Angiotensinogen and AT\textsubscript{1} antisense inhibition of osteopontin translation in rat proximal tubular cells

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Ricardo, Sharon D., David F. Franzoni, Colleen D. Roesener, Jacqueline M. Crisman, and Jonathan R. Diamond. Angiotensinogen and AT\textsubscript{1} antisense inhibition of osteopontin translation in rat proximal tubular cells. Am J Physiol Renal Physiol 278: F708–F716, 2000.—Antisense oligonucleotide inhibition of angiotensinogen and ANG II type I receptor (AT\textsubscript{1}) mRNA translation in rat proximal tubules (PT) was examined to provide direct evidence for a role of the renin-angiotensin system (RAS) in upregulated osteopontin expression observed following mechanical cell stretch. Male Sprague-Dawley rats underwent unilateral ureteral obstruction (UUO) under Brevital anesthesia. In situ hybridization and Western blot analysis demonstrated angiotensinogen mRNA and angiotensin converting enzyme (ACE) protein localized to PTs and upregulated in obstructed kidneys, respectively, confirming an increased expression of renal RAS in vivo. In vitro studies were performed to provide mechanistic insight into ANG II-dependent osteopontin expression following mechanical cell stretch, which putatively mimics the increased PT luminal pressure post-UUO. A cationic transfection method was used to introduce either angiotensinogen or AT\textsubscript{1} antisense oligonucleotide into cultured rat PT cells prior to 1 h of cyclic mechanical cell stretch. Northern blot analysis revealed that PT cells subjected to cyclic mechanical stretch with/without prior transfection with a sense oligonucleotide exhibited increased osteopontin mRNA expression compared with unstretched cells. Blockade of either angiotensinogen or AT\textsubscript{1} mRNA translation by antisense oligonucleotide inhibition prior to cell stretch was found to significantly decrease osteopontin mRNA levels 2.4-fold (P < 0.004) and 1.6-fold (P < 0.001), respectively, compared with values observed in control unstretched cells. This study provides evidence that stretch-induced upregulation of osteopontin mRNA expression is mediated, in part, via production of ANG II. These results lend insight into upregulation of osteopontin via a local PT RAS leading to macrophage infiltration in the tubulointerstitium in experimental hydronephrosis.

ureteral obstruction; angiotensin II; transfection; renin-angiotensin system; cyclic cell stretch

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ties as early as 1–2 h post-UUO (28). Evidence continues to accumulate in support of a local, independent proximal tubular RAS (12, 16). ANG II binding sites are found in high concentrations in proximal tubules, and the presence of renin, angiotensinogen, and angiotensin converting enzyme (ACE) has been identified (3). ACE is found in greatest concentrations in the kidney in the proximal tubules with smaller amounts in arterioles and glomeruli (33). ACE is present in the proximal tubular brush border and could convert ANG I to ANG II.

The present study will first delineate the expression and localization of ACE and angiotensinogen following experimental hydronephrosis in the rat. With these components being upregulated, we further postulate that proximal tubular ACE facilitates the epithelial generation of ANG II that then stimulates, in an autocrine manner, the production of macrophage recruitment factors, such as osteopontin, by these cells. Expression of markers of the RAS, namely renin, will be investigated in vitro to determine whether renin is increased following mechanical stretch of proximal tubular cells. Oligonucleotide antisense therapy, a novel strategy designed to bind specifically and efficiently to the complementary sequence of a targeted mRNA and lead to translational arrest, has been used extensively in both in vivo and in vitro systems. Interference of both the initial and terminal sites of the RAS cascade will be investigated in both in vivo and in vitro systems. The focus of the study will be to demonstrate the effectiveness of antisense therapy in vivo and to determine whether renin is increased following mechanical stretch of proximal tubular cells.

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**METHODS**

**Experimental animals.** Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 150–200 g, were used in this study. Animals were fed standard rodent chow (Purina Chows, St. Louis, MO) and were given water ad libitum. Following intraperitoneal Brevital (50 mg·kg$^{-1}$·body wt$^{-1}$; ip; E. Lilly, Indianapolis, IN), anesthetized animals (n = 10 per time point) underwent either left proximal ureteral ligation or sham operations. Both the obstructed kidney and the contralateral unobstructed kidney (CUK) as well as normal kidneys from sham-operated animals were harvested from UUO rats at 6, 12, 24, and 96 h postureteral ligation or sham procedure.

RNA extraction and Northern hybridization. The kidney cortex was removed and homogenized in ice-cold GIT buffer solution D containing 4 M guanidinium thiocyanate, 0.5% sarcosyl, 1 M sodium citrate, and 0.1 M mercaptoethanol. Total cellular RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (5). For Northern analysis, total RNA (5 µg/lane) was denatured and electrophoresed through 1.2% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters (Nytan; Schleicher and Schuell, Keene, NH) by capillary blotting. RNA was immobilized by baking at 80°C for 30 min. The blots were examined under ultraviolet light in the presence of ethidium bromide to determine the position and integrity of the 28S and 18S ribosomal RNA bands. The blots were hybridized as previously described (30) using a 28S cDNA probe for osteopontin (kindly provided by Dr. C. Giachelli, University of Washington, Seattle, WA) or a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA), which yields 1.6-kb and 1.3-kb mRNA transcripts, respectively. Following hybridization, blots were washed in conditions of increasing stringency, and mRNA signals on autoradiographs were quantified by laser densitometry and evaluated with an IBM-AT-compatible computer (Quantity One; PDI, Huntington, NY). GAPDH was used as a reference probe to correct for variations in loading of RNA samples. The mRNA levels for osteopontin were expressed as ratios of the optical density of osteopontin to that of GAPDH. The peak optical density of reading of each band on the autoradiograph is arbitrarily reported as densitometric units.

**Western blot analysis.** Specimens from renal cortex containing ~500 mg of protein were sonicated in extraction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and a minicomplete protease inhibitor cocktail tablet (Boehringer Mannheim, Indianapolis, IN) made up to a 10-ml volume. Following extraction, the homogenates were stored at −70°C after boiling for 10 min. The protein concentration of the homogenates was determined with the Bio-Rad assay. Samples containing 10 µg of protein were diluted in SDS-PAGE Laemmli buffer and loaded onto a prestained gradient gel. For Western blotting, the gel was removed and placed on a Hybond transfer membrane (Amersham, Cleveland, OH) using filter paper saturated with 300 mM Tris buffer (pH 10.4) in 5% methanol, then covered with additional filter paper saturated with 25 mM Tris, 40 mM glycine, and 20% methanol. This transfer unit was placed in a Milliblott-SDE transfer system (Millipore, Bedford, MA) and run for 30 min. Transfer was judged by the standard bands (New England Biolabs, Beverly, MA) on the transfer membrane. The gel was stained in 0.1% Ponceau solution (Sigma Chemical, St. Louis, MO) in 5% acetic acid to determine the transfer efficiency. The membrane was washed in 20 mM Tris-HCl buffer (pH 7.6) with 137 mM NaCl, 0.1% Tween-20, and 5% wt/vol nonfat dry milk for 1 h at room temperature before incubation with either osteopontin, ACE, or renin antibody. A monoclonal mouse anti-rat osteopontin antibody (MIP11B10 at 1:4,000 dilution; Developmental Studies Hybridoma Bank, Univ. of Iowa) was incubated for 1 h in phosphate-buffered saline (PBS) containing 1% horse serum, peroxidase-conjugated anti-mouse antibody (1:2,000 dilution, Amersham) at room temperature to demonstrate a 66-kDa osteopontin protein. A rabbit anti-goat ACE monoclonal antibody (1:12,000 dilution; kindly provided by Dr. J. Ingelfinger, Massachusetts General Hospital, Boston, MA) was used to demonstrate a 62-kDa ACE protein, and a rabbit anti-rat IgG antibody (1:4,000 dilution; kindly provided by Dr. T. Inagami, Vanderbilt Univ. School of Medicine, Nashville, TN) was used to demonstrate the 60-kDa and 61-kDa renin doublet. A monoclonal mouse anti-rat osteopontin antibody (MIP11B10 at 1:4,000 dilution; Developmental Studies Hybridoma Bank, Univ. of Iowa) was incubated for 1 h in PBS containing 1% horse serum, peroxidase-conjugated anti-mouse antibody (1:2,000 dilution, Amersham) at room temperature to demonstrate a 66-kDa osteopontin protein. A rabbit anti-goat ACE monoclonal antibody (1:12,000 dilution; kindly provided by Dr. J. Ingelfinger, Massachusetts General Hospital, Boston, MA) was used to demonstrate a 62-kDa ACE protein, and a rabbit anti-rat IgG antibody (1:4,000 dilution; kindly provided by Dr. T. Inagami, Vanderbilt Univ. School of Medicine, Nashville, TN) was used to demonstrate the 60-kDa and 61-kDa renin doublet. A mouse anti-rat monoclonal antibody obtained from Chemicon (Temecula, CA) was used to demonstrate the 49-kDa AT1. The membrane was washed in an Amersham ECL detection mixture layered on the surface of the membrane. To measure differences between samples, the bands on the membrane were scanned for optical density and compared with other bands. Densitometric quantification with statistical analysis was performed on autoradiographs from at least three Northern and Western blots using the same sample batch.

In situ hybridization. A 35S-labeled antisense cRNA for angiotensinogen was prepared using the Promega transcription protocol for synthesis of high specific activity RNA probes with T7 RNA polymerase and 35S-UTP (NEN, Boston, MA), following linearization with EcoRI. An angiotensinogen sense cRNA was transcribed from Hind III-cut angiotensinogen cDNA using SP6 RNA polymerase.
Six, 12, 24, and 96 h after obstruction, UUO and CUK kidney specimens were immersion fixed in 10% neutral buffered formalin for 3 h, processed and embedded in paraffin, and sectioned at 4 µm. Sections were alcohol dehydrated and incubated with prehybridization solution containing, 1.2 M NaCl, 0.02 M Tris, 0.04% Ficoll, 0.04% BSA, 0.04% polyvinylpyrrolidone, 0.002 M EDTA, 0.1% salmon sperm DNA, and 0.1 mg/ml of yeast tRNA (final concentrations). Tissue sections were hybridized at 50°C overnight in an identical solution, containing, 25% formamide, 10 mM DTT, 0.1% SDS, and the 35S-labeled cRNA probe at a specific interstitial activity of 4 × 106 counts·min⁻¹·µl⁻¹. After hybridization, the slides were rinsed in a series of washes, including RNase posttreatment. The final wash was in 2× SSC for 2 h at 60°C. The slides were exposed to BioMax autoradiography film (Eastman Kodak, Rochester, NY) and then dipped in diluted Kodak NTB-2 emulsion and stored at 4°C for 1–2 wk. Sections were developed and counterstained with hematoxylin.

Controlled cyclic mechanical cell stretch. Continuous cycles of stretch/relaxation utilizing a rat plasmid-transformed immortalized proximal tubule cell line (Ref. 37; kindly provided by Dr. J. Ingelfinger, Harvard Medical School, Boston, MA) were performed. This specific cell line was chosen since it has all the components of the RAS to locally generate ANG II as well as AT1 (37). Rat proximal tubule cells were plated at a density of 3 × 10⁴/ml in DMEM containing 5% FBS using 6-well plates with a flexible (experimental stretch/relaxation)-bottomed type I collagen-coated membrane (Flexcell, McKeesport, PA) according to our previously published methods (8). Cells were grown to confluence over 72 h and then subjected to cyclic stretch (i.e., stretch and relaxation/cycle) at 15 cycles/min for 1 h using a computer-driven, vacuum-operated, stress-providing instrument (Flexcell Strain Unit FX-2000, Flexcell). An applied vacuum of 20 kPa was used resulting in an elongation of the membrane by ~15%. One 6-well plate constituted an n = 1. Studies were performed in triplicate. As a control, rat proximal tubule cells were grown to confluence on the similar type I collagen-coated flexible-bottomed plates but were not subjected to repetitive cycles of stretch/relaxation, although these cells were cultivated under identical conditions and durations to the cyclic/stretch counterparts.

Angiotensinogen and AT1 antisense oligonucleotide delivery to rat proximal tubular cells. For angiotensinogen, transfection was performed using phosphorothioate angiotensinogen antisense oligonucleotide synthesized on an automated solid phase synthesizer using standard phosphoramidite chemistry. The sequence of antisense oligonucleotide used was 30-mer human preangiotensinogen starting from position 934 to 963 (5'-CAC-TGA-GGT-GCT-GTT-CTC-CCA-GAA-CTC-3') and a control scrambled sequence (20). The sequence of this region is homologous in humans and rats (20). The AT1 antisense oligonucleotide was obtained from Chemicon in the form of a phosphorothioate DNA sodium salt. The control consisted of a CG-matched randomized-sequence phosphorothioate oligonucleotide.

A cationic transfection method was used to introduce the antisense and scrambled oligonucleotides into rat proximal tubular cells plated on type I collagen-coated flexible plates as described above. Two micrograms of DNA were added to 100 µl of Opti-MEM serum-free media (GIBCO, Life Technologies, Grand Island, NY). This solution was then added to either 16 µl or 8 µl of Lipofectamine (GIBCO, Life Technologies, Grand Island, NY) for angiotensinogen or AT1, respectively, in 100 µl of Opti-MEM and incubated for 45 min at room temperature. After DNA-liposome complexes were formed, the subconfluent cells were washed and 0.8 ml of Opti-MEM added to each of the culture wells before the addition of the DNA solution. Cells were incubated with the antisense and sense scrambled oligonucleotides for 24 h before the addition of growth medium and initiation of cell stretch. Two six-well plates constituted an n = 1 for the mechanical cell stretch experiments as described above. Oligonucleotide uptake, efficiency, the desired DNA:Lipofectamine ratio, and incubation times for transfection, were determined using a fluorescein isothiocyanate (FITC)-labeled oligonucleotide at the 5'-amine to estimate cellular fluorescein uptake (Fluo-Reporter Oligonucleotide Phosphate labeling kit; Molecular Probes, Eugene, OR). RNA and protein were extracted from the cells for Northern and Western blot analysis, respectively.

Statistics. All values are expressed as means ± SD. When multiple groups were compared, one-way ANOVA was performed initially to confirm the presence of significant differences. Then individual comparisons were performed with Student’s t-test, and multiple pairwise comparisons were according to the method of Bonferroni as appropriate. Statistically significant differences between groups were defined as P < 0.05.

RESULTS

Expression of components of the RAS in UUO. To discern the role of the RAS in proximal tubular osteopontin expression in experimental hydronephrosis, both the obstructed and CUK specimens from UUO rats were examined for ACE and angiotensinogen protein and mRNA expression, respectively. On Western blot analysis there was a significant increase of ACE protein in the renal cortex of obstructed kidneys in comparison to the CUK specimens (0.78 ± 0.10 vs. 0.25 ± 0.10, P < 0.002). Figure 1 is a representative Western blot analysis demonstrating an increased 62-kDa protein corresponding to ACE, in the renal cortex of 96 h obstructed kidneys in comparison to the CUK specimens from the same animals. This ACE antibody was previously tested on rat lung and testis to ensure specificity to ACE protein (not shown).

![Fig. 1. Western blot analysis of angiotensin converting enzyme (ACE) protein (62 kDa) in 96 h obstructed and contralateral unobstructed kidney (CUK) specimens from unilateral ureteral obstruction (UUO) rats (10 µg protein/lane). Lanes 1–3: specimens of renal cortex from 96-h CUK specimens. Lanes 4–6: corresponding obstructed kidneys from the same animals. There was a significant 3.1-fold increase in ACE protein expression in renal cortex from 96-h obstructed kidneys compared with unobstructed control kidneys (0.78 ± 0.10 vs. 0.25 ± 0.10, P < 0.002). A rabbit anti-goat ACE monoclonal antibody (1:12,000 dilution; Dr. J. Ingelfinger, Boston, MA) was used to demonstrate the 62-kDa ACE protein.](image_url)
In situ hybridization using a rat angiotensinogen riboprobe revealed localization of angiotensinogen mRNA to the rat proximal tubule epithelium of only the obstructed kidney, as early as 6 h post-UUO in comparison to the CUK from the same animals. Figure 2 demonstrates angiotensinogen expressed in the proximal tubules of the renal cortex as evidenced by localization of the 35S-labeled antisense probe (Fig. 2, A and B). Renal cortical arterioles also demonstrated angiotensinogen expression, whereas glomerular tufts demonstrated relatively low angiotensinogen expression. A marked decrease in the number of silver granules representing mRNA transcription was observed in the corresponding CUK section (Fig. 2D). The specificity of the antisense probe hybridization signal was demonstrated by the lack of label in sections hybridized to the sense probe (Fig. 2C) or following predigestion of tissue sections with RNase.

Cyclic mechanical cell stretch. To gain mechanistic insight into osteopontin's increased synthesis in the obstructed kidney cortex, we evaluated the response of cultured proximal tubular epithelial cells to controlled cyclic mechanical stretch in specific regards to increased renin expression. We have previously reported that rat proximal tubule cells subjected to cyclic mechanical cell stretch for 1 h exhibited a 2.1-fold increment in osteopontin mRNA levels, which was decreased to control values following pretreatment with losartan (8). The present study demonstrated that proximal tubules subjected to mechanical cell stretch for 1 h exhibited a significant 3.0-fold increase in renin protein expression compared with unstretched cells at the same time point (0.13 ± 0.04 vs. 0.04 ± 0.01, P < 0.007).

Fig. 2. Photomicrograph of in situ hybridization for angiotensinogen, which was linearized with EcoRI and HindIII to generate antisense and sense 35S-labeled riboprobes with T7 and SP6 polymerase, respectively. A–C: representative cortical field from an obstructed rat kidney 6 h after ureteral ligation. In A and B, the antisense riboprobe was utilized, and as is evident, there is marked angiotensinogen mRNA localization (large arrowheads) throughout the entire cytoplasm of the proximal tubule epithelium. In B, angiotensinogen is localized to the cortical arterioles (small arrowheads), whereas glomeruli remain relatively free of angiotensinogen expression. C: use of sense riboprobe and exhibits only faint background. D: representative cortical field from the CUK specimen of the same rat as shown in A–C using the antisense riboprobe. As is shown, there is only faint angiotensinogen mRNA localization in the unobstructed kidney and faint background in the sense control specimens.

Fig. 3. Western blot analysis of renin protein (60- and 61-kDa doublet) from cultured rat proximal tubular cells exposed to cyclic mechanical stretch using a Flexcell strain unit for 1 h. Lane 1: unstretched control rat proximal tubular cells grown on type 1 collagen-coated plates. Lane 2: protein from proximal tubular cells following 1 h of cyclic mechanical stretch. A 3.0-fold elevated renin protein expression was observed in proximal tubular cells exposed to mechanical stretch compared with unstretched cells at the same time point (0.13 ± 0.04 vs. 0.04 ± 0.01, P < 0.007). A rabbit anti-rat IgG antibody (1: 4,000 dilution; kindly provided by Dr. T. Inagami, Vanderbilt Univ. School of Med., Nashville, TN) was used to demonstrate the 60- and 61-kDa protein.
Fig. 3). This data provides evidence that mechanical cell stretch can induce upregulation of components of a local, independent renin-angiotensin cascade within the proximal tubular epithelial cell.

Oligonucleotide delivery to proximal tubule epithelial cells. To investigate whether stretch-induced osteopontin upregulation is dependent on the renin-angiotensin cascade, the impediment of angiotensinogen and AT₁ mRNA translation was performed by antisense oligonucleotide delivery to cultured proximal tubular epithelial cells. Figure 4 shows fluorescent micrographs of FITC-labeled angiotensinogen and AT₁ antisense oligonucleotides in proximal tubular epithelial cells. Rapid uptake of the oligonucleotides was observed after incubation of the liposome-DNA complexes for 12 h. From 12–24 h strong fluorescent signal was observed in the nucleus and perinuclear organelles (Fig. 4). Following incubation, the cells were cultured for 24 h in serum-containing media before initiation of mechanical cell stretch.

Effect of the blockade of angiotensinogen and AT₁ mRNA translation on osteopontin expression. By Northern blot analysis, rat proximal tubular cells exhibited a 2.0-fold increase in osteopontin mRNA expression following 1 h of cyclic mechanical cell stretch, compared with unstretched cells grown on the same substratum for an identical duration (0.10 ± 0.00 vs. 0.05 ± 0.00, P < 0.001), as we have previously described (8). As shown in Fig. 5, osteopontin mRNA expression was significantly decreased 2.4- and 1.6-fold in stretched proximal tubular epithelial cells following transfection with antisense oligonucleotide for either angiotensinogen (0.042 ± 0.02 vs. 0.10 ± 0.00, P < 0.004) or AT₁ (0.10 ± 0.00 vs. 0.16 ± 0.00, P < 0.001), respectively, compared with stretched cells with/without prior transfection with the control scrambled oligonucleotide.

On Western blot analysis, proximal tubular epithelial cells transfected with angiotensinogen antisense oligonucleotide exposed to cyclic mechanical cell stretch exhibited osteopontin protein levels comparable to unstretched cells at the same time point. These stretched proximal tubular epithelial cells transfected with antisense oligonucleotide demonstrated a significant decrease in osteopontin protein expression compared with cells stretched for an hour (0.16 ± 0.08 vs. 0.42 ± 0.11, P < 0.01; Fig. 6). Figure 7 is a representative Western blot demonstrating osteopontin and AT₁ protein in cultured rat proximal tubular epithelial cells following the introduction of the AT₁ antisense or scrambled oligonucleotide. Proximal tubular epithelial cells transfected with the AT₁ antisense oligonucleotide had a significant 2.2-fold decrease in osteopontin (0.68 ± 0.10 vs. 0.31 ± 0.19, P < 0.01) and 4.0-fold less AT₁ (0.22 ± 0.02 vs. 0.053 ± 0.01; P < 0.001) protein expression compared with cultured stretched cells at the same time point. These data suggest that the
Our studies document increased cortical ACE expression and localization of angiotensinogen to proximal tubules in the renal cortex of the obstructed kidney as early as 6 h after UUO. A significantly increased renin expression was reported in cultured proximal tubule cells exposed to mechanical cell stretch. Angiotensino-

gen and AT₁ oligonucleotide antisense oligonucleotide delivery to cultured rat proximal tubule cells provides direct evidence that ANG II may mediate, in part, the activation of osteopontin following cell stretch. This data underscores the importance of the RAS in regulating proximal tubule osteopontin expression following the mechanical injury as a result of urinary tract obstruction.

Many recent clinical and experimental studies have clearly demonstrated that one of the initial events taking place in the process of progressive renal injury is monocyte infiltration of the glomerular and tubulo-interstitial compartments. Osteopontin is a macrophage chemotactic and adhesion molecule that may be involved in the macrophage infiltration of the renal interstitium. A number of inflammatory molecules including ANG II, transforming growth factor (TGF)-β, epidermal growth factor (EGF), and interleukin (IL)-1,

DISCUSSION

Our studies document increased cortical ACE expression and localization of angiotensinogen to proximal tubules in the renal cortex of the obstructed kidney as early as 6 h after UUO. A significantly increased renin expression was reported in cultured proximal tubule cells exposed to mechanical cell stretch. Angiotensinogen and AT₁ oligonucleotide antisense oligonucleotide were effectively taken up by the proximal tubular cells resulting in a significant decrease (0.68 ± 0.10 vs. 0.31 ± 0.19, P < 0.01) in osteopontin protein expression in cultured stretched cells transfected with AT₁ antisense oligonucleotide, compared with stretched cells at the same time point.

Fig. 5. Northern blot analysis of total RNA obtained from rat proximal tubular epithelial cells (5 µg/lane) probed for osteopontin (OP) (A and B; 1.6 kb) and GAPDH (C; 1.3 kb) in either cells transfected with AT₁ (A) or angiotensinogen (B) oligonucleotides. Lane 1: unstretched control rat proximal tubular epithelial cell RNA grown to confluence in DMEM/5% FCS on type I collagen-coated flexible-bottomed plates for 1 h. Lane 2: RNA from cells stretched for 1 h. Lane 3: rat proximal tubular epithelial cells that have been transfected with an antisense oligonucleotide prior to mechanical cell stretch, compared with stretched cells, which was transfected with the control scrambled oligonucleotide (lane 4). D: intact 28S and 18S ribosomal RNA bands. Osteopontin was statistically decreased in proximal tubular cells following cell stretch and transfection with either angiotensinogen (0.042 ± 0.02 vs. 0.10 ± 0.00, P < 0.004) or AT₁ (0.10 ± 0.00 vs. 0.16 ± 0.00, P < 0.001) antisense oligonucleotide, compared with stretched cells or cells transfected with the corresponding scrambled control oligonucleotide.

Fig. 6. Western blot analysis of osteopontin protein (OP, 66 kDa) in cultured rat proximal tubular epithelial cells following the introduction of an antisense oligonucleotide for angiotensinogen. Lane 1: corresponds to unstretched control rat proximal tubular cells grown on type 1 collagen-coated plates, whereas lane 2 corresponds to proximal tubular epithelial cells exposed to cyclic mechanical cell stretch for 1 h. Lane 3: corresponds to proximal tubular epithelial cells transfected with angiotensinogen antisense oligonucleotide prior to 1 h of cyclic mechanical cell stretch. There was a statistically significant decrease in osteopontin protein expression in cultured stretched cells transfected with angiotensinogen antisense oligonucleotide compared with stretched cells at the same time point (0.16 ± 0.08 vs. 0.42 ± 0.11, P < 0.01). A monoclonal anti-rat osteopontin antibody (1:4,000 dilution) was used to demonstrate the 66-kDa osteopontin protein.

Fig. 7. Western blot analysis of osteopontin (OP, 66 kDa, top) and AT₁ (49 kDa, bottom) protein in cultured rat proximal tubular epithelial cells following a cationic transfection method used to introduce the antisense oligonucleotide for AT₁. Lane 1: unstretched control rat proximal tubular cells. Lane 2: proximal tubular epithelial cells exposed to cyclic mechanical cell stretch for 1 h. Lane 3: proximal tubular epithelial cells transfected with an AT₁ antisense oligonucleotide prior to cyclic mechanical cell stretch. Lane 4: cells transfected with the corresponding scrambled antisense oligonucleotide. There was a statistically significant 2.2- and 4.0-fold decrease in osteopontin (0.68 ± 0.10 vs. 0.31 ± 0.19, P < 0.01) and AT₁ (0.22 ± 0.02 vs. 0.05 ± 0.01; P < 0.001) protein expression, respectively, in cultured stretched cells transfected with AT₁ antisense oligonucleotide compared with stretched cells at the same time point.
can enhance osteopontin transcription in tubular epithelium (5, 11, 23, 26, 41). In a model of focal tubulointerstitial injury with ANG II infusion, Giachelli et al. (11) noted an elevated expression of osteopontin occurring early followed by monocyte/macrophage influx. The macrophages localized almost exclusively to sites of tubular osteopontin, suggesting that elevated expression of osteopontin by kidney tubules may be an important chemoattractant mechanism in directing the inflammatory response (11). Pichler et al. (26) demonstrated that the degree of osteopontin expression in chronic cyclosporin nephropathy is associated with osteopontin expression macrophage accumulation and with TGF-β expression. More recently, Yu et al. (41) demonstrated that IL-1 can directly upregulate osteopontin expression in glomerular crescentic formation and tubulointerstitial fibrosis in a rat anti-glomerular basement membrane (anti-GBM) glomerulonephritis. Our laboratory has previously noted elevated osteopontin expression predominantly in proximal tubular epithelium as early as 12 h after unilateral ureteral ligation (7). Studies using isolated proximal tubules provided evidence that ANG II may regulate osteopontin expression due to a mechanical disturbance in the proximal tubules occurring shortly after ureteral ligation as a result of the transient hydrodynamic perturbations involving increased proximal tubular pressure and membrane stretch (8).

The vasoactive effects of intrarenal ANG II in altered renal hemodynamics have been demonstrated in a variety of models. Following UUO, there is a progressive increase in renal blood flow (RBF) in the obstructed kidney which peaks at ~2 h after ureteral obstruction, followed by progressive vasoconstriction of glomerular arterioles, principally due to ANG II, leading to a marked decline in glomerular filtration rate (GFR; 17). Studies by Pimental et al. (27, 28) and El-Dahr et al. (10) have demonstrated that acute UUO results in profound changes in the expression of genes that encode for components of the RAS. It is accepted that an intrarenal RAS exists that modulates renal function by the paracrine and autocrine effects of ANG II synthesized locally (24). ANG II is synthesized by the proximal tubule cell (16, 31). Ingelfinger et al. (15) demonstrated that the expression of angiotensinogen mRNA by Northern analysis in the renal cortex and medulla is regulated by dietary salt intake. The present study used in situ hybridization to localize angiotensinogen to the cytoplasm of the proximal tubule and cortical arterioles as early as 6 h following ureteral obstruction, compared with CUK specimens from the same animals. Localization and regulation of angiotensinogen in the proximal tubule suggest that angiotensinogen could be released into the lumen or interstitium of the obstructed kidney cortex, providing a source of substrate for the intrarenal generation of ANG I and II. Also, ACE is expressed in great abundance on the proximal tubular brush border, with smaller amounts in glomeruli and arterioles (2, 33). The present observations that ACE protein is statistically elevated in the obstructed kidney suggest that proximal tubular brush border ACE may facilitate epithelial generation of ANG II, leading to osteopontin synthesis and secretion by these cells.

The present study used oligonucleotide antisense transfection targeted at both the initial and terminal portions of the RAS, namely angiotensinogen and AT₁, respectively, to provide direct evidence for ANG II-mediated osteopontin expression. Two main ANG II receptors have been cloned, AT₁ and AT₂ (6). All known actions of the RAS are mediated by AT₁, belonging to the superfamily of G protein-coupled receptors it is the predominant receptor subtype found in adult rat kidneys (40). The role of AT₂ receptors and their signaling mechanisms are unclear; however, it has been speculated that the AT₂ receptor may counterbalance the actions of AT₁ (36). The present study used antisense oligonucleotides to hybridize AT₁ or angiotensinogen to lead to translational arrest of the specific protein in cultured rat proximal tubule epithelial cells. Our observations with fluorescence microscopy confirm that the oligonucleotides were taken up by the proximal tubular cells optimally by 24 h of culture. Li et al. (21) used an AT₁ oligonucleotide to determine the cellular uptake in bovine adrenal cells. In dose-response studies of both uptake and receptor inhibition, they noted a close correlation between uptake and effect; however, characteristic of antisense inhibition, there was never a 100% decrease in binding (20). Following mechanical cell stretch, the present study showed a significant decrease, but not complete inhibition, of osteopontin expression following AT₁ and angiotensinogen transfection of proximal tubular cells at both the RNA and protein level. The use of antisense oligonucleotide therapy for the incomplete blockade of gene expression may be important therapeutically, as the attempt of targeting antisense oligonucleotides to specific genes is to inhibit overactive systems rather than inhibit the physiological activity completely.

Cyclic mechanical cell stretch was used to mimic the increased proximal tubular luminal pressure as a result of the early changes following the onset of ureteral obstruction as previously used in our laboratory (8). Several investigators have demonstrated the role that mechanical stretch may play in a variety of disease models and an increasing number of reports focus on ANG II as an initial mediator for stimuli of mechanical stretch. Harris and colleagues (1) have extensively studied the responses of cultured rat glomerular mesangial cells subjected to mechanical cyclic stretch based on the premise that the increased mechanical tension, generated by elevated GFR and glomerular capillary pressure, noted in a variety of models of progressive renal disease, may be a primary cause of perturbed mesangial cell biology. Interestingly, they noted that ANG II augmented many of the mesangial cell derangements induced by mechanical cell stretch (1). Yamazaki et al. (39) demonstrated that ANG II plays an important role in mechanical stress-induced cardiac hypertrophy using studies with candesartan cilexetil, an AT₁ antagonist, in cultured rat cardiac myocytes. Angio-
tensinogen gene expression has been reported to be elevated following mechanical cell stretch of rat cardiomyocytes (34). More recently, Li et al. (20) used angiotensin antisense oligonucleotide transfection to demonstrate that stretch-induced proliferation of cultured smooth muscle cells is mediated by the RAS and a subsequent upregulation of platelet-derived growth factor-β mRNA. Further evidence for a role of the RAS cascade comes from the present finding of increased renin mRNA expression in proximal tubule cells following 1 h of mechanical cell stretch. We have previously shown that mechanical perturbation of proximal tubular cells in this cyclic stretch model downregulated catalase mRNA expression and increased osteopontin mRNA expression, suggesting that mechanical disturbances resulting from increased proximal tubular pressure post-UUO may lead to amplification of the proinflammatory state of ureteral obstruction (30). It has also been postulated that following UUO, there is increased cortical interstitial pressure in the obstructed kidney (32). We have recently observed that renal decapsulation of the obstructed kidney in UUO rats did not alter the production of inflammatory chemokines or elevated expression of the RAS, suggesting that cortical interstitial pressure does not contribute to macrophage infiltration and the development of fibrogenesis in experimental hydropnephrosis (unpublished observations).

In summary, this study provides direct evidence for the role of a local RAS in the secretion of ANG II from cultured proximal tubular cells following cell stretch. Our data indicate that ANG II acts as an initial mediator of stretch-induced osteopontin release from proximal tubular epithelial cells. Perturbation of proximal tubule cells after ureteral ligation may increase ANG II synthesis and ATı stimulation in an autocrine manner leading to osteopontin release. The elucidation of this mechanism provides new insights into the role of proximal tubular cell injury and the resulting chemotactant-facilitated macrophage influx into the tubulointerstitium in experimental hydropnephrosis. Further studies are needed to ascertain the importance of other cellular and molecular events resulting from the initial insult to proximal tubule cells as major codeterminants of renal scarring and fibrosis.

This study was supported by a Research Grants-in-Aid and an Initiator Investigator Grant from the American Heart Association (National and Pennsylvania Affiliate), and by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-DK-53163-01A1. A research supply award was provided by the Section of Surgical Sciences, Department of Surgery. The osteopontin monoclonal antibody in the present study was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Studies, Iowa City, IA 52242, under National Institute of Child Health and Human Development Contract NO1-HD-73263.

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Received 5 August 1999; accepted in final form 9 December 1999.

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