Bone morphogenetic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models

Michael Zeisberg, Cindy Bottiglio, Navin Kumar, Yohei Maeshima, Frank Strutz, Gerhard A. Müller, and Raghu Kalluri. Bone morphogenetic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. Am J Physiol Renal Physiol 285: F1060–F1067, 2003. First published August 12, 2003; 10.1152/ajprenal.00191.2002.—Tubulointerstitial fibrosis is a hallmark feature of chronic renal injury. Specific therapies to control the progression of renal fibrosis toward end-stage renal failure are limited. Previous studies have demonstrated that expression of endogenous bone morphogenetic protein-7 (BMP-7) is reduced in the kidneys of several inducible mouse models of acute and chronic renal disease and that administration of exogenous recombinant human BMP-7 (rhBMP-7) has a beneficial effect on kidney function. Here we report that treatment with rhBMP-7 leads to improved renal function, histology, and survival in mice deficient in the α3-chain of type IV collagen and MRL/MpJ-lpr/lpr lupus mice, two genetic models for chronic renal injury and fibrosis. Such therapeutic benefit is also associated with a significant decrease in the expression of profibrotic molecules, such as type I collagen and fibronectin, in renal fibroblasts. Additionally, rhBMP-7 induces expression of active matrix metalloproteinase-2, which is potentially important for removal of fibrotic matrix. Collectively, these studies provide further evidence for rhBMP-7 as an important bone-associated protein with protective function against renal pathology.

Tubulointerstitial fibrosis; fibroblasts; osteogenic protein-1; matrix metalloproteinases

Despite significant advances in understanding the mechanistic pathways mediating the progression of chronic renal disease toward end-stage renal failure (ESRF), options for effective pharmacotherapy are very limited (7, 18, 25, 37). In progressive chronic renal disease, a self-preserving mechanism dominates independent of the underlying disease, further validating the notion that a potential common pathway leading to ESRF exists (13, 39). Progressive renal disease is almost invariably characterized by the triad of glomerular injury, tubulointerstitial fibrosis, and tubular atrophy (4, 16).

Bone morphogenetic protein-7 (BMP-7, also known as osteogenic protein-1) is a member of the transforming growth factor-β (TGF-β) superfamily, which plays a crucial role in renal development (12, 28, 34). Expression of BMP-7 correlates with condensation of the metanephric mesenchyme to generate epithelium, which eventually leads to formation of tubules and glomeruli (28, 36, 40, 50, 51). In the adult mouse kidney, BMP-7 is expressed in the collecting duct, thick ascending limb, distal convoluted tubule, and podocytes within glomeruli (17). Acute renal injury associated with tubular necrosis leads to reduction in the expression of tubular BMP-7, and after recovery of tubular and glomerular damage, BMP-7 expression is restored (42, 49). Administration of exogenous recombinant human BMP-7 (rhBMP-7) restores normal tubule architecture in rat models of ischemic acute renal injury, and it also restores tubular homeostasis in a mouse model of nephrotoxic serum nephritis (42, 49, 56). Furthermore, administration of exogenous rhBMP-7 inhibits progression of chronic renal disease in a rat model of unilateral urethral obstruction and in a rat model of streptozotocin-induced diabetic nephropathy (21, 24). Here we report that rhBMP-7 exhibits inhibitory effects on chronic renal disease and fibrosis in MRL/MpJ-lpr/lpr lupus mice and mice deficient in the α3-chain of type IV collagen (Col4A3−/− mice). We further report that rhBMP-7 exerts antifibrogenic effects on renal fibroblasts in vitro and in vivo. We also demonstrate that treatment of mice with rhBMP-7 over a 4-mo period does not induce significant side effects and is nontoxic. Collectively, these results suggest that rhBMP-7 is a potential candidate for the treatment of chronic renal failure.

METHODS

Materials. For in vivo studies, rhBMP-7 homodimer, noncovalently attached to prodomain protein (referred to as soluble rhBMP-7; Curis, Cambridge, MA) was used (21). In cell culture experiments, rhBMP-7 dissolved in 24 mM sodium acetate (pH 4.5) containing 1% mannitol (Curis) was
used (17). Details regarding these recombinant proteins have been published previously (17, 21).

**Mice.** Female normal mice on an MRL/MpJ background and autoimmune disease-prone MRL/MpJ Palestinian mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the Beth Israel Deaconess Medical Center animal facility (11). Col4A3⁻/⁻ mice on a mixed background of C57BL/6 and 129/Sv were maintained at the Beth Israel Deaconess Medical Center animal facility. Homozygous deletion of Col4A3⁻/⁻ was confirmed by PCR, as described previously (30). The Col4A3⁻/⁻ mice were obtained from Jackson Laboratories or were kindly provided by Dr. Jeffrey Miner (St. Louis, MO). Mice were fed standard mouse Chow and water ad libitum. All mouse studies followed the standards approved by the institutional animal care committee.

**Experimental design.** Normal mice on an MRL/MpJ background (n = 24) were treated with vehicle buffer alone or received rhBMP-7 at 30, 100, and 300 μg/kg for 16 wk to assess long-term toxicity of rhBMP-7. MRL/MpJ Palestinian mice (lupus nephritis mice) were treated for 12 wk, beginning at 4 wk of age, and the study was terminated when the mice were 16 wk of age. Mice were divided into a control group (n = 6 each), which received vehicle buffer alone, and three experimental groups (n = 6 each), which received rhBMP-7 at 30, 100, and 300 μg/kg. Because of the different kinetics of disease progression, COL4A3⁻/⁻ mice were treated for 8 wk and the study was terminated when the animals were 14 wk of age. Col4A3⁻/⁻ mice (n = 14) were divided into a control group (n = 7), which received vehicle buffer alone, and a treatment group, which received 300 μg/kg rhBMP-7. rhBMP-7 was administered intraperitoneally three times a week. Serum was obtained from all mice every 2 wk from baseline to death. Urine was collected every 2 wk in metabolic cages.

**Histological assessment of renal injury.** Tissue from kidneys, liver, heart, spleen, and brain was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin, Masson’s trichrome, and periodic acid-Schiff. The extent of renal injury was estimated by morphometric assessment of the tubulointerstitial injury and glomerular damage. The relative interstitial volume was evaluated by morphometric analysis using a 10-mm² graticule fitted into the microscope. Five randomly selected cortical areas, which included glomeruli, were evaluated for each animal (5, 45). Tubules were evaluated for their widened lumen and thickened basement membranes to estimate percentage of atrophic tubules (21). In each kidney, 100 glomeruli were evaluated for crescent formation; the crescentic index reflects the percentage of crescentic glomeruli (47). Glomerulosclerosis was scored for severity (0–4, with 0 representing normal and 4 representing maximum severity) as described by Raji et al. (38). Nuclei in 50 glomeruli of similar size in each kidney were counted to estimate glomerular cellularity (29).

**Immunohistochemistry.** Kidney tissue samples were frozen in liquid nitrogen and processed by indirect immunofluorescence technique, as previously described (45). Frozen sections were stained with the primary antibody raised against type I collagen (Southern Biotechnology Associates, Birmingham, AL), matrix metalloproteinase (MMP)-2 (Chemicon, Temecula, CA), MMP-9 (Chemicon), or BMP-7 (Curis). After the sections were washed with Tris-buffered saline, they were incubated with FITC- and rhodamine-conjugated secondary antibodies (Sigma, St. Louis, MO). Deposition of IgG was determined by using FITC-conjugated anti-mouse IgG antibody (Sigma). Negative controls were performed by substituting the primary antibody with an irrelevant preimmune serum.

**Cell culture.** Human interstitial fibroblasts (TK-173) were maintained in culture as previously described (27, 32). For stimulation of fibroblasts, media were replaced with serum-free Iscove’s modified Dulbecco’s medium (IMDM) (45).

**Direct ELISA.** We performed ELISAs for estimation of type I collagen and fibronectin, as described previously (43). Quiescent fibroblasts (8 × 10⁴ cells/well) were stimulated with rhBMP-7 (1.0, 10.0, and 100 ng/ml) in IMDM that was supplemented with 50 μg/ml ascorbic acid and aminopropionitrile (Sigma) for 48 h. Supernatants were analyzed in Maxisorp (Nunc) plates using primary antibodies raised against type I collagen (Southern Biotechnologies, Birmingham, AL) and fibronectin (Sigma). All assays were performed in triplicate and repeated at least three times. The working range was 10–1,000 ng/ml for type I collagen and 0.1–10 μg/ml for fibronectin.

**Zymography.** Zymography was performed as described previously (57). Briefly, cells (1 × 10⁵/well) were plated in six-well plates and grown for 6 h in DMEM containing 10% FCS. The medium was replaced with IMDM or IMDM containing 1, 10, or 100 ng/ml rhBMP-7. After incubation, medium was removed, cells were counted, and the volume of medium was normalized to cell counts. Electrophoresis was performed using 20 μl of medium per lane in 10% gelatin zymogram ready-cast gels (Bio-Rad, Hercules, CA). Gels were washed twice for 10 min at room temperature in renaturing buffer [2.5% (vol/vol) Trition X-100] and then incubated for 18 h at 37°C in development buffer [50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, and 0.02% (vol/vol) Brij 35]. Bands were visualized by staining the gel with Coomassie blue.

**Statistical analysis.** Values are means ± SE unless specified. Analysis of variance was used to determine statistical differences between groups using Sigma-Stat software (Jandel Scientific, San Rafael, CA). Further analysis was carried out using Student’s t-test with Bonferroni’s correction to identify significant differences. P < 0.05 was considered statistically significant.

**RESULTS**

**Long-term treatment with rhBMP-7 does not induce significant side effects.** rhBMP-7 is used in the United States and Europe as an injectable drug to enhance bone formation when applied locally to fractured areas. Hence, we performed initial 4-mo safety and toxicology studies with rhBMP-7 administered intraperitoneally in normal mice (MRL/MpJ). After 4 mo of rhBMP-7 administration at three different doses, provided three times weekly, the normal MRL/MpJ mice were killed, and tissues and body fluids were examined for any toxic side effects. Histopathology from brain, heart, lung, liver, spleen, kidney, and muscle revealed insignificant toxicity. Ectopic bone formation at the injection site was not observed. Only 4 of 24 mice displayed protein casts in a few medullary collecting ducts. One mouse from the group treated with rhBMP-7 for 4 wk at 300 μg/kg also showed mild symptoms of hepatitis. Two mice displayed mildly dilated lateral ventricles, a lesion that is usually detected as an incidental finding in many mouse studies (Table 1).
Decrease of tubular endogenous BMP-7 in progression of renal disease in MRL/MpJFloplpr and Col4A3⁻/⁻ mice. Recent studies suggested that renal injury was associated with a decrease of endogenous BMP-7 expression and that administration of exogenous rhBMP-7 served to restore renal function and morphology (21, 52, 54, 56). In the present study, we attempted to explore the potential of rhBMP-7 to prevent progression of renal disease in genetic models of renal disease. We first assessed endogenous BMP-7 in the normal and diseased kidneys of these mice. Because of a mutation in the fas gene, MRL/MpJFloplpr mice develop a lupus-like disease with progressive renal fibrosis (23). Although kidneys from MRL/MpJ control mice displayed BMP-7 in tubular cells and glomeruli, confirming previous observations, BMP-7 was notably decreased in injured kidneys from MRL/MpJFloplpr mice (17, 54) (Fig. 1, A and B). Additionally, we used mice that lack the type IV collagen α3 gene (Col4A3⁻/⁻) as a second model for progressive renal disease. Col4A3⁻/⁻ mice develop progressive renal disease, which leads to death between week 12 and week 16 (1, 30). Although C57BL/6 control mice displayed tubular and glomerular BMP-7 localization, progression of renal disease in Col4A3⁻/⁻ mice was associated with a decrease in endogenous BMP-7 expression (Fig. 1, C and D). We thus speculated that supplementation of exogenous rhBMP-7 could improve renal pathology and function in both of these genetic mouse models for chronic renal disease.

rhBMP-7 ameliorates progression of chronic renal disease in MRL/MpJFloplpr mice. To evaluate a potential role for rhBMP-7 in the treatment of chronic renal disease, we first utilized MRL/MpJFloplpr mice (11). We initiated intraperitoneal injection of rhBMP-7 at 4 wk of age. At this age, very early signs of disease could be demonstrated in these mice. After termination of the study at 16 wk of age, rhBMP-7-treated mice displayed reduced relative interstitial volume as well as a reduced number of atrophic tubular structures compared with untreated control mice (Fig. 2, A–D). Animals that were treated with 300 μg/kg rhBMP-7 also displayed reduced glomerular crescents, markedly reduced glomerulosclerosis, and reduced glomerular hypercellularity (Fig. 2, E and F). Serum creatinine levels were significantly reduced in mice that were treated with 300 μg/kg rhBMP-7 after 4 wk of treatment, whereas by the end of study, results failed to reach significance because of wide variance in the treatment groups (Fig. 2G).

These findings also correlated with reduced interstitial staining for type I collagen in the treated mice compared with control mice (Fig. 3, A and B). Localization for IgG in glomeruli did not show substantial difference between untreated and treated mice (Fig. 3, C and D).

Treatment with rhBMP-7 inhibits progression of renal disease in Col4A3⁻/⁻ mice. We next attempted to investigate whether administration of exogenous rh-

### Table 1. BMP-7 treatment of normal MRL/MpJ mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Protocol</th>
<th>Histopathological Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle buffer, start week 4</td>
<td>No histopathological abnormalities</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle buffer, start week 8</td>
<td>Protein casts in a few collecting ducts in kidney in 1 mouse</td>
</tr>
<tr>
<td>3</td>
<td>BMP-7 (30 μg/kg), start week 4</td>
<td>Protein casts in a few collecting ducts in 1 mouse</td>
</tr>
<tr>
<td>4</td>
<td>BMP-7 (100 μg/kg), start week 4</td>
<td>Mild form of hepatitis in 1 mouse</td>
</tr>
<tr>
<td>5</td>
<td>BMP-7 (300 μg/kg), start week 4</td>
<td>Protein casts in a few collecting ducts in 2 mice; 1 mouse with mild form of hydrocephalus</td>
</tr>
<tr>
<td>6</td>
<td>BMP-7 (30 μg/kg), start week 8</td>
<td>Protein casts in a few collecting ducts in 1 mouse</td>
</tr>
<tr>
<td>7</td>
<td>BMP-7 (100 μg/kg), start week 8</td>
<td>Protein casts in a few collecting ducts in 1 mouse; mild hydrocephalus in 1 mouse</td>
</tr>
<tr>
<td>8</td>
<td>BMP-7 (300 μg/kg), start week 8</td>
<td>No histopathological abnormalities</td>
</tr>
</tbody>
</table>

Brain, lung, kidney, liver, spleen, heart, and skin were evaluated for histopathological abnormalities after treatment with bone morphogenetic protein-7 (BMP-7). A mild form of hydrocephalus and incidental findings of mild hepatitis have been observed in many mouse studies. The MRL/MpJ strain was selected to serve as the most appropriate control for MRL/MpJFloplpr lupus-nephritis mice.
BMP-7 had a similar role in the progression of renal disease in Col4A3−/− mice. Treatment with rhBMP-7 was initiated after 6 wk of age until the study was terminated at 14 wk of age (30). In this study, we treated mice with 300 μg/kg rhBMP-7, which was established by our previous study as the optimum dose and was also confirmed by other groups (21, 52). Treatment with 300 μg/kg rhBMP-7 led to reduction of tubulointerstitial fibrosis as determined by reduced relative cortical interstitial volume and significantly reduced tubular atrophy (Fig. 4, A–C). Reduction of renal damage led to decreased renal pathology-related...
mortality rate in the rhBMP-7-treated group (Fig. 4C). Five of seven untreated control mice died because of renal failure before week 14, whereas none of the treated mice died as a result of uremia and kidney disease. Treated mice lived to week 26. Renal function, as assessed by serum creatinine (Fig. 4D), blood urea nitrogen (Fig. 4E), and urine protein (Fig. 4F) revealed significant improvement in BMP-7-treated mice compared with untreated Col4A3<sup>−/−</sup> mice.

**rhBMP-7 has antifibrogenic effects on renal fibroblasts.** We next attempted to evaluate the effect of rhBMP-7 on interstitial fibroblasts to gain further insights into the antifibrotic action of rhBMP-7. Interstitial fibroblasts play a central role in the progression of chronic renal disease as the main mediators of extracellular matrix (ECM) deposition, the characteristic feature of fibrosis (13, 39). We utilized a human interstitial fibroblast cell line (TK-173), which displays an activated fibroblast-like state in vitro, to evaluate the potential of rhBMP-7 to inhibit profibrotic contributions of these cells (27, 32). Synthesis of type I collagen and synthesis of fibronectin are considered key features of activated fibroblasts, and we show that administration of rhBMP-7 decreases synthesis of type I collagen and fibronectin in the TK-173 cells in a dose-dependent manner, without significant alteration in the proliferative capacity of these cells (Fig. 5, A and B) (13, 39). In addition to increased synthesis, a decrease in ECM degradation is considered important for shifting the balance of ECM homeostasis toward enhanced matrix deposition during fibrogenesis (13, 44). Human interstitial fibroblasts secrete substantial levels of MMP-2 and MMP-9, which possess the capacity to cleave basement membranes and interstitial ECM constituents (8, 10, 26, 48). Treatment of TK-173 cells with rhBMP-7 led to upregulation of MMP-2 and MMP-9 (Fig. 5C). In the MRL/MpJ<sup>lpr/lpr</sup> mice, upregulation of MMP-2 by the interstitial fibroblasts by administration of rhBMP-7 was also observed (Fig. 5, D and E). Although MMP-2 was absent in the interstitial areas containing abundant myofibroblasts, MMP-2 was markedly increased in the interstitium of rhBMP-7-treated MRL/MpJ<sup>lpr/lpr</sup> mice (Fig. 5, D and E). In the rhBMP-7-treated kidneys, MMP-2 mainly colocalized with activated myofibroblasts expressing α-smooth muscle actin, further suggesting a potential of rhBMP-7 to induce MMP-2 in the interstitial fibroblasts (Fig. 5, D and E). MMP-9 was significantly present in the interstitium of untreated control kidneys; thus, further increase of MMP-9 could not be demonstrated after treatment with BMP-7 (Fig. 5, F and G). Further-
more, MMP-9 in the interstitium rarely colocalized with α-smooth muscle actin, suggesting that rhBMP-7 in interstitial fibroblasts activates mainly MMP-2 in the injured kidneys (Fig. 5, F and G).

DISCUSSION

Progression of chronic renal disease toward ESRF still represents one of the biggest problems in nephrology, inasmuch as it leads to an increasing number of patients who require long-term renal replacement therapy, such as dialysis or kidney transplant (9, 35). Although angiotensin-converting enzyme inhibitors are the most promising therapeutic agents to inhibit progression of renal fibrosis in clinical use, specific therapeutic options are not available (2, 7, 15, 25, 55).

Several recent studies have demonstrated a beneficial role for administration of exogenous rhBMP-7 in different animal models of chronic renal injury (21, 31, 52, 56). BMP-7 is a member of the TGF-β superfamily and has an important function during kidney development (20). It is associated with condensation of the metanephric mesenchyme, leading to the formation of tubules and glomeruli (20, 41). In the adult kidney, BMP-7 expression can be detected in tubular epithelial cells and in podocytes (17, 54). Endogenous BMP-7 expression significantly decreases during acute renal injury, and administration of exogenous rhBMP-7 accelerates the repair of the injured kidney, suggesting that BMP-7 plays a role in the maintenance of kidney homeostasis (42, 49). Similarly, BMP-7 expression is decreased in several induced animal models of chronic renal injury, and administration of exogenous rhBMP-7 in these models inhibits progression or enables recovery of chronic renal injury (21, 52, 54, 56).
In the present study we establish for the first time a role of rhBMP-7 as a therapeutic agent in two genetic mouse models that mimic long-term chronic renal disease. In MRL/MpJp<sup>lpr/lpr</sup> mice, which develop lupus nephritis-like renal disease associated with significant tubulointerstitial fibrosis after 3 mo, treatment with rhBMP-7 inhibits progression of renal disease in a dose-dependent manner. In mice deficient in the α<sub>3</sub>-chain of type IV collagen, ESFR associated with interstitial fibrosis after 14 wk is observed. rhBMP-7 prevents renal fibrosis and renal-related mortality in these mice and increases their survival by 12 wk. Thus we demonstrate the antifibrotic effect of rhBMP-7 in two long-term mouse models for chronic renal fibrosis, providing further evidence for the use of rhBMP-7 as a therapeutic agent for chronic renal injury.

Previous studies have suggested that rhBMP-7 exerts its antifibrotic effect mainly on tubular epithelial cells, where it inhibits the release of proinflammatory chemokines (17). It is also shown to reverse epithelial-to-mesenchymal transition, while it acts as an antagonist of TGF-β-induced E-cadherin downregulation (56). In chronic renal fibrosis, activated interstitial fibroblasts, which can derive from resident interstitial fibroblasts, from tubular epithelial cells via epithelial-to-mesenchymal transition, or from bone marrow-derived mesenchymal precursor cells, are considered the main pathogenic mediators of renal disease (13, 19, 22, 39). Renal fibrosis is characterized by an excessive deposition of interstitial ECM, which results in destruction of kidney architecture and impairment of renal function. Activated interstitial fibroblasts are the main mediators of enhanced ECM synthesis (3, 6, 14, 16, 33). In the present study, we provide evidence that rhBMP-7 functions (in addition to its effects on tubular epithelial cells) by inhibiting profibrotic contributions of activated interstitial fibroblasts. We hypothesize that upregulation of MMP-2 by rhBMP-7 reflects increased ECM degradation and, thus, potentially a decrease in scar tissue in the renal interstitium (46, 58). TGF-β1, the main profibrotic growth factor involved in renal fibrogenesis, mediates these three profibrotic features, which are inhibited by rhBMP-7 in our studies, in renal fibroblasts (6, 46). Previous studies have also demonstrated the potential of rhBMP-7 to counteract TGF-β1 action in tubular epithelial and mesangial cells (53, 56). Our research, coupled with these previous important studies, suggests that rhBMP-7 functions on tubular epithelial cells, mesangial cells, and fibroblasts to restore the health of kidney tissue. These studies provide further evidence that rhBMP-7 should be tested in human clinical trials involving kidney disease patients.

**DISCLOSURES**

This study was supported by a sponsored research project from Curis, in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-51711 and DK-55901, the American Society of Nephrology Carl Gottschalk Research Award, and Deutsche Forschungsgemeinschaft Grants Mu523/7-1 (to G. A. Müller) and ZE5231/1 (to M. Zeisberg).

**REFERENCES**

25. Komers R and Anderson S. Optimal strategies for preventing progression of renal disease: should angiotensin-converting en-


