 1E, F), and prevention of renal hypertrophy. Furthermore, 1D11-treated mice had improved survival and increased weight gain. While these data do not definitively prove cause and effect, it is striking that neutralization of TGF-β led to decreased renal apoB accumulation, decreased albuminuria, and improved mortality, suggesting that renal apoB accumulation contributes to renal disease development.

**TGF-β and renal diseases**

Extensive work has demonstrated a key role for TGF-β in the pathogenesis of renal diseases, including DN. The TGF-β superfamily has three isoforms, named TGF-β1, -β2, and -β3. All three isoforms are expressed in the kidney, as are all three TGF-β receptor isoforms. TGF-β signaling within the kidney induces both pro-fibrotic Smads (Smad2 and Smad3), as well as the anti-inflammatory and anti-fibrotic inhibitory Smad7. Diabetes increases renal TGF-β as well as systemic TGF-β levels. The elevations in TGF-β are due to hyperglycemia directly, as well as through increased protein traffic in the glomerulus and increased angiotensin II [for review see (29)]. The timing of the second elevation of TGF-β seen in 1D11-treated biglycan-deficient mice coincides with the onset of albuminuria, implying that the onset of renal injury led to this increase in TGF-β. Inhibition of TGF-β has been shown to limit development of DN and other renal diseases (30–33); however, studies have not consistently shown effects of TGF-β antagonism on proteinuria. Addition of agents antagonizing the renin angiotensin pathway provide further benefits in DN, including reduction in proteinuria. Decorin and biglycan are closely related proteoglycans that are thought to be natural inhibitors of TGF-β. Indeed, prior studies have used decorin as a TGF-β-neutralizing agent and found renal protection (34, 35). We and others have previously demonstrated increases in both systemic and tissue TGF-β in biglycan-deficient mice (18, 23, 36). We have now demonstrated that suppression of this elevation in TGF-β provides further reduction in DN in this murine model of biglycan deficiency. Thus, this data provides further evidence in support of biglycan playing a major role in the regulation of TGF-β activity, and adds to the literature demonstrating renal protective effects of TGF-β inhibition.

**Decreased renal apoB accumulation**

In previous studies using cultured cells, we and others have demonstrated that TGF-β leads to upregulation of biglycan core protein and elongation of the GAG chains on many proteoglycans (28, 37–39). We have further shown that proteoglycans from TGF-β-treated cells have increased lipoprotein binding (4, 10, 37). Proteoglycans bind lipoproteins via ionic interactions between negatively charged sulfate and carboxylic acid groups on GAG chains and positively charged residues on lipoproteins. Elongation of GAG chains is accompanied by increased sulfation, which accounts for the increased lipoprotein binding affinity of proteoglycans synthesized by cells stimulated with TGF-β (37). In previous studies, we demonstrated that suppression of TGF-β using 1D11 decreased lipoprotein binding to primary vascular smooth muscle cells (25); we now show a similar decrease in lipoprotein binding in primary mesangial cells. We propose that the decrease in renal apoB content observed in 1D11-treated mice is due to prevention of GAG chain elongation and, thus, decreased lipoprotein binding affinity. We did not find any compensatory increase in other renal proteoglycans in biglycan-deficient compared with biglycan wild-type mice in the present study; thus, it remains unclear which proteoglycans contribute to renal apoB accumulation. However, the lack of decreased renal apoB retention or LDL binding in biglycan deficiency implies redundancy of proteoglycans. Indeed all proteoglycans are capable of binding to lipoproteins, and binding is driven mainly by elongated GAG chains [for review see (5)]. However, given that inhibition of TGF-β alone does not consistently reduce proteinuria (40–42), we speculate that the decreased renal apoB accumulation observed in this study contributed to the decreased proteinuria observed.

**Elevated triglycerides**

Somewhat surprisingly, we found elevated triglycerides in mice that received 1D11. High triglycerides have been found to be a risk factor for the development of chronic kidney disease (43, 44) and one study found that high triglycerides were associated with altered levels of urea nitrogen, glomerular filtration rate, and creatinine, as well as an increased risk of chronic kidney diseases (45). Thus, perhaps the benefit of TGF-β neutralization is negated, at least in part, by the effect of elevated triglycerides in 1D11-treated mice. The cause of elevated triglycerides in 1D11-treated mice is unclear and this finding is inconsistent with the literature. In a previous study using 1D11 in nondiabetic LDLR/− mice, we did not find effects of 1D11 on triglycerides (45). Furthermore, another study reported a decrease in triglycerides (along with decreased adiposity and insulin resistance) using 1D11 (46). Further work is necessary to determine whether inhibition of TGF-β has negative impacts on triglyceride metabolism.

**1D11 doses**

The 1D11 is a pan-TGF-β-neutralizing antibody that has been extensively studied in a number of animal models and a closely related molecule, fresolimumab, has been examined in clinical studies. Inhibition of TGF-β has potential therapeutic impact in a number of pathologies, including renal diseases and several forms of cancer, systemic sclerosis, myelofibrosis, and others. In most of the animal studies, the antibody is injected multiple times per week (47–49).
Prevention of renal apoB retention in DN 2273

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