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Antibody to transforming growth factor-β ameliorates tubular apoptosis in unilateral ureteral obstruction

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Antibody to transforming growth factor-β ameliorates tubular apoptosis in unilateral ureteral obstruction.

Background. Unilateral ureteral obstruction (UUO) is characterized by progressive renal atrophy, renal interstitial fibrosis, an increase in renal transforming growth factor-β (TGF-β), and renal tubular apoptosis. The present study was undertaken to determine the effect of a monoclonal antibody to TGF-β (1D11) in UUO.

Methods. Mechanical stretch was applied to tubular epithelial cells (NRK-52E) by a computer-assisted system. Three doses of 1D11 (either 0.5, 2, or 4 mg/rat) were administered to rats one day prior to UUO and every two days thereafter, and kidneys were harvested at day 13. Fibrosis was assessed by measuring tissue hydroxyproline and mRNA for collagen and fibronectin. Apoptosis was assessed with the terminal deoxy transferase uridine triphosphate nick end-labeling assay. TGF-β levels were determined by bioassay. Western blot and immunostaining were used to identify proliferating cell nuclear antigen (PCNA), p53, bcl-2, and inducible nitric oxide synthase (iNOS).

Results. Stretch significantly induced apoptosis in NRK-52E cells, which was accompanied by an increased release of TGF-β; 1D11 (10 μg/mL) totally inhibited stretch-induced apoptosis. Control obstructed kidney contained 20-fold higher TGF-β as compared with its unobstructed kidney; 1D11 neutralized tissue TGF-β of the obstructed kidney. Control obstructed kidney exhibited significantly more fibrosis and tubular apoptosis than its unobstructed counterpart, which was blunted by 1D11. In contrast, 1D11 significantly increased tubular proliferation. p53 immunostaining was localized to renal tubular nuclei of control obstructed kidney and was diminished by 1D11. In contrast, bcl-2 was up-regulated in the 1D11-treated obstructed kidney. Total NOS activity and iNOS activity of the obstructed kidney were increased by 1D11 treatment.

Conclusion. The present study strongly suggests that an antibody to TGF-β is a promising agent to prevent renal tubular fibrosis and apoptosis in UUO.

Key words: renal tubular obstruction, cell death, fibrosis, progressive renal atrophy, monoclonal antibody.

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In unilateral ureteral obstruction (UUO), the obstructed kidney is characterized by interstitial fibrosis and an increase in transforming growth factor-beta (TGF-β). TGF-β is known to be profibrotic in several models of fibrosis, and the use of anti–TGF-β antibody can ameliorate fibrosis in glomerulonephritis [1]. Approximately 80% of the renal volume is occupied by renal tubules [2], and a positive correlation was found between the histologic presence of tubular atrophy and a decreased glomerular filtration rate in glomerulonephritis [3]. In UUO, it has been suggested that renal tubular apoptosis is related to renal tissue loss and dysfunction [4]. TGF-β has been found to stimulate apoptosis in human gastric cancer cells [5], uterine epithelial cells [6], and hepatocytes [7], although it has also been shown to inhibit apoptosis induced by serum deprivation, Fas, or β-amyloid peptide [8–10]. Moreover, TGF-β has been shown to modify specifically p53 expression in hepatocytes [11], although TGF-β–induced apoptosis is operative in a p53-independent manner in myeloid leukemia cells [12]. TGF-β is also shown to decrease levels of bcl-2–related protein in B lymphoma cells [13]. However, the effect of TGF-β on renal tubular apoptosis is not known.

Nitric oxide (NO) is a multifunctional mediator, synthesized by the action of NO synthase (NOS), which has also been implicated in the fibrosis of UUO. It has been shown that increasing NO through dietary arginine decreases fibrosis in UUO [14] and, conversely, that iNOS −/− mice with UUO have increased fibrosis (abstract; Hochberg et al, *J Urol Suppl* 159:131, 1998). NO and inducible NOS (iNOS) are shown to increase in the kidney of TGF-β −/− mice, suggesting that TGF-β is a negative regulator of NO in the kidney [15].

In the present experiments, we examined the role of TGF-β in renal tubular fibrosis and apoptosis by using 1D11, a monoclonal antibody to TGF-β. We examined the effect of stretch and 1D11 on tubular apoptosis, in vitro. In UUO, renal tubules may be stretched because of changes in intrarenal pressure. Therefore, we used 1D11 in vivo to assess its effect on apoptosis in UUO. In addition, since TGF-β is a negative regulator of NO, the effects of the antibody on NO expression in UUO were examined. We utilized 1D11 in rats that were subjected to UUO for 13 days.

METHODS

Materials

NRK-52E cells, a clonal line established from normal rat kidney tubular epithelial cells (CRL1571), were obtained from the American Type Culture Collections (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO BRL (Grand Island, NY, USA).

Cell culture

NRK-52E cells were carried in a monolayer cell culture and maintained in 75 cm^2 culture flask (Corning, Cambridge, MA, USA) with DMEM containing 5% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2/95% O_2 at 37°C.

Applying mechanical stretch to cells

Cells were seeded on six-well plates (25 mm diameter) of flexible silicone or on rigid-bottom plates coated with collagen type I (Flex I and II culture plates, respectively; Flexcell Corp., Mckeesport, PA, USA) and DMEM containing 10% FCS. After five hours, the supernatant was replaced with serum-free medium. NRK-52E cells were subjected to mechanical stretch using a Flexcell Strain Unit FX-2000 (Flexcell Corp.). A microprocessor controlled negative pressure to the flexible bottoms, resulting in reproducible deformation of the silicone rubber and the attached cells, providing a maximal elongation of 20%. All experiments were carried out using alternate cycles of five seconds of stretch and five seconds of relaxation at a rate of 6 cycles per minute at 37°C, 5% CO_2 in a humidified incubator. All treatments were done in triplicate, and all experiments were replicated three to five times. Cells were collected for analysis by flow cytometry. Supernatant was collected for the TGF-β bioassay.

Flow cytometric detection of DNA strand breaks

After various treatments, cells were removed from the bottom with trypsinization. After washing cells with phosphate-buffered saline (PBS), cells were fixed and permeabilized with 70% ethanol at 4°C for one hour [16]. Fragmented DNA was then detected by the terminal deoxy transferase uridine triphosphate nick end-labeling (TUNEL) assay (Intergen Co., Purchase, NY, USA). For flow cytometry, FITC-labeled antidigoxigenin Ab was applied. Then cells were analyzed on the flow cytometer after washing. During analysis, cells were gated on forward versus side scatter, and apoptosis-associated fluorescence was measured using a log scale.

In vivo unilatereral ureteral obstruction model

Sprague-Dawley rats ($N = 10$ per group) underwent left unilateral ureteral ligation with 4-0 silk suture through an abdominal midline incision under sterile conditions. Animals were anesthetized intraperitoneally with 0.35 cc pentobarbital (6.5 mg/mL). Animals were housed in a group of three to five and were fed standard chow and water ad libitum. Monoclonal antibody to TGF-β (1D11) was generously provided by the Genzyme Corp. (Cambridge, MA, USA). This antibody has been previously tested, and it was shown that doses as high as 2.5 mg per mouse, administered daily for three weeks, showed no histopathology in any organs of the body (unpublished observations, B. Pratt, S. Ledbetter, Genzyme Corp.). Three doses of 1D11 (0.5, 2, and 4 mg/rat) were administered by intraperitoneal injection. An antiverotoxin antibody (2 mg/rat) was administered as an isotype control. 1D11 was injected to each animal at days −1, 0, 2, 4, 6, 8, 10, and 12, and animals were euthanized at day 13. After the administration of intraperitoneal pentobarbital, both obstructed and unobstructed kidneys were harvested. Animal treatment adhered to approved institutional guidelines.

TGF-β bioassay

Transforming growth factor-β in cell supernatants or in tissue was assayed using a TGF-β bioassay. Tissue TGF-β was extracted as previously described [17]. Mink lung epithelial cells (MLECs), which were transfected with a plasminogen activator inhibitor-1 (PAI-1) promoter driving expression of luciferase in a TGF-β–responsive manner, were used to analyze TGF-β activity [18]. These cells were a generous gift from Dr. Daniel Rifkin. Transfected MLEC cells (1.6×10^4 per well) were plated in 96-multiwell plate, and they were allowed to attach for three hours at 37°C in a humidified atmosphere of 5% CO_2/95% O_2. After three hours, the medium was replaced with the test sample (either tissue extract or cell supernatant) and were incubated overnight at 37°C. For the bioassay, the TGF-β in the test sample was activated as follows: Samples were acidified to pH 1.5 with 1N HCl (1 hour at 4°C), followed by equilibration at neutral pH with 1N NaOH. At the end of the incubation, supernatants were discarded, and cells were washed twice with PBS. Cells were extracted with cell lysis buffer (Promega Co., Madison, WI, USA) for 30 minutes at room temperature. Cell extracts were

added to 100 μL of luciferase reagent (Promega). Luciferase activity was measured in a luminometer. A TGF-β1 standard curve was carried out in the range of 0.01 to 2.0 ng/mL. All assays were performed in triplicate. Results are normalized to cell number and are expressed as the active form of TGF-β in pg/mg tissue.

Assessment of tubular atrophy and interstitial fibrosis

Slides were examined by a board-certified pathologist (R.A.S.). Tubular atrophy was assessed by the presence of luminal dilation and flattened tubular epithelial cells. The general presence of interstitial fibrosis was assessed in slides stained with Masson's trichrome, when they were examined for tubular atrophy. In addition, interstitial fibrosis was assessed by measurements of interstitial volume, using a point-counting method as previously described [17]. Changes in the mRNA for collagen III and fibronectin were assessed by RNAse protection assays.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction was done on total cellular RNA using synthesized oligonucleotide primers that are complementary to the fibronectin cDNA sequence [19] for EDA domain of fibronectin and collagen type III cDNA sequence [20].

Preparation of riboprobe. Linearized cDNA was transcribed in vitro using the Maxiscript kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. T7 polymerase and p^{32}CTP (3000 Ci/mmol; DuPont-NEN, Boston, MA, USA) were included in the reaction mixture to generate P^{32}-labeled riboprobe. The reaction mixture was incubated for 60 minutes at 37°C, and then the DNA template was removed by digestion with 0.5 U RNAse-free DNAse. Full-length probes were purified from the transcription reaction by electrophoresis on 6% polyacrylamide/TBE gel (Novax, Novel Experimental Technology, San Diego, CA, USA), followed by autoradiography, excision from the gel band, and passive diffusion into probe elution buffer (Maxiscript kit) overnight at 37°C. The activity of the probe was quantitated by scintillation counting.

Ribonuclease protection assays. Total cellular RNA from whole kidney tissue was obtained using the RNAqueous kit (Ambion). RNAse protection assay was performed using the hybspeed RPA kit (Ambion) according to the manufacturer's instructions. Briefly, radiolabeled antisense RNA probe for fibronectin and collagen type III was combined and hybridized with 10 μg of total cellular RNA from different kidney samples. A probe for 18S RNA (Ambion) was also included in each hybridization mixture to normalize total RNA in individual sample. For negative control, yeast RNA alone was combined with probes. Digestion with RnaseA/RnaseT1 mix was performed to degrade unhybridized RNAs. Hybridized RNA protected from digestion was resolved by electrophoresis and visualized by using fusifilm BAS-1500 phosphoimager.

Bands on the phosphoimager representing collagen type III and fibronectin genes were quantitated using Mac BAS Version 2.4 software. In all samples, the expression of each gene was corrected by dividing probe-specific signal by that obtained for the housekeeping gene.

Measurement of NO synthase activity

After harvesting obstructed and contralateral kidney from rats, samples were stored in a −80°C freezer until assay. Samples were homogenized with a glass-glass Dounce homogenizer (Kontes, Vineland, NJ, USA) in homogenization buffer [1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L egtazic acid (EGTA), and protease inhibitor cocktail, pH 7.4]. The samples were centrifuged for 30 minutes (10,000 × *g*) at 4°C and were resuspended. Samples were then incubated in buffer containing 1 mmol/L nicotinamide adenine dinucleotide phosphate; Sigma, St. Louis, MO, USA), 10 μmol/L tetrahydrobiopterin, 60 nmol/L calmodulin (Calbiochem, CA, USA), 200 μmol/L $CaCl_2$, 20 μmol/L L-arginine, and tritium-labeled arginine (0.1 μCi/sample; DuPont) at 37°C for one hour in the presence and absence of 1 mmol/L EGTA (Ca chelator) or L-NMA (NOS inhibitor) [21]. Reactions were terminated with stop buffer ($C_2H_3O_2Na$, citrulline, EDTA) and then passed over 1 mL of an exchange resin, DOWEX 50WX8-100 (Sigma). Tritiated citrulline was then measured by counting in a scintillation counter. Measured NOS activity was expressed as picomole of citrulline per hour per milligram of protein.

Western blot for iNOS, proliferating cell nuclear antigen, p53, and bcl-2

Samples were homogenized and centrifuged at 3000 r.p.m. for 30 minutes. The supernatant was separated, and the protein concentration of the lysate was determined with the Bradford protein assay (Bio-Rad, Hercules, CA, USA). For Western blot analysis, equal amounts of protein were loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred to a nitrocellulose membrane (Bio-Rad). The nitrocellulose membrane was blocked with PBST (PBS with 0.1% Tween-20) solution containing 5% nonfat milk, and incubated overnight at 4°C with primary antibody against iNOS (polyclonal, 1:5000; Transduction Laboratories, Lexington, KY, USA), proliferating cell nuclear antigen (PCNA; monoclonal, 1:1000; Dako, Carpinteria, CA, USA), p53 (polyclonal, 1:2000; Santa Cruz Technology, Santa Cruz, CA, USA), or bcl-2 (monoclonal, 1:1000; Santa Cruz Technology). Subsequently, a one-hour incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) was carried out. After washing, streptavidin-HRP was applied to the membrane for 30 minutes, followed by washing and applica-

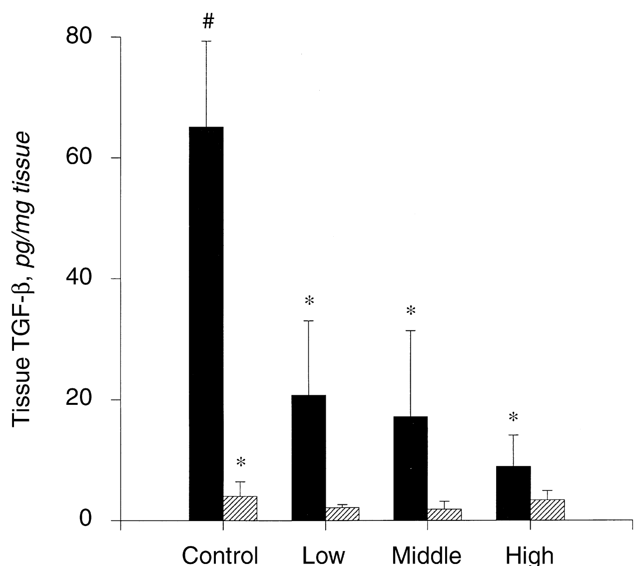


Fig. 1. Tissue transforming growth factor-β (TGF-β) concentration at 13 days of unilateral ureteral obstruction (UUO) after different doses of 1D11. Tissue TGF-β was measured by bioassay as in the **Methods** section. Symbols are: (■) obstructed kidney; (▨) unobstructed kidney. *$P < 0.01$ compared to obstructed kidney in control group. #$P < 0.01$ compared with the unobstructed kidney of the control group.

tion of Opti-4CN (4-chloro-1 naphthol, HRP substrate) until development. The membrane was scanned, and the intensity of each band was quantitated by NIH image (software downloaded from the Internet). iNOS, PCNA, p53, and bcl-2 were detected as a band at 130, 36, 53, and 26 kD, respectively. Intensity was expressed as an arbitrary unit.

Immunohistochemistry to stain iNOS, PCNA, p53, and bcl-2

Wedges of hemisected obstructed and contralateral kidneys were placed in neutral buffered formalin and were embedded in paraffin. Paraffin-embedded sections (5 μm) were cut onto glass slides. Sections were deparaffinized with Hemo-De for 30 minutes, rehydrated in decreasing concentrations of ethanol, and washed three times in PBS for 10 minutes. Endogenous peroxidase was quenched for 30 minutes with 0.3% hydrogen peroxide in methanol. To retrieve antigen, slides were boiled with 10 mmol/L citrate buffer (pH 6.8) for 10 minutes. After washing in filtered water and PBS, a blocking step was included using 5% species-appropriate serum in conjunction with avidin and biotin blocking solutions (Vector Kit; Vector Laboratories, Burlingame, CA, USA) for a total of 30 minutes. Primary antibody to either iNOS (polyclonal, 1:100; Transduction Laboratories), PCNA (monoclonal, 1:50; Dako), p53 (polyclonal, 1:100; Santa Cruz) or bcl-2 (monoclonal, 1:25; Santa Cruz Biotechnology) was then applied at room temperature for one hour. Negative controls were carried out with BSA instead of primary antibody. Biotinylated rabbit anti-mouse secondary antibody (Vector) was incubated for 30 minutes. Sections were then incubated with avidin-biotin peroxidase complex (Vector Laboratories) and developed with diaminobenzedene (DAB). After washing slides, counterstaining was done with 10% hematoxylin for one to two minutes. PCNA-positive cells of renal tubules, interstitium, and glomerulus were separately counted in 10 high-power fields (×400) by two different independent investigators in a blind fashion.

In situ detection of DNA strand breaks

To quantitate nuclei with fragmented DNA, the TUNEL assay (Intergen Co., Purchase, NY, USA) was performed according to the method of Gavrieli, Sherman, and Ben-Sasson [22]. After deparaffinization and quenching endogenous hydrogen peroxide as described previously in this article, sections were treated with 10 μg/mL proteinase-K for 15 minutes. The sections were rinsed with PBS and incubated with deoxynucleotidyl transferase (TdT) with digoxigenin-dNTP for one hour. The reaction was stopped with terminating buffer. Slides were then washed with PBS, and peroxidase-conjugated antidigoxigenin antibody was applied at room temperature for 30 minutes. After three washes in PBS, slides were developed with DAB and counterstained with 10% hematoxylin. Positive cells in renal tubules were quantitated in the same fashion as PCNA staining.

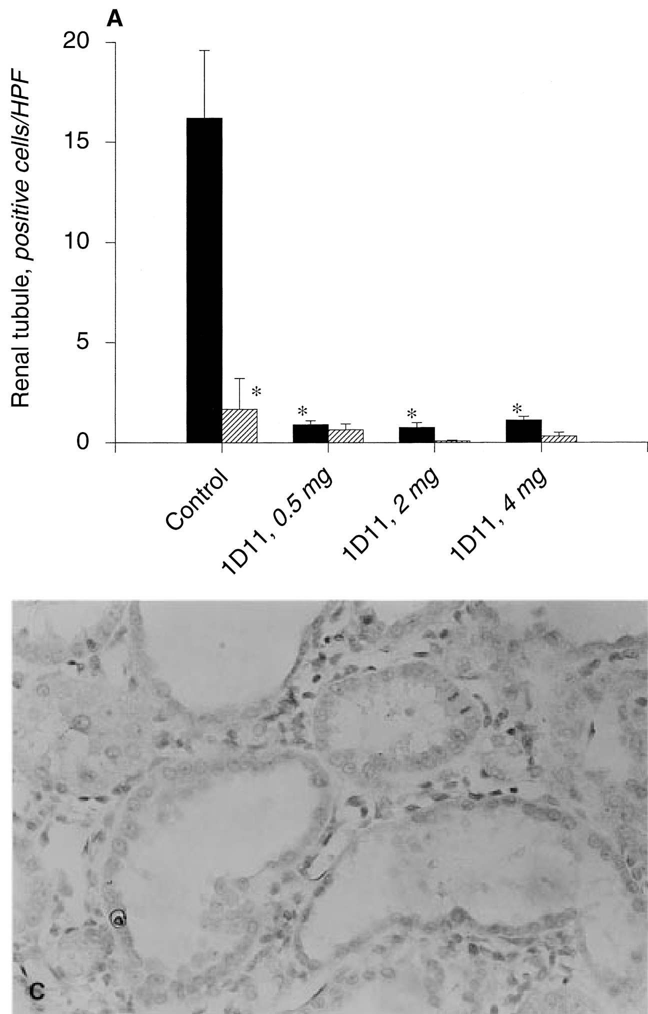
Statistical analysis

All results are expressed as the mean ± SE and were analyzed for significance by the one-way analysis of variance (ANOVA) and multiple comparison tests.

RESULTS

Effects of 1D11 on stretch-induced apoptosis in NRK-52E cells

We sought to determine whether mechanical stretch induces apoptosis in NRK-52E cells. Cells were stretched at 6 cpm and 15% elongation for 48 hours. These parameters were chosen because a significant difference of apoptotic index between unstretched cells and stretched cells was observed. Apoptosis was analyzed by flow cytometry using the TUNEL assay, as described in the **Methods** section. Stretched NRK-52E cells showed significantly higher apoptosis (6.06 ± 0.41%, $P < 0.01$, $N = 4$) as compared with unstretched cells (3.10 ± 0.47%). The addition of 1D11 (10 μg/mL) significantly inhibited apoptosis (2.66 ± 0.27%, $P < 0.01$, $N = 4$) compared with stretch alone, whereas control antibody had no effect. In the absence of stretch, no difference in an apoptosis was observed between the 1D11 treated and untreated cells.



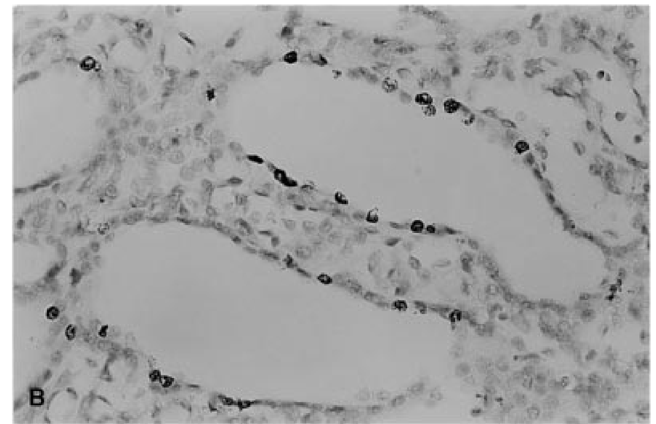


Fig. 2. (*A*) Determination of positive tubular cells in the TUNEL assay. Symbols are (■) obstructed kidney; (▨) unobstructed kidney. $*P < 0.01$ compared with the obstructed kidney of the control group. (*B*) TUNEL assay for the obstructed kidney in the control group (×400). (*C*) TUNEL assay for the obstructed kidney in the 1D11 group (4 mg; ×400).

Effects of 1D11 on TGF-β secretion from NRK-52E cells exposed to mechanical stretch

To determine whether TGF-β is secreted by NRK-52E cells exposed to mechanical stretch, we measured TGF-β concentration in the supernatant from NRK-52E cells with the bioassay. Whereas mechanical stretch significantly induces TGF-β (1.60 ± 0.25 ng/mL, $P < 0.01$, $N = 4$) compared with unstretched cells (1.04 ± 0.13 ng/mL), the addition of 1D11 (10 μg/mL) to stretched cells significantly reduced the TGF-β concentration (0.88 ± 0.09 ng/mL, $P < 0.01$, $N = 4$) compared with cells stretched without 1D11.

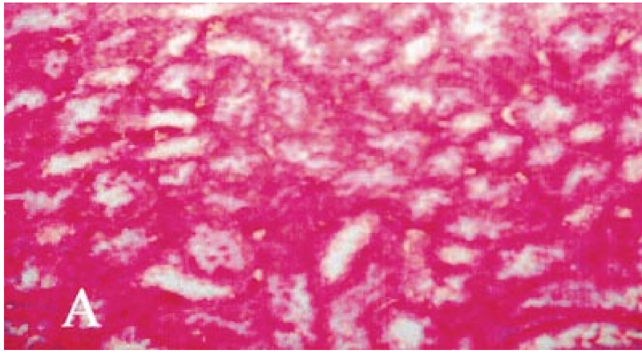
In UUO, renal tubules may be stretched because of changes in intrarenal pressure. Therefore, we were interested in using 1D11 in vivo to assess its effect on apoptosis in UUO. 1D11 was administered as described in the **Methods** section, and both obstructed and contralateral kidneys were harvested on day 13 after UUO.

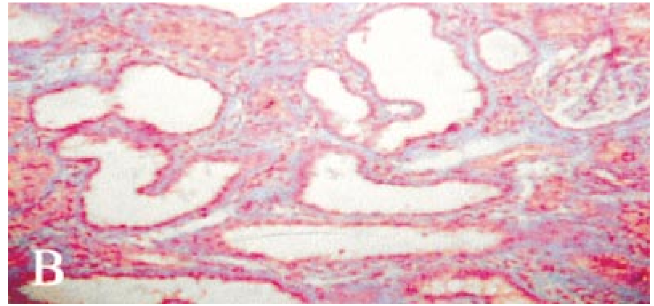
Measurement of kidney tissue TGF-β concentration in rats with UUO

Transforming growth factor-β concentration of kidney tissue was measured with the bioassay. As shown in Figure 1, tissue TGF-β content of the obstructed kidney in the control group (65.1 ± 14.3 pg/mg tissue) was significantly higher than the unobstructed kidney in the control group (4.0 ± 2.4 pg/mg tissue). However, the tissue TGF-β content of the obstructed kidney in each 1D11 group was not significantly different from that of its unobstructed counterpart. Moreover, tissue TGF-β of the obstructed kidney in each 1D11 group (1D11-4 mg, 8.9 ± 5.2 pg/mg tissue) was significantly lower than that of the obstructed kidney in the control group. In the unobstructed kidney, no significant difference was observed between the control group and any of the 1D11 groups.

In situ TUNEL

To determine renal tubular apoptosis, the TUNEL assay was performed in paraffin-embedded sections. The obstructed kidney of the control group showed significantly higher tubular apoptosis [16.2 ± 3.4 nuclei/high power field (HPF); Fig. 2A, B), as compared with the unobstructed kidney of the control group (1.7 ± 1.5 nuclei/HPF). This was readily apparent from apoptotic cell counts (Fig. 2A) and the in situ TUNEL assay (Fig. 2B).





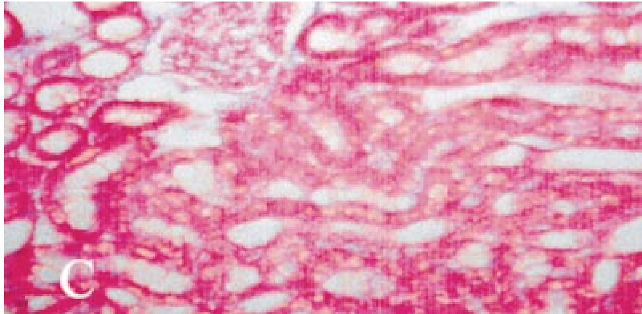
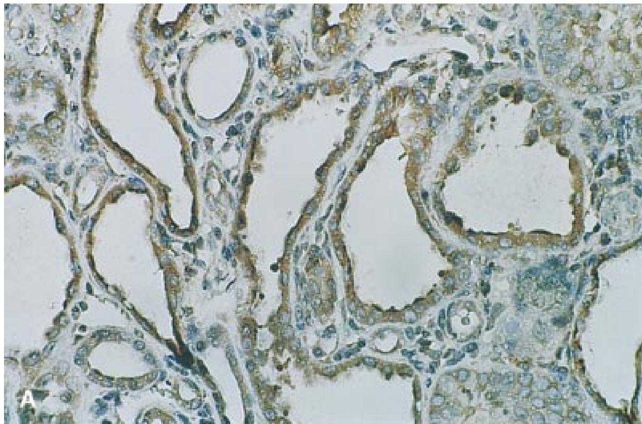


Fig. 3. Representative trichrome staining of kidneys with or without 1D11 treatment. (*A*) Control, unobstructed kidney. (*B*) Control, obstructed kidney. (*C*) Obstructed kidney following 1D11 treatment (4 mg).



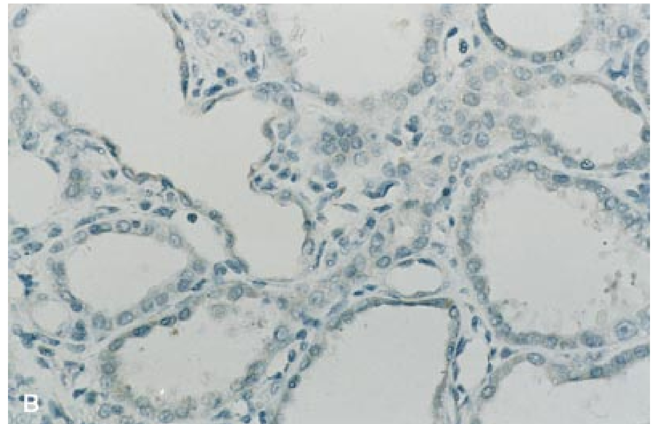
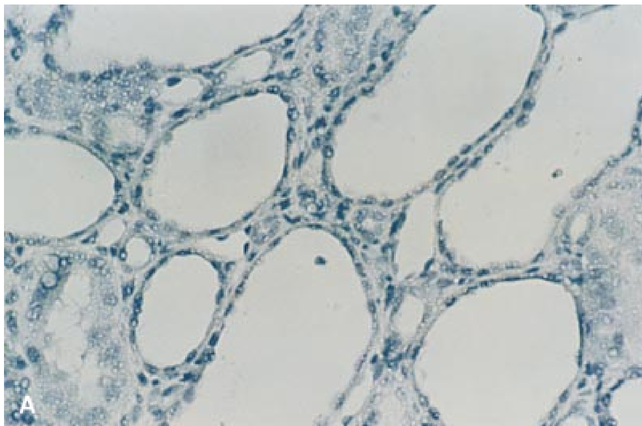


Fig. 6. Immunohistochemistry for p53. (*A*) Obstructed kidney, control group. (*B*) Obstructed kidney with 1D11, 4 mg.



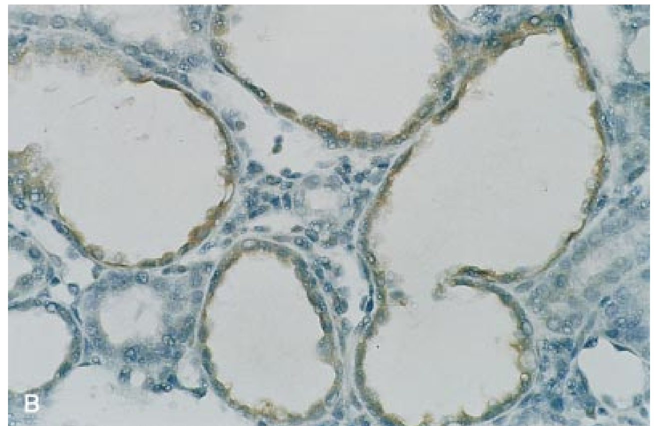


Fig. 7. Immunohistochemistry for bcl-2. (*A*) Obstructed kidney, control group. (*B*) Obstructed kidney with 1D11, 4 mg.

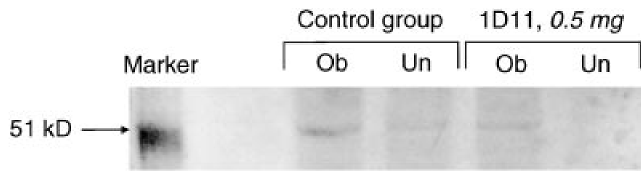


Fig. 6. (*C*) Western blot analysis for p53. Abbreviations are: Ob, obstructed kidney; Un, contralateral unobstructed kidney; Marker, 51 kD.

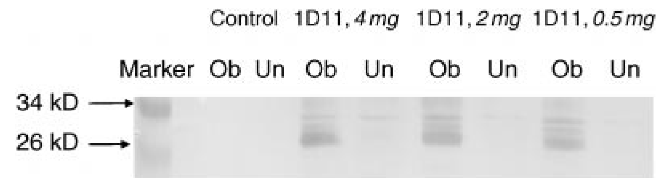


Fig. 7. (*C*) Western blot analysis for bcl-2. Abbreviations are: Ob, obstructed kidney; Un, contralateral unobstructed kidney; Marker, 26 kD.

Table 1. mRNA expression for collagen III and fibronectin with or without 1D11 treatment

	Control		1D11 low dose		1D11 high dose	
	Unobstructed	Obstructed	Unobstructed	Obstructed	Unobstructed	Obstructed
Collagen III	0.03 ± 0.01	0.05 ± 0.01[a]	0.06 ± 0.01	0.06 ± 0.02[a]	0.05 ± 0.01	0.04 ± 0.01
Fibronectin	0.12 ± 0.02	0.16 ± 0.02[a]	0.07 ± 0.01	0.07 ± 0.01	0.12 ± 0.01	0.10 ± 0.01

[a] $P < 0.05$, as compared to control unobstructed kidney

Following 1D11 treatment, renal tubular apoptosis was almost completely eliminated. The obstructed kidney of all 1D11 groups showed significantly lower tubular apoptosis (1D11-4 mg: 1.1 ± 0.2 nuclei/HPF; Fig. 2A, C) than that of the control group. In all 1D11 groups, there was no significant difference between the obstructed and the unobstructed kidney. There was no significant difference in the unobstructed kidneys between 1D11 and the control group.

Detection of tubular atrophy and interstitial fibrosis

Tubular atrophy was determined by examination of Masson's trichrome slides, as described in the **Methods** section. Figure 3 contains sections from either control kidneys (contralateral and obstructed) or following treatment with 1D11 (4 mg/rat). In the control contralateral kidney (Fig. 3A), it can be seen that the tubular lumens are not dilated, and the epithelial cells are cuboidal in shape. The interstitium is not apparent, and the tubules are back to back. The control obstructed kidney (Fig. 3B) exhibits significant blue staining, signifying interstitial fibrosis and resulting in separation of the tubules from one another. Furthermore, note the significant luminal dilation and epithelial flattening. In contrast, the kidney treated with a high dose of 1D11 (Fig. 3C) exhibits very mild epithelial cell flattening and only minimal interstitial fibrosis.

The change in interstitial volume was assessed by a point-counting technique. In the control cortex, interstitial volume was increased from 6.5 ± 0.5% to 26.7 ± 3.9%. In the presence of high-dose 1D11, cortical interstitial volume in obstructed kidneys was only 12.1 ± 1.4%. In the medulla of the obstructed kidney, the increase in interstitial volume was decreased by 46% at the highest dose of 1D11. We also assessed the expression of mRNA for collagen III and fibronectin in control and 1D11-treated kidneys. The results of the RNAse protection assay are shown in Table 1. As can be seen, expression of mRNA for both collagen III and fibronectin was significantly increased in control obstructed kidneys; this increase was abolished with 1D11 treatment.

Detection of PCNA expression

To determine the effect of TGF-β on renal cell proliferation, immunostaining for PCNA was carried out. Both renal tubules and interstitial cells showed positive PCNA staining (Fig. 4A, B). To determine whether tubules, interstitium, or glomerulus are affected by 1D11, positive cells were counted in each compartment. In both the control group and the 1D11 group, renal tubules in the obstructed kidneys show significantly more proliferation than in the unobstructed kidney (Fig. 4C). Renal interstitial proliferation in the obstructed kidney was significantly higher than in the unobstructed kidney (Fig. 4D). Proliferation of the glomeruli showed no difference between the obstructed and the unobstructed kidney in all groups (data not shown). Proliferation of renal tubules in both the obstructed and the unobstructed kidney treated with 1D11 was significantly higher than in each counterpart without 1D11 treatments (Fig. 4C). Proliferation of neither interstitium nor glomerulus in both obstructed and contralateral kidneys was affected by treatment with 1D11.

To determine whether total PCNA expression is increased in UUO, Western blot was performed for PCNA (Fig. 5). In the control group, PCNA expression of the obstructed kidney was significantly higher than the unobstructed kidney (Table 2). The obstructed kidney of the 1D11 group showed significantly higher PCNA expression than unobstructed kidney of the 1D11 group. In

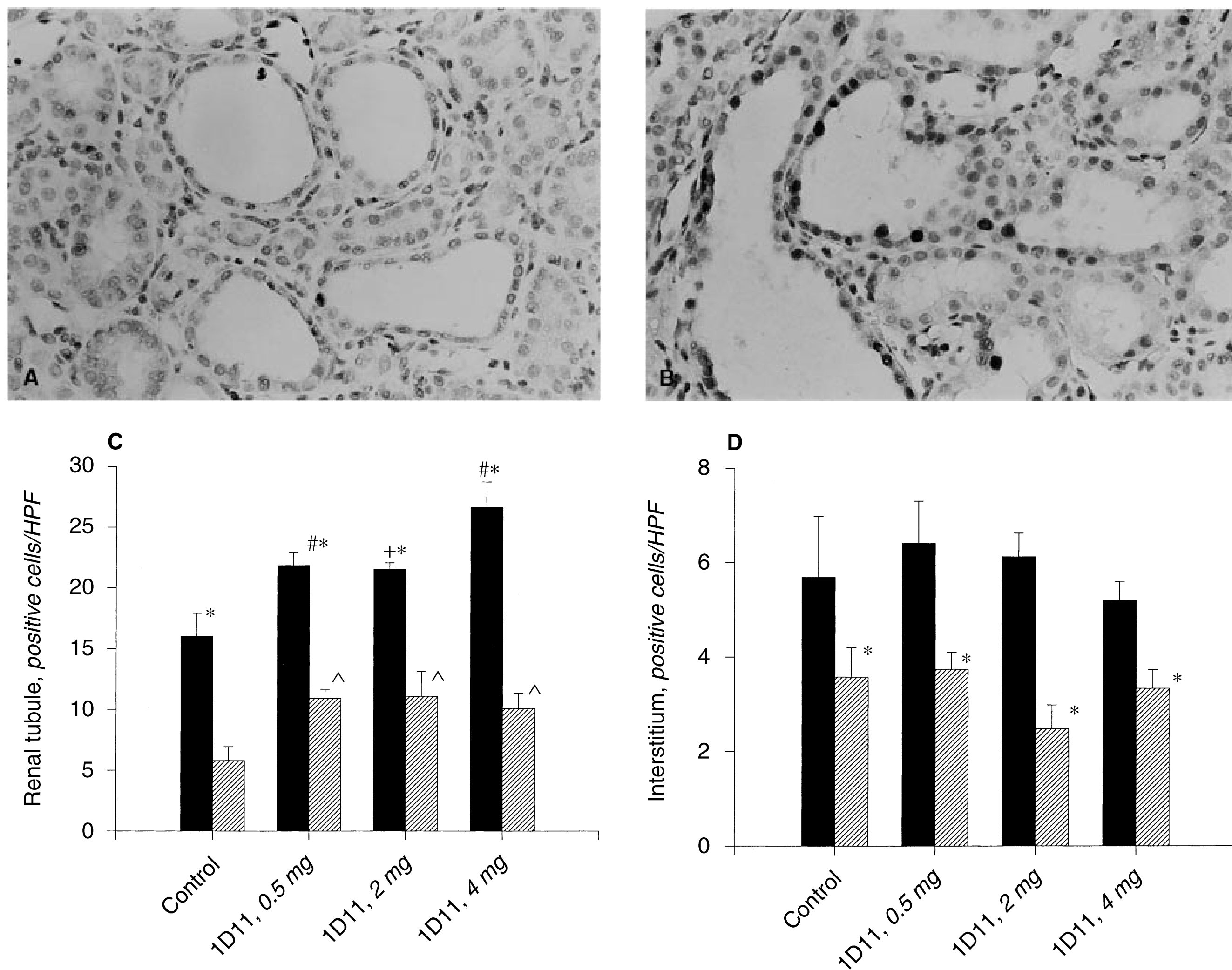


Fig. 4. Proliferating cell nuclear antigen (PCNA) expression in kidneys. (*A*) Immunostaining for PCNA in the obstructed kidney of the control group (×400). (*B*) Immunostaining for PCNA in the obstructed kidney with 1D11 (4 mg; ×400). (*C*) Determination of positive renal tubular cells in PCNA staining. Symbols are: (■) obstructed kidney; (▨) unobstructed kidney. $*P < 0.01$ compared with the unobstructed counterpart; $\#P < 0.01$ compared with the obstructed kidney of the control group; $+P < 0.05$ compared with the obstructed kidney of the control group; $\wedge P < 0.05$ compared with the unobstructed kidney of the control group. (*D*) Determination of positive renal interstitial cells in PCNA staining. $*P < 0.01$ compared with the obstructed counterpart.

addition, the obstructed kidney of the 1D11 group had higher PCNA expression than that of the control group.

Detection of p53

To determine the localization of p53 in the obstructed kidney, immunohistochemistry was carried out. Immunostaining for p53 showed positive staining only in renal tubules in the obstructed kidneys, while the unobstructed kidney expressed no p53 (data not shown). p53 staining was prominent in dilated renal tubules in the control obstructed kidney (Fig. 6A). Treatment with 1D11 markedly decreased staining for p53 in the obstructed kidney (Fig. 6B).

Western blot for p53 (Fig. 6C) showed that the obstructed kidney in either the control group or the 1D11 group demonstrated significantly more p53 expression than their unobstructed counterparts, and that p53 expression of the obstructed kidney in the control group was significantly higher than that of the 1D11 group (Table 2).

Detection of bcl-2

Immunohistochemistry was undertaken to determine the localization of bcl-2 expression. Neither obstructed (Fig. 7A) nor contralateral kidneys (data not shown) of the control group showed positive staining. In contrast, the obstructed kidney in the 1D11 group showed bcl-2 expression; only renal tubular epithelial cells stained positively (Fig. 7B).

Western blot for bcl-2 (Fig. 7C) demonstrated that

there was very little detectable expression of bcl-2 in any kidneys from the control group (Table 2) and that the obstructed kidneys of all 1D11 groups showed significantly higher bcl-2 expression than their counterparts.

Measurement of NOS activity in kidney tissue by citrulline assay

To determine NOS activity in the kidney, we performed an enzymatic assay in which [^{3}H] arginine is converted to [^{3}H] citrulline. With this assay, we were able to determine both total NOS activity and that portion which represents the activity of the iNOS (iNOS activity is Ca^{++} independent and is detected when the calcium chelator EGTA is present). As shown in Figure 8, the obstructed kidney in the control group showed a significant decrease in both total NOS activity and iNOS activity (8.7 ± 0.7 and 0.5 ± 0.5 ng/h/mg protein, respectively) compared with the unobstructed kidney of the control group (10.9 ± 0.5 and 3.2 ± 0.6 ng/h/mg protein, respectively). In contrast, when rats were treated with 1D11, there was no significant difference in either total NOS activity or iNOS activity between the obstructed kidney and the unobstructed kidney. In the obstructed kidney, total NOS activity and iNOS activity of each 1D11 group (high dose: 12.3 ± 0.4 ng/h/mg protein, 3.8 ± 0.8 ng/h/mg protein, respectively) was significantly higher than those of the control group, respectively. Furthermore, no significant difference in total NOS activity and iNOS activity was observed between the unobstructed kidney of the control group and that of the 1D11 groups.

Detection of iNOS

To determine where iNOS is expressed, immunostaining was performed for iNOS. Immunostaining for iNOS showed that renal tubules express iNOS in both obstructed and contralateral kidneys (data not shown), although the obstructed kidney of the control group showed less expression of iNOS (data not shown). In contrast, the obstructed kidney treated with 1D11 revealed more iNOS expression in renal tubules (Fig. 9A), compared with the obstructed kidney of the control group (Fig. 9B).

To quantitate iNOS protein expression in the kidney, we carried out Western blots for total protein extracted from each kidney (Fig. 9C). In the control group, the obstructed kidney showed significantly less iNOS expression compared with the unobstructed kidney. In contrast, the obstructed kidneys of the 1D11 group have significantly higher iNOS expression compared with the obstructed kidney of the control group. In addition, the obstructed kidneys of the 1D11 group are not different from unobstructed kidneys of the 1D11 group. There was also no significant difference between unobstructed kidneys of the control group and those of the 1D11 group (Table 2).

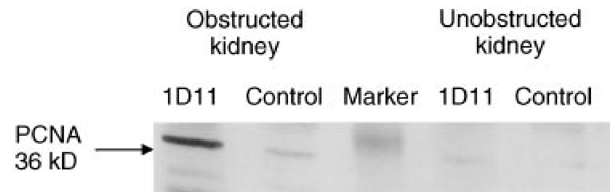


Fig. 5. Western blot analysis for PCNA in the obstructed kidney and unobstructed kidney. 1D11 is treatment with an antibody to TGF-β, 4 mg. Marker is 36 kD.

DISCUSSION

Clinically, UUO occurs in a wide variety of diseases. It is reported that approximately 166 out of 1 million patients had a presumptive diagnosis of UUO on admission to hospitals in the United States [23, 24]. Relief of the obstruction requires intervention to release the obstruction. In experimental obstruction, the time course has been studied, and it has been shown that the effects of obstruction on renal function in dogs are reversible if the obstruction is reversed at four weeks [25]. However, in patients, the duration of the obstruction is unknown. An obstructed kidney could be predisposed to further injury, even after release of the obstruction. Thus, we often encounter patients who sustain renal functional damage subsequent to the release of obstruction. The identification of factors that mediate such damage could lead to development of pharmacological agents that could be used to preserve renal recoverability of the obstructed kidney until, or subsequent to, release. In the present study, monoclonal antibody to TGF-β (1D11) was used prior to and throughout two weeks of obstruction, and the effects on renal fibrosis and tubular apoptosis were measured.

The present study demonstrates that the control obstructed kidney expressed significantly more tissue TGF-β, more tubular apoptosis and fibrosis, less iNOS expression, and less total NOS activity compared with its contralateral unobstructed kidney. Treatment with 1D11 significantly decreased tissue TGF-β concentration, tubular apoptosis, and fibrosis. Decreased apoptosis was accompanied by decreased p53 and increased bcl-2. Furthermore, iNOS expression in the obstructed kidney was restored by 1D11 treatments, and tubular proliferation in both kidneys was significantly increased by 1D11. These data suggest the possibility that 1D11 can preserve the obstructed kidney in UUO.

Mechanical stretch, TGF-β, and apoptosis

The changes in intrarenal pressure accompanying UUO may result in tubular mechanical stretch and mediator release [26]. The source of TGF-β is controversial and may be the renal tubular cells [17] and/or macrophages [27]; however, the function of TGF-β and other such

Table 2. Quantification of Western blot results in control and 1D11-treated rats

	Control		1D11-4 mg		1D11-2 mg		1D11-0.5 mg	
	Obstructed	Unobstructed	Obstructed	Unobstructed	Obstructed	Unobstructed	Obstructed	Unobstructed
PCNA	7943 ± 821[a]	2924 ± 390	15698 ± 1428[ab]	5719 ± 567[c]	ND	ND	ND	ND
p53	11137 ± 622[a]	1242 ± 308	1915 ± 197[b]	1168 ± 148	2528 ± 228[b]	1279 ± 230	4006 ± 266[b]	1552 ± 301
bcl2	462 ± 243	408 ± 219	8521 ± 200[ab]	1631 ± 468	8899 ± 554[ab]	1468 ± 566	8581 ± 170[ab]	1591 ± 617
iNOS	2464 ± 302[c]	5385 ± 391	5496 ± 1201	4942 ± 690	5116 ± 967	5599 ± 1813	4235 ± 680	5306 ± 820

Abbreviation is ND, not determined.
[a] $P < 0.01$, compared to the unobstructed counterpart
[b] $P < 0.01$, compared to the obstructed kidney of the control group
[c] $P < 0.05$, compared to the unobstructed kidney of the control group

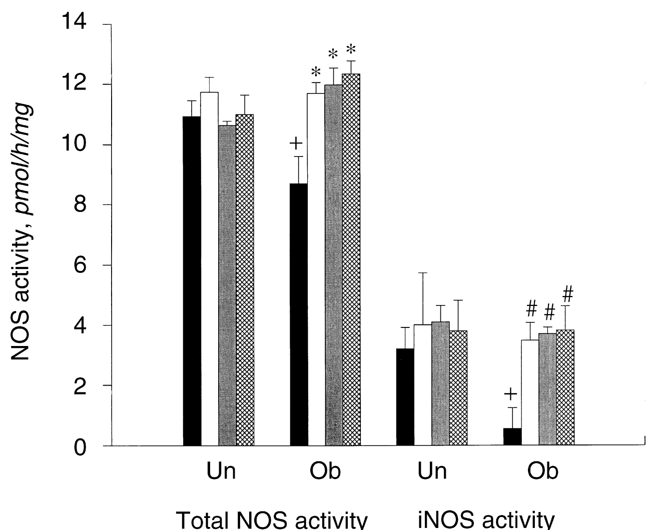
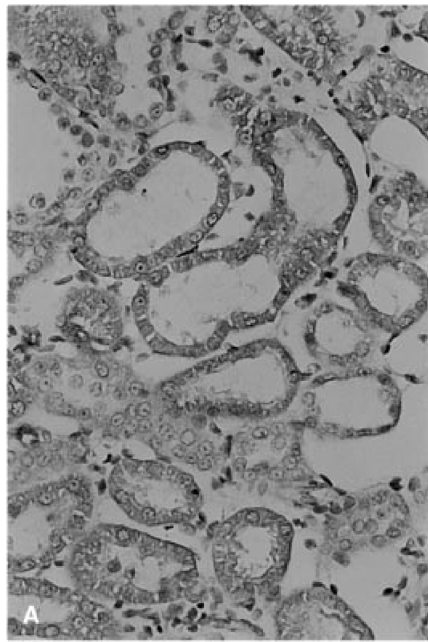


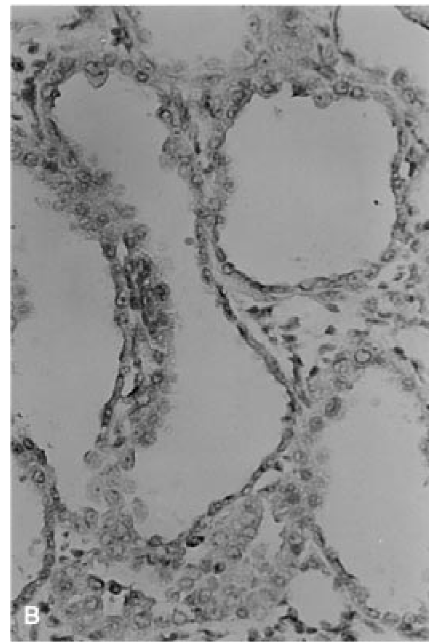
Fig. 8. Nitric oxide synthase (NOS) activity assay. NOS was measured as the conversion of ^{3}H-arginine to citrulline as described in the **Methods** section. Abbreviations are: Ob, obstructed kidney; Un, contralateral unobstructed kidney; iNOS, inducible NOS. Symbols are: (■) control group; (□) 1D11-0.5 mg; (▩) 1D11-2 mg; (▨) 1D11-4 mg $*P < 0.05$ compared with total NOS activity in the obstructed kidney of the control group; $\#P < 0.05$ compared with iNOS activity in the obstructed kidney of the control group; $+P < 0.05$ compared with the unobstructed kidney of the control group ($N = 5$).

mediators in the sequelae of UUO is still unclear. To elucidate the function of TGF-β in renal tubular apoptosis, we stretched tubular epithelial cells and determined both the TGF-β concentration of the supernatant and apoptosis in those cells. Our results show that mechanical stretch induces TGF-β and apoptosis significantly. The addition of 1D11, in contrast, neutralized TGF-β and reduced apoptosis. This suggests that TGF-β is involved in apoptosis in renal tubular cells exposed to mechanical stretch. TGF-β has been previously implicated in stretch responses of mesangial and aortic cells. It has been shown that stretch of mesangial cells results in increased TGF-β secretion, as well as collagen synthesis and breakdown. An antibody to TGF-β was able to reverse these changes, when cells were stretched in a high-glucose medium [28]. Similarly, in aortic smooth muscle cells, stretch induced increased TGF-β release and collagen synthesis, which were also reversed by an antibody to TGF-β [29]. The apoptotic effects of stretch were not noted in these studies.

Mechanical stretch and TGF-β appear to be proapoptotic in vitro; however, it was not known whether TGF-β could be implicated in the apoptosis of UUO in vivo. Apoptosis in the obstructed kidney has been shown to be increased compared with the unobstructed kidney in control animals [4]. In the present study, we confirmed that apoptosis is increased in UUO. The administration of the antibody to TGF-β strongly decreased renal levels of TGF-β in the obstructed kidney. Concomitantly, apoptosis of tubular cells was dramatically decreased; even the lowest dose (0.5 mg) of the antibody to TGF-β decreased renal tubular apoptosis by greater than 90%. p53 is known to be associated with cell proliferation and regulation of apoptosis [30], and TGF-β–induced apoptosis is also reported to involve p53 up-regulation [11]. In contrast, bcl-2 is known to play an anti-apoptotic role in tubule damage [31]. We sought to determine whether p53 and bcl-2 are implicated in apoptosis of this model. Immunostaining, in the present study, showed that p53 is localized to the renal tubule of the obstructed kidney. Western blot showed that UUO increases p53 expression. This is consistent with the previous demonstration that p53 mRNA and protein were up-regulated at five days after UUO, although p53 localization was not determined in that study [32]. Treatment with the antibody to TGF-β decreased renal tubular expression of p53 in the obstructed kidney, suggesting that p53 expression in UUO is dependent on TGF-β.

On the other hand, preliminary data regarding bcl-2 in UUO had been contradictory. Chevalier reported, in the rat UUO model, bcl-2 expression in the contralateral control kidneys, which was lost in the obstructed kidneys [33]. Shappell et al showed that bcl-2 is detectable in the human obstructed kidney, especially in dilated tubules (abstract; *Lab Invest* 74:169A, 1996). Our immunostaining showed that bcl-2 is localized in the dilated renal tubule in the obstructed kidney with 1D11, not in the contralateral unobstructed kidney. However, we could not detect bcl-2 in both the obstructed and contralateral





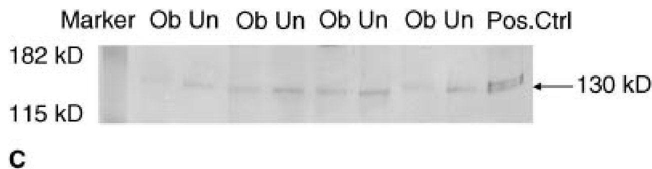


Fig. 9. Inducible nitric oxide synthase (iNOS) expansion in kidney. (*A*) Immunostaining for iNOS in obstructed kidney with 1D11 (4 mg; ×400). (*B*) Immunostaining for iNOS in the control obstructed kidney (×400). (*C*) Western blot analysis for iNOS. Abbreviations are: Ob, obstructed kidney; Un, contralateral unobstructed kidney; Pos.Ctrl, positive control (purified macrophage iNOS). Marker is 130 kD.

kidneys in the control group, which is partially consistent with the results of Chevalier. Bcl-2 in the human kidneys could be the result of UUO of longer duration than in the present study or species differences. Furthermore, Western blot showed that the obstructed kidney of control group expresses little bcl-2 and that the treatment with antibody to TGF-β increases bcl-2 expression in the obstructed kidney. Of particular interest is the finding that neutralizing TGF-β by 1D11 resulted in bcl-2 upregulation in the obstructed kidney, while the control contralateral kidney expresses little bcl-2. This demonstrates the appearance of bcl-2 in the obstructed kidney, but not in the contralateral unobstructed kidney, subsequent to TGF-β neutralization and removal of TGF-β's inhibition of bcl-2.

Fibrosis and TGF-β

The obstructed kidney is characterized by interstitial fibrosis and tubular atrophy [24]. In the present experiments, interstitial fibrosis was identified in several ways. We noted increases in interstitial volume, tissue hydroxyproline, and mRNA for collagen III and fibronectin. These parameters were significantly decreased by 1D11 treatment. Tubular atrophy was also diminished by 1D11 treatment. These results suggest a beneficial effect of inhibiting TGF-β in UUO. The effects of 1D11 on fibrosis are consistent with previous reports of use of antibody to TGF-β in models of glomerulonephritis or corneal fibrosis [1, 34].

Interaction of NO and TGF-β in UUO

Transforming growth factor-β is also known to be a negative regulator of iNOS [15]. The increased TGF-β, which is found after UUO, could be expected to downregulate iNOS in the obstructed kidney of the control group; 1D11 treatment should be associated with increased iNOS and NO in the obstructed kidney as compared with control. Both total NOS activity and iNOS activity were determined by citrulline assay, and iNOS protein was detected with a Western blot. These studies demonstrated that the obstructed kidney of control group at day 13 had significantly less NOS expression compared with the unobstructed kidney. This finding is consistent with our previous results that mice, at day 14 after UUO, had significantly less NOS activity in the obstructed kidney as compared with the unobstructed kidney [abstract; Miyajima et al, *J Urol* 163(Suppl):83, 2000]. In the groups with 1D11, NOS expression and

NOS activity of the obstructed kidney were similar to the unobstructed kidney. Therefore, 1D11 contributes to the maintenance of NOS expression in the obstructed kidney by neutralizing TGF-β, suggesting the existence of an interaction between TGF-β and NO in UUO.

Role of TGF-β and NO in renal tubular proliferation

It has been reported that TGF-β inhibits proliferation in tubular cells and glomerular endothelial cells [35]. NO has also been shown to be antiproliferative in renal tubules [abstract; Miyajima et al, *J Urol* 163(Suppl):83, 2000]. Proliferation of the obstructed kidney has previously been studied [4]. An increase in proliferation of both tubular and interstitial cells, with different time courses, was demonstrated. Glomerular proliferation was minimal. In the present study, we confirmed that there was more proliferation of both tubular and interstitial cells in the obstructed kidney compared with the contralateral side. Glomerular proliferation was unchanged. Treatment with 1D11 significantly increased proliferation in the obstructed and contralateral kidneys. As shown here, 1D11 treatment decreases the antiproliferative cytokine TGF-β and increases NO synthesis, which would increase tissue NO, the antiproliferative cytokine. Thus, the suppression of TGF-β appears to be more important to proliferative activity in UUO than the antiproliferative effects of NO.

Role of an antibody to TGF-β in UUO

Transforming growth factor-β is a multifunctional cytokine with sometimes opposing actions. It is profibrotic and, in most systems, pro-apoptotic. Conversely, it is considered to be anti-inflammatory because of its effects on inflammatory cell migration and mediator release, and effects on leukocyte adhesion [36, 37]. Thus, use of an antibody to TGF-β may be associated with multiple and, possible conflicting, effects. Nevertheless, in the present study, we demonstrate that an antibody to TGF-β can modify an interaction between TGF-β, NO, p53, and bcl-2, all contributing to the prevention of renal tubular apoptosis and the increase in tubular proliferation in UUO. It is concluded that an antibody to TGF-β is a promising agent for kidney preservation in UUO. When patients present with obstructed kidney, a significant amount of time can elapse between the diagnosis and release of obstruction. In this time, the kidney can deteriorate further. In addition, even if the obstruction is released, events occurring in the kidney can predispose it to further damage. Understanding the biochemical events that induce renal apoptosis can lead to development of pharmacological agents, which could ameliorate, or reverse, the damage.

NOTE ADDED IN PROOF

The abstract by Hochberg et al on p. 2301 is now an article in press. The complete citation is: Hochberg D, Johnson CS, Chen J, Cohen D, Stern J, Vaughan ED Jr, Poppas DP, Felson D: Interstitial fibrosis of unilateral obstruction is exacerbated in kidneys of mice lacking the gene for inducible nitric oxide synthase. *Lab Invest* 80:1721–1728, 2000.

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