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Acute and Chronic Renal Effects of Recombinant Human TGF-β2 in the Rat

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Abstract. The expression of transforming growth factor-β (TGF-β) correlates with the incidence of renal glomerular and interstitial injury, however, nothing is known of the effect of these proteins on renal hemodynamics. This study examines the renal hemodynamic and morphologic effects of recombinant human TGF-β2 in normal male Sprague Dawley rats. Acute infusion of TGF-β2 (1.2 μg/kg per min) induced no hemodynamic changes, except for a modest though significant fall in mean arterial pressure. Administering TGF-β2 at varying doses (20, 100, and 400 μg/kg) for 9 wk caused modest increases in systolic BP and proteinuria and minimal tubular interstitial fibrosis, however, renal hemodynamic end points were not significantly altered. TGF-β2 (800 μg/kg) was also administered to volume-depleted rats for 7 consecutive days. In contrast to the findings in volume-replete animals, administration of TGF-β2 to volume-depleted rats caused a marked reduction in GFR and medullary blood flow. Histologic fibrosis of the medullary vasa recta and cortical interstitium was seen, but glomeruli were unaffected. Thus, acute and short-term chronic TGF-β2 administration did not induce major renal changes in the volume-replete state, however, TGF-β2 combined with volume depletion caused medullary hypoperfusion and reduced GFR.

Transforming growth factor-β (TGF-β) are a group of genetically related, multifunctional cytokines that regulate diverse cellular activities (1). The three mammalian isoforms (TGF-β1, -β2, and -β3) share 70 to 80% of their amino acid sequences and appear to engage the same cell surface receptors to effect biologic responses. The biologic responses to TGF-β are pleiotropic. TGF-β protects cells from the damaging effects of ischemic reperfusion injury as best demonstrated in cardiac and neural tissues (2,3). TGF-β are also potent immunosuppressants and key regulators of immune responses (4). Conceivably, the promotion or neutralization of these activities will find widespread application in tumor biology, tissue transplantation, and the treatment of autoimmune diseases (5). TGF-β also play a central role in advancing wound closure and regulating connective tissue deposition at the site of wounds (6). These properties may prove useful in promoting the healing of intractable wounds such as those associated with chronic diabetes.

There are, however, concerns that pharmacologic application of any TGF-β isoform may cause tissue damage, pathologic scarring, and even organ failure (7). Elevated expression of TGF-β, particularly TGF-β1, has been associated with many examples of tissue fibrosis in animal models and human diseases (8). In the only clinical trial that has examined systemic administration of a TGF-β isoform, patients with chronic multiple sclerosis experienced a significant but reversible decline in renal function when treated with TGF-β2 (9).

Nephrotoxicity from exogenously administered TGF-β was not predicted from preclinical toxicologic studies performed in rodents and rabbits (10), even though increased production of TGF-β has been associated with the accumulation of extracellular matrix molecules including collagens, proteoglycans, and fibronectin in models of renal fibrosis as diverse as glomerulonephritis (11–13), diabetic mesangial sclerosis (14–16), and tubulointerstitial injury (17). Although not well studied, TGF-β may play a role in regulating renal vascular tone. TGF-β may be a renin secretagogue (18) and is known to stimulate the production of endothelin-1 (ET-1) by cultured renal collecting duct cells (19). Conversely, there is evidence that angiotensin II (AngII) stimulates the production of TGF-β1, and that TGF-β may be a prominent mediator of AngII-induced renal injury (20,21). Similarly, the glomerular sclerosis and interstitial fibrosis seen in ET-1 transgenic mice are likely due to TGF-β (22). Finally, TGF-β are also known to downregulate the production of nitric oxide, a vasodilator of particular importance to renal function (23).

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To better understand the role of TGF-β in progressive renal disease, its potential role in regulating renal hemodynamics, and the basis of its reported nephrotoxicity, we have studied the effects of administering recombinant human TGF-β2 to Sprague Dawley rats. TGF-β2 was chosen because it is available in amounts required for animal studies and because this isoform is currently in clinical development. Here, we report that TGF-β2 profoundly decreases GFR, induces medullary hypoperfusion, and stimulates fibrosis of the vasa recta and to a lesser degree tubular interstitial fibrosis in volume-depleted rats. Conversely, there were no hemodynamic changes and very little fibrosis was observed in volume-replete rats.

Materials and Methods

All studies were conducted in adult male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 290 to 375 g. They were housed in an animal facility with standard 12-h light/dark cycles. Unless otherwise specified, the rats were fed standard rat rations (Rodent Laboratory Chow 5001; Ralston Purina Co., Richmond, IN) and tap water *ad libitum*. Systolic BP (SBP) was measured by the awake tail-cuff method. In most studies, rats underwent 24-h urine collections for sodium ($U_{Na}V$), protein ($U_{prot}V$), and urinary excretion of the brush-border enzymes *N*-acetyl-β-glucosaminidase and alanine aminopeptidase. These studies were conducted in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, and were approved by the Portland Veterans Administration Institutional Animal Care and Use Subcommittee. Recombinant human TGF-β2 was produced and purified at Genzyme Corp. (Framingham, MA).

Protocol 1: Acute Studies

These studies were designed to examine the effects of acute intravenous infusions of TGF-β2 (n = 8) or vehicle (n = 16). Rats were anesthetized and studied for baseline renal function, as described below. After these measurements, infusions (0.1 ml/h) of either TGF-β2 (Genzyme Corp.) at 1.2 μg/kg per min, or vehicle buffer (4 mM sodium acetate at pH 6.5) were started. After 30 min of equilibration, all hemodynamic studies were repeated. Pilot studies were performed with incremental doses of TGF-β2 from 0.1 to 10 μg/kg per min, to confirm that TGF-β2 could be recovered from the systemic circulation in the activated form. The arterial pH was measured late in the experiment to ensure that the buffer did not induce a metabolic acidosis that might itself influence renal hemodynamics. At the end of the experiment, rats were sacrificed and blood was collected from cardiac puncture into ethylenediaminetetra-acetic acid tubes and processed for determination of plasma renin concentration (PRC), AngII, and TGF-β2. Plasma samples were stored at −80°C until assayed. The left kidney was harvested and homogenized in cold 100% methanol for AngII measurement, and the right kidney was placed in 10% neutral-buffered formalin and subsequently processed for histologic examination.

Protocol 2: Chronic Studies

To ascertain the nephrotoxic potential of TGF-β2 administered over a longer time course, two chronic studies were performed. For both studies, TGF-β2 (frozen stock) was diluted in 15% wt/wt propylene glycol, 20% wt/wt polyethylene glycol, 20 mM sodium phosphate, and 130 mM NaCl, pH 7.2 (PG buffer). Fresh PG buffer and TGF-β2 dilutions were made weekly. In the first study, rats were randomly assigned to one of three groups: vehicle control (n = 6), TGF-β2 at 20 μg/kg (n = 3), or TGF-β2 at 100 μg/kg (n = 10). All doses were given as intraperitoneal injections in a volume of 200 μl, three times per week for 9 wk. SBP measurements and 24-h urine collections were performed before initiation of the dosing regimen and were repeated at weeks 4 and 8. At the end of the study, rats were anesthetized (Inactin, 100 mg/kg, intraperitoneally) for renal function studies and blood collection. Blood samples were processed for determination of PRC, AngII, and TGF-β2. After the renal function studies, animals were sacrificed and tissue samples were collected within 10 min. The right kidney, adrenal glands, liver, lungs, and heart were placed into 10% neutral-buffered formalin and processed for histologic examination. The left kidney was placed in cold methanol for the determination of AngII.

In the second study, rats were given TGF-β2 at 400 μg/kg (n = 6) three times a week for 6 wk. SBP measurements and 24-h urine collections were performed before initiation of the dosing regimen and were repeated at weeks 4 and 6. At the end of the study, renal function studies and sample harvesting were performed as described above. Renal function studies were also performed at this time on a group of weight-matched control animals (n = 6).

Protocol 3: Volume Depletion Studies

In the third protocol, the effects of administering daily, high dose TGF-β2 were examined in the setting of volume depletion. After baseline SBP and 24-h urine collections, rats were randomly assigned to one of three groups. Group 1 (euvolemic control, n = 12) was fed synthetic rat rations, with normal (0.4%) sodium composition (PMI Feeds, Inc., Richmond, IN) for the duration of the study (16 d). Groups 2 (n = 8) and 3 (n = 7) were subjected to volume depletion, produced by injection of furosemide (20 mg/kg subcutaneously, once daily for 2 d) and were fed low (0.02%) sodium rat rations for the duration of the study. On days 10 through 16, group 2 rats received daily intraperitoneal injections of TGF-β2 (800 μg/kg in 200 μl of PG buffer) and group 3 rats received PG buffer control injections on the same schedule. On day 14, all rats had repeat measurements of SBP and 24-h urine collections. On day 16, they underwent renal function studies followed by blood and tissue sample collection for histology and renin and AngII levels. In addition, one half of the left kidney was snap-frozen in liquid nitrogen for subsequent measurement of ET-1 levels. Also, a sample of whole blood was collected, using the Unopette Microcollection System (Becton-Dickinson, Rutherford, NJ) for white blood cell counts.

Renal Function Studies

Rats were anesthetized with Inactin (100 mg/kg, intraperitoneally) and placed on a thermoregulated table. The left femoral artery was cannulated, and a baseline sample of blood was collected for determination of hematocrit (Hct), and inulin and para-aminohippurate (PAH) blanks. This arterial catheter was used for subsequent blood sampling and for measurement of mean arterial pressure (MAP) via an electronic transducer connected to a direct writing recorder. After tracheostomy, bilateral internal jugular catheters were inserted for infusions of rat serum and 4 to 10% inulin with 0.2 to 0.8% PAH in saline (1.2 ml/h). The left femoral vein was cannulated for drug administration, and the left ureter was catheterized for urine collections. To maintain euvolemia, rat serum was infused at 0.1 ml/min for a total equal to 1% of the body weight, followed by a reduction in infusion rate to 0.42 ml/h, to maintain a constant Hct. After a 1-h period of equilibration, two timed (20 min) urine collections were made for determination of flow rates, and inulin and PAH concentra-

tions. Blood was obtained simultaneously for measurement of Hct, inulin, and PAH.

Laser Doppler Blood Flow Measurements

These experiments were performed on male Sprague Dawley rats dedicated for flowmetry end points, and were volume-depleted and dosed with TGF-β2 as described for protocol 3. On the day of the acute experiment, rats were anesthetized with ketamine (30 mg/kg, intramuscularly) and Inactin (50 mg/kg, intraperitoneally). A cannula was placed in the femoral artery for measurement of MAP, and the left kidney was exposed using a midline incision and placed in a stainless steel kidney micropuncture cup to minimize respiratory motion. Three fiber-optic probes (0.5 mm outer diameter) were inserted approximately 6 mm deep into the kidney to monitor outer medullary blood flow at multiple sites using a Pf3 laser Doppler flowmeter (Perimed Corp., Stockholm, Sweden) as described previously (24). Cortical blood flow was measured by placing a large integrating fiber-optic probe (Pf 347) approximately 1 mm from the cortical surface and recording the flow signal. After surgery, 30 min were allowed for equilibration of the preparation. Then, MAP and the laser Doppler flow signals obtained from the three implanted medullary fiber-optic probes and readings from five random sites on the surface of the left kidney were recorded.

Biochemical Studies

For the calculation of GFR, inulin concentrations in plasma and urine were determined by the macro-anthrone method. Effective renal plasma flow was determined by PAH clearance. PAH concentrations in plasma and urine were determined by colorimetric methodology. For determination of PRC, 100 μl of rat plasma was incubated with 100 μl of rat anephric plasma and 400 μl of 0.2 M maleate buffer, pH 6.0, at 37°C for 1 h, and the generation of AngI was then determined by RIA using commercially available reagents (New England Nuclear, Boston, MA). AngII was measured using methods established by Fox *et al.* (25) and quantified with a competitive single antibody RIA, using rabbit anti-AngII antibody (Peninsula, Belmont, CA) and monoiodinated ^{125}I-labeled AngII (Amersham, Arlington Heights, IL). All samples were assayed in duplicate, and mean values were plotted against a curve generated from AngII standards (Sigma). Urine protein was measured spectrophotometrically after precipitation with 3% sulfosalicylic acid. Urinary *N*-acetyl glucosaminidase was measured using a commercially available kit (Boehringer Mannheim, Indianapolis, IN), and alanine aminopeptidase was measured by colorimetric assay (26). Circulating TGF-β2 was measured on serum samples using a TGF-β2-specific enzyme immunoassay (Genzyme Corp.). For determination of ET-1, kidney homogenates were prepared in 1 M acetic acid containing 1 μg/ml pepstatin A and a protease inhibitor cocktail (Sigma) and processed according to the method of Wong and Jeng (27). ET-1 and thromboxane B2 were measured by enzyme immunoassay following the manufacturer's directions (Cayman Chemical, Ann Arbor, MI).

Tissues were prepared for morphologic examination by immersion in 10% neutral-buffered formalin and routine processing for light microscopy. Paraffin sections were stained with hematoxylin-eosin or Masson's trichrome.

Statistical Analyses

Values are reported as means ± SEM. Statistical analysis was performed by paired *t* test (for studies before and after an intervention), by unpaired *t* test (for studies of two groups), or ANOVA followed by computation of modified *t* values according to the method of Bonferroni (for multiple groups), as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Protocol 1: Acute Studies

Results of the acute hemodynamic studies are shown in Table 1. There were no differences between groups in any baseline parameter. When compared with pretreatment baseline, TGF-β2 induced a small but statistically significant fall in MAP. TGF-β2 did not cause any renal hemodynamic changes. TGF-β2 could be detected from rats infused with exogenous TGF-β2, but was not detectable in the vehicle group. Neither group developed systemic acidosis during the experiments; arterial pH remained at 7.43 to 7.45 (data not shown).

Protocol 2: Chronic Studies

Results of the chronic studies in the vehicle, 20 μg/kg, and 100 μg/kg groups are summarized in Table 2. There were no differences in baseline parameters among the groups. All rats

Table 1. Acute hemodynamic effects of intravenous TGF-β2 (1.2 μg/kg per min) in volume-replete rats[a]

Variable	Vehicle (n = 16)		TGF-β2 (n = 8)	
	Before	After	Before	After
BW (g)	336 ± 7		352 ± 8	
LKW (g)	1.34 ± 0.03		1.29 ± 0.06	
MAP (mmHg)	133 ± 3	130 ± 4	129 ± 4	124 ± 4[b]
GFR (ml/min)	1.71 ± 0.08	1.80 ± 0.07	1.71 ± 0.08	1.82 ± 0.20
ERPF (ml/min)	5.20 ± 0.20	5.32 ± 0.16	5.21 ± 0.34	5.34 ± 0.59
FF	0.33 ± 0.01	0.34 ± 0.01	0.34 ± 0.02	0.33 ± 0.01
UV (ml/min)	3.8 ± 0.2	4.1 ± 0.1	4.6 ± 1.0	4.5 ± 0.5
Serum TGF-β2 (pg/ml)		ND		116 ± 48

[a] Values are means ± SEM. BW, body weight; LKW, left kidney weight; MAP, mean arterial pressure; ERPF, effective renal plasma flow; FF, filtration fraction; UV, urine volume; PRC, plasma renin concentration; AngII, angiotensin II; ND, not detectable. There were no differences between groups at baseline.

[b] $P < 0.05$ *versus* baseline.

Table 2. Effects of chronic TGF-β2 in volume-replete rats[a]

Variable	Vehicle (n = 6)	Low Dose (20 μg/kg) (n = 10)	Medium Dose (100 μg/kg) (n = 10)
BW1 (g)	324 ± 6	332 ± 6	313 ± 5
BW2 (g)	457 ± 31[b]	442 ± 12[b]	380 ± 14[b,c,d]
Change in BW (g)	134 ± 26[b]	110 ± 13[b]	67 ± 10[b,e]
LKW (g)	1.29 ± 0.10	1.33 ± 0.07	1.23 ± 0.04
LKW/100 g BW	0.28 ± 0.01	0.30 ± 0.01	0.33 ± 0.01[c]
SBP1 (mmHg)	130 ± 7	119 ± 8	112 ± 3
SBP2 (mmHg)	113 ± 8	122 ± 3	127 ± 5
Change in SBP (mmHg)	−13 ± 7	+10 ± 8	+16 ± 5[b,e]
$U_{prot}V1$ (mg/d)	8 ± 1	5 ± 1	10 ± 4
$U_{prot}V2$ (mg/d)	9 ± 1	7 ± 1	9 ± 2
Change in $U_{prot}V$ (mg/d)	2 ± 1	3 ± 1[b]	2 ± 1
$U_{NAG}V1$ (U/d)	1.8 ± 0.5	2.1 ± 0.2	1.7 ± 0.3
$U_{NAG}V2$ (U/d)	2.1 ± 0.3	1.7 ± 0.2	1.2 ± 0.3
Change in $U_{NAG}V$ (U/d)	0.3 ± 0.3	−0.3 ± 0.3	−0.5 ± 0.4
$U_{AAP}V1$ (U/d)	2.9 ± 0.7	3.2 ± 0.6	2.8 ± 0.3
$U_{AAP}V2$ (U/d)	5.3 ± 0.7[b]	4.3 ± 0.4	3.0 ± 0.7
Change in $U_{AAP}V2$ (U/d)	2.8 ± 0.3	1.2 ± 0.7	0.5 ± 0.9
PRC (ng AngI/ml per h)	25 ± 7	25 ± 4	23 ± 3
Plasma AngII (fmol/ml)	13 ± 4	7 ± 2	10 ± 2
Renal AngII (fmol/g)	73 ± 23	64 ± 17	95 ± 12

[a] Values are means ± SEM. SBP, systolic blood pressure; $U_{prot}V$, 24-h urinary protein excretion; $U_{NAG}V$, 24-h urinary excretion of *N*-acetyl-β-glucosaminidase; $U_{AAP}V$, 24-h urinary excretion of alanine aminopeptidase. 1 = initial; 2 = final (9 wk for first three groups; 6 wk for 400 μg/kg group). There were no significant differences between changes in the low- and medium-dose groups.
[b] $P < 0.05$ *versus* initial value in same group.
[c] $P < 0.05$ *versus* vehicle.
[d] $P < 0.05$ *versus* low dose.
[e] $P < 0.05$ *versus* change in vehicle group.

gained weight, although the absolute weight gain in the 100 μg/kg TGF-β2 group was lower than that in the other groups. Absolute left kidney weights did not differ among the groups, however, the kidney/body weight ratio in the 100 μg/kg TGF-β2 group was higher than that in the vehicle group. Systolic BP was comparable in the vehicle and 20 μg/kg TGF-β2 group, both pre- and posttreatment. However, in rats receiving 100 μg/kg TGF-β2, there was a statistically significant increase in SBP compared with their own baseline values. TGF-β2 did not significantly affect urinary excretion of *N*-acetyl-β-glucosaminidase or alanine aminopeptidase, plasma levels for renin and AngII, or renal AngII. Urinary protein levels were significantly elevated in the 20 μg/kg TGF-β2 group but were slightly decreased in the 100 μg/kg TGF-β2 group. Morphologic examination of all three groups by light microscopy revealed no changes in glomerular, tubular, or tubulointerstitial morphology. Similarly, there were no histopathologic changes in the heart, liver, adrenal glands, or lung tissue at any of the doses. Thus, the 100 μg/kg dose of TGF-β2 was associated with some retardation of body growth and a modest but significant systemic pressor effect.

Results of the 400 μg/kg TGF-β2 group are summarized in Table 3. Compared with controls, rats receiving this dose of TGF-β2 had significantly higher awake SBP values, MAP (under anesthesia), and urinary protein excretion rates. There were no statistically significant differences in renal functional parameters between these groups, although the TGF-β2 group had slightly lower GFR values. Values for PRC and plasma and renal AngII in both groups were higher than those seen in Protocol 1, due to more prolonged anesthesia and surgery. However, there were again no differences between the groups. Histologic examination (data not shown) of kidneys from these two groups provided evidence of sporadic hypercellularity and connective tissue deposition in the vascular bundles (vasa recta) of the outer medulla with some suggestion of medullary tubular interstitial fibrosis. There was no evidence of glomerular or cortical tubular fibrosis. Thus, the 400 μg/kg dose of TGF-β2 was associated with increased BP and proteinuria. GFR in this group was lower, although the decrease did not achieve significance.

Protocol 3: Volume Depletion Studies

The results of the studies performed in the setting of volume depletion are summarized in Tables 4 and 5. Table 4 summarizes body weight, SBP, and urinary end points. Both volume depletion and the administration of TGF-β2 affected body weight gain. Control rats gained 53 g during the 2 wk of study, whereas volume-depleted rats receiving vehicle showed no

Table 3. Effects of chronic TGF-β2 (400 μg/kg) on renal hemodynamic function in volume-replete rats[a]

Variable	Control (n = 6)	TGF-β2 (n = 6)
SBP (mmHg)	106 ± 5	128 ± 3[b]
$U_{prot}V$ (mg/d)	7 ± 1	20 ± 5[b]
BW (g)	382 ± 4	406 ± 8[b]
LKW (g)	1.22 ± 0.02	1.27 ± 0.04
LKW/100 g BW	0.32 ± 0.004	0.31 ± 0.01
MAP (mmHg)	113 ± 4	130 ± 5[b]
GFR (ml/min)	1.82 ± 0.15	1.59 ± 0.09
ERPF (ml/min)	5.93 ± 0.32	5.62 ± 0.50
FF	0.31 ± 0.02	0.29 ± 0.02
UV (nl/min)	4.0 ± 0.2	4.4 ± 0.3
PRC (ng AngI/ml per h)	85 ± 37	86 ± 14
Plasma AngII (fmol/ml)	78 ± 43	66 ± 17
Left kidney AngII (fmol/g)	370 ± 61	523 ± 76

[a] Values are means ± SEM. Abbreviations as in Tables 1 and 2.
[b] $P < 0.05$ *versus* vehicle group.

weight change and volume-depleted rats receiving TGF-β2 experienced a significant decline (30 g) in weight. There were no significant differences in SBP, either within or between groups, before or after dosing. Sodium restriction caused significant and equivalent reductions in urinary sodium excretion in the two volume depletion groups. Changes in urinary protein excretion and brush-border enzyme excretion did not follow any consistent patterns. However, it should be noted that these measurements did not take into account changes in GFR (see below), which would be expected to change urinary protein excretion by altering the filtered protein load.

Results of the hemodynamic studies in the three groups are depicted in Table 5. In contrast to the essentially negative findings in the volume-replete animals (Protocol 2), TGF-β2 administered to volume-depleted rats caused significant systemic and renal effects. At the time of renal function studies, the TGF-β2-treated rats demonstrated marked cachexia and lethargy. Kidney weight was also reduced in the TGF-β2 group, however, the kidney/body weight ratio did not change in any group. Their BP were also unstable initially, especially during laparotomy. As a result, longer equilibrium periods were required to reestablish stable BP. Despite the initial instability, MAP was stable and equivalent among all groups at the time of renal function studies. There were no differences in Hct among the groups. Leukocytosis was apparent in the TGF-β2 group. In contrast to the previous protocols, administration of TGF-β2 in the context of volume depletion had a profound effect on GFR. Values for GFR in volume-depleted rats receiving vehicle did not differ from those in volume-replete controls, however, there was a marked (57%) fall in GFR in the group receiving TGF-β2. There were no differ-

Table 4. Effects of chronic TGF-β2 (800 μg/kg per d) and volume depletion in rats[a]

Variable	Controls (n = 12)	Volume Depletion + Vehicle (n = 7)	Volume Depletion + TGF-β2 (n = 8)
BW1 (g)	309 ± 5	322 ± 4	306 ± 4
BW2 (g)	362 ± 5	323 ± 2[b]	276 ± 10[c]
Change in BW (g)	53 ± 4[d]	1 ± 4[e]	−30 ± 7[d,f]
SBP1 (mmHg)	132 ± 5	147 ± 7	141 ± 7
SBP2 (mmHg)	131 ± 4	147 ± 5	141 ± 6
Change in SBP (mmHg)	−1 ± 7	0.1 ± 10	−3 ± 7
$U_{Na}V1$ (mEq/d)	1.2 ± 0.1	1.4 ± 0.3	1.3 ± 0.2
$U_{Na}V2$ (mEq/d)	1.2 ± 0.2	0.04 ± 0.1[b]	0.2 ± 0.04[b]
Change in $U_{Na}V$ (mEq/d)	0.05 ± 0.2	−1.4 ± 0.3[d]	−1.1 ± 0.2[d]
$U_{prot}V1$ (mg/d)	7 ± 1	11 ± 2	7 ± 1
$U_{prot}V2$ (mg/d)	9 ± 1	14 ± 2	4 ± 1[c]
Change in $U_{prot}V$ (mg/d)	2 ± 2	3 ± 2	−2 ± 2
$U_{NAG}V1$ (U/d)	2.2 ± 0.2	2.1 ± 0.2	1.4 ± 0.2[b]
$U_{NAG}V2$ (U/d)	2.7 ± 0.2	2.3 ± 0.3	1.6 ± 0.1[b]
Change in $U_{NAG}V$ (U/d)	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.2
$U_{AAP}V1$ (U/d)	2.6 ± 0.6	4.8 ± 0.6	3.7 ± 1.0
$U_{AAP}V2$ (U/d)	4.1 ± 0.7	2.1 ± 0.4[b,d]	3.5 ± 0.3
Change in $U_{AAP}V2$ (U/d)	1.5 ± 1.1	−2.7 ± 0.6[e]	−0.3 ± 0.8

[a] Values are means ± SEM. $U_{Na}V$, 24-h urinary sodium excretion. Other abbreviations as in Tables 1 and 2. 1 = initial; 2 = final.
[b] $P < 0.05$ *versus* controls.
[c] $P < 0.05$ *versus* volume depletion/vehicle.
[d] $P < 0.05$ *versus* initial value.
[e] $P < 0.05$ *versus* change in controls.
[f] $P < 0.05$ *versus* change in volume depletion/vehicle.

Table 5. Effects of TGF-β2 (800 μg/kg per d) and volume depletion on renal hemodynamic function in rats[a]

Variable	Controls ($n = 12$)	Volume Depletion + Vehicle ($n = 7$)	Volume Depletion + TGF-β2 ($n = 8$)
LKW (g)	1.29 ± 0.04	1.19 ± 0.02	1.05 ± 0.03[b]
LKW/100 g BW	0.36 ± 0.01	0.37 ± 0.01	0.38 ± 0.01
MAP (mmHg)	126 ± 5	130 ± 6	126 ± 2
Hct (vol/dl)	0.46 ± 0.004	0.45 ± 0.01	0.46 ± 0.01
WBC (cell/mm^3)	5566 ± 564	5462 ± 471	10,378 ± 1469[b,c]
GFR (ml/min)	1.76 ± 0.08	1.87 ± 0.10	0.76 ± 0.09[c]
ERPF (ml/min)	6.20 ± 0.44	6.61 ± 0.29	5.94 ± 1.39
FF	0.29 ± 0.02	0.28 ± 0.01	0.14 ± 0.01[b,c]
UV (nl/min)	7.1 ± 2.2	4.7 ± 0.3	8.0 ± 1.4
PRC (ng AngI/ml per h)	75 ± 20	84 ± 19	65 ± 31
Plasma AngII (fmol/ml)	112 ± 31	84 ± 19	65 ± 31
Left kidney AngII (fmol/g)	600 ± 88	785 ± 145	530 ± 95
Renal ET-1 (pg/g)	1594 ± 337	1601 ± 595	3024 ± 617[b,c]
$U_{TxB2}V2$ (ng/d)	6.5 ± 0.6	10.6 ± 2.0	14.2 ± 1.7[b]

[a] Values are means ± SEM. Hct, hematocrit; WBC, white blood cell count; ET-1, endothelin-1; U_{TxB2}, urinary thromboxane B2. Other abbreviations as in Table 1.
[b] $P < 0.05$ *versus* controls.
[c] $P < 0.05$ *versus* volume depletion/vehicle.

ences in effective renal plasma flow among the groups. Accordingly, the filtration fraction was markedly reduced in the TGF-β2 group. Urine volumes did not differ. Renin-angiotensin system (RAS) parameters, taken at the end of the experiment, did not differ among the three groups.

Results of laser doppler flowmeter measurements are given in Figure 1. In this study, as with the results from Protocol 3, body weight was significantly decreased in the TGF-β2 group when compared with the volume-depleted, vehicle control group. Mean arterial pressure under anesthesia also tended to be lower in rats treated with TGF-β2 (118 + 7 mmHg) than values measured in the vehicle control rats (133 + 4 mmHg), but this difference was not significant. Cortical blood flow was approximately 20% lower in the rats treated with TGF-β2 than the control group. In contrast, outer medullary blood flow was reduced by a much greater extent, approximately 70%, in the rats treated with TGF-β2 compared with control animals.

At the time that the kidneys were surgically exposed for functional measurements, obvious subcutaneous fibrous nodules and thickened vessel walls were observed. Often, the kidneys were adherent to the dorsal abdominal wall by thickened, sclerotic peritoneum and retroperitoneal fibrosis. The ureters were universally thickened and relatively immotile.

Histologic examination of trichrome-stained kidney sections prepared from the TGF-β2 group and the volume-depleted control group of Protocol 3 are shown in Figure 2. In the cortex of TGF-β2 treated, volume-depleted rats, interlobular arteries and glomerular arterioles were prominently stained (Figure 2A). An occasional Bowman's capsule was also stained; however, there was no evidence of glomerular fibrosis and rarely tubular interstitial fibrosis. In contrast, the dense capillary network of the medullary vasa recta was filled with trichrome-positive deposits (Figure 2, B and C). The additional connective tissue appeared to fill the intervascular space, significantly reducing the number of red blood cells seen in a given field of view. For comparison, a representative image of vasa recta taken from a volume-depleted control rat is shown in Figure 2D.

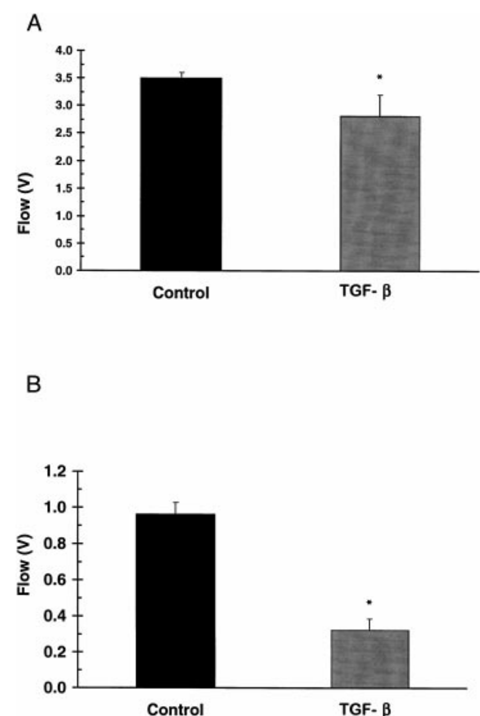

Figure 1. Laser doppler blood flow measurements in cortex (A) and medulla (B) of rat kidneys prepared from normal rats or volume-depleted transforming growth factor-β2 (TGF-β2)-treated rats. Shown are the blood flow means with SD. *$P < 0.05$.

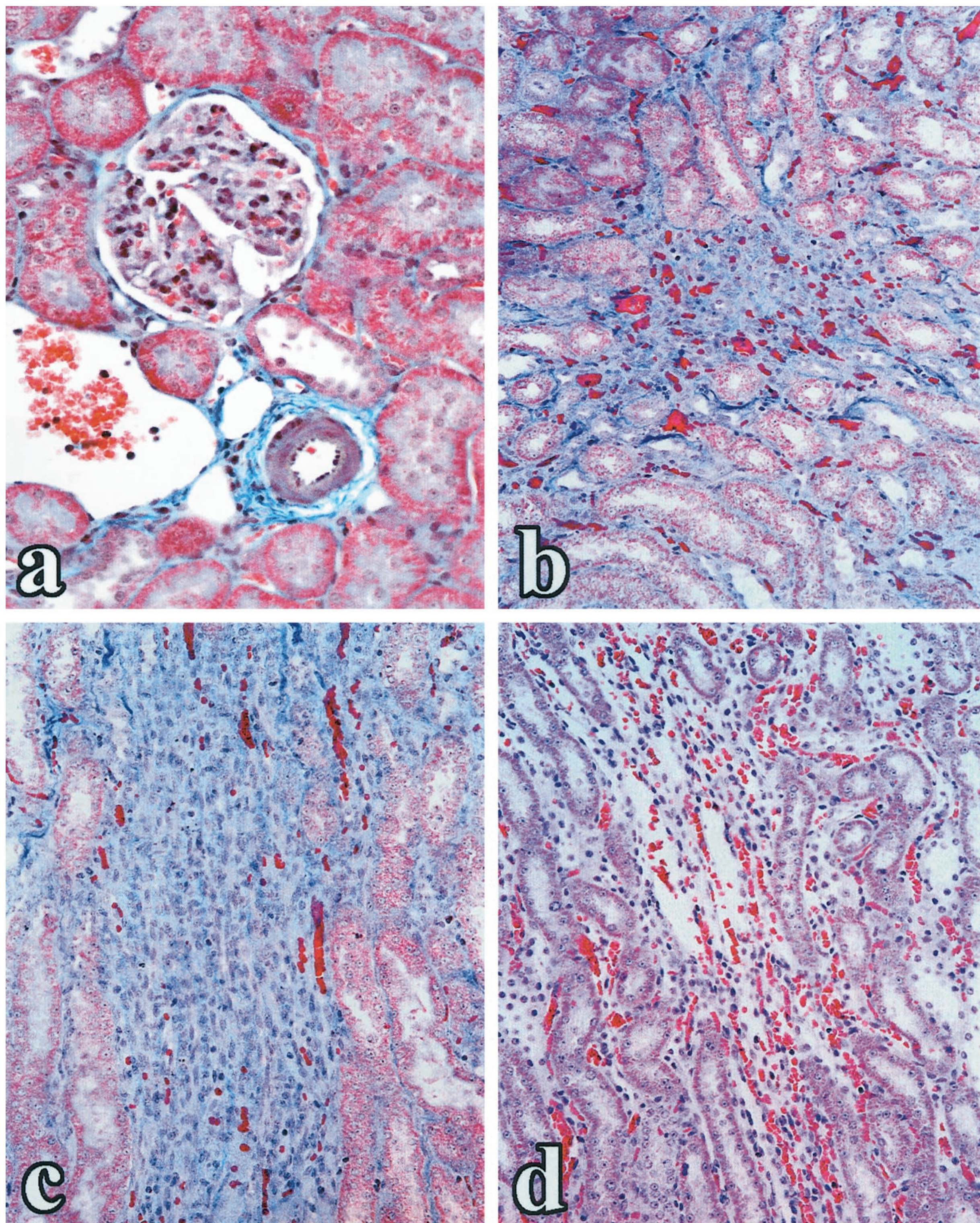

Figure 2. Light microscopic sections of kidney from TGF-β2 volume-depleted rats stained with Masson's trichrome. Deposits of additional connective tissue deposition are seen in the adventitia of interlobular arteries and, although not shown here, glomerular arterioles (A). However, adjacent glomeruli show no evidence of fibrosis. The luminal vascular spaces of medullary vasa recta appear markedly diminished as viewed in cross section or longitudinally (B and C). The vascular bundles are positively stained for connective tissue and the relative number of red blood cells are significantly reduced. A section of vasa recta from a volume-depleted control rat, stained with trichrome, is shown for comparison (D). Magnification: ×400 in A; ×200 in B through D.

Discussion

TGF-β are potent, multifunctional cytokines that regulate many cellular processes, including cell growth and differentiation, vascular tone, inflammation and immunosuppression, and the production of extracellular matrix proteins (1,4,6,8). With the expression and appearance of TGF-β intimately tied to the major themes of tissue repair, we and others believe that the exogenous administration of these molecules may have clinical utility. However, TGF-β have been widely implicated in pathologic tissue fibrosis (8), raising concerns that pharmacologic administration may have accompanying toxicity. To address these concerns, Terrell and colleagues (10) administered recombinant human TGF-β1 chronically to rats and rabbits but did not observe pathologic renal or hepatic fibrosis. Furthermore, there were no fibrotic complications upon installation of TGF-β2 following vitrectomy for the treatment of macular holes (28). It was therefore unexpected when administration of TGF-β2 (2 μg/kg) to patients with chronic multiple sclerosis caused significant but reversible reduction in GFR (9). This observation prompted us to reexamine the toxicity of recombinant human TGF-β2 with an emphasis on its potential nephrotoxicity.

Our acute studies in the volume-replete rat demonstrated little effect on renal hemodynamic measurements, despite confirmation of detectable circulating TGF-β2 at the end of the experiment. The slight reduction in MAP was not due to a volume effect because there was no diuresis or change in Hct. Since this finding was not observed in the chronic studies, its biologic importance is uncertain.

We also found relatively little effect of chronic TGF-β2 dosing on renal hemodynamics in the volume-replete animal. The 100 and 400 μg/kg doses both produced a modest, although significant, pressor effect. RAS measurements did not differ. While all forms of anesthetized RAS measurements are higher than those in conscious animals, the brief duration of anesthesia in the chronic studies should have allowed detection of prominent changes. Therefore, we believe it unlikely that TGF-β2, at the doses examined, exerts significant effects on the systemic and intrarenal RAS. It has been reported that water deprivation induces a marked increase of TGF-β2 immunolocalization in the juxtaglomerular apparatus of normal mice, and that this staining colocalizes with staining for renin (29). In a child with hypertension, hyper-reninemia, and renal artery stenosis, intense juxtaglomerular apparatus staining for TGF-β3 and renin was noted (30). Thus, although our studies did not find evidence of increased AngII at the level of the whole kidney, it remains possible that redistribution or localized increases occurred within specific kidney compartments.

We did note a slight increase in proteinuria after 6 wk dosing at 400 μg/kg TGF-β2. This observation may suggest early renal injury. Because urinary enzyme studies are not available for this group, an effect of TGF-β2 on tubular function resulting in proteinuria cannot be excluded. Additional studies will be needed to determine the nature of the protein leak, as there is currently little information regarding the effects of TGF-β on glomerular permeability or tubular reabsorption. It is worth noting that no histologic changes were observed in the glomeruli of the TGF-β2-treated animals. This is a somewhat unexpected finding in light of the strong correlation noted between upregulation of mesangial cell expression of TGF-β1 and glomerular extracellular matrix deposition, which has been noted for several other models of renal injury (11,12,31). Transgenic mice with increased plasma levels of TGF-β1 also develop progressive renal disease with accompanying glomerular sclerosis (13). These differences in glomerular response to increases in TGF-β may be a reflection of cellular accessibility to TGF-β, isoform-specific cellular responses, or other less obvious aspects of glomerular cell physiology.

The most dramatic findings were those in the volume-depleted animals receiving TGF-β2. These animals exhibited thickened skin and their peritoneal membranes were markedly fibrotic, often restricting organ mobility. This peritoneal fibrosis is consistent with the known effects of TGF-β on extracellular matrix synthesis. However, since peritoneal fibrosis was not seen in the 400 μg/kg group of Protocol 2, it seems likely that sodium and volume depletion enhanced the fibrotic effects of TGF-β2, possibly through regulating TGF-β receptors. These findings are consistent with published observations that sodium depletion markedly accelerates renal fibrosis in animals with cyclosporine toxicity (32), a drug that is itself known to stimulate TGF-β1 synthesis (33).

The marked fall in GFR in the TGF-β2-treated, volume-depleted animals was striking, and also at marked variance with our findings in the volume-replete animals. The higher doses of TGF-β2 in the volume depletion studies may have contributed to this finding, although as a sole cause this seems unlikely since all TGF-β2 doses were well above physiologic levels. As in some other forms of acute renal failure, *e.g.*, nephrotic syndrome and some toxicities, there was no fall in renal plasma flow. The reduced filtration fraction may, in part, reflect a reduction in the glomerular capillary hydraulic pressure gradient (ΔP), but it could also reflect an increase in proximal tubular pressure in an attempt to stabilize GFR, as proposed by Karlsen *et al.* (34). We are intrigued by the speculation that the marked fall in GFR was due to suppression of K_f, the glomerular capillary ultrafiltration coefficient. In those models in which GFR falls without a fall in RPF, a reduction in K_f has been the most consistent micropuncture finding (35). Numerous mediators may suppress K_f, including AngII (36) and ET-1 (37). Our data are not consistent with a major increase in AngII, but the elevated ET-1 levels in kidneys of the volume-depleted rats receiving TGF-β2 raise the possibility that this mediator played a role in the suppression of GFR, via a reduction in K_f.

These studies bear an interesting similarity to findings in experimental cyclosporine toxicity. Although cyclosporine is clearly nephrotoxic in humans, investigators have had difficulty inducing nephrotoxicity in volume-replete animals. However, the drug is reproducibly nephrotoxic in rats when administered in the setting of volume depletion (32,38,39). Striped interstitial fibrosis (fibrosis of the vasa recta), an early and defining feature of cyclosporine nephropathy (38), may be mediated through upregulation of TGF-β (33). As shown here, TGF-β2 also selectively promoted vascular fibrosis particu-

larly in the vasa recta, an area prone to ischemic injury (40). The mechanism underlying cyclosporine- and TGF-β2-induced tissue ischemia is likely to include increased production of vasoconstrictive agents (41). Cyclosporine increases the production of paracrine-acting vasoconstrictors, including AngII, endothelin, and prostaglandins (42,43); TGF-β increased production of ET-1 and thromboxane. Additionally, the effect of stimulating vasoconstrictor production might be exacerbated by reduced nitric oxide production, a potent vasodilation whose production is decreased by TGF-β2 (23).

Both cortical and medullary blood flow were significantly lower in rats treated with TGF-β2, but medullary blood flow was reduced by a far greater extent. It is concluded that, when coupled with the histologic evidence of medullary vascular fibrosis, the renal toxicity of TGF-β2 is a reflection of medullary hypoperfusion and consequent hypoxic injury. The mechanism involved remains to be determined, but most likely reflects a shift in the balance of vasoconstrictive and vasodilatory pathways. From our studies, it is not clear whether medullary hypoperfusion precedes or is a consequence of altered GFR. However, it is very clear that volume depletion enhances the susceptibility of kidneys to nephrotoxins. An obvious conclusion is to avoid combining risks such as volume depletion and the use of agents such as TGF-β that stimulate the production of vasoactive molecules. This combination is likely to have been responsible for the reversible fall in GFR experienced by patients with chronic multiple sclerosis to whom TGF-β2 was administered (9), since these patients tend to keep themselves voluntarily volume-depleted.

In summary, acute intravenous infusion of TGF-β2 into normal, volume-replete rats led to a slight fall in BP, without significant effects on renal function, urine volume, or RAS parameters. When administered chronically, at doses up to 400 μg/kg, there was a modest increase in systemic BP and proteinuria. In contrast, administration of 800 μg/kg in a context of volume depletion was associated with selective fibrosis and a profound reduction in GFR. The reversibility or prevention of these effects of TGF-β2 on renal function is currently under investigation. The data presented here suggest that renal vasodilation, improvement of medullary circulation, and avoiding the combination of agents known to enhance nephrotoxicity could aid in managing TGF-β2-mediated renal toxicity. Given the growing number of potential clinical applications of TGF-β2, greater understanding of the impact of TGF-β on renal function is clearly important.

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