Renal Bone Morphogenetic Protein-7 Protects against Diabetic Nephropathy

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Longstanding diabetes causes renal injury with early dropout of podocytes, albuminuria, glomerular and tubulointerstitial fibrosis, and progressive renal failure. The renal pathology seems to be driven, in part, by TGF-β and is associated with a loss of renal bone morphogenetic protein-7 (BMP-7) expression. Here, the hypothesis that maintenance of renal (especially podocyte) BMP-7 by transgenic expression reduces diabetic renal injury was tested. Diabetic mice that expressed the phosphoenolpyruvate carboxykinase promoter–driven BMP-7 transgene and nondiabetic, transgenic mice as well as diabetic and nondiabetic wild-type controls were studied for up to 1 yr. Transgenic expression of BMP-7 in glomerular podocytes and proximal tubules prevents podocyte dropout and reductions in nephrin levels in diabetic mice. Maintenance of BMP-7 also reduces glomerular fibrosis and interstitial collagen accumulation as well as collagen I and fibronectin expression. Diabetic wild-type mice develop progressive albuminuria, which is substantially reduced in transgenic mice. These effects of the BMP-7 transgene occur without changing renal TGF-β levels. It is concluded that maintenance of renal BMP-7 during the evolution of diabetic nephropathy reduces diabetic renal injury, especially podocyte dropout. The findings also establish a role for endogenous glomerular BMP-7 as an autocrine regulator of podocyte integrity in vivo.


B one morphogenetic protein-7 (BMP-7) is a member of the BMP-family that forms together with several other protein families, including the TGF-β family, growth differentiation factors, Müllerian inhibitory substance, activins/inhibin, lefty and nodal, the TGF-β superfamily of cytokine growth factors. BMP-7 plays pivotal roles in kidney and eye development (1). In adult organisms, BMP-7 is selectively expressed in only a few tissues, and the kidneys are among the organs with the highest expression. In the kidney, BMP-7 is expressed mainly in tubules of the outer medulla and in glomerular visceral epithelial cells (podocytes).

Members of the TGF-β superfamily of proteins use a series of type I and II receptors for initiation of cellular signals. Binding of a ligand to the type II receptor with constitutive serine/threonine kinase activity causes recruitment of a type I receptor into a heterodimeric receptor complex. Activation by phosphorylation of the type I receptor serine/threonine kinase induces serine phosphorylation of cellular substrates, notably receptor Smad. In most tissues, TGF-β causes Smad2/3 activation, whereas BMP activate Smad1, 5, and 8 (2,3). In renal epithelial cells, BMP-7 uses preferably or exclusively Smad5 (4).

Whereas TGF-β is the single most prominent cytokine in renal fibrosis, pharmacologic BMP-7 has emerged recently as an antifibrogenic agent in the kidney. In experimental, unilateral obstructive nephropathy or diabetic glomerular sclerosis, exogenously administered rhBMP-7 reduces renal fibrosis (5,6). The mechanisms of BMP-7’s antifibrogenic actions have been explored in vitro and involve BMP-7–induced Smad signals that block activation and nuclear translocation of TGF-β–induced Smad2/3 (4). Thus, in cultured cells, BMP-7 acts as a counter-regulator of TGF-β’s profibrogenic actions (7). In experimental diabetes in rodents, renal levels of BMP-7 decrease early, predating onset and progression of structural damage to the kidneys (8). The decrease of renal BMP-7 may favor TGF-β–driven fibrogenesis. In these in vivo experimental studies, we examined whether endogenous renal BMP-7 prevents early podocyte injury and has antifibrogenic activity in glomeruli and the renal tubulointerstitium. We tested the hypothesis that overexpression of BMP-7 during diabetes by means of a phosphoenolpyruvate carboxykinase (PEPCK) promoter–driven transgene retards onset and progression of diabetic nephropathy in mice with special emphasis on early glomerular injury. PEPCK–BMP-7 transgenic (TG) mice were made diabetic with streptozotocin and studied for up to 1 yr along with nondiabetic BMP-7 transgenic mice and diabetic and nondiabetic, wild-type (WT) controls.

Materials and Methods

Animal Model

Studies were performed in male FVB/N mice that expressed the human BMP-7 transgene under the control of the rat PEPCK promoter...
and in FVB/N WT mice from the same colony (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) to ensure identical genetic background. TG mice were generated using the rat PEPCK promoter element (−2086 to 65) to drive expression of human BMP-7 (9). The construct was generated in two stages. First, the 2.1-kb rat PEPCK promoter element (provided by Sarah Comerford, UT Southwestern Medical Center, Dallas, TX) was subcloned into the SalI site of pBSV-PEPK. This contains the 2.1-kb PEPCK promoter element 5′ of a 1.1-kb spliced SV40 poly A tail on a B5-SK+ backbone. Second, the full-length human BMP-7 cDNA was amplified by reverse transcription–PCR (RT-PCR) and shutting into BglII/ClaI sites 3′ of the PEPCK promoter to generate PBSV–PEPK–hBMP-7. The KpnI/NotI linearized construct then was purified and microinjected into FVB/N ooocytes. Genotyping of the offspring was performed by Southern blot analysis of HindIII-digested DNA probed with the 0.9-kb HpaI/ERV SV40 poly A fragment from PBSV-PEPK. Subsequent genotyping then was performed by slot blot using the same probe or by tail DNA PCR using SV40 poly A–specific primers that spanned the intron segment (forward primer act tct gtg tga cat aat tg; reverse primer aga ttc aga gcc agg atg).

Seven phenotypically normal founder lines were identified and mated onto WT FVB/N mice to generate hemizygous F1 mice. Transgene expression was detected in three of these lines (D1, D3, and D5) by Western blot analysis using the BMP-7 mAb 12G3 (provided by Kuber Sampath, Curis Corp., Cambridge, MA) and/or by Northern blot analysis of whole kidney, fat, and liver RNA using the SV40 poly A tail probe. The strongest transgene expression was seen in line D1, and homozygous male descendants were used in these studies.

Forty TG and WT mice were made diabetic with repeated intraperitoneal injections of streptozotocin (Sigma, St. Louis, MO), 50 mg/kg in 50 mM citrate buffer (pH 4.8), on five consecutive days. Two days after the last injection, tail-tip blood glucose was measured with a glucometer (Encore; Bayer Corp., Elkhart, IN). Mice with blood glucose ≥350 mg/dl were maintained with free access to laboratory food and drinking water. Similar numbers of TG and WT, nondiabetic mice were maintained in parallel as controls. Thus, four groups of mice were studied: WT, nondiabetic controls (WT-Co), WT diabetic mice (WT-D), BMP-7–TG, nondiabetic controls (TG-Co), and BMP-7–TG mice with diabetes (TG-D). Periodically, blood glucose was measured and timed urine was collected and cleared by centrifugation, and urine aliquots were frozen at −80°C for quantitative measurement of albuminuria. At 4, 16, and 52 wk, aliquots of mice from each of the four groups (n = 8 to 11) were anesthetized, blood was obtained by aortic puncture, and kidneys were removed. One-millimeter coronal kidney sections were obtained and processed.

One hundred glomerular tufts that were free of Bowman’s capsule and tubular elements were microdissected manually on ice under a dissecting microscope from aliquot renal sections from six animals in each group at each of the three time points in recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI) for subsequent RNA extraction. Total RNA also was extracted from whole kidney sections using RNA Stat-60 reagent and the manufacturer’s recommended procedure (Tel-Test, Friendswood, TX). Additional kidney aliquots were shock-frozen in liquid N2 and stored at −80°C. Additional coronal kidney slices were fixed in buffered paraformaldehyde for histologic and immunohistologic studies. For protein analysis, the wet weights of aliquots of kidney were measured with an analytical microbalance; tissues were minced and homogenized in 10 μl/mg extraction buffer (50 mM HEPES, 50 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, and 10 mM EDTA [pH 7.4], containing 10 mM Na-orthovanadate and protease inhibitor cocktail [Roche, Penzberg, Germany]), kept on ice for 15 min, cleared by centrifugation, and stored at −80°C.

Serum levels of total and glycated albumin were measured with the BCG-albumin assay (BioAssay Systems, Haywood, CA) and a specific ELISA assay (Glyco-Albumin M; Exocell Inc., Philadelphia, PA), respectively. Albumin was measured in triplicate in diluted urine with a mouse-specific, sensitive, double-antibody ELISA assay (Bethyl, Montgomery, TX). Urea nitrogen and creatinine concentrations in sera were measured with the urease-glutamate dehydrogenase and the kinetic Jaffe methods, respectively, on the Cobos Mira automated assay system (Roche Diagnostic Systems, Montclair, NJ). The studies that involved animals were performed with adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by institutional review boards.

Cell Cultures

Cultured murine mesangial cells and mouse podocytes were used to assess the expression of the PEPCK enzyme in combination with isolated glomeruli and mouse whole kidney. Murine mesangial cells (ATCC, Manassas, VA) were cultured in DMEM/F12 that contained 10% FCS. Murine podocytes were donated by K. Endlich (University of Heidelberg, Heidelberg, Germany) and have been characterized in detail previously (10). Podocytes were grown at 33°C in collagen I–coated culture flasks in RPM1 1640 that contained 10% FCS and IFN-γ (10 U/ml) and then thermo-shifted and incubated at 37°C in IFN-free medium for 2 wk for differentiation.

Biochemical Assays

BMP-7 levels in sera and renal extracts and total renal TGF-β were measured with ELISA assays using commercially available kits and the manufacturers’ instructions (R&D Systems, Minneapolis, MN; Ray BioTech, Norcross, GA). Serum TGF-β was measured with a highly sensitive and specific bioassay as described previously (11). mRNA levels that encoded TG (human) BMP-7 in several different organs from TG, nondiabetic control mice were assessed by RT-PCR using hBMP-7–specific primers (Table 1) and optimized PCR conditions avoiding co-amplification and recognition of endogenous, murine BMP-7. In whole-kidney RNA extracts from each mouse in the four groups, human and murine BMP-7 mRNA, respectively, was measured separately by competitive RT-PCR using selective primer sets (Table 1) and co-amplification of 18S rRNA as internal standard. Expression of transcription factor inhibitor of differentiation-1 (Id-1), plasminogen activator inhibitor-1 (PAI-1), a2-chain of collagen I, and fibronectin also was assessed by competitive RT-PCR using mouse-specific primers (Table 1).

The expression of mRNA encoding the PEPCK enzyme was assessed by semiquantitative RT-PCR in RNA extracts from normal FVB/N mouse whole kidney; microdissected glomeruli; cultured, differentiated podocytes; and cultured murine mesangial cells using specific primers (Table 1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for loading control. Murine and human (transgene-derived) BMP-7 mRNA were measured in extracts from microdissected glomeruli by real-time RT-PCR using mouse or human BMP-7–specific primers in separate assays and commercially available reagents (Quantitect SYBR green PCR; Qiagen, Valencia, CA) and the ABI Prism 7000 sequence detection system cycler (Applied Biosystems, Foster City, CA). As an internal control, GAPDH mRNA was measured in parallel in each sample.

Levels of phosphorylated Smad2/3 and Smad1/5 were assessed by Western blot analysis of 50 μg of whole-kidney protein extracts per sample.
lane. After electrophoretic separation and transfer onto nitrocellulose, membranes were incubated with anti-pSmad2/3 or anti-pSmad1/5 (Santa Cruz Biotechnology, Santa Cruz, CA) and appropriate horseradish peroxidase–conjugated second antibodies. Bands were visualized with chemiluminescence. Stripped membranes were rebotted with anti-GAPDH for loading control.

Total acid-soluble renal collagen was measured in frozen samples that were homogenized in 0.5 M acetic acid (50 μl/mg wet wt) and extracted on ice for 24 h. Samples were cleared by centrifugation. The collagen concentration in the extracts was measured in duplicate with a sircol dye precipitation method and collagen type I as standard using a commercially available kit (Biocolor Ltd., Newtonabbey, Northern Ireland, UK).

The activity of matrix metalloproteases/gelatinases was examined by gelatin zymography and with a quantitative activity assay. Zymography was performed on renal tissue extracts that were electrophoretically separated in SDS-PAGE gels (25 μl/mg). Gels then were acrylamide-separated in SDS-PAGE gels (25 μg/lane). After electrophoretic separation and transfer onto nitrocellulose, membranes were incubated in 2.5% Triton X-100 in water to remove SDS and then incubated in buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij-35) for 30 h at 37°C. Gels then were stained with 0.5% Coomassie Blue G250 in water that contained 30% methanol and 10% glacial acetic acid and then destained in the same solution.

Assessment of Podocyte Number and Glomerular and Interstitial Fibrosis

Podocytes were visualized in 4-μm kidney sections by immunofluorescence with anti–WT-1 (Santa Cruz Biotechnology) in 3% BSA/PBS for 16 h at 4°C after blocking with 10% normal serum. Nephrin was assessed by immunofluorescence with guinea pig polyclonal anti-nephrin antibody (Research Diagnostics Inc., Flanders, NJ). Slides then were incubated with fluorescein-conjugated second antibody. Quantification of WT-1–positive podocytes and of nephrin was performed by digital histomorphometry. Fifty glomeruli in each mouse were microphotographed in a Nikon Eclipse E400 microscope with a Nikon DXM1200 digital camera. In each glomerulus, the number of WT-1–positive podocytes was counted, and the color-specific pixel number reflecting nephrin was counted with Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA) and recorded as a fraction of the cross-sectional area of the glomerulus. Podocyte proliferation activity was assessed by double staining of sections with monoclonal anti–proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology), biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA), and Alexa Fluor 488–conjugated streptavidin (Molecular Probes) followed by staining with rabbit anti–WT-1 and Alexa Fluor 568–conjugated anti-rabbit IgG (Molecular Probes). Double-positive cells were counted in 50 glomerular cross-sections in each mouse.

Glomerular fibrosis was examined in 50 glomerular cross-sections of trichrome-stained slides and defined as the relative area of blue-stained collagens. Interstitial collagen was assessed in 20 nonoverlapping cortical interstitial high-power fields in each sample, and the pixel number of blue-stained collagen was counted.

### Statistical Analyses

Group data are expressed as mean ± SE. Group comparisons were performed with ANOVA followed by Newman-Keuls multicomparison test. Statistical significance of differences between group means was defined at a probability of <5% (P < 0.05).

### Results

**PEPCK Promoter–Driven Transgene Upregulates hBMP-7**

BMP-7–TG mice had normal appearance, and observations during the life span and at autopsy did not reveal any specific phenotype. WT mice express endogenous, murine BMP-7 in kidney and was virtually absent in pancreas, spleen, ileum, and colon (Figure 1).

**Effect of Diabetes on BMP-7 Expression**

Diabetic mice remained hyperglycemic throughout the period of study (up to 52 wk), and maintenance of the diabetic milieu also is ensured by two- to three-fold elevated levels of glycated albumin (Figure 2). Hyperglycemia and the increases in the levels of glycated serum albumin were similar in diabetic WT and TG mice.

The renal expression and levels of total (sum of human and mouse) BMP-7 were measured with real-time PCR. Levels were expressed as fold change from WT mice. The renal expression of BMP-7 was increased in diabetic mice compared to control mice (Table 1).

#### Table 1. Primers used in PCR assays

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Product Size (bp)</th>
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<tr>
<td>hBMP-7</td>
<td>5'-AGG ATG AGC TTG GAG CTG TC-3'</td>
<td>268</td>
</tr>
<tr>
<td>mBMP-7</td>
<td>5'-GGG CAG TGA GAG ACT TAG-3'</td>
<td>425</td>
</tr>
<tr>
<td>Id-1</td>
<td>5'-GCC GAA CCG CAA AGT GAG CAA G-3'</td>
<td>497</td>
</tr>
<tr>
<td>PAI-1</td>
<td>5'-AGC ACA GGC ACT GCA AAA GGT C-3'</td>
<td>141</td>
</tr>
<tr>
<td>C1A2</td>
<td>5'-AGT TCG TGG TTC TCA GGG TAG-3'</td>
<td>254</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5'-AGT ACC TTG TCA ACA GA-3'</td>
<td>428</td>
</tr>
<tr>
<td>PEPCK</td>
<td>5'-AGT CCT GAC AGC AGC TGT GC-3'</td>
<td>346</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCA TGG AGA AGG CCG GGG-3'</td>
<td>195</td>
</tr>
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- **C1A2, α2-chain of collagen I:** GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hBMP-7, human bone morphogenetic protein-7; mBMP-7, mouse BMP-7; Id-1, transcription factor inhibitor of differentiation-1; PAI-1, plasminogen activator inhibitor-1; PEPCK, phosphoenolpyruvate carboxykinase.
murine) BMP-7 tended to decrease in diabetic WT mice compared with nondiabetic controls at 16 and 52 wk (Figure 3). In contrast, the BMP-7 transgene raised the renal BMP-7 expression and levels approximately two-fold compared with WT controls. Addition of the diabetic milieu, however, did not further increase PEPCK promoter–driven hBMP-7 or total kidney BMP-7 levels. Nevertheless, compared with diabetic WT mice, renal BMP-7 levels were substantially greater in diabetic TG mice (*P < 0.05 versus WT-Co).

Figure 1. Assessment of transgenic (TG) expression of human bone morphogenic protein-7 (BMP-7) in different tissues of human BMP-7 (hBMP-7)-TG mice by reverse transcription–PCR (RT-PCR). A kidney sample from a wild-type (WT), non-TG mouse is included (right lane) as a negative control.

Figure 2. Serum glucose and serum glycated albumin levels in the four groups of mice. ▲, BMP-7–TG mice with diabetes (TG-DM); △, WT diabetic mice (WT-DM); ■, BMP-7–TG, nondiabetic controls (TG-Co); □, WT, nondiabetic controls (WT-Co). *P < 0.05 versus WT-Co.

Figure 3. BMP-7 levels and expression in kidney of WT and TG control and diabetic mice. (a) Representative immunofluorescence with anti–BMP-7 at 16 wk. Each image depicts a glomerulus in the center and few distal tubular and several proximal tubular cross-sections. In WT mice, BMP-7 immunofluorescence is limited to distal tubules and some glomerular podocytes. In TG mice, BMP-7 is increased in glomerular podocytes and visualized in proximal tubules in addition to distal tubules. (b) Kidney BMP-7 levels by ELISA (c) Renal total (sum of human and murine) BMP-7 mRNA levels by quantitative RT-PCR at the three time points in the four groups of mice. *P < 0.05 versus WT-Co.

BMP-7 expression in microdissected glomeruli was quantitatively assessed with real-time PCR using primers that allowed for separate assessment of endogenous mBMP-7 and transgenic hBMP-7 mRNA. Endogenous expression of mBMP-7 in glomeruli was lowered by the diabetic state in WT mice at 52 wk (Figure 4a). In TG, nondiabetic mice, the glomerular BMP-7 mRNA levels were increased approximately two-fold compared with WT controls (Figure 4a). However, TG hBMP-7 tended to decrease in diabetic compared with nondiabetic TG mice, indicating that insulin deficiency or hyperglycemia failed to raise but, in fact, tended to lower the transcriptional activity...
of the PEPCK promoter in glomeruli. Nevertheless, total BMP-7 mRNA levels in glomeruli were greater in diabetic TG mice compared with diabetic WT mice (Figure 4). Attempts were made to measure BMP-7 levels in sera by ELISA assay. However, we were not able to detect BMP-7 in sera by ELISA in any of the mice, suggesting that circulating BMP-7 levels were below the detection limits of the assays (<50 pg/ml).

The increase in expression of hBMP-7 in mouse glomeruli follows the pattern of PEPCK enzyme expression in this anatomic location (Figure 4b). PEPCK is expressed in WT-FVB/N mouse kidney as well as in Bowman’s capsule–free glomeruli that were isolated by manual microdissection (Figure 4b). PEPCK mRNA also is expressed in cultured podocytes but not in murine mesangial cells (Figure 4b), consistent with glomerular expression of PEPCK enzyme and PEPCK promoter–driven hBMP-7 in podocytes.

Transgenic BMP-7 Does Not Affect TGF-β Levels

Fibrogenesis in diabetic nephropathy has been shown in different laboratories to be TGF-β driven, at least in part. In these studies, total TGF-β levels were measured in kidney lysates and sera by ELISA and bioassay, respectively. In diabetic mice, renal TGF-β levels tended to be elevated already at 4 wk and rose progressively further during the subsequent 48 wk; serum TGF-β levels also were elevated in diabetic mice (Figure 5). TGF-β was not affected by TG–BMP-7, consistent with the previous finding that BMP-7 does not regulate TGF-β levels (4).

Smad1/5 and Smad2/3 Phosphorylation and the Renal Id-1/PAI-1 Ratio as Integrative Assessments of BMP-7/TGF-β Activities

In diabetic WT and TG mice, renal pSmad2/3 levels were increased compared with nondiabetic mice, consistent with increased TGF-β signaling (Figure 6c). Phosphorylation of BMP-signaling substrates Smad1/5 was increased in TG mouse kidney, indicative of increased bioactivity of the transgene-driven BMP-7.

The Id-1 is a downstream target of BMP-Smad1/5 signals and is nonspecifically transcriptionally upregulated by BMP-7 (12). PAI-1 is transcriptionally regulated by TGF-β through Smad2/3-responsive promoter elements. Thus, the expression ratio of Id-1/PAI-1 can be used as an integrative measure for the renal activities of BMP-7 and TGF-β in the context of these studies (13). Id-1 is expressed primarily in the glomerular and peritubular capillary endothelium, and its levels are visibly decreased in diabetic WT mice compared with nondiabetic WT animals (Figure 6a). The BMP-7 transgene raises Id-1 levels in nondiabetic mice; although induction of diabetes in BMP-7–TG mice lowers Id-1 expression, levels are still comparable to those seen in WT control mice and greater compared with WT diabetic mice. In WT mice, the diabetic state causes a significant

Figure 4. (a) Expression of total (human, TG and murine, endogenous) BMP-7 in microdissected, Bowman’s capsule–free glomeruli at the three time points in the four groups of mice as measured by quantitative RT-PCR. *P < 0.05 versus WT-Co. (b) Semiquantitative RT-PCR of murine phosphoenol-pyruvate carboxykinase (PEPCK) mRNA in normal FVB/N mouse whole kidney, microdissected glomeruli, cultured and differentiated murine podocytes, and cultured murine mesangial cells.

Figure 5. Serum total (active and heat-activated) TGF-β were measured in sera by bioassay and were increased in diabetic mice already at 4 wk. Renal TGF-β (ELISA) tended to increase in diabetic mice at 4 wk and were increased two- to three-fold at 16 and 52 wk. *P < 0.05 versus WT-Co.
decrease in Id-1 mRNA levels by 27% and an increase in PAI-1 mRNA levels by 34% at 16 wk compared with nondiabetic WT controls, leading to a substantial decrease in the Id-1/PAI-1 ratio (Figure 6b). In BMP-7–TG mice that were not diabetic, Id-1 mRNA levels were increased by 46% compared with nondiabetic WT-Co, resulting in a substantial increase in the Id-1/PAI-1 ratio (Figure 6b). The BMP-7 transgene in diabetic mice maintains the Id-1/PAI-1 ratio at normal WT-Co levels and prevents the decrease in this ratio below baseline levels that was caused by the diabetic state in WT mice that did not express the BMP-7 transgene (Figure 6b). Although neither Smad1/5 phosphorylation nor Id-1 transcriptional up-regulation is a specific effect of BMP-7 compared with other BMP, in concert and in the context of these studies, these findings can serve as indications for bioactivity of the transgene-derived BMP-7.

Fibrosis also was assessed separately in glomeruli and the cortical interstitium as the trichrome-positive sectional area. In the absence of the transgene, the glomerular fibrosis index increased by 45% and the interstitial fibrosis index by 43% in diabetic mice but was substantially lessened in diabetic mice that expressed the BMP-7 transgene (P < 0.05; Figure 7, b and e).

In WT mice, diabetes reduced renal matrix metalloprotease-1 (MMP2) and MMP9 activity at 1 yr, on average by 44 and 46%, respectively, compared with nondiabetic controls (P < 0.05 for each comparison). TG–BMP-7 per se had no effect on the activity of these two proteases; in BMP-7–TG mice with superimposed

Transgenic BMP-7 Ameliorates Renal Fibrogenesis in Diabetes

Total acid–extracted collagen was increased at 52 wk compared with 4 wk in kidneys from all mice, including WT and BMP-7–TG, nondiabetic controls. In nondiabetic mice, total renal collagen increased late, after 16 wk (Figure 7a). In diabetic mice, total renal collagen was increased already at 16 wk, but subsequent increases were blunted in diabetic animals that expressed the BMP-7 transgene (Figure 7a). At 52 wk, the mRNA encoding the α2-chain of collagen type I or fibronectin were especially increased in WT diabetic mice, on average by 70% and approximately 4.5-fold, respectively (P < 0.05), compared with WT controls, and these diabetes-induced rises were virtually quantitatively prevented by the BMP-7 transgene (Figure 7, c and d).

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Figure 6. The downstream efficacy of renal TGF-β and transgenic BMP-7 was assessed by transcription factor inhibitor of differentiation-1 (Id-1) immunofluorescence and as the ratio of (BMP-7 driven) Id-1 and (TGF-β driven) plasminogen activator inhibitor-1 (PAI-1) mRNA as well as Smad2/3 and Smad1/5 phosphorylation. (a) Id-1 (red) is expressed in endothelial cells and is substantially increased in TG but reduced in WT-DM mice at 16 wk. (b) Id-1/PAI-1 mRNA ratio in kidney extracts at 16 wk. (c) Western blot of phosphorylated Smad2/3 and Smad1/5 in kidney extracts. *P < 0.05 versus WT-Co.

Figure 7. Quantitative assessment of renal and glomerular fibrosis in the four groups of mice. (a) Total acid–extractable collagen in whole kidney. (b) Quantitative glomerular fibrosis score measured as the ratio of trichrome-stained glomerular collagen area per glomerular cross-sectional area and expressed in percentage of WT control. (c) Renal mRNA levels encoding the α2 chain of collagen I as measured by competitive RT-PCR. (d) Whole-kidney fibronectin mRNA. (e) Quantitative interstitial fibrosis score in percentage of WT-Co. *P < 0.05 versus WT-Co; *P < 0.05 versus WT-Co, TG-Co, or TG-DM.
diabetes for 1 yr, the decrease in MMP2 and MMP9 activity was nominally less than in WT diabetic animals, on average 22 and 20%, respectively, but statistically not different from the decrease in WT diabetic mice. Total renal collagenase activity at 1 yr was substantially decreased in WT diabetic mice and tended to decrease less in TG mice with diabetes, albeit the difference between these two groups of animals was statistically indi-

Transgenic BMP-7 Preserves Podocyte Number and Integrity

In diabetic nephropathy, a progressive loss of podocytes occurs early in experimental animals as well as in humans (14–16). At 16 wk of diabetes, the number of WT-1–positive podocytes per glomerular cross-section tended to decrease in diabetic WT mice and decreased further subsequently. The decreases in podocytes per glomerulus were less in BMP-7–TG, diabetic mice, especially at 52 wk (Figure 9, a and b). The amount of nephrin per glomerular cross-section area followed a similar pattern, and estimates of the amount of nephrin per podocyte are similar among the four groups at 1 yr (Figure 9, c and d). Therefore, the decrease in nephrin seems to be largely or exclusively due to a loss of podocytes.

The loss of podocytes in diabetic glomerulopathy has been ascribed to increased rates of apoptosis (17). This is balanced insufficiently by podocyte proliferation, as indicated by the num-

Figure 8. Renal matrix metalloprotease-2 (MMP2) and MMP9 activities as measured by semiquantitative gelatin zymography (in percentage of WT-Co) as well as by quantitative activity assay at 52 wk. *P < 0.05 versus WT-Co.

Figure 9. Assessment of podocyte number and nephrin at 52 wk. (a) Representative visualization of podocytes by WT-1 immunofluorescence. (b) Quantitation of podocyte number per glomerular cross-section. (c) Representative visualization of nephrin by immunofluorescence. (d) Quantitative assessment of nephrin levels per glomerular cross-sectional area. (e) Quantification of proliferating cell nuclear antigen–positive (PCNA+) podocytes per glomerular cross-section. *P < 0.05 versus WT-Co; #P < 0.05 versus WT-DM.

Transgenic BMP-7 Ameliorates Renal Functional Deterioration in Diabetes

Glomerular proteinuria (microalbuminuria) is an early functional hallmark of diabetic glomerulopathy. Diabetic WT as well as BMP-7–TG mice developed onset of albuminuria at approximately 10 to 16 wk of diabetes, which progressed subsequently. However, albuminuria worsened much more steeply in WT compared with TG mice with diabetes (Figure 10), and after 1 yr, the difference was approximately 4.5-fold. In contrast to nondiabetic WT and TG controls, both groups of diabetic mice were modestly azotemic at 1 yr (i.e., had elevated blood urea nitrogen [Figure 10]). This likely reflects prerenal azotemia given osmotic diuresis from hyperglycemia in these
mice. Serum creatinine levels also tended to be moderately elevated in diabetic mice, reaching statistical significance only in diabetic WT mice but not in the group of diabetic mice that expressed the transgene. This rise in serum creatinine is indicative of renal insufficiency, perhaps as a result of diabetic nephropathy in addition to some degree of prerenal azotemia. Nevertheless, the BMP-7 transgene seems to protect renal functional integrity.

Discussion

Long-standing diabetes causes diabetic nephropathy with diabetic glomerular and interstitial fibrosis in a substantial proportion of patients. TGF-β is a major mediator of diabetic renal fibrogenesis (18). Several pieces of experimental evidence indicate that BMP-7 reduces the fibrogenic activity of TGF-β (4–7,19). Early findings in diabetic renal disease include dropout of podocytes and onset of albuminuria. In streptozotocin-induced diabetic nephropathy in rats, reduced renal expression of BMP-7 predates onset of these and other abnormalities, and exogenous, pharmacologic therapy with human recombinant BMP-7–transgene in glomeruli, specifically podocytes, by RT-PCR and immunofluorescence microscopy, respectively. In conjunction with the seemingly very low circulating BMP-7 levels as indicated by the negative ELISA, these findings indicate that local expression of the BMP-7 transgene in podocytes accounts for the glomeruloprotective effects in diabetes.

Diabetes not only lowers renal BMP-7 levels but also decreases BMP-7 activity as illustrated by the reduction in renal pSmad1/5 levels and Id-1 and the Id-1/PAI-1 expression ratio. Id proteins are dominant negative inhibitors of basic helix-loop-helix transcription factors that play important roles in phenotype regulation of some cells. Whether Id-1 somehow contributes to reduce the onset and the progression of diabetic nephropathy by BMP-7 is unknown. Previous in vitro studies have shown that BMP-7–induced Smad5 reduces Smad2/3 signaling downstream of TGF-β and, hence, lowers TGF-β–dependent profibrogenic events in cultured cells (4,7). Evidence of this previous in vitro finding also is seen in these in vivo studies: The BMP-7 transgene leads to elevated renal levels of phosphorylated Smad1/5 and moderately reduced pSmad2/3 levels (Figure 6c) without affecting renal TGF-β (Figure 5). In addition to reduced expression and levels of fibrosis-associated extracellular matrix proteins in glomeruli and the cortical interstitium, maintenance of BMP-7 in podocytes improves podocyte survival, which may account for lesser albuminuria in diabetic mice that express the BMP-7 transgene compared with their WT counterparts. BMP-7 may be a survival factor for podocytes as it is for neuronal cells (23). Given that TGF-β reduces survival and increases apoptosis in podocytes through mechanisms that involve TGF-β–induced Smad7, this beneficial effect of BMP-7 also may be explained by its antagonism to effects of TGF-β (24).

Conclusion

Maintenance of renal and glomerular podocyte BMP-7 levels by means of a transgene reduces onset and progression of nephropathy in diabetic mice. BMP-7 does not affect the diabetes-induced increase in TGF-β but reduces early accumulation of collagens and fibronectin and maintains renal collagenase activity, consistent with a TGF-β–activity–opposing action. Moreover, transgenic BMP-7 preserves podocyte number and gene would maintain renal and podocyte BMP-7 levels and preserve renal functional and structural integrity during diabetes. PEPCK is a rate-limiting enzyme that regulates gluconeogenesis. It is expressed in diverse tissues but not ubiquitously; in the kidney, strongest expression has been described in proximal tubules (20) but also in podocytes, as shown in these studies.

The rat PEPCK promoter has been used to regulate transgene expression of other proteins, including TGF-β and human growth hormone in mice (9,21). Levels of PEPCK promoter transgene expression in various tissues can differ substantially. Reasons for these differences in transgene expression may include environmental factors and genetic background of the animals; moreover, transgene integration sites are random with substantial effects on expression efficiency (22). In these studies, we demonstrate expression of the PEPCK enzyme and the BMP-7–transgene in glomeruli, specifically podocytes, by RT-PCR and immunofluorescence microscopy, respectively. In conjunction with the seemingly very low circulating BMP-7 levels as indicated by the negative ELISA, these findings indicate that local expression of the BMP-7 transgene in podocytes accounts for the glomeruloprotective effects in diabetes.

Figure 10. Assessment of renal function at 52 wk in the four groups of mice. ∆, WT-DM; ▲, TG-DM; □, WT-Co; ■, TG-Co. *P < 0.05 versus WT-Co.
lessens albuminuria, suggesting functions of endogenous BMP-7 as a podocyte survival factor. The loss of endogenous renal BMP-7 that has been observed in early diabetes in rodents contributes to diabetic nephropathy, and its prevention is beneficial.

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