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NEPHROLOGY FORUM

Treatment of chronic tubulointerstitial disease: A new concept

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CASE PRESENTATION

A 41-year-old white man was referred to the Washington University renal stone center 3 years ago following a ureteroscopic lithotripsy for an obstructing right mid-ureteral stone 2 months earlier. The patient was asymptomatic and had an unremarkable dietary history.

His medical history was long and complicated. He had had recurrent nephrolithiasis since his childhood, and he carried the diagnosis of cystinuria. He had undergone a left nephrectomy 24 years ago at age 17 for complications during a nephrolithotomy for a staghorn calculus. He had stopped forming renal stones until 11 years ago, when he developed an ileocolic fistula and was diagnosed with Crohn's disease. After several intestinal resections and development of various fistula tracks, he underwent an ileostomy 6 years ago. Following recovery, his inflammatory bowel disease was controlled by medications. He gained weight and developed a stable ileostomy excretory volume of approximately 1.5 L/day. However, he experienced recurrent nephrolithiasis. Four years ago, he was treated for renal colic in the Department of Urology at the University of Alabama. He had a 2.5 cm stone in the renal pelvis, which required percutaneous nephrolithotomy; the extracted stone was 97% cystine. At that time, his serum creatinine was 1.6 mg/dL; creatinine clearance, 90 mL/min; and protein excretion, 100 mg/24 h.

The patient moved to St. Louis 4 years ago and was stable until he presented with right flank pain 3 years ago. His medications at the time of his visit to the stone center included mesalamine and loperamide. His family history was notable only for a brother with cystinuria. He did not drink alcohol except socially on occasion; he smoked one pack of cigarettes/day for 20 years. He did not use illicit drugs.

The physical examination was normal except for multiple surgical scars and an ileostomy appliance containing liquid feces.

Laboratory tests were ordered, and the patient entered Barnes Hospital for ureteroscopic laser lithotripsy of a right mid-ureteral stone before he could begin therapy. He was discharged to the renal stone center, where laboratory data revealed a serum creatinine of 1.6 mg/dL; creatinine clearance of 93 mL/min; urinary cystine excretion, 620 mg; and 1 to 2 white blood cells/high-power field on urinalysis. Treatment was begun with N-(2-mercaptoproprionyl) glycine (tiopronin), 100 mg qid, and potassium citrate, 10 mEq bid.

Four months later, the patient was seen in follow-up at the stone center. The physical examination revealed a blood pressure of 135/90 mm Hg; serum creatinine, 1.7 mg/dL; creatinine clearance, 80 mL/min; urine volume, 2.7 L/day; and cystine excretion, 400 mg/day. A small mixed disulfide peak (cysteine-tiopronin) was apparent in the amino acid analysis. Urinalysis revealed 5 to 7 white blood cells/high-power field.

One month later, he presented to the Barnes Hospital emergency department because of anuria of two days duration and low back pain on the right side. Sonography revealed ureteral obstruction and a 1 cm stone at the pelvic brim just above the bladder. The patient underwent cystoureteroscopic extraction of the stone.

At the stone center one month later, evaluation revealed: blood pressure, 140/95 mm Hg; serum creatinine, 2.1 mg/dL; creatinine clearance, 40 mL/min; protein excretion, 500 mg/day; and urine volume, 1.9 L/day. Urinalysis demonstrated 15 to 20 white blood cells/high-power field. Enalapril was added to his therapeutic regimen, 10 mg/day, and the tiopronin was increased to 200 mg qid.

The patient still did not achieve remission of his nephrolithiasis. An episode of right flank pain 2 years ago prompted another sonogram, which demonstrated a ureteropelvic junction stone. Extracorporeal shockwave lithotripsy was performed. His serum creatinine after the procedure and ureteral stent removal was 1.8 mg/dL.

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At the renal stone center 4 months later, his blood pressure was 130/80 mm Hg. Urinalysis revealed 5 to 7 white blood cells/high-power field. Sonography disclosed no stones. The right kidney measured 11.8 cm. The tiopronin was increased to 1200 mg/day; enalapril and potassium citrate therapy were continued unchanged. Six months later, his blood pressure was 130/80 mm Hg; urinalysis revealed 2 to 4 white blood cells/high-power field and no crystals. The serum creatinine was 1.6 mg/dL; creatinine clearance, 96 mL/min; and urine volume, 2.7 L. Sonography revealed no stones and a right kidney measuring 12.1 cm.

The patient was last examined three months ago. His blood pressure was 130/80 mm Hg; urinalysis revealed 0 to 2 white blood cells/high-power field, and sonography revealed no stones. The serum creatinine was 1.5 mg/dL; creatinine clearance, 95 mL/min; urine protein excretion, 275 mg/day; cystine excretion, 250 mg/day; there was a large mixed disulfide peak in the amino acid profile. Liver function tests were normal. Therapy with tiopronin, 1200 mg/day; enalapril, 10 mg/day; potassium citrate, 10 mEq tid; mesalamine; and loperamide was continued.

DISCUSSION

Dr. Keith A. Hruska (*Professor of Pediatrics, Medicine, Cell Biology and Biophysics, and Chief, Division of Pediatric Nephrology, Washington University, St. Louis, Missouri, USA*): The patient for discussion today almost certainly had active chronic tubulointerstitial nephritis for at least two years due to repeated bouts of obstructive uropathy, cystinuria, and nephrolithiasis involving a single kidney. He was successfully treated with angiotensin-converting-enzyme inhibition and conversion of cystine to a mixed disulfide of cysteine-mercaptopropionylglycine. These agents restored the glomerular filtration rate (GFR) to baseline, decreased his proteinuria, and alleviated his pyuria.

Today's patient is atypical in that recurrent nephrolithiasis is not a common cause of tubulointerstitial nephritis. However, this patient was at risk because of his single kidney and the recurrent episodes of obstructive uropathy and instrumentation. Pyuria and a reduction in creatinine clearance, which developed insidiously between episodes of urinary tract obstruction due to ureteral stones, heralded the onset of tubulointerstitial nephritis. At its peak, the serum creatinine was twice the baseline level, and the creatinine clearance was reduced by 50%. The development of hypertension, heavy pyuria without hematuria, and protein excretion of less than 1 g/24 h is indicative of the presence of tubulointerstitial nephritis. This patient also was at risk for interstitial inflammation from calcium oxalate crystalluria, which often complicates Crohn's disease and can cause tubulointerstitial inflammation [1]. However, at no time was there evidence of calcium oxalate crystalluria. Cystinuria as a cause of tubulointerstitial inflammation is less clear than calcium oxalate crystalluria because the defect in cystinuria is decreased cystine transport [2], and specific epithelial receptors for the crystals of cystine are not known, as they are for calcium oxalate. Most likely, recurrent ureteral obstruction is the cause of interstitial nephritis in cystinuria [1].

This is not the first Nephrology Forum dedicated to chronic tubulointerstitial disease and ureteral obstruction. The topic was discussed by Saulo Klahr in 1998 on the occasion of the twentieth anniversary of the Nephrology Forum [3]. That Forum presented an excellent review of the pathophysiology of renal fibrosis stimulated by obstructive uropathy, and I will not repeat the points he made. I refer the reader to Dr. Klahr's Forum. Obstructive uropathy can manifest as a sudden decrease in renal function. But gradual and insidious decreases in renal function similar to those seen in today's patient are also observed. The decrease in renal function can be halted or even reversed if obstruction is relieved and prevented, and if the tubulointerstitial inflammation resolves spontaneously or with treatment. Thus, obstructive uropathy is a potentially treatable form of chronic tubulointerstitial nephritis. Today's case presentation highlights the treatable nature of tubulointerstitial nephritis, and it directs attention to our current therapeutic arsenal, which is limited to relief of obstruction and inhibition of angiotensin II either by angiotensin-converting enzyme (ACE) inhibition or angiotensin II receptor (AT1 and AT2) blockade. A new therapeutic agent and its potential to mitigate the pathophysiology of tubulointerstitial nephritis will be the focus of my discussion today.

Pathophysiology of obstructive uropathy

Obstructive uropathy can be due to anatomic or functional abnormalities of the renal pelvis, ureters, bladder, or urethra. The result of the obstruction, however produced, is hydrostatic-pressure-induced injury to the tubular epithelium, especially that of the collecting duct (Fig. 1). As a result of injury, the epithelium loses features of the differentiated phenotype. Epithelial cells can move into the cell cycle and apoptose or produce dedifferentiated daughter cells that express increased production of extracellular matrix. They can even undergo epithelial-mesenchymal transdifferentiation, migrate from the epithelium, and contribute to the pool of interstitial myofibroblasts. The tubular injury leads to tubulointerstitial inflammation, tubular atrophy, and fibrosis, eventually producing end-stage renal failure unless the process is reversed [4].

Interstitial fibrosis is a common consequence of long-standing obstructive uropathy. Fibrosis likely develops due to an imbalance between extracellular matrix synthesis and deposition, and matrix degradation. The mechanisms underlying fibrogenesis of obstructive uropathy have been extensively investigated [4–7]. Nearly 3 decades ago, Nagel and Bulger reported widening of the interstitial space following 7 days of ureteral obstruction

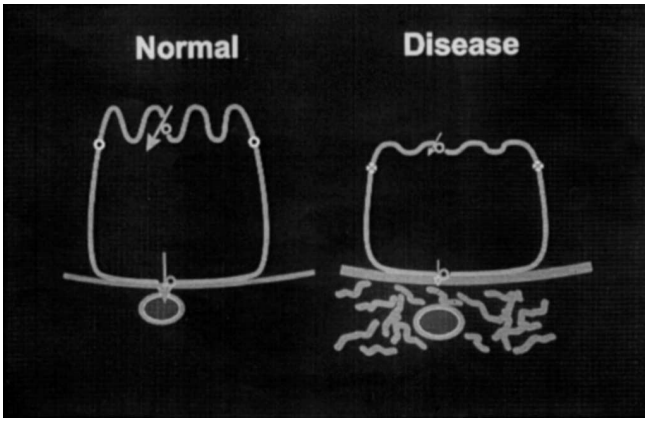

Fig. 1. Effects of obstruction-induced injury on the collecting duct epithelium. The epithelium undergoes rapid changes involving loss of many features of its terminally differentiated phenotype. The apical microvilli become effaced; cell height is reduced; the tight junctions lose many of their properties and functions; ion transport is reduced and can become non-vectorial. Extracellular matrix production is increased possibly because of transdifferentiation to interstitial myofibroblasts.

in rabbits, with an increase in collagen fibers and fibroblasts. By day 16 of obstruction, collagen was greatly increased and arranged in large bundles. Nagel and Bulger also described a mononuclear cell infiltrate and proliferation of interstitial cells in the renal parenchyma following chronic unilateral ureteral obstruction [6]. Interstitial fibrosis and thickening of the tubular basement membrane following unilateral obstruction are associated with increased deposition of several extracellular matrix molecules, including collagen types I, III, IV, XV, and XVIII, fibronectin, and heparan sulfate proteoglycans [7, 8].

Animal models have illuminated our understanding of the complex milieu of renal diseases associated with interstitial fibrosis. Moller et al utilized the model of unilateral ureteral obstruction (UUO) to study the pathophysiology of tubulointerstitial disease [9]. Unilateral ureteral obstruction in the rat and mouse produce tubulointerstitial inflammation and fibrosis that seem to mirror the human condition produced by obstructive uropathy. In this model, hypertension, proteinuria, and lipid dysregulation do not contribute to progressive nephron destruction [3, 10–13], and glomerular injury is not prominent early in the course of the injury produced. Uremia is avoided by the function of the contralateral kidney, which undergoes hypertrophy and hyperplasia as the obstructed kidney is destroyed. The renal injury of UUO is mediated in part through stimulation of renal angiotensin II production, which activates TGF-β in a cascade of events culminating in tubulointerstitial inflammation and fibrosis [14–18]. Inhibition of angiotensin II production by ACE inhibitors decreases the expansion of the renal interstitium associated with fibrosis [19, 20]. We have shown that ACE inhibitors also decrease transformation of renal cells to interstitial myofibroblasts and diminish infiltration of the interstitial compartment by inflammatory cells [19, 20]. However, limiting angiotensin II production even through direct modulation of angiotensinogen expression does not attenuate tubular atrophy [16]. We recently demonstrated that a new potential therapy for chronic renal disease, bone morphogenic protein (BMP-7), was more effective than ACE inhibition in preserving renal structure and function in rats with UUO [21]. I will return to a discussion of BMP-7 later.

Before that, I would like to focus on leukocyte infiltration of the renal interstitium. The few resident macrophages in the cortex of normal kidneys are mainly in glomeruli [22]. The normal medulla is completely devoid of leukocytes. In obstructive uropathy, mononuclear cells are present in both the renal cortex and medulla [23]. A cellular-rich infiltrate composed mainly of macrophages was detected as early as 4 hours after the onset of ureteral obstruction in rats, but the peak response occurred after 24 hours [23]. The second most abundant cells of the interstitial infiltrates were T-lymphocytes of the cytotoxic suppressor cell subclass [23]. T-lymphocytes of the helper type did not account for a significant portion of the infiltrate, despite their predominance in the peripheral circulation. Ureteral obstruction for less than 24 hours produced only a scant infiltration of B lymphocytes or neutrophils in the kidney [23]. The mononuclear cell infiltrate disappears slowly following release of obstruction in animals, returning to basal levels only after several days. The macrophage content of the cortical interstitium increases moderately in the first two days after release of obstruction and then decreases over the next four days to pre-obstruction levels. T-lymphocytes in the cortex were diminished to less than 20% of their value during obstruction within two days of release of ureteral obstruction [23].

The signals responsible for recruiting macrophages and suppressor T-cells into the renal interstitium of rats following unilateral ureteral obstruction appear to be specific for these cells, as neutrophils were not detected in the compartment. The factors involved in cellular infiltration of the interstitium of the obstructed kidney have been partially characterized. Supernatants prepared from renal cortices of rats with UUO had greater chemotactic activity for peritoneal macrophages than did supernatants from the contralateral kidney of the same rats [24]. This macrophage chemotactic activity peaked between 4 and 12 hours of obstruction and declined after longer periods, 24 to 72 hours. Among the molecules responsible for this chemotactic activity, monocyte chemoattractant peptide-1 (MCP-1), which is expressed in tubular epithelium at 12 hours following ureteral obstruction and persists as long as 96 hours, is a key participant [15]. Other members of the chemokine family such as RANTES and interleukin-8 (IL-8) also play a role.

Osteopontin, a multifunctional protein with potent chemotactic activity, is upregulated by ureteral obstruction, and macrophage infiltration following UUO in the osteopontin knockout mouse is diminished [8]. Other substances such as transforming growth factor beta (TGF-β), intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which are overexpressed following ureteral obstruction, also play roles in macrophage recruitment [25, 26].

The volume of the renal cortical interstitium increases rapidly following UUO in the rat kidney [19, 21, 27]. By three days following unilateral obstruction, interstitial volume was increased from 10% of the cortex to 29% (abstract; Duan et al, *J Am Soc Nephrol* 7:1697, 1996) and to 47% by five days following obstruction [21]. Deposition of collagen types I, III, and IV was increased in the tubulointerstitium by the third day of obstruction. In addition, the level of mRNA for collagen α1 (IV) was significantly greater in the obstructed kidney at that time. Thus, events leading to interstitial fibrosis occur promptly following the onset of obstruction. By contrast, the amounts of collagen I, III, and IV did not change in the glomeruli of the obstructed kidney, even after five days of unilateral obstruction. These results were consistent with the finding that glomeruli appear normal by light microscopy after seven days of obstructive nephropathy.

Renal tubular cells contribute to the increased production and deposition of type IV collagen in tubular basement membranes and interstitium following obstruction. Renal tubular cells in culture produce collagen types I, III, and IV. The expression of collagen α1 (IV) mRNA is substantially increased in the tubules of the obstructed kidney. Nagel and Bulger reported that fibroblasts migrated to, and proliferated in, the interstitium of the obstructed kidney during UUO [6]. In addition, Kuncio and colleagues found that several cytokines secreted by infiltrating macrophages and T-lymphocytes stimulate fibroblast proliferation, and that interstitial fibroblasts produce collagen types I, III, and IV [28]. The substantial increase in collagen types I and III found in the interstitium of the obstructed kidney at days 3, 4, or 5 following UUO is consistent with the increased cellularity due to fibroblast proliferation and infiltrating mononuclear cells. Thus, interstitial fibroblasts probably contribute to the increase in collagen production in the obstructed kidney of rats. Our group [29] and others [30] have reported that α-smooth muscle actin mRNA and protein and the intermediate filament desmin are upregulated in the obstructed kidney of rats following UUO. The overexpression of α-smooth muscle actin and desmin indicates the myofibroblast phenotype. The origin of the myofibroblast is likely bimodal following ureteral obstruction. A significant source of the myofibroblast derives from transformation of interstitial fibroblasts. However, epithelial-mesenchymal transdifferentiation is an important source of the myofibroblast phenotype and a component of the tubular pathology produced by ureteral obstruction.

Table 1. Factors exhibiting increased expression in kidneys with ureteral obstruction

Vasoactive compounds
Angiotensinogen, angiotensin II, endothelin, thromboxane A_2
Growth factors
Transforming growth factor-β (TGF-β1), basic fibroblast growth factor (bFGF)
Cytokines
Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), platelet activating factor (PAF)
Chemoattractants/chemokines
Monocyte chemoattractant peptide-1 (MCP-1), osteopontin
Immediate early response genes
c-fos, c-jun, jun B, c-myc, cH-Ras
Cell cycle proteins
Protein 53 (p53), protein 21 (p21, WAF-1)
Proteins associated with apoptosis
Clusterin (SGP-2), osteopontin
Transcription factors
Nuclear factor-κB (NF-κB)
Extracellular matrix proteins
Collagen types I, III, IV, XV, and XVIII, tissue inhibitor of metalloproteinases-1 (TIMP-1), decorin, fibronectin, alternate splice forms
Adhesion proteins
Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)

What pathways lead to interstitial fibrosis in obstructive uropathy? A number of cytokines, vasoactive compounds, chemoattractant molecules, and growth factors are upregulated after the onset of obstructive uropathy (Table 1). Several studies [20, 29, 31, 32] suggest that the renin-angiotensin system is activated after ureteral obstruction. Increasing levels of angiotensin II stimulate the expression of other factors, such as TGF-β1 and TNF-α, which instigate a damage program in the renal interstitium (Table 1). These instigating molecules represent the early steps in a signal transduction pathway that culminates in interstitial fibrosis.

A multifunctional cytokine and growth factor, transforming growth factor-β (TGF-β), participates widely in embryonic development and in regulating repair and regeneration following tissue injury [33, 34]. Transforming growth factor-β also is involved in angiogenesis, regulation of inflammation, integrin expression, protease activity, and apoptosis [35]. Of the three main TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, TGF-β1 is most important in interstitial nephritis. Synthesized as a 391 amino acid procytokine, TGF-β1 comprises a C-terminal TGF-β sequence and a larger N-terminal region that, after processing, forms a protein called latency-associated peptide (LAP). As TGF-β is secreted in an inactive (latent) form because of its association with the LAP, binding to receptors is prevented. The LAP-TGF-β1 complex (the small latent complex) is stored at the cell surface

in the extracellular matrix, and it is associated with another LAP, which in most cases is the latent TGF-β binding protein-1 (LTBP1). This protein has sequence similarity to the fibrillins. The complex of all three proteins is called the large latent complex, and through LTBP1 it can be linked to the extracellular matrix. In the case of the renal interstitium, perhaps the proteoglycan decorin serves as the link [15, 36].

The LAP–TGF-β complex in the extracellular matrix can be activated by binding of the small LAP to thrombospondin or to cell surface integrins. Latency-associated peptide-β1 contains an arginine-glycine-aspartic acid (RGD) sequence, which is a binding site motif for a subset of integrins. Binding changes the confirmation of the LAP protein, releasing the mature TGF-β molecule for binding with cell surface receptors [37] or with thrombospondin in an active state. Biologically active TGF-β1 is a 25 kD dimer composed of two processed mature proteins linked by a disulfide bond. Active TGF-β1 is a critical factor involved in tubulointerstitial fibrosis (Table 1). It increases matrix protein synthesis, inhibits matrix protein degradation, and upregulates integrin matrix adhesion factors. Transforming growth factor-β stimulates production of a wide variety of proteins found in the extracellular matrix during obstructive uropathy including fibronectin, collagen types I, III, IV, thrombospondin, osteonectin, tenascin, elastin, hyaluronic acid, SPARC, and proteoglycans such as biglycan and decorin (Table 1). Transforming growth factor-β1 inhibits matrix degradation by increasing the activity of tissue inhibitors of metalloproteinases (TIMPS) and decreasing the activity of metalloproteinases (MMPs). It also stimulates the synthesis of receptors for extracellular matrix proteins. Furthermore, TGF-β1 is a chemoattractant for fibroblasts [38] and stimulates fibroblast proliferation.

Bone morphogenic protein-7

Bone morphogenetic protein-7 (BMP-7), also known as osteogenic protein-1 (OP-1), is a member of the TGF-β superfamily and a key morphogenic signal for renal development [39]. The protein continues to be produced in adult mammals [40–43], predominantly within the kidney [44, 45], but also is found in the circulation (Sampath TK, unpublished observations). Deletion of BMP-7 in mice results in failure of differentiation of the metanephric mesenchyme and leads to loss of mesenchymal cell condensation around the ureteric bud and eventually to glomerular and tubular agenesis and severely hypoplastic kidneys [39, 40, 46]. Animals deficient in BMP-7 die shortly after birth because of uremia. Many features of development are recapitulated during repair of renal injury, and BMP-7 might be important both in preservation of function and resistance to injury [47]. Bone morphogenic protein-7 decreases the loss of renal function associated with acute ischemic injury [47]. Although the function of BMP-7 in the kidney and its role in renal injury are unknown, this protein might have a cytoprotective effect, or it might regulate chemotactic cytokines involved in monocyte infiltration associated with a variety of renal diseases. In addition to its role as a renal morphogen, BMP-7 probably is a critical renal tubular differentiation factor determining epithelial cell phenotype. Therefore, we analyzed a potential role of BMP-7 in preventing tubulointerstitial fibrosis.

To assess the effect of BMP-7 on preservation of renal function following injury, we performed renal clearance studies in each of the kidneys of rats after 5 days of UUO and 5 days of recovery. We used enalapril-treated animals (25 mg/kg) as a positive control for comparison purposes. As Figure 2A illustrates, renal function did not recover in the post-obstructed kidneys of vehicle- or enalapril-treated animals. However, 50% of the post-obstructed kidneys in the 100 μg/kg and 300 μg/kg BMP-7 treated groups had return of urine flow following release of UUO. The mean glomerular filtration rate (GFR) in the post-obstructed kidneys of animals in the 100 and 300 μg/kg BMP-7 groups was 0.19 and 0.21 mL/min/100 g bw, or 34% and 38% of normal GFR values, respectively. The GFR of the contralateral kidneys tended to be increased in both the enalapril-treated group and vehicle group when compared to sham animals; this finding suggested compensatory hypertrophy. Contralateral renal hypertrophy was less in the BMP-7–treated groups. Renal blood flow was assessed by para-amminohippurate (PAH) clearances; as a result, assessment of effective renal blood flow was limited to the animals with return of urine flow. Mean single-kidney PAH clearances were 1.0 and 1.2 mL/min/100 g bw in the 100 and 300 μg/kg bw BMP-7 groups, respectively, or 58% and 68% of the values obtained in sham-operated normal rats. Renal blood flow in the contralateral kidneys was not altered, with the exception of a modest but significant ($P < 0.05$) increase in the enalapril-treated group; this increase indicated contralateral renal hypertrophy.

To improve the comparison of renal function protection among the various groups, in a subsequent study we decreased the severity of the renal injury to three days of UUO and seven days of recovery. Either BMP-7 or enalapril was given intraperitoneally beginning at the time of ureteral obstruction, and the investigators treating the animals as well as those performing the clearance studies were blinded to the treatment group; hence, a double-blind, placebo-controlled format was used. Figure 2B shows that urine flow was re-established in the vehicle-treated (placebo) group ($N = 8$), and the GFR was 0.16 mL/min/100 g bw, compared to 0.33 in the sham-operated normal animals. Thus the experimental window was narrowed in this study. The GFR in the enalapril group ($N = 8$) was 0.21 mL/min/100 g bw, not statistically better than that in the vehicle group. The GFR of the

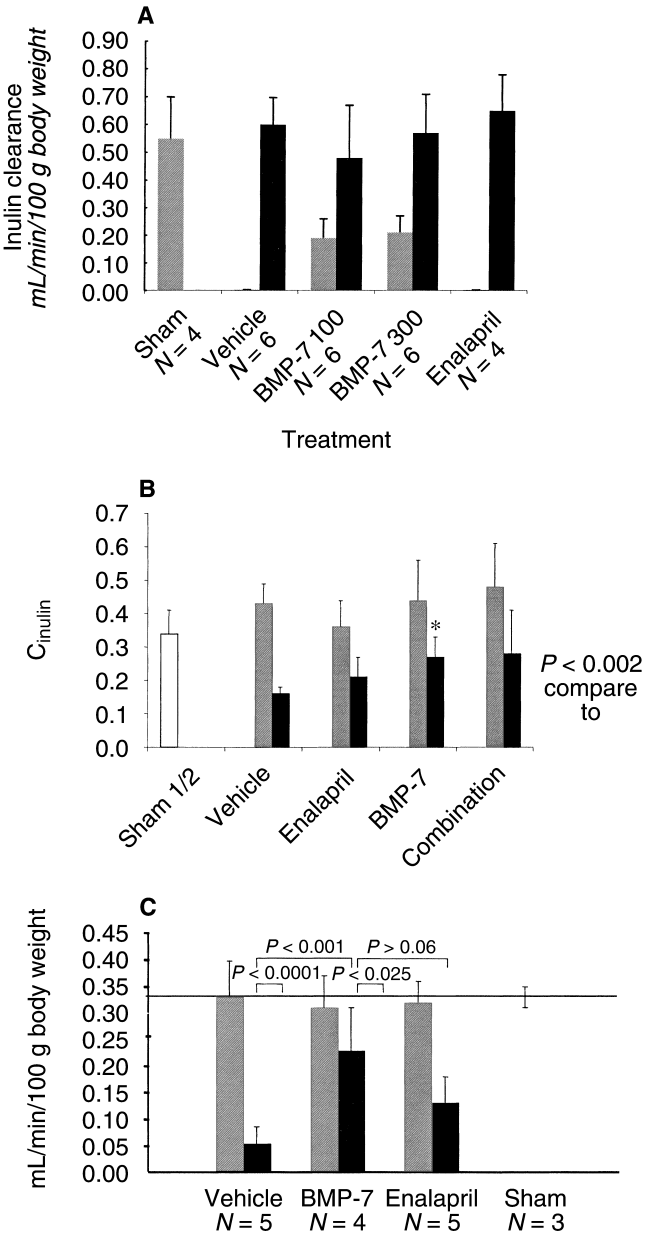

Fig. 2. Recovery of renal function following release of unilateral ureteral obstruction in three protocols. *A*. Inulin clearances following 5 days of obstruction and 5 days of recovery in obstructed (■) and contralateral (■) kidneys. BMP-7 and enalapril were begun at the time of UUO (prevention protocol). *B*. Inulin clearances following 3 days of obstruction and 7 days of recovery in obstructed (■), control (■), and sham-operated ( ) kidneys. BMP-7 and enalapril were begun at the time of UUO. *C*. Inulin clearances following 3 days of obstruction and 7 days of recovery in obstructed (■), contralateral (■), and sham-operated kidneys. BMP-7 and enalapril were begun at the time of release of the UUO (treatment protocol). In each protocol, BMP-7, 100 μg/kg bw, was significantly more effective than enalapril in restoring GFR toward normal.

two groups given doses of BMP-7 did not differ, and the groups were combined for analysis. The GFR was 0.28 mL/min/100 g bw ($P < 0.02$) in the BMP-7–treated animals, significantly higher than that in the vehicle-treated animals. Combination of BMP-7 and enalapril did not produce an additional benefit, probably because of the maximal doses of both agents used.

To further test the therapeutic potential of BMP-7, we changed the protocol from a prevention to a treatment strategy by delaying institution of the test agents until release of the obstruction at day 3. In this treatment trial (Fig. 2C), the GFR of the post-obstructed kidneys of animals in the BMP-7 treated group, 0.22 mL/min/100 g bw, was significantly higher than that in post-obstructed kidneys of the vehicle-treated group, and also greater ($P < 0.06$) than that in the post-obstructed kidneys of animals treated with enalapril. Thus, in three separate trials including a treatment trial, BMP-7 significantly protected renal function and was more potent than a maximal dose of an ACE inhibitor.

Treatment with BMP-7 prevented the infiltration of the renal parenchyma by macrophages following UUO. Using a monoclonal antibody (ED-1) to a macrophage-specific antigen, Diamond et al quantitated the cellular infiltration of the interstitium following UUO [15]. Using this techinque, we demonstrated that administration of BMP-7 at 100 or 300 μg/kg bw reduced the macrophage infiltration by 55% and 72%, respectively, in the UUO kidneys from rats in which obstruction was released at day 5 prior to analysis at day 10. Although enalapril also was effective, the protective effect of 300 μg/kg bw BMP-7 every other day was significantly greater.

We analyzed the degree of tubulointerstitial fibrosis in cortical sections of rat kidneys subjected to either 5 or 10 days of UUO. Masson trichrome-stained sections were used to analyze the accumulation of collagen in the interstitium. As Figure 3 shows, collagen was apparent in the Bowman's capsule of normal kidneys, the perivascular adventitia (Fig. 3A), and in the renal capsule (Fig. 3B). In comparison, kidneys subjected to UUO for five days, with release and recovery for five days prior to analysis at day 10, had massive accumulation of collagen in the expanded interstitium along with cellular interstitial infiltrates (Figs. 3C and 3D). Contralateral, non-ligated kidneys in all the experimental groups were indistinguishable from normal kidneys. Kidneys from rats given 100 μg/kg bw of BMP-7 with five days of UUO prior to release of the obstruction and examination at day 10 had minimal interstitial collagen deposition (Figs. 3E and 3F). Rats treated with 300 μg/kg bw of BMP-7 had almost no renal interstitial collagen deposition following UUO (Figs. 3G and 3H). Expansion of the interstitial space after UUO is apparent in Figure 3 (C and D), and this was quantitated. Unilateral ureteral obstruction with release at five days and analysis at 10 days revealed a fivefold expansion of the interstitial space, from 9.7% to 47.4% of the cortical volume ($P < 0.001$). In the group of rats given 300 μg/kg bw of BMP-7, the expansion of the interstitial space was only 17.1% (less than twofold above normal). Administration of 300 μg/kg bw of BMP-7

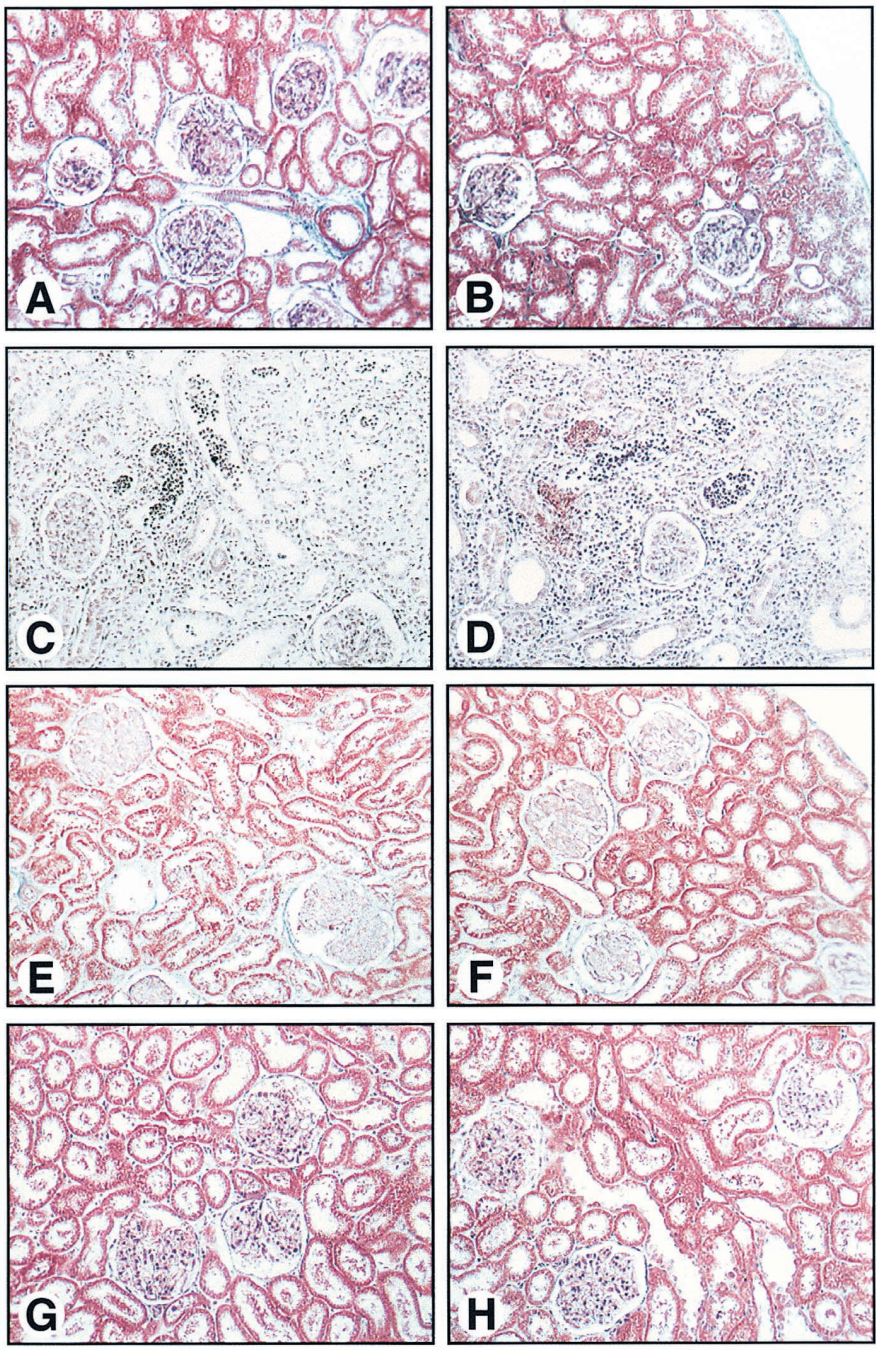

Fig. 3. BMP-7 prevents renal fibrogenesis following UUO. Coronal sections of kidneys stained with the Masson trichrome stain for collagen. With this stain, collagen fibrils stain blue, whereas the cells stain red. *A*, *B* are sections from the cortices of both kidneys of sham-operated animals. Collagen is detected in the renal capsule around larger vessels and Bowman's capsule. Little collagen is detectable in the renal interstitium. *C*, *D* are sections from two different areas of the obstructed kidney of a vehicle-treated animal. There is widespread and diffuse blue stain of the interstitial collagen, and a major interstitial cellular infiltrate. *E*, *F* are two sections of the cortex of an obstructed kidney from an animal treated with 100 μg/day bw of BMP-7. The amount of interstitial collagen is only slightly more than that in the sham-operated kidneys. *G*, *H* are two sections from the cortex of a kidney from an animal treated with 300 μg/day bw of BMP-7. The amount of interstitial collagen is indistinguishable from the interstitial collagen deposition in the sham-operated animals. [Reprinted with permission of the American Physiology Society from *Am J Physiol* (Renal Physiol) 280:F130-F143, 2000.]

was significantly more effective than the administration of 100 μg/kg bw of BMP-7 ($P < 0.03$). Treatment with BMP-7 at either dose afforded greater protection than did enalapril ($P < 0.01$), which our group has previously shown to be renoprotective in this model of fibrogenesis [19, 20].

We further examined the expansion of the renal interstitium following UUO by analyzing the deposition of type IV collagen. Type IV collagen is a normal component of tubular basement membranes, which frequently abut basement membranes of neighboring tubules in normal and contralateral kidneys. In obstructed kidneys, expansion of the interstitial compartment increases the distance between tubular basement membranes. Type IV collagen staining of the tubular basement membrane was decreased in UUO, and type IV collagen was aberrantly expressed as a component of the interstitial collagen accumulation. The interstitial myofibroblast responsible for deposition of the increased interstitial collagen matrix in tubulointerstitial fibrosis secretes type IV

collagen [48]. Thus, type IV collagen staining serves both as a measure of interstitial volume and as a marker of interstitial collagen accumulation. Treatment with BMP-7 diminished the distance between tubular basement membranes, thus confirming the interstitial volumetric measurements shown in Figure 3, and decreased the deposition of type IV collagen in the interstitium.

We next examined the expression of alpha smooth muscle actin (α-SMA), a marker of the tubulointerstitial myofibroblast responsible for a large component of interstitial collagen deposition following UUO. In the normal and contralateral kidneys, α-SMA was expressed only in the blood vessels, but it was prominently expressed in the interstitium of the kidneys subjected to 5 days of UUO and released prior to analysis at day 10. Administration of BMP-7 significantly reduced interstitial α-SMA, as demonstrated by a lesser histologic immunostaining score of α-SMA. The effect of BMP-7 on α-SMA histologic scores was dose dependent, and the 300 μg/kg bw dose was significantly more effective than enalapril in preventing accumulation of cells expressing α-SMA in the interstitium.

We also analyzed the protective action of BMP-7 during a longer period of UUO sufficient to produce a severe injury that would be irreversible in the experience of most investigators. Ten days of sustained UUO in vehicle-treated rats further increased interstitial volume from 47.4% in the 5-day obstruction/5-day release group to 57.2%. This increase, while significant, represents some leveling off in the rate of interstitial volume increase. Concordant with this observation, we noted no further induction of α-SMA or increase in the interstitial type IV collagen score when comparing values from the release at 5 days to the 10-day sustained UUO animals. Treatment with BMP-7, especially using the 300 μg/kg bw dose, significantly decreased interstitial volume, collagen IV matrix score, and expression of α-SMA. Visual inspection of the immunocytochemistry for collagen IV and α-SMA confirmed the scoring results. In rats with sustained UUO for 10 days, administration of BMP-7 reduced cellular infiltration. The administration of BMP-7, especially the 300 μg/kg bw dose, afforded greater protection than did enalapril administration in the rats with UUO of 10 days duration.

To assess the mechanism of protection by BMP-7 against UUO-stimulated tubulointerstitial fibrosis, we first examined the effects of UUO on tubular atrophy. We quantitated tubular atrophy as described by Chevalier et al [49]. Unilateral ureteral obstruction for 5 days with 5 days of recovery (with analysis at day 10) significantly increased the number of atrophic tubules (Fig. 4B) over those in a sham-operated kidney (Fig. 4A). The BMP-7 dose dependently decreased tubular atrophy (Fig. 4C). As in other studies [16], enalapril had no effect on the tubular atrophy produced by UUO (Fig. 4D). These data demonstrate a significant difference between the mechanism of renal protection with BMP-7 treatment and that with enalapril treatment. The information suggests that the mechanism of BMP-7–induced renal protection is related to preservation of renal tubular epithelial integrity, and that this mechanism might have suppressed the tubulointerstitial inflammation and fibrosis due to the UUO-stimulated, angiotensin II-mediated damage cascade.

We further investigated the mechanism of renal tubular epithelial preservation following UUO by examining whether apoptotic loss of epithelial cells, which contributes to tubular atrophy and is increased by UUO [50], was affected by BMP-7 treatment. We examined sections of kidney, similar to those in Figure 4, using the TUNEL assay for apoptotic nuclei. In vehicle-treated rats that had undergone 5 days of UUO with analysis on day 10, a significant increase ($P < 0.001$) in apoptotic nuclei of epithelial cells was found, from an average of 1.7 per 200× microscopic field in the kidney of sham-operated animals to 30.1 apoptotic nuclei per 200× field. Both doses of BMP-7 significantly reduced the number of apoptotic nuclei by about one-third ($P < 0.001$). Enalapril produced a modest but significant decrease (<0.05) of about 10% in tubular epithelial cell apoptosis.

Tubular epithelial morphology in the medulla was preserved by BMP-7 treatment. After UUO for 5 days with recovery for 5 days following release, the medullary collecting duct was characterized by flattened, effaced epithelia. The BMP-7 dose dependently preserved epithelial morphology. Associated with preservation of epithelial cell morphology, expression of BMP-7 was protected. The BMP-7 was expressed in the normal collecting duct epithelia [44, 45] and its expression was preserved by BMP-7 treatment but not by enalapril. Because the half-life of BMP-7 is short, and the last dose in our studies was administered 48 hours prior to analysis, exogenous BMP-7 was not detected by immunostaining.

In summary, BMP-7 administration protects against the tubulointerstitial fibrosis and renal destruction produced by UUO. The mechanism of action is partly related to inhibition of renal tubular atrophy through the prevention of tubular cell apoptosis and de-differentiation, which preserve tubular epithelial integrity as well as epithelial cell phenotype, and to inhibition of interstitial infiltration and interstitial fibroblast transformation. Secondary actions on preservation of renal blood also were contributory. Furthermore, BMP-7 administration at the time of relief of obstruction was therapeutic and more potent than ACE inhibition. Our results clearly suggest that BMP-7 is an important renal homeostatic factor that can suppress the impact of renal injury.

QUESTIONS AND ANSWERS

Dr. John T. Harrington (*Dean, Tufts University School of Medicine, Boston, Massachusetts, USA*): Let

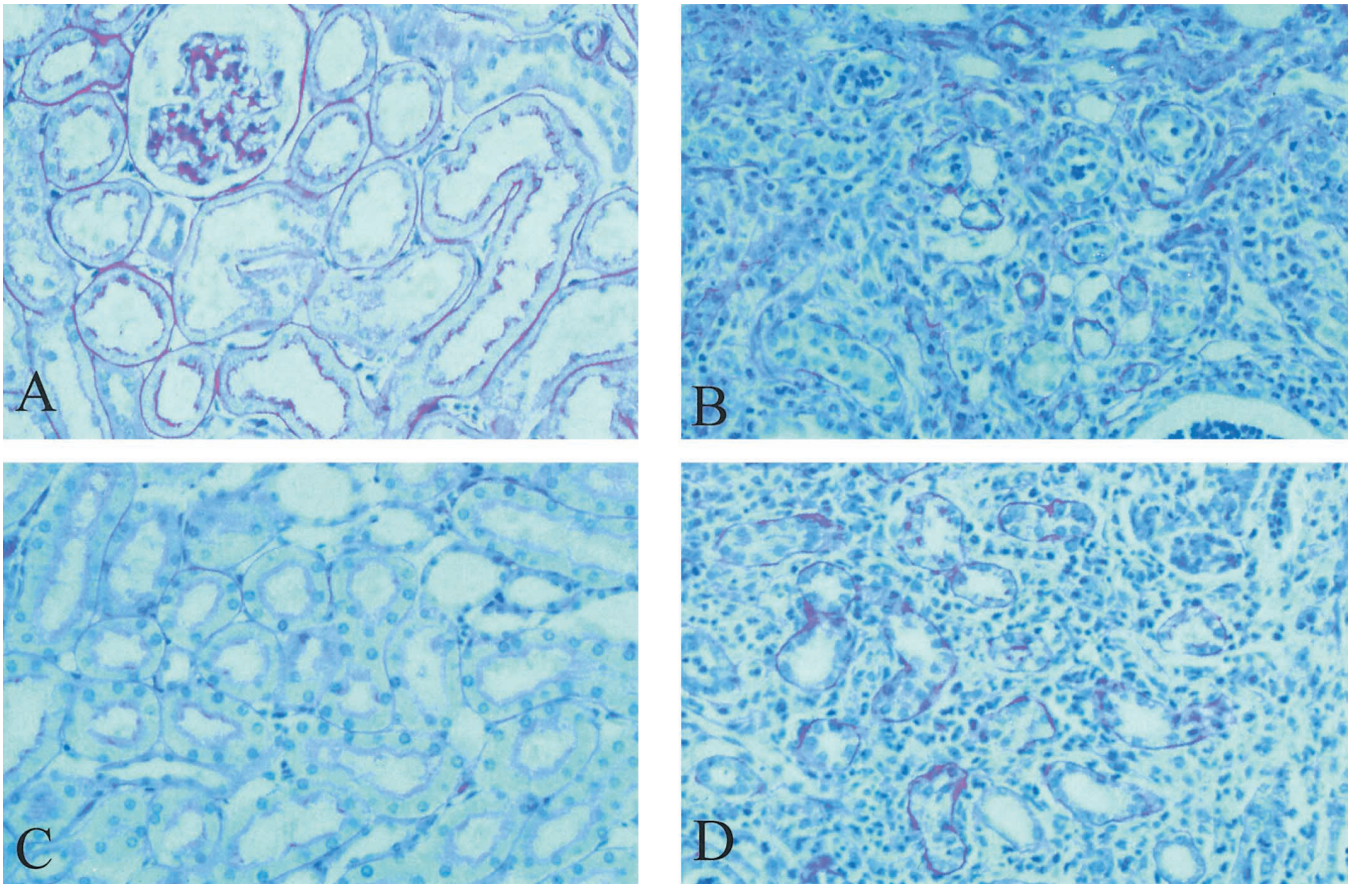

Fig. 4. BMP-7 blocks development of tubular atrophy following UUO. Coronal sections of kidneys stained with the periodic-acid Schiff stain to highlight basement membranes. *A*. Sham-operated kidney. Basement membranes (BMs) are thin, BMs of different tubule segments often abut each other, and no interstitial infiltrate is present. *B*. Vehicle-treated kidney. UUO for 5 days with release and analysis at day 10. Heavy interstitial infiltrate and small atrophic tubules with thickened basement membranes. Some tubules have white blood cells in the lumens. *C*. 300 μg/kg bw of BMP-7 every other day. The pattern of basement membrane expression has basically been preserved as normal. *D*. Enalapril, 25 mg/kg daily. The cellular infiltrate is less than that with vehicle treatment (B), but the tubular atrophy is essentially unchanged compared with the vehicle-treated group.

me start by going back to your comments about ACE inhibitors and their ability to block fibroblast proliferation. How does that work?

Dr. Hruska: That action appears to be indirect in that fibroblast proliferation is a direct action of TGF-β. Inhibition of TGF-β transcription by ACE inhibition is the means of inhibiting fibroblast proliferation.

Dr. Harrington: Does BMP-7 have a role in preventing fibrotic processes in other diseased organs, or is the effect restricted to the kidney?

Dr. Hruska: A very interesting paper was published by Ed Nord's group in the *Journal of Clinical Investigation* demonstrating that primary interstitial nephritis is associated with the development of the Epstein-Barr genome in the proximal tubule [51]. There is no difference in terms of ACE inhibition between primary chronic interstitial nephritis versus secondary forms of interstitial nephritis, such as obstructive nephropathy, so we would expect that in the setting of EB virus in the proximal tubule, BMP-7 would be very effective.

But is BMP-7 effective in organs other than the kidney during chronic renal disease? As BMP-7 is a newly described hormone, an analogy to epoetin and calcitriol is appropriate. These are both renal hormones with physiologic actions outside the kidney that are diminished during chronic kidney disease. What are the extrarenal physiologic actions of BMP-7? One of them is that it organizes and participates in osteoblast differentiation. Renal osteodystrophy due to secondary hyperparathyroidism produces a pathologic skeletal state called osteitis fibrosa. The fibrosing cells in their bone marrow are osteoblast progenitors that cannot get into the differentiation programs because the marrow has been constricted. If you treat hyperparathyroidism, which is a high turnover bone disease, with BMP-7, you further increase bone formation rate, increase mineralization rates, and decrease bone reabsorption. The most impressive thing is the disappear-

ance of all of the fibroblastic cells and the bone marrow fibrosis. Thus, at the level of the skeleton, there's a marked anti-fibrotic effect of BMP-7. At the level of the vasculature, there's also an amazing effect that I think will be important.

Dr. Ronald D. Perrone (*Division of Nephrology, New England Medical Center, Boston*): Is it known precisely how the signaling pathway of BMP-7 functions?

Dr. Hruska: No, it is not. Perhaps the most intriguing possibility is that BMP-7 could inhibit TGF-β–induced signaling. The BMPs and TGF-β signal through transcription factors activated by their serine kinase receptors. These transcription factors are called SMADs. There are active SMADs, SMADs that form a dimer and transit to the nucleus, and inhibitory SMADs. One possible mechanism by which a member of the superfamily inhibits the signal transduction produced by another member of the family is that BMP-7 might stimulate an inhibitory SMAD, thus decreasing TGF-β-stimulated active SMADs. Another possibility is that a primary inhibitory action of BMP-7 signaling decreases expression of angiotensin II and TGF-β; that remains to be seen.

Dr. Perrone: Tubulointerstitial fibrosis is a substantial component of the pathology of autosomal dominant polycystic kidney disease. Is anything known about BMP-7 and cystic kidneys? Do we have any data on the use of BMP-7 to alter cyst progression in animal models?

Dr. Hruska: We know that renal injury in general decreases BMP-7 expression. We also know that BMP-7 expression in the collecting duct decreases in renal cystic diseases, but we have no idea what BMP-7 might do in polycystic kidney disease.

Dr. Andrew J. King (*Division of Nephrology, New England Medical Center*): Is there a difference between enalapril and the AT-1 receptor blocker with respect to effects following unilateral ureteral obstruction.

Dr. Hruska: There are two immediate possibilities but both appear not to be the case. The most likely possibility was that this difference was a kinin effect. The second possibility was that it was mediated through the AT2 receptor. The data that I discussed showed that it wasn't an AT2 receptor or a kinin effect [52]. One possibility is that the difference is due to angiotensin IV and the novel receptor. The angiotensin converting enzyme would block that pathway, but the AT1 receptor would not. All we know is that the two obvious explanations were not the case.

Dr. Nicolaos E. Madias (*Executive Academic Dean, Tufts University School of Medicine*): Are there known examples of renal injury in which BMP-7 is spontaneously upregulated?

Dr. Hruska: None that I know of. I haven't come across anything that would indicate a stimulation of BMP-7 expression. In cells that are terminally differentiated, the key differentiation factor is likely one of the constitutive molecules of that cell. You might not see tremendous regulation, and regulation might be an all or none switch. Renal injury seems to eliminate BMP-7 expression. In the UUO model, the injury affects the collecting duct, where BMP-7 is made. Replacement of BMP-7 protects against the injury. Is there a broader role for BMP-7 in chronic renal disease? The answer to that is yes, and the basis for that answer is that specific BMP-7 receptors exist not only in the collecting duct, but also in the proximal tubule and the glomerulus. The presence of the receptor in the proximal tubule defines a paracrine role of BMP-7, but the presence of the receptor in the glomerulus according to renal development suggests a hormonal role. The hormonal role that I was talking about for BMP-7 regulating osteoblast differentiation would be further substantiated by protection of the glomerular compartment in chronic renal disease and a differentiation function for BMP-7 probably in the glomerular compartment. Receptors for BMP-7 in compartments other than the collecting duct might indicate additional physiologic functions for BMP-7.

Dr. Madias: Angiotensin II activates transcription factor NF-κB, which is central in the pathophysiologic cascade of injury in tubulointerstitial disease. Do we know the mechanism by which such activation occurs? Also, what is the effect of BMP-7 on NF-κB activation?

Dr. Hruska: I don't know the effect of BMP-7 on NF-κB activation. Those experiments are in progress. The function of angiotensin II in activation of NF-κB is twofold. There is a direct effect and also an indirect effect that is most prominent through TGF-β.

Dr. Andrew S. Levey (*Chief, Division of Nephrology, New England Medical Center*): I think it's particularly interesting that you've shown the effect of BMP-7 in a model of interstitial disease for two reasons: interstitial disease is a component of most kinds of chronic renal disease, and also because the effects of ACE inhibitors appear to be greater in patients with proteinuria, which is most often associated with glomerular diseases rather than interstitial diseases. For example, our work using meta-analysis of controlled trials shows a benefit of reducing proteinuria and blood pressure, and an additional benefit independent of those two. Hypertension and proteinuria are more common in glomerular disease. Perhaps the benefit of ACE inhibitors is independent of lowering blood pressure and urinary protein, and occurs in the interstitium, as you have shown. What else needs to be done before bringing drugs like BMP-7 or BMP-7 itself to clinical development?

Dr. Hruska: The next step on the basis of a series of studies that have been completed but not reported (although submitted as abstracts for the 2001 ASN/ISN World Congress of Nephrology: Davies MR, et al; Lund RJ et al; Wang S et al) is that BMP-7 is ready to go from preclinical studies to human trials. While chronic

tubulointerstitial disease is very significant, it might be too limited of an indication, and BMP-7 might not be developed solely for that indication. A broader context of chronic renal disease in general would be a stronger indication for which BMP-7 could be developed as a therapy.

Dr. Harrington: Are there toxicity studies in humans?

Dr. Hruska: No, only in animals. In the model of diabetic nephropathy, we used repeated tail vein injections of BMP-7 for 16 weeks, 2 injections per week. We did not see a single local sight reaction, nor was there any vascular calcification, in 125 animals. We found no toxicity. We performed a study related to heterotopic calcification, which might be expected to be stimulated by BMP-7, to directly look at this issue. Our hope was that BMP-7 would not stimulate heterotopic calcification. The BMP-7 prevented vascular calcification in chronic renal disease, a state of severe vascular calcification.

Dr. King: Is BMP-7 detectable in urine?

Dr. Hruska: Yes, it's detectable in urine and in blood. You can measure changes in the urine with ELISAs, but the threshold of the detection limit for serum is such that you can't measure any decrease. Thus, we don't have an ability to demonstrate that renal injury produces a decrease in blood levels, but it does decrease urinary BMP-7 excretion. You can ask, why is BMP-7 excreted in the urine? Perhaps urinary BMP-7 has a differentiation role in the urinary tract and the bladder.

Dr. King: Have you or others performed immunostaining for BMP-7 using human tissue? Fibrosis is prominent in chronic renal allograft rejection, and TGF-β has been implicated. Is any information available on the effects of BMP-7 in cyclosporine nephrotoxicity?

Dr. Hruska: Immunostaining for the BMPs is complicated by multiple molecular forms of these substances and by prominent degradation products. Immunostaining for the BMP family in general has been hindered by these problems.

Dr. King: How about cyclosporine nephrotoxicity?

Dr. Hruska: The pathophysiology of the cyclosporine nephrotoxicity is likely to be BMP-7-responsive. However, the issue becomes how many pre-clinical studies should we do before we undertake human studies? I proposed a protocol on cyclosporine nephrotoxicity, but the proposal wasn't funded. If I were to move from studying obstructive uropathy to a different disease that most likely would respond to BMP-7, I would study cyclosporine nephrotoxicity.

Dr. Harrington: You've discussed the role of BMP-7 from ureteral obstruction to allograft rejection to bone repair to diabetes. You also talked briefly about chronic renal failure from a variety of tubular interstitial diseases. What do we know about BMP-7 in any of the models of acute renal failure?

Dr. Hruska: It has been difficult to bring therapeutic agents effective in animal models of acute renal failure to the clinic. The models of acute renal failure are not very reproducible from laboratory to laboratory. Second, our models of acute renal failure do not correspond well with human disease. Development of BMP-7 as a therapy for acute renal failure was discontinued for these reasons. Some studies demonstrated promising results.

Dr. Madias: Whereas controlled apoptosis might represent a mechanism of repair of renal injury, unchecked apoptosis can have injurious consequences. You mentioned that BMP-7 decreased apoptosis in your experiments. Does this effect offer insights into the mechanism of dysregulated apoptosis?

Dr. Hruska: Probably, but I do not have any experimental data.

Dr. Harrington: You showed a list of many proteins whose expression are increased in unilateral ureteral obstruction (Table 1). Yet we focus on BMP-7. What are the effects of these other overexpressed molecules as they dance along, and why is BMP-7 so effective?

Dr. Hruska: Most of those proteins listed in the table are involved in the destructive process downstream of TGF-β and NF-κB. The hypothesis of BMP-7's mechanism of action is that BMP-7 functions early in the destructive pathway and that the proteins that are upregulated will be inhibited by BMP-7.

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