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BMP-7 regulates chemokine, cytokine, and hemodynamic gene expression in proximal tubule cells[1]

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BMP-7 regulates chemokine, cytokine, and hemodynamic gene expression in proximal tubule cells.

Background. Proximal tubule epithelial cells (PTEC) play a central role in the response of the kidney to insult by virtue of their production of chemokines and cytokines that signal an inflammatory response. Bone morphogenic protein-7 (BMP-7/OP-1), a member of the transforming growth factor-β (TGF-β) superfamily, has previously been demonstrated to reduce macrophage infiltration and tissue damage in animal models of acute and chronic renal failure. The present study was designed to define the molecular mechanism of BMP-7 action in human PTEC.

Methods. Expression of BMP-7 in the adult mouse kidney was determined indirectly through X-gal staining of heterozygous BMP-7/lacZ mice in combination with cell-type specific markers. Primary human PTEC were cultured in the presence of the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α), with and without BMP-7. RNA isolated from these two populations was then used to identify differentially regulated genes via gene-array analysis. Modulation of potential target genes was subsequently confirmed through ELISA and/or quantitative PCR.

Results. Expression from the BMP-7/lacZ transgene was detected in the collecting duct, thick ascending limb, distal convoluted tubule, and podocytes within glomeruli. No expression was detected within PTEC; however, these cells were found to express mRNA for BMP receptors including, ActR-I, BMPR-IA, ActR-II, ActR-IIB, and BMPR-II. BMP-7 significantly reduced TNF-α stimulated increases in mRNA for the pro-inflammatory genes, interleukin-6 (IL-6) and interleukin-1β (IL-1β), and the chemoattractants monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) in primary human PTEC. In addition, BMP-7 also reduced the expression of mRNA for endothelin-2 (ET-2), a vasoconstrictor, and increased the expression of mRNA for heme oxygenase-1 (HO-1), a vasodilator, although the latter was not statistically significant. In experiments designed to examine MCP-1 and IL-6 protein levels in response to additional TGF-β superfamily members, TGF-β1 was unable to mimic the effects of BMP-7 in reducing IL-6 production. However, the closely related BMP-6 exhibited similar properties to those of BMP-7. Each of the factors reduced MCP-1 expression.

Conclusions. BMP-7 represses the basal and TNF-α–stimulated expression of the pro-inflammatory cytokines IL-6 and IL-1β, the chemokines MCP-1 and IL-8, and the vasoconstrictor ET-2 in PTEC. This data are consistent with the in vivo observations that BMP-7 administration in a model of chronic and acute renal failure results in a reduction in the infiltration of macrophages in the renal interstitium. Taken together, these observations suggest that BMP-7 may be a novel therapeutic agent for kidney disorders involving inflammation and ischemic damage of PTEC.

[1] **See Editorial by Kopp, p. 351.**

Key words: bone morphogenic protein-7, proximal tubule, anti-inflammatory, hemodynamic, IL-6, IL-1β, MCP-1, IL-8, ET-2, TGF-β1.

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The most common cause of injury leading to acute renal failure is ischemia. Moreover, proximal tubular epithelial cells play an important role in ischemia-induced disease [1]. Proximal tubule epithelial cells (PTEC) cells are the major site of necrosis associated with renal ischemia, and the extent of tubulointerstitial damage is a better predictor of kidney function and clinical outcome than the extent of glomerular damage in cases of chronic renal failure [2]. Tubular epithelial cells (TECs) produce a number of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), chemokines such as monocyte chemotactic protein-1 (MCP-1), RANTES (regulated on activated normal T-cell expressed), and interleukin-8 (IL-8), and cytokines such as transforming growth factor-β1 (TGF-β1) [3–6]. Release of pro-inflammatory cytokines and chemokines by tubular epithelial cells contribute to the extent of tissue damage by attracting and activating infiltrating leukocytes in the renal interstitium [4].

Bone morphogenic protein-7 is a member of the TGF-β superfamily of ligands that is required during embryogenesis for normal skeletal, kidney, and eye development [7–9]. In the kidney BMP-7 is thought to be required in the metanephric mesenchyme for continued epithelial tubule development in the embryonic kidney [7]. The expression of BMP-7 continues in the adult kidney and this expression has been suggested to decrease in response to injury [10, 11]. Administration of BMP-7 to animals in a model of renal ischemia has been reported to increase survival [12]. The increase in survival was found to correlate with a decrease in the re-

sulting infarct size, necrosis, and infiltration and activity of neutrophils following reperfusion [12]. Similar results, including reductions in monocyte/macrophage accumulations, have recently been reported following administration of BMP-7 in a model of unilateral ureteral obstruction that mimics some aspects of progressive chronic renal failure including interstitial fibrosis [13].

Our study was initiated to identify the mechanisms by which BMP-7 promotes recovery from, or reduces injury associated with such renal insults. We describe the detailed cellular expression of BMP-7 in the adult murine kidney and use gene array analysis to identify genes regulated by BMP-7 in primary human proximal tubule cells. We have found that BMP-7 acts to significantly reduce the expression of pro-inflammatory cytokines including IL-6 and IL-1β, the chemokines IL-8 and MCP-1, and the vasoactive peptide endothelin-2 (ET-2). The ability to reduce the production of MCP-1 and IL-6 protein in PTEC was shared by BMP-6, a closely related BMP family member, whereas TGF-β1 was found to reduce MCP-1 production, but not IL-6 production by PTEC. These observations support a mechanism by which BMP-7 reduces renal injury by decreasing the infiltration and activation of neutrophils through reduced chemokine and cytokine expression, and by maintaining blood flow to the kidney through a reduction in vasoconstrictive peptide gene expression.

METHODS

BMP-7/lacZ transgene localization

BMP-7/lacZ mice were generously provided by Dr. Elizabeth Robertson [8]. Transgene expression was detected via β-gal staining as described [8]. Following postfixation in 4% paraformaldehyde/phosphate-buffered saline (PBS), kidneys were embedded in paraffin through a graded ethanol series into xylenes keeping the total time in xylenes under 20 minutes. Serial 3-μm sections were cut and processed for further histochemical analysis according to standard protocols. Proximal tubules were identified by staining for alkaline phosphatase (AP) via an initial equilibration in AP buffer [100 mmol/L Tris-HCl, pH 9.5; 50 mmol/L $MgCl_2$; 100 mmol/L NaCl; 0.1% Tween 20 (vol/vol)] for 15 minutes at room temperature followed by an incubation in the same buffer containing fast red (1 fast red tablet/2 mL; Roche Molecular Biochemicals, Indianapolis, IN, USA) for two hours at 37°C. Collecting ducts were identified by immunostaining with biotinylated *Arachis hypogea* lectin (Sigma, St. Louis, MO, USA) at 10 μg/mL for one hour at room temperature. Biotinylated lectin was then visualized using streptavidin-horseradish peroxidase (HRP) and diaminobenzidine (DAB) chromogenic substrate according to the manufacturer's recommendations (Histostain-Plus; Zymed, South San Francisco, CA, USA). Thick ascending limb were identified by immunoreactivity with rabbit anti-Tamm-Horsfall protein (THP) antisera (Biodesign International, Saco, ME, USA) at 34 μg/mL for one hour at room temperature followed by visualization with biotinylated anti-rabbit secondary, streptavidin-HRP, and DAB chromogenic substrate (Histostain-Plus; Zymed). Whole-mount specimens were postfixed as above and processed through a graded glycerol series to 80% glycerol for photography.

Cell culture and RNA isolation

Human proximal tubule epithelial cells (PTEC) were obtained from Clonetics (Walkersville, MD, USA) and passaged in the recommended media, REBM™ containing 0.1 mg/mL recombinant human epithelial growth factor (rhEGF), 0.05 mg/mL insulin, 5 μg/mL hydrocortisone, 5 μg/mL epinephrine, 0.56 mg/mL triiodothyronine, 0.1 mg/mL transferrin, 0.25 mg/mL gentimycin, and 0.25 mg/mL amphotericin-B, except that it contained 2% fetal bovine serum (FBS) instead of 0.5% FBS. In all cases cells were used between passages 4 and 5. For gene array and quantitative polymerase chain reaction (PCR) analysis PTEC were plated on standard tissue culture plastic and grown to 70 to 80% confluence prior to treatment. BMP-7 (mature formulation; Curis, Inc.) and/or TNF-α (R&D Systems, Minneapolis, MN, USA) was then added to REBM™ media without additives and containing 0.5% FBS. For analysis of dose dependence, time course analysis, and enzyme-linked immunosorbent assay (ELISA), PTEC cells were plated on collagen-I coated culture dishes (Becton Dickenson, Franklin Lakes, NJ, USA). Analysis of a select set of markers revealed no significant differences in response between cells grown on collagen-I coated plates and those grown on tissue culture plastic. RNA isolations were carried out via the Atlas Pure Total RNA isolation system (Clontech, Palo Alto, CA, USA) or by the Stratagene 96-well total RNA isolation kit (Stratagene, La Jolla, CA, USA) following the manufacturer's recommendations.

Gene array analysis

Thirty micrograms of PTEC total RNA was used to generate ^{33}P-labeled probes using the Atlas Pure Total RNA labeling system (Clontech). Probes were hybridized to the Atlas Human 1.2 array containing 1176 known genes and hybridization detected with a Cyclone phosphoimager (Packard Instruments, Meriden, CT, USA). Quantitation was performed using the Atlas Image analysis software package (V 1.01; Clontech). A ratio of ±1.8 was taken as the minimum threshold for reporting significant differences between blots. Within a single blot genes were considered expressed if they had values greater than twice background. Blots were normalized for probe activity based on the hybridization signal of 40S ribosomal protein S9. A value of "undefined" indi-

Table 1. Primers used for polymerase chain reaction (PCR)

Gene	Forward	Reverse
18S rRNA	5′-GCCGCTAGAGGTGAAATTCTTG-3′	5′-CATTCTTGGCAAATGCTTTCG-3′
HO1	5′-GCCCTGCCCTCCAGCAT-3′	5′-TTGAGACAGCTGCCACATTAGG-3′
IL-1β	5′-ATGTGCACGATGCACCTGTAC-3′	5′-TCATATGGACCAGACATCACCAA-3′
Id-1	5′-AGAACCGCAAGGTGAGCAA-3′	5′-TCCAACTGAAGGTCCCTGATG-3′
Id-2	5′-CCACCCTCAACACGGATATCA-3′	5′-ACACAGTGCTTTGCTGTCATTTG-3′
Id-3	5′-GCGGCAGAGCTGGTCTTC-3′	5′-TCAGGGCAACAGAACCTTTCTC-3′
Sky	5′-GAAGCCCGGACTGACCAA-3′	5′-CATCAAGCCAAGGCCTAAACTG-3′
Smad6	5′-GCCACTGGATCTGTCCGATT-3′	5′-CACCCGGAGCAGTGATGAG-3′
MCP-1	5′-CGCCTCCAGCATGAAAGTCT-3′	5′-GGGAATGAAGGTGGCTGCTA-3′
IL-6	5′-GGCTGCAGGACATGACAACTC-3′	5′-TGAGGTGCCCATGCTACATTT-3′
IL-8	5′-AGATATTGCACGGGAGAATATACAAA-3′	5′-TCAATTCCTGAAATTAAAGTTCGGATA-3′
AODRA1	5′-CCACAAGCCCAGCATCCTT-3′	5′-GGGTTCATGGCCGAGTTG-3′
ET-1	5′-CCCTCCAGAGAGCGTTATGTG-3′	5′-TGGAGGCTATGGCTTCAGACA-3′
ET-2	5′-AAGGCTGAGGGACATTTCC-3′	5′-TGTTGTCGCTTGGCAAAG-3′
ICAM-1	5′-CATGTGTAGCATCAAAACACAAAGG-3′	5′-GGTAAAATAACAAATGAATAAATACATATCATCA-3′
Groβ	5′-GGAGGCCCTGCCCTTACA-3′	5′-CAAACACATTAGGCGCAATCC-3′
ActR-IIB	5′-CAAGCAGTCGTGGCAGAG-3′	5′-ATGGTCACACAAAGTGGG-3′
ActR-II	5′-GGATGTCTCTTGGAAATGTTAAGA-3′	5′-TTTATGTACACAGAACAGCAGCA-3′
BMPR-IA	5′-TGAAATCAGACTCCGACC-3′	5′-TGCTTGAGATGCTCTTGC-3′
BMPR-IB	5′-GTTCCCTTTATGATTATCTGAAGTCC-3′	5′-TTAGACACTCATCACTGCTCCAC-3′
ActR-I	5′-ATGATTGCTCTCCCCTCC-3′	5′-CACCATACCTGCCTTTCC-3′
BMPR-II	5′-ATCCGTATCAGCAAGACC-3′	5′-GCAGTGACTCTCTCATCTCC-3′
Beta actin	5′-CTGGAACGGTGAAGGTGACA-3′	5′-CGGCCACATTGTGAACTTTG-3′

cates that one of the two groups being compared had no detectable signal (less than twice background) and therefore no ratio could be determined.

RT-PCR and quantitative RT-PCR

cDNA was generated by reverse transcribing 1 to 2 μg of DNAse-treated total RNA in a reaction volume of 20 μL using oligo-dT as a priming agent and a commercial reverse transcription system (Promega, Madison, WI, USA). cDNA was diluted to a 100 μL with water and 2 μL of this pool used as template for 25 μL PCR reactions. Quantitative real-time TaqMan PCR was performed using a PE Biosystems 5700 SDS with SYBR Green core reagents (PE Biosystems, Foster City, CA, USA). Reactions were performed in triplicate and analyzed via relative quantitation with data presented as differences from basal levels of expression in untreated PTEC. All data are presented after normalization to 18S rRNA to account for differences in cell number. cDNAs were further diluted 1:10 prior to analysis of 18S rRNA to maintain similar amplification profiles to those of other gene amplification products in the assay (Table 1). BMP receptor expression was determined via amplification of 2 μL of cDNAs using 50 μL reactions containing 15 mmol/L $MgCl_2$ with the following cycling parameters: 94°C for two minutes, 30 cycles of 94°C at 30 seconds, 60°C for one minute, and 72°C for 45 seconds. One tenth of the products were loaded on an agarose gel for visualization.

MCP-1 and IL-6 ELISA

Proximal tubule epithelial cells were plated on collagen-I coated 24 well plates (Becton Dickenson) in REBM™ containing 0.1 mg/mL rhEGF, 0.05 mg/mL insulin, 5 μg/mL hydrocortisone, 5 μg/mL epinephrine, 0.56 mg/mL triiodothyronine, 0.1 mg/mL transferrin, 0.25 mg/mL gentimycin, and 0.25 mg/mL amphotericin-B (Clonetics, Walkersville, MD) with 2% FBS. BMP-7 was added to REBM™ media containing 0.5% FBS without growth supplements. Media was withdrawn following 24 hours of BMP-7 treatment for ELISA analysis. MCP-1 was detected in PTEC culture media using a competitive sandwich ELISA (Biosource International) and IL-6 detected using a sandwich ELISA (R & D Systems, Minneapolis, MN, USA), both following the recommendations of the manufacturer.

RESULTS

Localization of BMP-7 in the adult kidney

To identify the mechanism by which administration of BMP-7 protects renal function following ischemic insult, we initially defined the cellular distribution of BMP-7 within the adult mouse kidney. Previous studies have documented the cellular distribution of BMP-7 during murine kidney development [7, 9, 14, 15], but the available data are less complete for distribution in adult kidneys. To precisely map BMP-7 expression in the adult kidney, mice heterozygous for a null BMP-7 allele (BMP-7/β-galactosidase insertion) were used, which allowed us to indirectly localize BMP-7 expression through the analysis of β-galactosidase expression [8]. Examination of a kidney stained for X-gal in whole-mount reveals abundant BMP-7 expression with increased expression in the medulla and ureter, relative to the cortex (Fig. 1 A, B). Analysis of sections of stained kidneys, in combination with cell-type specific markers, localized the majority of BMP-7 expression to the tubular epithelium (Fig. 1 C–H). BMP-7 was

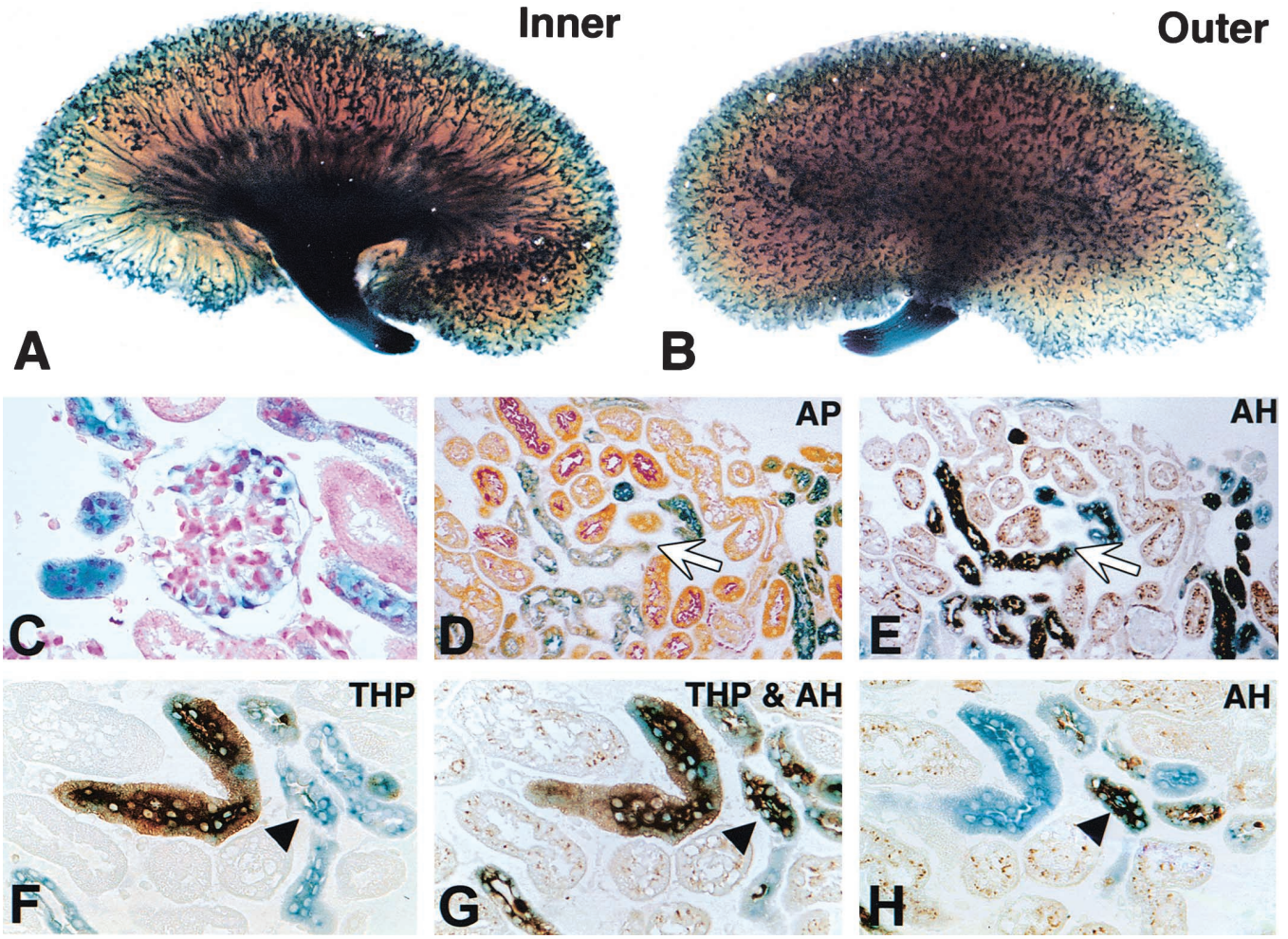

Fig. 1. Cellular localization of bone morphogenic protein-7 (BMP-7) in the adult mouse kidney. Heterozygous BMP7/lacZ mice were used to evaluate expression of BMP-7 transcripts in adult mice. X-gal staining in blue indicates BMP-7/lacZ expression. (*A* and *B*) Whole mount preparation of an adult kidney demonstrating abundant expression of BMP-7 in the medullary region and ureter. Expression is also detected in the cortical region albeit to a lesser extent. (*C*) A section of a similarly stained kidney demonstrating BMP-7/lacZ expression in podocytes within the glomerulus. (*D* and *E*) Adjacent sections stained for alkaline phosphatase (AP, red) marking proximal tubules and the lectin Arachis hypogea (AH) marking collecting ducts (brown). Note that the x-gal staining does not co-localize with the AP, demonstrating that the BMP-7/lacZ transcripts are not expressed in proximal tubules (pt). There is, however, overlap between AH staining and x-gal indicating that a subset of the BMP-7 expression includes collecting ducts (cd). (*F–H*) Adjacent sections stained for (F) Tamm-Horsfall protein (THP) marking the thick ascending limb (brown), (G) both AH and THP marking both collecting ducts and thick ascending limb, or (H) AH marking the collecting ducts. The arrowhead marks the same collecting duct present in each section.

detected in collecting ducts marked by Tamm-Horsfall protein (THP), the thick ascending limb, marked by the lectin *Arachis hypogea* (AH), and by virtue of the continuous expression between these two segments within the intervening distal convoluted tubule (Fig. 1 D–H). Additional BMP-7 was detected in the glomerulus within podocytes (Fig. 1C). No detectable expression of BMP-7 was observed in proximal tubules, thin descending limb, loop of Henle, or in the peritubular vasculature (Fig. 1D).

BMP receptor expression within PTEC cells

Interestingly, with the exception of the thick ascending limb, the epithelial structures exhibiting BMP-7 expression were those that have been reported to survive ischemia-induced renal damage. Proximal tubules, which lack detectable BMP-7 expression, and the thick ascending limb have been reported to be extremely sensitive to ischemic insults [1, 2, 16–19]. This observation raises the possibility that exogenous administration of BMP-7 provides proximal tubule cells with a source of BMP-7 that then serves to act as a survival factor for these cells. Therefore, we sought to characterize the molecular response of PTEC to BMP-7. As a first measure of response the BMP receptor complement present on human primary PTEC was defined. These cells were found to express all known type I and type II BMP

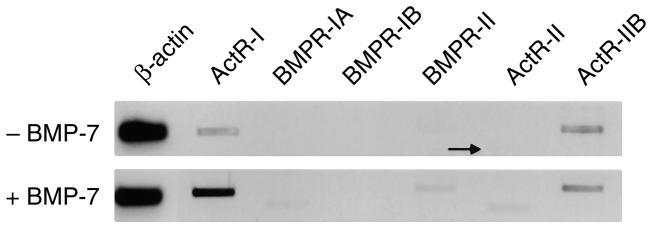

Fig. 2. Bone morphogenic protein (BMP) receptor expression in proximal tubule epithelial cells (PTEC). PTEC were treated for 6 hours in the presence of 0 or 100 ng/mL BMP-7 and analyzed for BMP receptor expression via RT-PCR. Unstimulated PTEC express detectable levels of ActR-I, BMPR-II, ActR-II, and ActR-IIB. Stimulated PTEC express these and in addition, contain detectable transcripts for BMPR-IA. Table 1 lists the primer sequences used for PCR.

receptors excluding the type I receptor, BMPR-IB as defined by RT-PCR (Fig. 2). Notably, no BMPR-IA was detected in PTEC prior to stimulation with BMP-7, and the levels of ActR-I, ActR-II, and BMPR-II appeared to increase following BMP-7 treatment. This semiquantitative analysis suggests that PTEC express the appropriate receptors for BMP-7 signaling and, based on the change in receptor expression following BMP-7 treatment, further suggests that these cells are capable of responding to this ligand.

Gene array analysis

The effects of BMP-7 treatment on PTEC were compared in the presence and absence of the pro-inflammatory cytokine TNF-α on the expression of a panel of 1176 known human genes. The percentage of genes on the blots with detectable expression (greater than twice background) ranged between 24% for untreated and 29% for BMP-7–treated representing 284 to 338 separate genes (data not shown). Addition of BMP-7 to cells treated with TNF-α caused a detectable (see **Methods** section for analysis thresholds) change in the expression of 40 genes, 8 that increased and 32 that decreased in response to BMP-7 (Table 2). Of the genes that increased most dramatically in response to BMP-7, three (Id-1, Id-2, and Id-3) belong to a family of negative regulators of bHLH transcription factors [20]. In addition to these genes, heme oxygenase-1 (HO-1), an enzyme implicated in vasodilation was found to increase threefold in response to BMP-7. Similar increases were noted in the absence of TNF-α suggesting that these genes may be directly regulated by BMP-7 (Table 2). Genes identified as having their expression reduced greater than 1.8-fold following BMP-7 treatment included the inflammatory or stress response genes, adenosine A1 receptor (ADORA1), monocyte chemotactic protein-1 (MCP-1), corticotropin-releasing factor receptor 1 (CRF-1), clusterin (CLU), intercellular adhesion molecule-1 (ICAM-1), IL-6 and TNF-α. In addition, BMP-7 treatment also reduced the expression of the vasoconstrictor, endothelin-2 (ET-2), and MAL, a protein involved in the apical transport pathway in epithelial cells to undetectable levels (Table 2).

Since it is possible that the changes in gene expression noted 48 hours following BMP-7 treatment are due to secondary factors released in response to BMP signaling, the time-course of BMP-7 effects was analyzed on a select set of genes identified by gene array. The effects of BMP-7 on Id-3, MCP-1, and ET-2 mRNA expression were noted from 0.5 (MCP-1 and Id-3) to 3.0 (ET-2) hours following BMP-7 treatment, suggesting that these changes in gene expression are likely due to a direct effect of BMP signaling (Fig. 3). Consistent with this observation, gene-array analysis performed on cells treated with BMP-7 and or TNF-α for six hours revealed a similar set of genes whose expression is modified as those identified following a 48 hour treatment, with the exception of ICAM-1 (data not shown).

BMP-7 reduces expression of chemotactic, inflammatory and vasoactive genes in PTEC

Given the prominent representation of genes involved in inflammatory and hemodynamic regulation regulated by BMP-7 detected by gene array, it seemed reasonable to confirm and extend these findings to other genes with similar function using more quantitative analyses than gene array. Quantitative real-time PCR was therefore performed using primer sets directed against pro-inflammatory, chemokine, and hemodynamic regulators. BMP-7 treatment for a period of six hours significantly reduced the TNF-α-induced expression of the pro-inflammatory genes IL-6, IL-1β, and ADORA1, and the chemokines MCP-1 and IL-8 (2.7-, 3.6-, 2.4-, 2.9- and 2.0-fold, respectively; Fig. 4A). BMP-7 also significantly inhibited the basal expression of these genes in the absence of TNF-α. BMP-7 had a similar effect on growth-related oncogene (Groβ), a chemokine, although this reduction was not statistically significant (Fig. 4A). No changes were noted in RANTES (data not shown). In addition, BMP-7 significantly reduced the level of endothelin-2 (ET-2, 4.6-fold) while increasing the expression of ET-1 by 3.2-fold. We also examined the expression of several other damage-related genes in this system and either failed to detect or found no changes in the expression of cyclooxygenase 2 (COX2), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), angiotensin II (Ang II), and angiotensin-converting enzyme (ACE). In addition, no changes in ICAM-1 expression were noted six hours following BMP-7 treatment. However, both the endogenous and TNF-α-induced expression of ICAM-1 were reduced 2.9-fold following 48 hours of BMP-7 treatment as assayed by quantitative PCR in close agreement with data obtained by gene array (data not shown).

Bone morphogenic protein-7 treatment stimulated the expression of Id-1 Id-2, Id-3, Smad-6, and SKY approximately equally in the presence or absence of TNF-α

Table 2. Genes identified by gene array

Gene	BMP-7/TNF-α	BMP-7	TNF-α
Helix-loop-helix protein; DNA-binding protein inhibitor ID-1; Id-1H	+ ud	+ ud	nc
Helix-loop-helix protein; DNA-binding protein inhibitor ID-3; Id-3	+ ud	+ ud	nc
GTP-binding protein ras associated with diabetes (RAD1)	+ ud	nc	nc
Heme oxygenase 1 (HO1)	3	+ ud*	0
Helix-loop-helix protein; DNA-binding protein inhibitor Id-2	2.58	+ ud	0
Endothelial plasminogen activator inhibitor-1 precursor (PAI1)	2.18	3.55	2.39
Tyrosine-protein kinase receptor tyro3 precursor; rse; sky; dtk	2.16	+ ud*	− ud*
Sodium/potassium-transporting ATPase alpha 1 subunit (Na^+/K^+-ATPase)	1.88	1.37*	−2.13*
Growth arrest & DNA-damage-inducible protein (GADD45)	−1.83	+ ud*	+ ud*
Golgi 4-transmembrane spanning transporter; MTP	−1.85	0	−2.78
Calcium/calmodulin-dependent protein kinase I (CAMKXI)	−1.86	nc	+ ud*
Activated RNA polymerase II transcriptional coactivator p15	−1.86	+ ud*	+ ud
Alpha-1-antitrypsin precursor	−1.86	1.10*	2.14
Cellular nucleic acid binding protein (CNBP)	−1.87	nc	+ ud*
Interleukin enhancer-binding factor (ILF)	−1.87	nc	+ ud*
Cyclin-dependent kinase 4 inhibitor (CDK4I)	−1.88	1.09*	−1.92*
Prothymosin alpha (ProT-alpha)	−1.88	1.32*	1.14*
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	−1.90	−1.35	−2.50
Major prion protein precursor (PRP)	−1.93	nc	− ud*
LIM domain kinase 1 (LIMK-1)	−1.94	nc	+ ud*
ras-related protein RAB2	−1.94	+ ud*	+ ud*
Transcription factor ZFM1	−1.94	1.1*	−2.32*
Matrix metalloproteinase 7 (MMP7)	−1.95	−1.45*	1.90
Alpha1 catenin (CTNNA1)	−1.98	−1.12	1.11*
Tumor necrosis factor precursor (TNF-α)	−2.06	−1.45*	1.62
Tissue inhibitor of metalloproteinases 2 (TIMP2)	−2.06	nc	+ ud*
B94 protein	−2.12	1.04*	1.80
acyl-CoA-binding protein (ACBP)	−2.13	−1.02	2.36
Interleukin-6 precursor (IL-6)	−2.14	1.23*	1.4
Brain-specific tubulin alpha 1 subunit (TUBA1)	−2.14	1.33	1.39
Transcriptional regulator interferon-stimulated gene factor 3 gamma subunit	−2.19	−1.11*	−1.47*
14-3-3 protein beta/alpha; protein kinase C inhibitor protein-1 (KCIP-1)	−2.19	+ ud*	− ud*
Sodium- & chloride-dependent taurine transporter	−2.31	+ ud*	+ ud*
Intercellular adhesion molecule-1 precursor (ICAM-1)	−2.52	−1.35	2.33
Clusterin precursor (CLU)	−2.53	−1.81*	−1.03*
Monocyte chemotactic protein 1 precursor (MCP-1)	−2.88	−1.43	3.78
Corticotropin releasing factor receptor 1 precursor (CRF-1)	−2.98	−1.37*	1.78*
Adenosine A1 receptor (ADORA1)	−4.10	−1.82	−2.0
T-lymphocyte maturation-associated protein MAL	− ud	nc	− ud*
Endothelin 2 (ET-2)	− ud	1.04*	−1.69*

Results from gene array analysis. Only those genes whose expression was altered greater than 1.8-fold in response to addition of BMP-7 in the presence of TNF-α are listed (BMP-7/TNF-α). Comparisons are presented as a ratio of treated/untreated. In cases where one of the two samples contained undetectable (less than twice background) signal and no ratio could be determined the value is stated as + or − undetermined (ud) depending on whether the treated (+ ud), or the untreated sample contained detectable signal (− ud). Data obtained from BMP-7-treated and TNF-α-treated cells also are presented for comparison. Ratios marked by asterisk do no fulfill the requirements for significance (signal greater than twice background and a ratio of greater than +/−1.8).

(19.8-, 14.0-, 25.4-, 15.5-, and 7.2-fold, respectively, in the presence of TNF-α, Fig. 4B). There was a trend for higher HO-1 expression following BMP-7 treatment, however, these differences were not significant. No changes were noted in HO-2 expression in response to BMP-7 (data not shown).

The reduction of IL-6 and MCP-1 production by BMP-7 is shared by BMP-6 but not by TGF-β1

To determine if these responses in PTEC were unique to BMP-7 or were shared by other members of the TGF-β superfamily, since it has been reported that TGF-β1 can reduce MCP-1 expression in PTEC [21], the effects of BMP-7, BMP-6, CDMP-1, and TGF-β1 were compared on IL-6 and MCP-1 protein production in PTEC by ELISA (Fig. 5). BMP-6 was the most effective in reducing IL-6 production, 2.0-fold, compared to 1.9-, 1.5-, and 0.9-fold for BMP-7, CDMP-1, and TGF-β1, respectively. BMP-6 was also the most effective TGF-β superfamily member in reducing MCP-1 production in PTEC (3.5-fold) followed by TGF-β1, BMP-7, and CDMP-1 (2.7-, 2.4-, and 1.8-fold, respectively). Therefore, all but CDMP-1 were effective in reducing MCP-1 expression by PTEC. However, only BMP-7 and the closely related BMP-6 were effective in reducing IL-6 production, demonstrating some level of specificity among TGF-β superfamily members.

DISCUSSION

Bone morphogenic protein-7 has been demonstrated to maintain kidney function in models of acute and

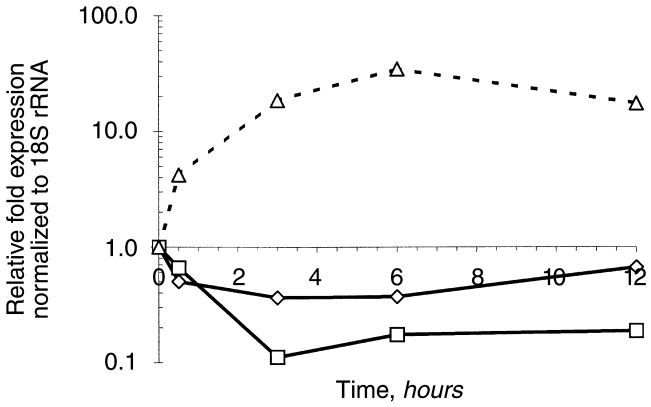

Fig. 3. Time course of BMP-7 repression of Id-3, MCP-1, and ET-2 expression. PTEC were cultured in the presence or absence of BMP-7 (100 ng/mL) and the levels Id-3 (△), MCP-1 (◇), and ET-2 (□) were analyzed by quantitative real-time PCR. Values are represented as fold change relative to untreated PTEC.

chronic renal failure [12, 13]. BMP-7 treated animals have significantly increased renal blood flow and glomerular flow rate following ureteral obstruction [13], and lower blood urea nitrogen and serum creatine levels following ischemic damage [12]. In both models BMP-7 treatment increased survival and histologic parameters, including a significant reduction in the accumulation of neutrophils within the tubular interstitium. These data combined with the requirement of BMP-7 for proper embryonic kidney formation [7, 9] suggest that BMP-7 acts as a maintenance or differentiation factor in kidney development and pathophysiology. Despite these observations, little is known about the mechanism of BMP-7's therapeutic action within the kidney.

BMP-7 expression (BMP-7/lac-Z mRNA) was higher in the medullary region of the kidney than in the cortical region (Fig. 1 A, B). A similar medullary distribution of BMP-7 in the kidney has been reported by others [10, 11]. At the cellular level BMP-7/LacZ expression was detected in the tubular epithelium of the thick ascending limb, distal convoluted tubule, collecting duct, and in podocytes within the glomerulus (Fig. 1). No detectable expression of the BMP-7/LacZ transgene was found in the proximal tubular epithelium (PTEC), confirming what has been observed in a proximal tubule cell-line, HK-2 [22]. Given the increased sensitivity of proximal tubules and thick ascending limb segments to ischemic injury [16, 17, 23], we hypothesize that exogenously added BMP-7 may provide a maintenance or survival signal for these cells.

Our results demonstrate that proximal tubule epithelial cells (PTEC) express BMP receptors and respond to BMP-7 both in the presence or absence of the pro-inflammatory cytokine TNF-α. BMP-7 significantly reduced the basal and TNF-α–stimulated expression of the pro-inflammatory cytokines IL-6 and IL-1β, and the chemokines MCP-1 and IL-8. Given the important role that PTEC play in renal inflammation, these changes in expression provide a potential mechanism by which BMP-7 exerts its therapeutic effects. Reducing the expression of pro-inflammatory cytokines may help limit the inflammatory cascade triggered by the initial insult. The finding that BMP-7-treatment altered the expression of MCP-1, ET-2, and Id-3 within three hours of addition also suggests that these changes could be due to a direct action of BMP-7. The BMP-7 induced reduction in chemokine expression including MCP-1 is of particular interest. MCP-1 is produced by PTEC in response to TNF-α and IL-1α [3], and by plasma proteins associated with proteinuric urine [24], and serves as a chemotactic factor for monocytes and T-cells. Elevated levels of MCP-1 have been detected in renal tissue in a number of pathologic conditions [25–28], strongly supporting a role for MCP-1 in renal inflammation and interstitial fibrosis. Indeed, recent data generated in MCP-1 −/− knockout mice suggest that elimination of this single chemokine can reduce peritubular macrophage accumulation in a model of nephrotoxic serum nephritis by 40% [29]. Taken together with the observation that BMP-7 reduced the expression of two additional chemokines, IL-8, and growth-related oncogene-β (Gro-β) in PTEC, it is tempting to speculate that such effects may underlie the reductions in macrophage accumulation observed following renal injury [12, 13]. Blocking the influx of inflammatory cells would then reduce the local release of pro-inflammatory cytokines and help arrest the progression of the inflammatory response.

In addition to significant reductions in the pro-inflammatory cytokines and chemokines, a significant reduction in the expression of adenosine receptor A1 (ADORA1) was observed. Adenosine receptors play a role in inflammatory response and also have been implicated in vasoconstriction in the renal system. A flavonoid found in *Microtea debilis*, cirsimarin, has been used to treat proteinuria and has more recently found to be an antagonist of the A1 receptor [30, 31]. BMP-7 caused a significant reduction in the expression of ADORA1 in PTEC, suggesting another possible mechanism for the observed reduction in neutrophil accumulation and increased GFR in animal models of renal disease [12, 13]. Renal blood flow may be enhanced by BMP-7 treatment through the observed reduction in endothelin-2 (ET-2) expression by PTEC. Similarly, BMP-7 was found to increase the expression of heme oxygenase 1 (HO-1), a vasodilator, further supporting the idea that treatment with BMP-7 could lead to an increase in GFR. Recent work has suggested that HO-1 expression in human renal epithelial cells is up-regulated by TGF-β1, and that this could serve as a protective mechanism in renal disease [32]. Our data show that BMP-7 may act in a similar fashion during renal disease. Interestingly, ET-2 also has been implicated in stimulating renal fibrosis and BMP-7 has

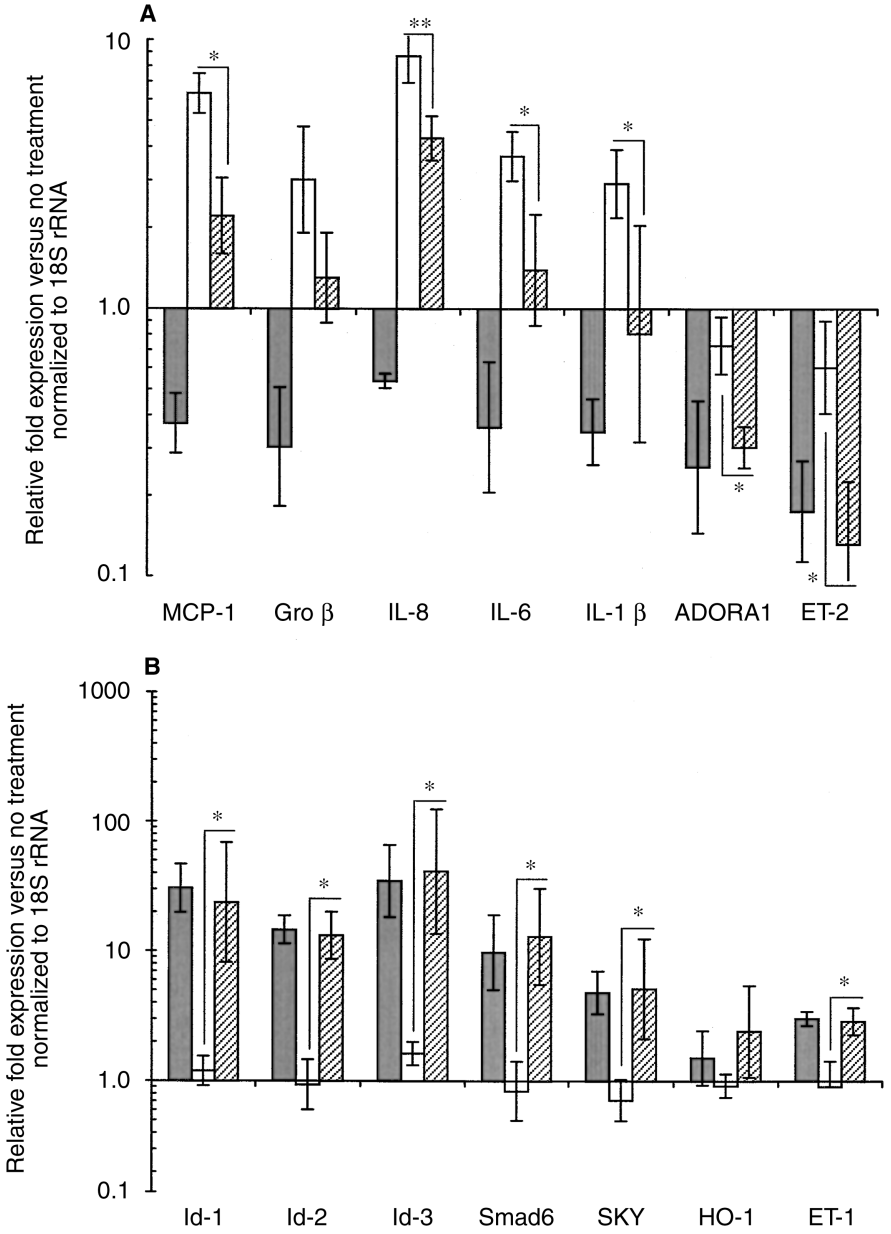

Fig. 4. Quantitative analysis of pro-inflammatory, chemokine, and vasoactive gene expression in PTEC in response to BMP-7. PTEC were grown for 6 hours in the presence or absence of BMP-7 (100 ng/mL) and or TNF-α (2 ng/mL). Results are the mean of three independent experiments each sampled in triplicate by quantitative real-time PCR and presented as average fold change compared to no treatment after normalization to 18S rRNA. Data are plotted on a log scale with the value for untreated PTEC set at 1.0. (*A*) Genes down-regulated in response to BMP-7. (*B*) Genes up-regulated in response to BMP-7. *Significant difference where $P = <0.05$, and **where $P = <0.005$ as determined by the Student *t* test. Table 1 lists the primer sequences used. Symbols are: (▨) BMP-7; (□) TNF-α; (■) BMP-7 and TNF-α.

been demonstrated to reduce fibrosis associated with unilateral obstruction, a model of chronic renal failure [12, 13]. Furthermore, BMP-7 has been shown to reduce the expression of the endothelin receptor, ET(A), in primary rat osteoblasts [33], suggesting a broader context in which BMP-7 may regulate endothelin activity. We also observed a significant increase of ET-1 in response to BMP-7 in contrast to the effects of on ET-2. Similar results have been reported for TGF-β, where TGF-β enhanced ET-1 expression while inhibiting ET-2 expression [34]. Future studies are warranted to extend these in vitro observations into animal models to clearly demonstrate an effect of BMP-7 on blood flow within the kidney.

In addition to repressing transcription of genes in PTEC, BMP-7 stimulated the expression of several genes including Id-1, Id-2, and Id-3, negative regulators of helix-loop-helix transcription factors, and Smad-6, a factor involved in down-regulating BMP signaling. The finding that BMP-7 dramatically up-regulated Id gene expression is not unexpected as BMP-2 has been reported to regulate the expression of Id-1 in embryonic stem cells [35], osteoblast like cells [36, 37], and in the breast cancer cell line, MCF-7 [38]. Interestingly, in the normal kidney abundant Id-1 immunoreactivity has been detected in both the distal convoluted tubule and thick ascending limb [39] both areas found to express high levels of BMP-7/LacZ mRNA.

Taken together, the data suggest a model by which

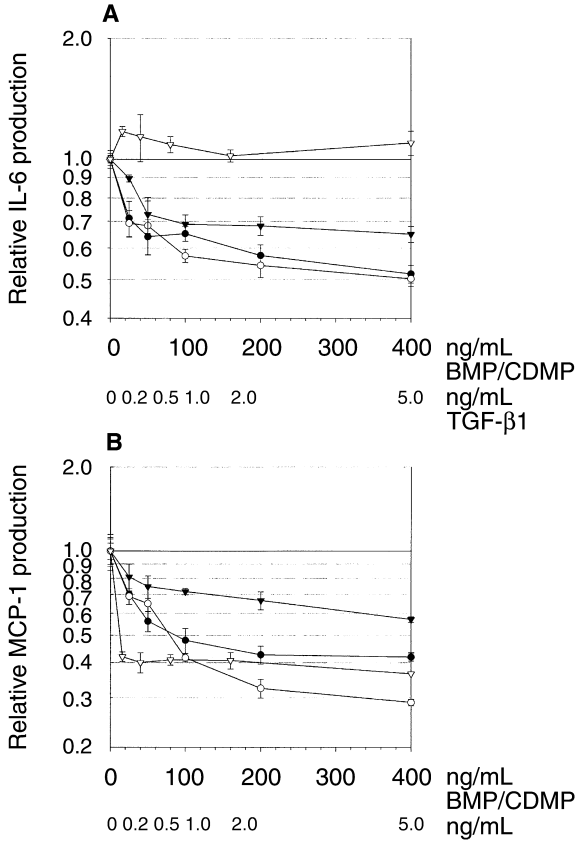

Fig. 5. Comparison of the effects of BMP-6 (○), CDMP-1 (▼), and TGF-β1 (▽) to that of BMP-7 (●) in PTEC. PTEC were treated for 24 hours with or without BMP-7, BMP-6, CDMP-1, or TGF-β1 and the media assayed by ELISA for IL-6 and MCP-1. Data are represented as relative fold change from untreated PTEC.

BMP-7 reduces damage by (*1*) limiting the inflammatory cascade and subsequent fibrosis that follows the initial insult by reducing the release of pro-inflammatory cytokines and chemokines, and (*2*) helping to maintain renal blood flow through its actions on ET-2, HO1, and ADORA1 expression. Importantly, it is not yet known whether any of these changes in gene expression observed in vitro, occur in vivo after BMP-7 administration. Such studies will help clarify the role of BMP-7 in preventing damage to the kidney.

Finally, a number of reports also indicated that BMP-7 has therapeutic effects when administered following stroke [40–42], improving recovery as well as reducing the infarct size. These observations are interesting in that MCP-1, endothelins, and adenosine receptors have all been suggested as therapeutic targets for stroke and other ischemic injury [43–48]. It would be of value to determine whether the same genes regulated by BMP-7 in PTEC are similarly modulated in neuronal cell lines. If so, this would suggest a broader role for BMP-7 in treating other ischemic and inflammatory diseases.

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