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Heparin-Binding EGF-Like Growth Factor Is Up-Regulated in the Obstructed Kidney in a Cell- and Region-Specific Manner and Acts to Inhibit Apoptosis

Hiep T. Nguyen, Samuel H. Bride, Abdel-Basset Badawy, Rosalyn M. Adam, Jianqing Lin, Anna Orsola, Paul D. Guthrie, Michael R. Freeman, and Craig A. Peters

From the Urologic Laboratory, Department of Urology, Children's Hospital, Boston; and the Department of Surgery, Harvard Medical School, Boston, Massachusetts

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Abstract

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The expression of certain growth factors in the epidermal growth factor (EGF) family is altered in response to renal injury. Recent studies have demonstrated that heparin binding EGF-like growth factor (HB-EGF) expression may be cytoprotective in response to apoptotic signals. The purpose of this study was to investigate the potential role of HB-EGF in the upper urinary tract following unilateral ureteral obstruction. We present evidence that: i) ureteral obstruction induced cell-specific but transient activation of HB-EGF gene expression; ii) HB-EGF expression in renal epithelial cells increased under conditions where mechanical deformation, such as that caused by hydronephrotic distension, induces apoptosis, but HB-EGF expression did not increase in renal pelvis smooth muscle cells under identical conditions; and iii) enforced expression of HB-EGF served to protect renal epithelial cells from stretch-induced apoptosis. These results suggest a potential mechanism by which the kidney protects itself from apoptosis triggered by urinary tract obstruction.

In human and experimental animal models, urinary obstruction causes renal parenchyma changes including loss of tubular cells, cell proliferation, myofibroblastic transformation of interstitial fibroblasts, and expansion of the extracellular matrix (reviewed by Chevalier[1] and by Nguyen and Kogan[2]). These changes are likely to be mediated by alterations in the expression of specific growth, differentiation, and survival factors. Experimental ureteral obstruction in rats has demonstrated that the expression of epidermal growth factor (EGF), a potent renal epithelial cell mitogen, is markedly suppressed in the obstructed kidney.[3] Administration of exogenous EGF has been shown to increase renal tubular cellular proliferation and to reduce tubular apoptosis in the obstructed kidney,[4] indicating that signaling through the EGF receptor (ErbB1 receptor tyrosine kinase) may antagonize the process of kidney damage.

A variety of other peptide growth factors are structurally homologous to EGF and signal through the EGF receptor. Heparin-binding EGF-like growth factor (HB-EGF), a 20- to 22-kd glycoprotein, is a member of the EGF-like growth factor family and a potent epithelial cell, fibroblast, and smooth muscle cell (SMC) mitogen.[5] In the urinary system, HB-EGF is synthesized by bladder SMCs and urothelial cells and serves as an autocrine growth factor for both cell types.[6,7] In the kidney, HB-EGF

mRNA is expressed predominantly in the epithelial cells of the proximal tubules in the outer medulla[8] and is a potent mitogen for renal epithelial cells.[9] Recent studies have demonstrated that HB-EGF expression is increased following acute ischemia or nephrotoxin-induced renal injury,[9-11] suggesting that HB-EGF may serve a protective or regenerative role in the response of renal epithelial cells to injury. Ureteral obstruction also induces renal injury and apoptosis, in part through mechanical distension of renal cellular elements. However, a potential role for HB-EGF in the context of urinary obstruction has not been explored. In this study, we present evidence that HB-EGF serves a cytoprotective role in the obstructed kidney.

Materials and Methods

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Ureteral Obstruction

Complete unilateral ureteral obstruction was performed on 12 adult female CD-1 mice (Charles River Laboratories, Cambridge, MA) using methods approved by the Animal Research Committee at Children's Hospital. The adult mice, weighing 22 to 24 g, were anesthetized with ketamine (45 mg/kg) and xylazine (5 mg/kg) intraperitoneally. The right kidney and ureter were exposed via a midline incision. Under optical magnification, the right ureter was ligated close to the vesico-ureteral junction with a 6–0 nonabsorbable suture. The incision was then closed in two layers with a 4–0 absorbable suture. The mice recovered from anesthesia and were maintained with an *ad libitum* supply of standard mouse diet and water. Eight mice underwent a sham operation in which the right kidney and ureter were manipulated without ligation of the ureter. Two unoperated mice served as additional negative controls (t = 0 hours). Kidneys were harvested at 3, 6, 12, and 24 hours after complete obstruction (n = 3) or sham operation (n = 2) for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical analysis.

Semiquantitative RT-PCR

The relative levels of HB-EGF and GAPDH mRNAs were determined in whole kidney, renal cortex, and renal pelvis/calyces specimens by semiquantitative RT-PCR. Total RNA was extracted from the specimens using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Reverse transcription and polymerase chain reaction were performed using methods described by Nguyen et al.[12] Primers were selected based on published gene sequences for mouse HB-EGF[13] and GAPDH[14] found in the GenBank database. A 279-nucleotide (nt) HB-EGF product was amplified using a sense 5′-TTT GGA GAG TCC TTT GCA GA-3′ (nt 4–23) and an antisense 5′-TGT GAC AAT GAG ATT CCT TGT G-3′ (nt 282–261) primer pair. A 571-nt GAPDH product was amplified using a sense 5′-TCA CCA TCT TCC AGG AGC G-3′ (nt 245–263) and an antisense 5′-CTG CTT ACC ACC TTC TTG A-3′ (nt 816–797) primer pair. PCR amplification was performed for 30 cycles at 94°C (denature), 58°C (anneal), and 72°C (extend) for 30 seconds each. Normalization to GAPDH expression and a limiting dilution method were used to make semiquantitative comparisons between samples. Relative mRNA levels were assessed by comparing band density using the IS-100 Image Analysis System (Alpha Innotech Corp., San Leandro, CA). Means and standard deviations were calculated from data of three independent experiments for each experimental condition.

Immunohistochemistry

The kidney specimens were fixed in 10% buffered formalin, dehydrated through graded alcohols, cleared with toluene, and embedded in paraffin. Tissue sections 5 μm thick from the harvested kidney were mounted and fixed on aminoalkylsilane-treated slides. After deparaffinization and rehydration, the tissue sections were incubated with 1% hydrogen peroxide to eliminate endogenous peroxidase activity, followed by incubation in 1.5% pre-immune serum from species in which the secondary

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antibody was raised. The kidney sections were then incubated with the primary antibody, a chicken polyclonal IgY (produced for our laboratory by Aves Laboratories, Tigard, OR) and a goat polyclonal antibody raised against the C-terminus of HB-EGF (M18, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:250 and 1:100 dilutions, respectively. Slides were then incubated with species-specific biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized using the Vectastain Elite ABC kit and DAB Substrate Kit (Vector Laboratories) per manufacturer's protocols. As controls for nonspecific immunoreactivity, pre-immune serum was substituted in place of the HB-EGF antibodies at equivalent dilutions. The slides were counterstained with hematoxylin, and the results of immunostaining were analyzed qualitatively.

Isolation of Primary Renal Epithelial and Pelvic/Ureteral SMC Cultures

Using an enzymatic dispersion method modified from Taub et al[15] and Park et al,[16] renal epithelial cells and SMC were isolated from renal cortex and from the renal pelvis/upper ureters of unmanipulated Zucker rats (n = 50). Briefly, the kidneys were stripped of their capsules and surrounding fatty tissues. The renal cortex and renal pelvis/upper ureters were isolated, divided into 1- to 3-mm pieces, and incubated separately in phosphate buffered saline (PBS) supplemented with 0.125 mg/ml elastase (type III, 90 U/mg), 1.0 mg/ml collagenase (Type I, 150 U/mg), 0.250 mg/ml soybean trypsin inhibitor (type 1-S), and 2.0 mg/ml crystalline bovine serum albumin suspended in PBS (pH 7.4) for 30 to 60 minutes at 37°C (all chemicals purchased from Sigma Chemicals, St. Louis, MO). The tissue suspensions were then filtered through a 100-μm cell strainer and centrifuged to pellet cells. The cell pellet obtained from the renal cortical tissues was resuspended in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12, GIBCO, Gaithersburg, MD) supplemented with bovine insulin (5 μg/ml, Collaborative Biomedical Products, Bedford, MA), bovine transferrin (5 μg/ml, Sigma), 3,5,3′-triiodothyronine (5 pmol/L, Sigma), prostaglandin E_1 (25 ng/ml, Sigma), hydrocortisone (50 nmol/L, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml, GIBCO). The cell pellet obtained from the renal pelvis/ureter was resuspended in medium 199 (GIBCO) supplemented with 20% fetal bovine serum (Hyclone Laboratory, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml, GIBCO). Cells were grown in a humidified 5% CO_2/95% air atmosphere at 37°C. All experiments were performed on cells between passages 1 and 3.

Characterization of Primary Renal Epithelial Cells and Pelvic/Ureteral SMC Cultures

Immunohistochemical analysis and enzymatic assays were performed to characterize the phenotype of cultured cells. Cells were grown on polystyrene glass chamber slides (Becton Dickinson Labware, Franklin Lakes, NJ). Using methods described previously, renal epithelial cells and pelvic/ureteral SMC were evaluated for the expression of α-smooth muscle actin (α-SMA) and cytokeratin, using a mouse monoclonal antibody raised against α-SMA and a mouse monoclonal antibody raised against pan-cytokeratin (both from Sigma) at 1:1000 and 1:100 dilutions, respectively.

In addition, characterization of the renal epithelial phenotype also included assay for two renal epithelial cell enzymes. Leucine aminopeptidase and γ-glutamyl transpeptidase (GGT) enzyme activities were assessed in the cultured cells using methods modified from Chung et al.[17] Approximately 1×10^5 renal epithelial cells or renal pelvic/ureteral SMC per well were plated onto six-well plates. After the cells became 60 to 80% confluent, the culture medium was removed and the plates were washed twice with sterile PBS. Leucine aminopeptidase activity was assayed using L-leucine-p-nitroanilide as substrate. The cultures were incubated at 37°C with 2 ml of PBS containing 1 mmol/L L-leucine-p-nitroanilide. The release of p-nitroanilide was measured every 30 minutes by collection of the cell media and determination of absorbance at 405 nm. Each determination was made using duplicate wells, and the results were standardized with respect to protein concentration. The protein concentration was measured in replicate wells with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). GGT activity was assayed in a similar manner using L-γ-glutamyl-p-

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nitroanilide (20 mmol/L) as substrate and glycylglycine (0.3 mmol/L, all chemicals purchased from Sigma) as the acceptor molecule in 150 mmol/L NaCl/Tris buffer, pH 8.5.

Application of Cyclical Stretch-Relaxation

Approximately 1×10^5 renal epithelial cells or pelvic/ureteral SMC per well were plated onto six-well silicone elastomer-bottomed culture plates coated with collagen type I (Bioflex, Flexcell, Hillsborough, NC). Cells were grown to 80% confluence, were rendered quiescent by incubation for 24 hours in DMEM/F12 or medium 199 lacking supplements, and were then subjected to continuous stretch-relaxation cycles using the FX-3000 Flexercell Strain Unit (Flexcell). Each cycle consisted of 5 seconds of stretch and relaxation (0.1 Hz) with 25% maximum radial stretch at the periphery of the membrane. Cells were harvested at 0, 2, 6, 12, and 24 hours after stimulation for total RNA extraction.

Generation of Renal Collecting Duct Cells Expressing HB-EGF

A cDNA fragment encoding the complete coding sequence of human pro-HB-EGF was cloned into the expression vector, pcDNA 3.1-*myc*/His (Invitrogen, Carlsbad, CA), such that the carboxyl-terminus of the resulting protein was tagged with an epitope from c-*myc* and a polyhistidine sequence. Briefly, total RNA was extracted from normal human prostate epithelial cells using TRI reagent (Molecular Research Inc., Columbus, OH) and reverse transcribed using Superscript II reverse transcriptase and oligo $(dT)_{12–18}$ as first strand primer. Primers specific for pro-HB-EGF were used to subsequently amplify a 667-bp fragment corresponding to pro-HB-EGF using the high fidelity Expand DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The sense primer sequence was 5′-CGG TGC *GGA TCC* ATG AAG CTG CTG CCG TCG-3′ and incorporated a *Bam*HI restriction site (GGATCC), whereas the antisense primer sequence was 5′-AAG TCT *GGG CCC* GTG GGA ATT AGT CAT GCC-3′ and included an *Apa*I site (GGG CCC); *Bam*HI and *Apa*I restriction sites present in the resultant PCR product enabled subcloning of the fragment into the expression vector pcDNA 3.1-*myc*/His. Amplification of a PCR product of the correct size was confirmed by agarose gel electrophoresis and the product was purified using the High Pure purification system (Roche Molecular Biochemicals). The fragment was digested with *Bam*HI and *Apa*I and ligated in frame (Rapid Ligation Kit, Roche Molecular Biochemicals) into the expression vector, pcDNA 3.1-*myc*/His(A), which had also been digested with *Bam*HI and *Apa*I, and dephosphorylated by treatment with shrimp alkaline phosphatase. Competent bacteria were transformed with the ligation reaction and plated on LB/ampicillin agar. Putative recombinants were analyzed by agarose gel electrophoresis and analytical restriction digests, and the presence of the 667-bp insert corresponding to pro-HB-EGF was confirmed by sequencing. Transfection quality DNA was subsequently generated by large scale plasmid purification (Qiagen MaxiPrep, Valencia, CA) and quantitated before expression studies in mammalian cells.

Immortalized rat renal collecting duct cells (B7, gift of Dr. J. Kreidberg, Children's Hospital, Boston, MA) were transfected with the human pro-HB-EGF-*myc* epitope/polyhistidine tag plasmid using FuGene 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Stably transfected cells were selected with the antibiotic, G418 (0.4 mg/ml, GIBCO). Five clones were isolated and expanded *in vitro* by culturing in DMEM/F12 medium supplemented with bovine insulin (5 μg/ml), bovine transferrin (5 μg/ml), 3,5,3′-triiodthyronine (5 pmol/L), prostaglandin E_1 (25 ng/ml), hydrocortisone (50 nmol/L), penicillin (100 U/ml), streptomycin (100 μg/ml), and G418 (0.2 mg/ml).

RT-PCR was used to identify transfected clones with high levels of human HB-EGF mRNA. The relative levels of human HB-EGF, rat HB-EGF, and human pro-HB-EGF-*myc* epitope/polyhistidine tag mRNA were determined in the B7 untransfected cells and the five transfected clones. The human HB-EGF primers (a sense 5′-ACA AGG AGG AGC ACG GGA AAA G-3′, nt 521–542 and an antisense 5′-CGA TGA CCA GCA GAC AGA CAG ATG-3′, nt 796–773, primer pair) were selected based on

published gene sequences for human pro-HB-EGF[18] to amplify a 278 nt product. The rat HB-EGF primers (a sense 5′-TCC CAC TGG AAC CAC AAA CCA G-3′, nt 157–178 and an antisense 5′-CCC ACG ATG ACA AGA AGA CAG AC-3′, nt 570–548, primer pair) were selected based on the published gene sequence for rat HB-EGF[19] to amplify a 413-nt product. Of note, the human and rat HB-EGF primers were selected specifically to amplify sequences unique to their respective species. A third primer pair (sense 5′-GAG CTC GGA TCC ATG AAG CTG CTG CCG TCG-3′ and an antisense 5′-GCG GGT GGA TCC TCA ATG GTG ATG GTG ATG ATG-3′) was selected to amplify an approximately 700-bp product from the N-terminus of the pro-HB-EGF gene to the C-terminal *myc* epitope/polyhistidine tag.

Western blot analysis was used to identify transfected clones with high levels of human HB-EGF protein. Untransfected and transfected B7 cells were washed with cold PBS and lysed in a solution containing 62.5 mmol/L TrisCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), and 10% glycerol. Protein concentration of the cell lysates was measured with the Bio-Rad protein assay, after which, dithiothreitol (DTT, final concentration of 50 mmol/L) and bromophenol blue (final concentration of 0.1%) were added. Equivalent amounts of protein were then separated by electrophoresis using a 12% SDS-polyacrylamide gel and transferred to a PVDF Immobilon-P membrane (Millipore, Bedford, MA). The membranes were then probed with a goat polyclonal antibody raised against the C-terminus of human HB-EGF (C18, Santa Cruz Biotechnology, Santa Cruz, CA). Specific antigen-antibody complexes were detected with the Phototope-HRP Western Blot Detection System (New England Biolabs, Beverly, MA). The photograms were obtained in the linear range of detection and band densities were quantified using the IS-100 Imaging Analysis System.

Application of Apoptotic Stretch

To induce cell death, transfected and untransfected (control) cells were grown to 80% confluence on collagen-coated, silicon elastomer-bottomed culture plates. After incubation in DMEM/F12 without supplementation for 48 hours, the cells were subjected to continuous cycles of stretch/relaxation at high frequency and maximal radial stretch (1.0 Hz with a 30% maximum radial stretch at the periphery of the membrane) for 24 hours and were assessed for apoptosis. These stretch parameters were selected based on our observation that untransfected, renal epithelial cells had increased cell death after stretch stimulation at higher frequency. This response was most evident in untransfected, renal epithelial cells that were subjected to the highest frequency tested (1.0 Hz), which was augmented by the increase to 30% maximal radial stretch. To implicate overexpression of pro-HB-EGF in inhibition of apoptosis, transfected cells were also stretched in the presence of CRM197, a mutant diphtheria toxin that specifically inhibits human, but not rat, HB-EGF.[19]

Apoptosis Assay

Apoptosis was assessed by determining the extent of nucleosomal DNA fragmentation (laddering) using methods described by Herrmann et al.[20] Untransfected and transfected cells were scraped, pelleted, and lysed briefly in 1% IGEPAL-CA630 (NP-40, Sigma), 20 mmol/L EDTA, and 50 mmol/L Tris-HCl, pH 7.5. After centrifugation, the supernatants were incubated with 1% SDS and RNase A (5 μg/μl, Sigma) for 2 hours at 56°C, followed by digestion with proteinase K (2.5 μg/μl) for 2 hours at 37°C. The DNA was precipitated with 10 mol/L ammonium acetate and ethanol dissolved in gel loading buffer (15% Ficoll and 0.25% bromophenol blue, Sigma) and separated by electrophoresis in 1.8% agarose gels.

Apoptosis was quantified using the Cell Death Detection ELISA Plus assay (Roche) according to the manufacturer's protocol. The cytoplasmic fraction of the cell lysates from transfected and untransfected cells with and without exposure to apoptotic stretch for 24 hours were incubated with antibodies raised against DNA and histones and sequestered on a streptavidin-coated microtiter plate. Immunoreactivity was measured as absorbance at 415 nm with a background correction at 490 nm from two independent

experiments. An apoptosis enrichment factor was calculated as a ratio of the absorbance of treated (CRM 197 with or without stretch) cells to that of the untreated (no CRM197 or stretch) cells.

Results

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Ureteral Obstruction Transiently Up-Regulates HB-EGF mRNA and Protein Levels in the Renal Cortex

Semiquantitative RT-PCR demonstrated that HB-EGF mRNA levels were increased significantly in the obstructed kidneys within 3 hours after ligation of the ipsilateral ureter and reached maximal levels at 6 h (4.9 ± 1.0-fold increase compared to $t = 0$ hours, Figure 1, A and B). Interestingly, HB-EGF mRNA levels decreased toward baseline levels 24 hours after obstruction. Because we previously demonstrated that HB-EGF was up-regulated in the smooth muscle layer of the obstructed bladder,[7] we examined whether the increased HB-EGF gene expression was specific to the renal pelvis/calyces (predominantly SMC) or the renal cortex (predominantly epithelial cells). We found that there was a time-dependent increase in HB-EGF mRNA levels in the obstructed kidney cortex but not in the renal pelvis/calyces (Figure 1, A and B) . Of note, in animals in which bilateral renal obstruction was induced by urethral ligation, HB-EGF mRNA levels in the obstructed kidneys remained elevated 12 to 24 hours after obstruction and by 30 hours was slightly decreased (data not shown).

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Figure 1.

A: RT-PCR analysis of HB-EGF mRNA levels in mouse kidney, renal cortex, and renal pelvis/calyces after ipsilateral ureteral ligation or sham operation, representative result from three independent experiments. HB-EGF mRNA levels increased progressively following obstruction in the obstructed kidney. By 24 hours, HB-EGF mRNA returned to baseline levels. The increase in HB-EGF gene expression was specific to the renal cortex, not the renal pelvis/calyces. **B:** Densitometric analysis of HB-EGF mRNA expression as determined by RT-PCR in the obstructed kidney, renal cortex and renal pelvis/calyces. All samples were normalized to GAPDH mRNA levels and expressed as a dimensionless ratio to values at time 0. Means and standard deviations for each time point were calculated from three independent experiments. The results demonstrated a time-dependent increase in HB-EGF expression in the obstructed kidney and renal cortex but not in the renal pelvis/calyces.

Immunohistochemical analysis demonstrated similar changes in the expression of the HB-EGF protein following ureteral obstruction. Immunostaining for the C-terminus of pro-HB-EGF demonstrated increasing cytosolic staining in the obstructed kidney, peaking at 12 hours after obstruction (Figure 2, A -E). The increase in HB-EGF protein expression was localized to the tubular epithelial cells of the renal cortex and the collecting duct cells in the medulla (Figure 2, F -I). By 24 hours, however, immunostaining for the HB-EGF protein decreased and was not different from that of the sham-operated kidneys. Using a second anti-HB-EGF antibody, also raised against the C-terminus of pro-HB-EGF, an identical pattern of immunostaining was observed (data not shown).

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Figure 2.

Immunostaining for pro-HB-EGF in the mouse kidneys 6 (**A**), 12 (**B**), and 24 hours (**C**) following ureteral obstruction (50×). The results demonstrated an increase in HB-EGF protein expression at 12 h following ureteral obstruction. In comparison, kidney tissues obtained from animals 12 h following sham-operation (**E**) did not demonstrate any immunoreactivity. Replacement of the pro-HB-EGF antibody with pre-immune serum (**D**) did not demonstrate any non-specific immunoreactivity (negative control). By 24 hours, HB-EGF protein levels returned to baseline levels. The increase in HB-EGF protein expression was localized to renal epithelial (**F**) and collecting duct cells (**H**) 12 hours after ureteral obstruction (100×). By 24 hours, HB-EGF protein expression returned to baseline levels in renal epithelial (**G**) and collecting duct cells (**I**).

Mechanical Stretch Up-Regulates HB-EGF mRNA Levels in Primary Cultured Renal Epithelial Cells

We previously demonstrated that in bladder SMC, mechanical stretch can induce the expression of the HB-EGF gene.[12,16] Consequently, in this study we assessed whether mechanical stretch, such as that resulting from hydronephrotic distension, could also affect HB-EGF gene expression in renal epithelial cells.

We first established primary cultures of rat renal epithelial cells and of SMC from renal pelvis/ureter for comparison. In this series of experiments, rats were used instead of mice, because many of the available antibodies cannot be used directly with mouse tissues. Immunohistochemical analysis and enzymatic assays were used to characterize the cells. The predominant cell type in the renal cell primary culture was morphologically epithelial and expressed cytokeratin (Figure 3A) . However, the cell population was not homogenous, in that there were some cells that expressed α-SMA (Figure 3B) . After several passages, the cell population became more homogeneous, and nearly all cells expressed cytokeratin, not α-SMA (Figure 3C) . In comparison, the cultured SMC from renal pelvis/ureter were spindle-shaped and expressed only α-SMA (Figure 3D) . To further confirm the phenotype of these cells, we quantitatively evaluated the activity of two renal epithelial cell enzymes, leucine aminopeptidase and GGT.[17] As measured by the release of p-nitroanilide from the substrates placed in the media, the cultured renal epithelial cells demonstrated higher leucine aminopeptidase and GGT activity than the SMC (Figure 3, E and F) . Together with the results from immunohistochemical analysis, these results indicated that the cultured cells from the renal cortex were functional renal epithelial cells.

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Figure 3.

Immunostaining for pan-cytokeratin (**A**) and α-SMA (**B**) protein expression in cultured renal epithelial cells (100×). The initial population consisted predominantly of cytokeratin-expressing cells demonstrating an epithelial morphology with some intervening α-SMA-expressing stromal elements. After several passages, nearly all cells expressed cytokeratin and not α-SMA (**C**). In comparison, the cultured renal pelvis/ureteral SMC were spindle-shaped and expressed only α-SMA (**D**). Enzymatic analysis of the cells cultured from renal cortex and renal pelvis/ureter. The release of p-nitroanilide from L-leucine-p-nitroanilide and L-γ-glutamyl-p-nitoranilide placed in the media was measured by absorbance at 405 nm. Each determination was made using duplicate wells and the results were standardized to protein concentration. The results demonstrated greater leucine aminopeptidase (**E**) and GGT (**F**) activity in the isolated renal epithelial cells than in the renal pelvis/ureter SMC, indicating a functional epithelial phenotype.

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The renal epithelial cells and SMC from the renal pelvis/ureters were subsequently subjected to repetitive stretch-relaxation. RT-PCR analysis demonstrated that mechanical stretch induced an increase in HB-EGF mRNA levels in renal epithelial cells but not in the SMC (Figure 4) . At 12 hours after the initiation of continuous stretch, HB-EGF mRNA levels in renal epithelial cells reached maximal levels (11.4 ± 2.5-fold increase compared to unstretched controls). In contrast to that seen *in vivo*, the increased expression of HB-EGF *in vitro* was maintained at 24 hours after stretch. Moreover, HB-EGF mRNA levels returned to baseline levels in the renal epithelial cells that were subjected to 12 hours of continuous stretch and were then left undisturbed for 6 hours.

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Figure 4.

RT-PCR analysis of HB-EGF mRNA levels in renal epithelial cells and renal pelvis/ureter SMC subjected to continuous cycles of stretch-relaxation, representative results from three independent experiments. Means and standard deviations were obtained from densitometric analysis of the three experiments (after normalization to GAPDH mRNA levels and expression as a dimensionless ratio to values at time 0). The results demonstrated a sustained increase in HB-EGF mRNA levels following stretch-stimulation in renal epithelial cells but not in renal pelvis/ureter SMC.

The Expression of pro-HB-EGF Protects against Stretch-Induced Apoptosis

Recent studies have demonstrated that chronic ureteral obstruction results in apoptosis of the tubular, interstitial, and glomerular cells (reviewed by Truong et al[21]). Moreover, the expression of pro-HB-EGF in cultured renal epithelial cells has been shown to have a protective role by preventing cell death.[22] Consequently, using mechanical stretch as an *in vitro* model of obstruction, we investigated whether the enforced expression of HB-EGF in kidney cells can inhibit stretch-induced apoptosis.

We engineered immortalized renal collecting duct cells (designated B7) to express large amounts of human pro-HB-EGF by transfection of a pro-HB-EGF expression plasmid driven by the high efficiency, constitutive cytomegalovirus immediate-early promoter. A *myc* epitope and a polyhistidine tag were fused to the C-terminus of the pro-HB-EGF sequence. Stably transfected cells were obtained by antibiotic selection, and five clones were isolated and analyzed. RT-PCR analysis demonstrated that, whereas rat HB-EGF mRNA was detected in both the transfected and untransfected cells, human HB-EGF mRNA was expressed only in the transfected cells (Figure 5A) . Expression of the transfected human pro-HB-EGF gene in the B7 clones was also demonstrated by PCR amplification of mRNA from the N-terminus of the pro-HB-EGF cDNA to the *myc* epitope/polyhistidine tag. Western blot analysis using antibodies raised against the C-terminus of human HB-EGF demonstrated that B7 clones 1, 3, and 5 expressed high amounts of multiple HB-EGF isoforms (ranging from 22–30 kd,[23]) compared to untransfected parent cells (Figure 5B) .

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Figure 5.

A: RT-PCR analysis of rat HB-EGF, human pro-HB-EGF, pro-HB-EGF-myc epitope/polyhistidine tag mRNA levels in B7 cells transfected with the human pro-HB-EGF construct and untransfected B7 cells. **B:** Western blot analysis with antibodies raised against the C-terminus of human HB-EGF protein in transfected and

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untransfected B7 cells. Clone #1, 3 and 5 expressed high levels of HB-EGF isoforms (ranging from 22 to 30 kd), which were not seen in untransfected B7 cells.

B7 clones 1, 3, 5 and untransfected cells were subsequently subjected to continuous cycles of stretch-relaxation of high frequency and magnitude (ie, apoptotic stretch) to induce cell death. We observed the presence of nucleosomal DNA fragmentation (laddering) in untransfected B7 cells exposed to apoptotic stretch but not in the transfected cells (Figure 6A)▶ . However, when exposed to CRM197, a specific inhibitor of human but not rat HB-EGF,[19] the transfected cells underwent apoptosis, as evidenced by the presence of nucleosomal DNA fragmentation, in response to apoptotic stretch.

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Figure 6.

A: Pattern of nucleosomal DNA laddering in transfected and untransfected B7 cells with/without exposure to stretch-stimulation at high frequency and magnitude, and in the presence/absence of CRM197. Nucleosomal DNA laddering was present in untransfected B7 cells following apoptotic stretch-stimulation. In contrast, cells transfected with pro-HB-EGF did not undergo apoptosis in response to apoptotic-stretch. However, in the presence of CRM197 (a specific inhibitor of human HB-EGF), apoptotic stretch was able to induce apoptosis in these cells. **B:** Quantification of apoptosis using DNA fragmentation enzyme-linked immunosorbent assay in untransfected and transfected B7 cells with/without exposure to stretch-stimulation at high frequency and magnitude, and in the presence/absence of CRM197. Results were obtained from two independent experiments and expressed as a ratio to the values obtained in cells not exposed to stretch.

As an independent measure of apoptosis, we used an enzyme-linked immunosorbent assay to quantify the amount of cytoplasmic histone-associated DNA fragments after induced cell death. Untransfected B7 cells demonstrated a 4.6 ± 1.05-fold increase in the amount of apoptosis compared to unstretched cells after exposure to apoptotic stretch (Figure 6B)▶ . The presence of CRM197 did not further increase the amount of apoptosis (4.77 ± 0.68) in these cells. In comparison, cells transfected with the human pro-HB-EGF gene (Clone 5) demonstrated only a 1.81 ± 0.27-fold increase in the amount of apoptosis following apoptotic stretch. However, in the presence of CRM197, a 5.3 ± 0.73-fold increase in the extent of apoptosis was observed in these cells ($P = 0.024$ compared to cells not exposed to CRM197). Similar results were observed with Clones 1 and 3 (data not shown).

Discussion

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In this study, we observed that complete unilateral ureteral obstruction resulted in an up-regulation of HB-EGF within discrete cellular compartments of the upper urinary tract. Increased HB-EGF mRNA levels were found in the renal cortex but not in the renal pelvis/calyces. A corresponding increase in HB-EGF protein expression was localized to the renal tubular and collecting duct cells but was not seen in glomerular cells or SMC of the renal pelvis/ureters. Interestingly, these responses to obstruction were not sustained; within 24 hours after obstruction, HB-EGF mRNA and protein levels in the renal epithelial cells returned to baseline levels. These findings indicate that urinary obstruction results in regional, cell-specific, and time-dependent regulation of the HB-EGF gene.

Other studies have found that HB-EGF expression is up-regulated in the injured kidney. Homma et al[9] demonstrated induction of HB-EGF mRNA in renal epithelial cells of the inner cortical and outer medullary regions of the ischemic/reperfused rat kidneys. Similarly, Lee et al[24] found an increase in

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HB-EGF mRNA in the kidney tissues of streptozotocin-induced diabetic rats. Sakai et al[11] observed that HB-EGF protein is produced predominantly in the collecting ducts and cortical distal tubules of rat kidneys injured by either ischemia/reperfusion or aminoglycoside administration. With respect to HB-EGF expression in SMC, Borer et al[7] recently found that HB-EGF mRNA and protein levels were elevated in the bladder after complete obstruction of the urethra. Increases in HB-EGF expression were localized predominantly to the smooth muscle layer and not the urothelial layer of the bladder. Our finding that obstruction did not alter the level of HB-EGF gene expression in the SMC of the renal pelvis/calyces indicate that SMC from different regions of the urinary tract are not the same and respond differently to the same stimulus.

We also demonstrated in this study that renal epithelial cells subjected to mechanical stretch *in vitro* similarly demonstrated an increase in HB-EGF mRNA levels, albeit in a more sustained pattern. Of note, mechanical stretch did not significantly affect the expression of HB-EGF in SMC of renal pelvis/ureter. These findings reflect, in large part, those seen *in vivo*. Because in obstruction, mechanical forces from hydronephrotic distension are transmitted to renal pelvis, calyces, and subsequently to the renal collecting ducts and other tubular cells, the results from these *in vitro* experiments suggest that mechanical stretch may act as a stimulus for the up-regulation of HB-EGF expression in the obstructed kidney.

Studies in bladder SMC have similarly demonstrated that mechanical stretch directly activates HB-EGF expression. Previously, we demonstrated that HB-EGF is a stretch-responsive gene in rat SMC, establishing a link between mechanical forces and growth factor expression.[16] Using the same model, we subsequently demonstrated that in human bladder cells, mechanical stretch induces cell-specific activation of the HB-EGF gene[12] ; following stretch stimulation, HB-EGF mRNA levels were increased in bladder SMC but not in urothelial cells. In the present study, we again demonstrated that mechanical stretch results in cell-specific activation of the HB-EGF gene. However, in the kidney, the increase in HB-EGF expression occurs in epithelial cells and not in SMC. Together, these findings suggest a functional role for HB-EGF in renal pathology.

By stretching cells at high frequency and magnitude, we also sought to simulate the physical forces experienced by the obstructed kidney, which are severe enough to induce programmed cell death.[4] Using mechanical stretch as an *in vitro* model of obstruction, we further demonstrated in this study that renal collecting duct cells underwent apoptosis when exposed to stretch of high frequency and magnitude. When engineered to overexpress human HB-EGF, these cells were protected from stretch-induced cell death. By specifically inhibiting human HB-EGF with CRM197, the cells subsequently underwent apoptosis when subjected to stretch of high frequency and magnitude. These findings suggest a role for HB-EGF in preventing renal cell death induced by obstruction.

HB-EGF was originally identified as a heparin-binding mitogen secreted by cultured human macrophages.[5] Subsequently, HB-EGF has been shown to be mitogenic for many cell types including vascular[25] and bladder SMC,[7] fibroblasts,[5] intestinal epithelial cells,[26] urothelial cells,[6] and keratinocytes.[27] Similarly, Homma et al[9] have demonstrated that HB-EGF was mitogenic for renal proximal tubular cells and NRK-52E cells, a cell line derived from normal rat kidney. Because of its induction after acute renal injury and its mitogenic activity, it has been suggested that HB-EGF may be important in renal epithelial cell repair and regeneration in the early stages of recovery after acute renal injury.[11]

More recent studies have suggested additional roles for HB-EGF expression after acute renal injury. Using a remnant rat kidney model in which two-thirds of the kidney was infarcted, Kirkland et al[10] observed an increase in HB-EGF mRNA in renal tubular epithelial cells surrounding the infarcted zone and that HB-EGF strongly inhibited TGF-β-induced increases in α-SMA expression in fibroblasts. Because the tubular cells expressing high levels of HB-EGF mRNA were directly apposed to

fibroblasts, the authors suggested that the potential benefit to tubular cells expressing HB-EGF may be in reducing local myofibroblast transformation, and thus in preventing distortion of viable cellular architecture in areas that undergo contraction during scarring. Using NRK-52E cells engineered to express pro-HB-EGF, Takemura et al[22] demonstrated that HB-EGF prevented H_2O_2-induced apoptosis in renal epithelial cells. Interestingly, the authors also found that addition of conditioned media or exogenous HB-EGF did not replicate this cytoprotective effect of HB-EGF, suggesting that juxtacrine or tightly coupled paracrine interactions may be required for HB-EGF to achieve its effect. Our study provides additional evidence that HB-EGF has a functional role in regulating apoptosis in renal epithelial cells. In other cells such as LNCaP human prostate carcinoma cells[28] and AH66tc cells, a hepatoma cell line,[29] HB-EGF may similarly modulate apoptosis in response to cell stress or injury

In summary, we have observed that: i) *in vivo*, urinary obstruction transiently up-regulates the expression of HB-EGF specifically in renal epithelial cells; ii) *in vitro*, stretch, simulating the mechanical forces of obstruction, induces a more sustained increase in HB-EGF expression; and iii) HB-EGF protects renal epithelial cells from stretch-induced apoptosis. These findings suggest that the induction of HB-EGF gene expression in renal epithelial cells is activated by mechanical forces resulting from the hydronephrotic distension caused by urinary obstruction. This response, though attenuated with prolonged obstruction, may serve to protect the kidney from injury induced by obstruction. Further studies are needed to define the mechanism by which HB-EGF inhibits apoptosis and to identify the factors involved in regulating HB-EGF expression *in vivo*. Modulation of HB-EGF expression may help to prevent renal injury induced by urinary obstruction.

Footnotes

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Address reprint requests to Michael R. Freeman, Ph.D., Enders Research Laboratories, Room 1161, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. E-mail: freeman_m@a1.tch.harvard.edu.

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