Transforming growth factor-β in acute renal failure: receptor expression, effects on proliferation, cellularity, and vascularization after recovery from injury

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SECONDARY PHASE. In the second phase, early tubulointerstitial repair is evident as early as 1 day post-I/R. It is characterized by a proliferation of injured proximal tubules, as evidenced by positive S100A4 staining in 5–10% of proximal tubular cells at 3 days post-I/R. Moreover, the expression of TGF-β1 mRNA and protein is rapidly (within 3 days) increased in I/R-injured kidneys (28, 29). Recent studies suggested that these alterations may play a role in the later stages of reparative processes following acute renal failure (30).

For example, TGF-β1 was shown to stimulate epithelial-mesenchymal transition, which might also affect processes such as renal fibrosis (31). In addition, acute renal failure is associated with the activation of proinflammatory cytokines, such as interleukin-1β, which may promote renal fibrosis (32). However, the mechanisms by which TGF-β1 and other proinflammatory cytokines contribute to renal fibrosis remain to be fully elucidated. TGF-β1 is a key player in the pathogenesis of renal fibrosis (33). It is a multifunctional cytokine that regulates cell proliferation, differentiation, and apoptosis. In this regard, TGF-β1 may contribute to the development of renal fibrosis by stimulating fibroblasts to produce extracellular matrix proteins and matrix metalloproteinases (34).

ISCHEMIC ACUTE RENAL FAILURE (ARF) is characterized by a reduction in glomerular filtration rate, reduced renal blood flow, and tubular injury that is primarily evident in the proximal tubule (9, 33). Recovery from ARF occurs by the restoration of renal blood flow and the regeneration of the damaged tubular epithelium. The processes of injury and recovery have been studied extensively in rat models of ARF such as ischemia-reperfusion (I/R). In such models, if ischemia is prolonged, damage to the proximal tubule is severe. The renal regeneration response is characterized by multiple phases of recovery including an early proliferation of proximal tubule cells. A second, a more prolonged phase, is characterized by concurrent processes of basement membrane remodeling, cellular hypertrophy, differentiation, and apoptosis of hyperplastic epithelial cells that ultimately return the proximal tubule to normal morphology (9, 30, 35, 36, 40).

Although restoration of renal structure and function following I/R injury is impressive, the functional and structural recovery from this injury is not complete. It is clear that the renal vasculature is affected by I/R injury; there is a dysregulation of vascular tone, a breakdown in barrier function, and increased adherence of inflammatory cells (31, 32, 37). Damage to the vasculature is persistent following recovery of the tubular system. We reported that there is a permanent reduction in vascular density and a compromise in oxygen delivery in the postischemic kidney (4, 5). We suggested that these alterations in renal vascular structure underlie altered renal function following ARF (3).

There is considerable interest in identifying the factors that mediate repair of the injured kidney. It has been proposed that promitogenic growth factors and immediate early gene activation may play a role in the early, proliferative phase of regeneration (18, 29). However, factors involved in the later stages of proximal tubular repair are not well studied. Transforming growth factor-β (TGF-β) is a polypeptide growth factor with the potential to mediate many of the events that are observed during the later stages of renal repair (2). TGF-β inhibits proliferation of renal proximal tubule cells in vitro and stimulates extracellular matrix (ECM) synthesis, cell clustering, tubulogenesis, and apoptosis (2, 17, 26). Expression of TGF-β1 mRNA and protein is rapidly (<12 h) enhanced in damaged and regenerating proximal tubules and remains elevated for up to 14 days postischemia (8). There is a spatial and temporal relationship with TGF-β and several ECM-related genes postischemia, which is partially inhibited by the administration of a TGF-β neutralizing antibody in vivo (7). However, there is little evidence of fibrosis within the time frame that TGF-β expression returns to baseline values (i.e., ~4 wk).

It is possible that TGF-β represents an important mediator of the renal repair response. TGF-β may negatively regulate cellular proliferation, balancing the activity of promitogenic growth factors. TGF-β might also affect processes such as cellular hypertrophy and differentiation of regenerating proximal tubule cells. Conversely, it is also possible that the early increase in TGF-β may adversely affect vascular structure.

The current study was carried out to gain further insight into the potential role of TGF-β activity following I/R injury. We sought to characterize the expression of TGF-β receptors in rat kidney following I/R and to determine the effects of TGF-β immunoneutralization on the recovery of renal structure and function.
function following I/R injury. The data suggest that TGF-β activity following I/R affects multiple different activities at the level of the tubular epithelium, as well as a long-term effect on the vascular and interstitial structure of the postischemic kidney.

METHODS

Animals. Care of the rats before and during the experimental procedures was conducted in accordance with the policies of the Animal Resource Center, Medical College of Wisconsin, and National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols had received prior approval by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing ~250–300 g were housed with a 12:12-h light-dark cycle. Animals were fed standard laboratory rat chow (Purina, St. Louis, MO) with 0.8% Na content; food and water were available ad libitum.

Experiment 1 was designed to characterize the expression and localization of TβRI and TβRII during the recovery from ARF. ARF was induced by 45 min of bilateral renal artery clamping exactly as described previously (8, 25). Animals were killed at 1, 3, 7, 14, and 28 days postsurgery and sham-operated controls included at each time point. The extent of renal injury was determined by measuring the level of serum creatinine values 24 h postsurgery to ensure that all ischemic animals received similar levels of injury. An a priori range of 2.5–4.0 mg/dl was established as evidence of renal failure (8). All postischemic animals used in these studies recovered uneventfully from surgery; in animals allowed to recover for over 7 days, serum creatinine returned to normal levels (data not shown). Sham-operated animals had serum creatinine of ∼0.5 ± 0.1 mg/dl and these values were unchanged at all time points.

Experiment 2 was designed to assess the role of TGF-β immunoneutralization on elements of renal structure and function during the early repair phase following I/R at 1, 2, 3, and 9 days postschemia. All animals in these experiments received a neutralizing antibody against TGF-β, which recognizes all TGF-β isoforms (1D11, a gift from Genzyme Tissue Research, Cambridge, MA), or an equal amount of nonimmune mouse IgG as a control (1C34, Genzyme). The antibodies (0.5 mg·kg⁻¹·dose⁻¹) were administered via tail vein injection immediately following removal of renal artery clamps and once every 48 h thereafter. The 1D11 antibody is highly effective in vitro and has been used in vivo with the dosing schema outlined here based on data available to the supplier (Dr. S. Ledbetter, Genzyme, personal communication and Refs. 7, 13, 39). Sham-operated controls were treated only with control antibodies, in part, due to previous studies in which we demonstrated that 1D11 had no effect on gene expression in the kidney of sham-operated control rats (7). The determination of cell proliferation in experiment 2 was facilitated by administration of BrdU (Sigma, St. Louis, MO, 120 mg/ml ip) 90 min before death.

Experiment 3 was carried out to determine the effects of TGF-β immunoneutralization on renal tubular and vascular structure following more complete recovery from I/R injury at 5 wk following surgery. In these studies, animals were subjected to two separate surgeries. In the first surgery, animals were implanted with chronic venous jugular catheters to facilitate the intravenous administration of anti-TGF-β antibodies. The catheter was constructed from a ~1.5-cm microremanence (0.04 OD × 0.025 ID, Braintree Scientific, Braintree, MA) that was connected to 23-gauge tygon tubing (~12 cm) through a 23-gauge stainless steel hollow tube. The tygon tubing was connected to a PRN injection adaptor (REF 315110, Becton Dickinson, Sandy, UT) through a 23-gauge blunt-ended needle. Animals were anesthetized with ketamine hydrochloride (60 mg/kg), xylazine (6 mg/kg), and acepromazine maleate (0.9 mg/kg) by intraperitoneal injection. Chronic indwelling venous catheters were inserted into the jugular vein and secured. The distal ends of the catheters were tunneled around the neck and secured under the skin at the scapula. The catheters were filled with 1,000 U/ml heparin in sterile saline to prevent clotting. The placement of the injection adaptor-catheter below the skin allowed for repeated intravenous injection of anti-TGF-β antibodies through the skin and into the catheter under light halothane anesthesia; the overall time of anesthesia and administration of the antibody solutions was typically <3 min/animal. Animals were allowed to recover from catheter implantation for 5–7 days before they were subjected to I/R injury. Animals were treated every 48 h with anti-TGF-β antibody or control antibody for 35 days of recovery postsurgery. In addition, at the conclusion of experiment 3, renal tissue was prepared for microfil analysis.

Measurement of renal function. Renal functional parameters were measured at the indicated times. Tail blood samples (0.5 ml) were collected under light halothane anesthesia into heparinized tubes and plasma was obtained following centrifugation. Urine collection was for 24 h in metabolic cages (Nalgene). Serum and urine creatinine were determined using standard assays (Sigma creatinine kit 555A). Urine volume was determined gravimetrically. Creatinine clearance over 24 h was calculated using (Ucreatinine × V)/Pcreatinine, where Ucreatinine is the urinary concentration of creatinine, Pcreatinine is the plasma concentration of creatinine, and V is urinary flow rate. Urine sodium excretion was determined by flame photometry (Instrumentation Laboratories, Lexington, MA).

Isolation of kidney tissue. At the indicated times, rats were anesthetized with ketamine and pentobarbital sodium. Both kidneys were quickly excised and cut longitudinally, and half of each was frozen in liquid nitrogen and stored at ~70°C. The remaining halves were prepared for immunohistochemical analysis and in situ hybridization studies. For histochemical studies, we analyzed tissue from additional animals at 3 and 7 days postschemia that was subjected to the same degree of injury.

Ribonuclease protection assays. Total cellular RNA from whole kidney was obtained using the Ultraspec RNA kit (Biotecx, Houston, TX). Ribonuclease protection assays were carried out as previously described (8). Two sets of probes were used in these studies. Anti-sense mouse type I and type II probe template sets were obtained from Pharmingen (San Diego, CA). Riboprobes were generated with 100 µCi [α-32P]CTP (800 Ci/mM) and T7 RNA polymerase. A second set of cDNA were the corresponding to the type I receptor (ALK-5) and the type II receptor was cloned into the pSport vector (11, 12). Antisense RNA probes were synthesized with 50 µCi [α-32P]CTP (800 Ci/mM) following linearization of the plasmids with PstI (type I) or BglII (type II). Ribonuclease protection generation protected fragments of 356 and 547 nt, respectively. Using either probe set, data were quantitatively similar. For illustrative purposes, ribonuclease protection assays from the Pharmingen set are shown.

The intensity of the resulting signals was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). In each of the samples, the expression of each gene was corrected by dividing probe-specific signal by that obtained for the “housekeeping” gene; in this case, an antisense probe against L32 mRNA that was included in the probe set by the vendor. The data are expressed as relative expression, which was obtained by normalizing postischemic values to the mean values of the respective sham-operated control animals (8).

In situ hybridization. "Sense" and "antisense" RNA probes were prepared from the rat-specific templates described above and digoxigenin-labeled UTP. A BLAST search (1) of the GenBank database yielded no significant homology with other mRNAs. Kidneys from postischemic or control animals were fixed by immersion in Bouin’s solution overnight and prepared for paraaffin sectioning. In situ hybridization was performed on 5-µm sections exactly as described previously with a probe concentration of 1–2 ng/ml and hybridization at 52°C for 12–18 h (8). Posthybridization washing and immunological detection were performed exactly as described previously, using anti-digoxigenin-alkaline phosphatase with nitroblue tetrazolium (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate as substrates (X-phosphate, Boehringer Mannheim) (8).
Immunohistochemistry. Localization of TGF-β receptor protein was performed on 5-μm Bouin’s-fixed paraffin sections. Following deparaffinization and rehydration, tissue was prepared as follows: 1) endogenous peroxidase activity was blocked by incubation in 3% H2O2, 2) endogenous biotin blocked with sequential incubations with avidin and biotin (Avidin-Biotin Blocking kit, Zymed), and 3) non-specific sites blocked by incubation in 0.01 M PBS containing 0.3% Triton X-100, 10% goat serum, and 0.3% BSA. Rabbit primary antibodies specific for the TβRI (R20) and TβRII (C16) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). These were applied at a concentration of 1 μg/ml for 16 h at 4°C in blocking buffer. Detection was performed using a streptavidin-biotin immunoperoxidase technique with AEC as a substrate (Histostain SP, Zymed). The specificity of staining was verified by performing control experiments in which tissues were incubated with an equal concentration of nonimmune rabbit IgG.

Assessment of cell proliferation. Localization of BrdU was carried out using a BrdU detection kit from Zymed according to the manufacturer’s instructions. Sections were visualized using a Nikon Eclipse E400 microscope equipped with a Spot Insight color video camera (Diagnostic Instruments, Sterling Heights, MI). The images were captured on-line using Metamorph imaging software (Version 4.0, Universal Imaging). At least five random images of the outer stripe of the outer medulla of both kidneys (10 fields/animal) were stored using a ×20 objective lens and a field dimension of ~0.26 mm2. BrdU-positive cells were scored visually by a study group member who was blinded to the experimental groups. Data are expressed as BrdU-positive cells per visual field; statistical analysis was by Student’s unpaired t-test; P < 0.05 for two-tailed analysis was considered significant.

Assessment of interstitial cellularity and S100A4-positive cells. Determination of the degree of interstitial cellularity was carried out by examination of hematoxylin/eosin-stained sections following fixation in 10% formalin and paraffin embedding. A minimum of five random images from cortex and outer medulla were obtained from each animal using the same microscope and settings described above. All digitized images were stored and subsequently analyzed by a study group member who was blinded to the experimental groups. The analysis was performed based on previously published methods (34), used a point-counting technique in which the image is overlain with a reference grid (20 × 20). Of the 400 points/image represented by the intersection of the grid lines, each was classified as either being over tubular epithelium, tubular lumen, glomerular, interstitial, or acellular.

For determination of S100A4-positive cells, immunohistochemistry was carried out using a rabbit primary antibody that was obtained from Dako (Carpinteria, CA). Immunohistochemistry was carried out as described for the TβR with the following exceptions: 1) the tissues used were fixed in formalin, 2) an antigen retrieval step was included by microwaving tissues in 0.1 M citrate buffer, pH 6.0, for 10 min, and 3) dianisobenzidine was used as the substrate for the peroxidase reaction. For analysis, five random images of outer medulla and inner medulla of S100A4-stained tissues were analyzed by counting the number of positive cells per field exactly as described above for BrdU incorporation.

Microvessel density measurements. For analysis of the renal microvasculature, Microfil (Flow Tech, Carver, MA) infusion was carried out out at the time of death, as described previously (5). After the microfil, the kidneys were removed and placed in 5% formalin for several days. The kidneys were bisected and cleared with successive changes in graded alcohol and methyl salicylate according to the manufacturer’s instructions. Initial visualization was facilitated by placing a coverslip over the flat surface of the bisected kidney and examination under a stereomicroscope. For quantification of vessel density, kidneys were sectioned at 50 μm on a vibrotome, cleared again with methyl salicylate, and mounted in Permount. Quantification for each kidney was carried out by analysis of at least three tissue sections per kidney. Each section was visualized using a Nikon Eclipse E400 microscope equipped with a Spot Insight color video camera and Metamorph imaging software, as described above for each section, and at least five random images were stored for cortex, outer stripe of the outer medulla, and inner stripe of the outer medulla using a ×10 objective with a field dimension of ~0.48 mm2. For determination of vascular density, the sharp contrast between the opaque-filled vessels and adjacent translucent renal parenchyma facilitated image thresholding by the Metamorph software program and allowed for computer-generated determination of percent area occupied by microfil. In these analyses, as in our previous report (5), the contribution of glomerular vascular density was not considered; rather structures quantified represented small peritubular vessels.

RESULTS

mRNA expression of TβRI and TβRII following ischemic injury. Figure 1A illustrates a ribonuclease protection assay performed on total RNA from whole kidney of rats at various times following recovery from bilateral renal I/R injury or comparable sham surgery. The baseline signal obtained in kidneys of sham-operated rats 24 h post surgery for TβRII was 5.3 ± 0.3-fold higher than that obtained for TβRI; these signals did not change at any time following sham surgery. Ischemic injury affected the expression of both TβRI and TβRII in a similar fashion. The receptor mRNAs were unchanged when examined 24 h postischemia relative to corresponding sham-operated controls. Additional studies also failed to demonstrate an increase in mRNA expression at 2 days postischemia (data not shown). However, both receptor subtypes manifested a significant, approximately twofold increase by 3 days postischemia and these remained elevated until at least day 7 (Fig. 1, A and B). The mRNA expression of both receptor subtypes returned to levels observed in sham-operated controls by 14 days postinjury (Fig. 1B). Therefore, the mRNA expression of TGF-β receptors is elevated more slowly and returns to baseline more quickly than previously reported for TGF-β1, mRNA (8).

Localization of receptor subtypes in normal and postischemic kidneys. Figure 2 illustrates results obtained from in situ hybridization using an antisense riboprobe for the rat TβRI (Fig. 2, A–C) and the TGF-βRII (Fig. 2, D–H). The hybridization signal was difficult to detect in the cortex of kidneys from sham-operated control rats, whereas a faint signal was detected in the outer medulla (Fig. 2A). At 3 days postischemia, there was increased hybridization for TβRI mRNA in the outer medulla (Fig. 2, B and C). This signal was evident in cells of the flattened regenerating proximal tubules 3 days postischemia (thick arrow) as well as cells in the thick ascending limb (TAL; small arrow). TβRII mRNA expression was similarly increased in renal outer medullary tubular cells (Fig. 2, E and F, vs. G and H). No signal was evident in necrotic cellular debris. There was little evidence of hybridization in the interstitium of postischemic tissue (Fig. 2, B and C). No signal was evident following hybridization with a corresponding sense control probe (Fig. 2D).

Immunohistochemical analysis of TβRI and TβRII was consistent with results obtained from in situ hybridization studies (Fig. 3). Specific TβRI-like immunoreactivity was not prominent in tubular epithelial cells in kidneys from sham-operated controls but was present in the glomerulus (Fig. 3A). Increased immunoreactivity was evident in tubular epithelial cells in the outer medulla but not the cortex 3 days post-I/R.
TGF-β activity in the postischemic kidney, and these are localized primarily in the tubular epithelium.

Effects of TGF-β on restoration of renal function and the early repair response. The effects of TGF-β immunoneutralization on the early stages of renal recovery from I/R injury were addressed in experiment 2. The administration of anti-TGF-β antibody at the time of reperfusion did not affect either the initial loss of renal function nor did it affect the recovery of renal function as assessed by serum creatinine values (Fig. 4). Moreover, in this study, we could not distinguish other effects of anti-TGF-β antibodies on renal function using other measurements including creatinine clearance, urinary Na output, and urinary flow (data not shown).

The expression of TGF-βR in the regenerating epithelia on day 3 suggested that the local TGF-β/TGF-βR axis may modulate the early wave of cell proliferation that is characteristic of this model. In vehicle-treated postischemic rats, BrdU incorporation was substantially increased on day 1 and day 2 postischemia and decreased by day 3 (Fig. 5). There was no effect of TGF-β antibody on BrdU incorporation in the first 2 days postischemia; however, BrdU incorporation remained elevated on day 3 in antibody-treated animals vs. vehicle-treated postischemic animals (Fig. 5). Localization of BrdU-positive cells was generally in the regenerating tubular epithelium, although occasional cells were noted in tubulointerstitial space (curved arrow). In anti-TGF-β-treated animals, a small number of tubules with the morphological appearance of the TAL had BrdU-positive cells (Fig. 5C, arrows); this was not common in the vehicle-treated group. By day 9, BrdU proliferation was negligible in both vehicle- and antibody-treated postischemic groups.

Effects of TGF-β immunoneutralization on renal structure following I/R. Additional studies (experiment 3) were conducted to determine the possible effects of TGF-β immunoneutralization on renal microvessel density following recovery from ischemic injury. In these studies, animals were allowed to recover for 35 days; the characteristic features of the resultant renal injury and the effects of immunoneutralization from experiment 3 are shown in Table 1. Similar to the results in experiment 2, there was no effect of TGF-β antibodies on the initial (i.e., 24 h) extent of renal injury or recovery based on serum creatinine values. In addition, postischemic animals manifested a urinary concentrating defect, which was not affected by treatment with anti-TGF-β.

Kidney wet weight at the time of death was significantly higher in postischemic animals than sham-operated control animals; this hypertrophy was attenuated by administration of anti-TGF-β antibodies (Table 1). Histological examination of kidneys from postischemic animals treated with control antibody demonstrated largely normal renal tubular morphology, little evidence of scaring, but the consistent presence of interstitial cells (Fig. 6A, arrows). In contrast, kidneys from animals treated with anti-TGF-β antibody showed a normal renal morphology with a marked reduction in interstitial cells (Fig. 6B). Morphometric analysis of sections from kidneys of sham-operated and postischemic animals verified a prominent expansion of the interstitial compartment following I/R injury that was attenuated by anti-TGF-β antibody (Fig. 6C).

S100A4 calcium binding protein has been reported to be specific for renal interstitial fibroblasts (27). Immunohistochemical staining for S100A4 was not prominent in kidneys of...

Fig. 1. Ribonuclease protection assay of transforming growth factor (TGF)-β receptors type I and II mRNA in response to ischemic acute renal failure (ARF). A: TGF-β receptors type I and II (TβRI and TβRII) were detected in total cellular RNA originating from rat kidneys at various times (1–28 days) following sham operation or bilateral renal ischemia (ARF). L32 and GAPDH mRNA were included as internal standards. B: protected fragments from ribonuclease protection assays were quantified with a phosphorimager. Levels for each mRNA were standardized to signal due to L32 mRNA for each sample. The expression of mRNA from kidneys of rats rendered ischemic for each mRNA were standardized to signal due to L32 mRNA for each group at each time point and are expressed as means ± SE. *P < 0.05 vs. appropriate sham-operated control.
This study was conducted to gain greater insight into the TGF-β/TGF-β receptor system and their potential role in the renal repair response. We demonstrated the enhanced expression of both TGF-β receptor type I and II in the damaged and regenerating proximal tubule. In a previous study, we demonstrated a similar localization of TGF-β₁ mRNA and peptide (8); these data suggest that there is an enhanced TGF-β autocrine/paracrine loop localized predominantly in the proximal tubule of the injured and regenerating kidney. In this study, we failed to consistently identify the presence of TGF-β receptors in interstitial cells or in the peritubular vascular system. Therefore, the fact that the most prominent effects of TGF-β immunoneutralization occur in these regions is noteworthy. A possible explanation is that TGF-β immunoneutralization effects on the interstitial and vascular compartments may be mediated by interference with receptors contained within these regions that we failed to detect. Alternatively, the effects of immunoneutralization on these cells may be secondary to effects on regenerating tubular epithelial cells, which clearly express both TβRI and TβRII. It is worth noting that other investigators have identified TGF-β receptors within the renal vasculature (23).

TGF-β receptor expression is unchanged in the first 2 days following I/R but is enhanced thereafter when the rate of cell proliferation is known to decline. Our data are consistent with the concept that TGF-β receptor expression may modulate the early wave of proliferative activity. To our knowledge, TGF-β is the only extracellular factor whose activity is induced postischemia that inhibits proximal tubule cell proliferation. In this scenario, the postischemic milieu is dominated by promitogenic growth factors such as HB-EGF, HGF, FGF, and IGF-I in the first 1–2 days (15, 16, 18–21, 24) but thereafter becomes

sham-operated control rats at 35 days postsurgery (not shown) but was prominent in the interstitium of postischemic animals at 35 days post-I/R (Fig. 7A), suggesting that the expansion of the interstitial compartment is attributable, in part, to the presence of interstitial fibroblasts. TGF-β immunoneutralization during the 35-day recovery period resulted in a significant reduction in S100A4-positive cells when compared with the postischemic, vehicle-treated group (Fig. 7, B and C).

The effects of TGF-β immunoneutralization on renal vascular density following 35 days of recovery are shown in Fig. 8. Postischemic animals treated with control antibody manifested a significant reduction in vascular density as measured by microfil analysis (Fig. 8, A vs. B). Importantly, kidneys from animals treated with anti-TGF-β antibody manifested a marked attenuation of blood vessel loss following recovery from I/R (Fig. 8, C and D).

**DISCUSSION**

The potential role for TGF-β in setting ischemic ARF remains obscure. Many potential roles can be suggested from available data on the actions of TGF-β in vitro. TGF-β has been shown to inhibit in vitro “regeneration” of proximal tubule cells stimulated by exogenous mitogenic growth factors such as epidermal growth factor (EGF) or fibroblast growth factor (FGF) as a result of its antiproliferative effect (16, 18, 26, 38). However, renal injury and regeneration are much more complex processes in vivo (9, 16, 18, 30, 35, 36, 40). The expression of growth factors and their receptors in these in vitro models may not reflect the environment that is present in vivo. Moreover, in vitro studies on proximal tubule cells do not address activity in other potentially relevant cell types in the damaged kidney.

Figure 2. Localization of renal TβRI and TβRII mRNA by nonisotopic in situ hybridization. Five-micrometer Bouin’s-fixed kidneys were probed using a rat-specific antisense riboprobe corresponding to the TβRI through the cortex (cx) and outer medulla (om) of a sham-operated rat (A) and a postischemic rat (B), showing the increase in signal in outer medulla. Higher magnification shows incorporation within the tubular epithelium (C, arrow). Antisense hybridization showed little detectable signal (D). TβRII was moderately present in sham-operated control tissues (E, F); small arrows in F demonstrate signal in thick ascending limb. G (low magnification) and H (higher magnification): 3 days posts ischemia demonstrating an increase in the outer medulla, with signal detectable within the tubular epithelia. Bars = 100 μm.
more greatly influenced by the antiproliferative effects of TGF-β. Consistent with the suggestion, neutralizing TGF-β enhanced BrdU incorporation at 3 days but had no effect 1 or 2 days postinjury.

Despite our ability to measure alterations in cell proliferation (this study) and the mRNA expression of specific ECM-related genes (7), we did not observe any significant effect of anti-TGF-β antibody treatment on the functional course of ARF or recovery based on typically studied parameters. Immunoneutralization did not affect plasma creatinine values at any time point during recovery nor did it affect other parameters related to the later stages of tubular regeneration such as gross appearance of cellular hypertrophy or functional parameters related to redifferentiation such as %FeNa. It could be predicted that TGF-β immunoneutralization would result in a more severe degree of renal tubular hyperplasia due to its tendency to increase cell proliferation and potentially abrogate apoptosis. However, we were unable to demonstrate an effect of immunoneutralization on tubular apoptosis (data not shown), and tissues from antibody-treated animals showed no evidence of tubular hyperplasia that was different from vehicle-treated postischemic animals (see Fig. 6). Therefore, in contrast to our

Fig. 3. Immunohistochemical analysis of TβRI and TβRII in kidney postischemic injury. Five-micrometer Bouin’s-fixed kidney sections were probed using an antibody generated against the TβRI (A–C): sham-operated rat kidney at low magnification (A); staining is evident in the glomerulus. B: 3 days postischemia, TβRI-like immunoreactivity is observed primarily in tubular structures in the outer medullary (om) but is not prominent in the cortex (cx). C: higher magnification of outer medulla demonstrating immunoreactivity in regenerating proximal tubules (thick arrow) as well as distal nephron (thin arrow). D–F: tissues probed with an antibody against TβRII. D: little staining is observed in sham-operated controls. Increased tubular expression is present 3 days following ischemia-reperfusion (I/R) injury (arrows, E–F). G: no staining was evident in postischemic tissue when the antibody was replaced with an equal concentration of nonimmune rabbit IgG. Bars = 100 μm.
original hypothesis, these data indicate the overall effect of TGF-β activity in the repair of the tubular epithelium is minimal.

We and others described secondary progression of chronic renal disease following recovery from ARF (5, 14, 28). The chronic renal disease that ensues following recovery from ARF is characterized by the gradual development of interstitial scarring and the loss of renal microvessels. Under the conditions of ARF used in our study, renal scarring is not a prominent feature when animals are allowed to recover for 35 days and TGF-β levels have returned to baseline. However, we observed that there is an increase in the number of interstitial cells present at 5 wk postinjury and speculate that these cells may play an important role in the development of tubulointerstitial fibrosis. Therefore, the most important observations from

<table>
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<th>Time post-surgery (days)</th>
<th>Ischemic-anti-TGF-β</th>
<th>Ischemic-vehicle</th>
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<tr>
<td>1</td>
<td>3.2 ± 0.1</td>
<td>3.1 ± 0.2</td>
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<tr>
<td>7</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
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<tr>
<td>35</td>
<td>0.5 ± 0.1</td>
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Table 1. Effect of anti-TGF antibody on recovery from renal I/R injury for 35 days postsurgery; serum creatinine, urine flow, body weight, and kidney weight (experiment 3)

<table>
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<tr>
<th>Serum creatinine, mg/dl</th>
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<th>Vehicle Treated (n = 10)</th>
<th>45-min I/R (TGF n = 10)</th>
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<tr>
<td>7 day</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
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<td>35 day</td>
<td>0.5 ± 0.1</td>
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Data are means ± S.E. TGF, transforming growth factor; I/R, ischemia-reperfusion. *P < 0.05 vs. sham-operated control by Student’s t-test. †P < 0.05 postischemic antibody treated vs. postischemic vehicle treated by ANOVA and Student-Newman-Keuls post hoc test.

Fig. 4. Effect of ischemic injury and neutralizing TGF-β antiserum on renal function. Serum creatinine values were determined at the indicated times following recovery from I/R injury. Because animals were killed at different time points during the course of the study, the n for each group is indicated in parentheses above the data points. Thus, 24-h serum creatinine values derive from 52 vehicle-treated rats and 28 anti-TGF-β antibody-treated rats; at 9 days values derive from 8 vehicle-treated and 7 antibody-treated animals. Serum creatinine values from sham-operated control rats (not shown) were between 0.4 and 0.6 mg/dl at all time points. Data are means ± SE. *P < 0.05 vehicle ischemic vs. sham operated. †P < 0.05 anti-TGF-β postischemic vs. sham operated. Vehicle-treated postischemic values were not different from antibody-treated postischemic values at any time point.

Fig. 5. BrdU incorporation following ischemic ARF. A–D: cross sections of renal outer medulla of rat following sham surgery (A), 3 days postischemia treated with vehicle (B), and 3 days postischemia treated with anti-TGF-β (C); note that the majority of BrdU-positive cells are localized in tubular epithelium. C: (anti-TGF-β) BrdU-positive cell was apparently thick ascending limb (arrow). D: BrdU-positive cells were counted in sham-operated, postischemic animals, and in postischemic animals treated with anti-TGF-β. The number above each bar indicates the number of animals from which data originate. Data are means ± SE. *P < 0.05 vs. sham operated in days 1, 2, or 3 postinjury. *P < 0.05 day 3 antibody-treated vs. day 3 vehicle.
Fig. 6. Effect of α-TGF-β antibody on renal morphology following 35-day recovery from I/R injury. Shown are 5-μm hematoxylin/eosin-stained sections through renal outer medulla from a representative posts ischemic rat (35-day postsurgery, A) and a posts ischemic rat treated with α-TGF-β antibody (B). Note the increased cellularity in the tubulointerstitium of posts ischemic animals denoted by the arrows. C: morphometric analyses of kidneys from sham-operated, ischemic vehicle-treated, and ischemic α-TGF-β antibody-treated animals. Sections were analyzed in cortex and outer medulla by counting the number of arbitrary dots of a 20 × 20 matrix overlaying tissues; interstitial cells (ISC), tubular lumen, acellular (acell), and glomerular (glomer) structures were counted and expressed as mean dots/field (see METHODS). Values of dots overlying tubular epithelial cells were 5- to 8-fold higher than values of other structures and are not shown because of scale; values for tubular epithelial area were not different between groups. Data are means ± SE. *P < 0.05 vs. sham-operated control by Student’s t-test. †P < 0.05 ischemic α-TGF-β antibody-treated vs. ischemic vehicle-treated by Student’s t-test.

Fig. 7. Effect of α-TGF-β antibody on S100A4-positive cells following 35-day recovery from I/R injury. Five-micrometer formalin-fixed kidney sections were probed using an antibody generated against S100A4. Shown are representative sections through renal outer medulla from posts ischemic vehicle-treated rats (A) and posts ischemic anti-TGF-β antibody-treated rats (B) at 35 days postsurgery. Note the general presence of S100A4-positive cells localized within the tubulointerstitium. Magnification is shown. C: S100A4-positive cells were quantified in kidney from animals following either sham surgery or ischemic injury and recovery with either anti-TGF-β antibodies or vehicle at 35 days following recovery. Data are means ± SE. *P < 0.05 vs. sham-operated control by Student’s t-test. †P < 0.05 ischemic α-TGF-β antibody-treated vs. ischemic vehicle-treated by Student’s t-test.
these studies would appear to be those in which TGF-β immunoneutralization attenuated the degree of tubulointerstitial expansion and the loss of renal microvessels following 35 days of recovery from I/R.

The development of renal fibroblasts may be crucial in the ultimate development of interstitial fibrosis. With regard to the increased number of cells in the tubulointerstitium, it is noteworthy that gene profiling studies identified the fibroblast-specific S100A4 calcium binding protein as one of the most persistently upregulated genes at 5 wk post-I/R. This protein has also been referred to as fibroblast-specific protein-1, or FSP-1. S100A4 has been suggested to play an important role in epithelial-mesenchymal transdifferentiation (EMT), a process by which epithelial cells transdifferentiate into fibroblasts or myofibroblasts (27). S100A4-positive cells were occasionally identified in the tubular epithelium, suggesting that EMT may occur in the setting of renal regeneration. TGF-β, in conjunction with other factors, stimulates the expression of FSP-1 in cultured proximal tubule cells and stimulates EMT in vitro (27). Whether EMT actually occurs in the setting of ARF and whether fibroblasts are the result of EMT or promulgate through some other mechanism are not thoroughly addressed in the current study. However, our data clearly support the view that transient TGF-β activity postischemia may prime the kidney for the ultimate development of interstitial fibrosis by promoting the deposition of renal fibroblasts; the potential mechanism of this process warrants further investigation.

Likewise, the observation that TGF-β neutralization prevented the loss of renal microvessels following recovery from ARF is of potential significance in long-term renal function and the development of progressive renal disease. We hypothesized the potential ramifications of microvessel loss in the development of chronic renal failure and/or hypertension following ARF (3, 4). This hypothesis has been difficult to address, because little is known regarding the mechanism of blood vessel dropout or the factors that influence it. However, the results of the current study, as well as other reports, indicate that TGF-β may be one important factor regulating blood vessel stability. TGF-β has been shown to promote apoptotic cell death in endothelial cells (10) and chronic infusion of TGF-β2 resulted in a gradual decline of renal medullary blood flow, suggesting that TGF-β might influence vascular structure or tone (22). In recent studies, the administration of the anti-TGF-β antibody used in these studies appeared to protect the vasa recta bundles in hypertensive Dahl S rats (13). These data are consistent with the hypothesis that TGF-β predisposes the development of renal disease through effects on the vascular stability.

Because TGF-β receptors could not be definitively localized in renal blood vessels, it is unclear if the effects of TGF-β on the vasculature are direct or indirect. Although TGF-β could directly influence blood vessel stability, it may also indirectly influence blood vessel stability through the production of secondary factors. Factors with potential antiangiogenic activity that may be influenced by TGF-β include angiotatin or plasminogen activator inhibitor-I (3). Regardless of the mechanism, TGF-β antibody treatment may become a valuable tool

Fig. 8. Effects of α-TGF-β antibody on microvessel density following 35-day recovery from I/R injury. Shown are microfilled kidneys in whole mount from a sham-operated rat (A), a postischemic rat (B), and a postischemic rat treated with α-TGF-β antibody (C). Magnification is shown. D: morphometric analyses of renal microvessel density obtained by quantifying vibrotome sections as described in METHODS. Data are means ± SEM of percent area occupied by microfil in cortex, outer stripe of the outer medulla (OSOM), and inner stripe of the outer medulla (ISOM). *P < 0.05 vs. sham-operated control by Student’s t-test. †P < 0.05 ischemic α-TGF-β antibody-treated vs. ischemic vehicle-treated by Student’s t-test.
in understanding the long-term effects of renal injury and renal blood vessel loss.

In conclusion, TGF-β activity appears to influence cellular proliferation following I/R; however, there is no substantive evidence suggesting that it plays a critical role in the renal tubular repair response. In contrast, TGF-β activity promotes the deposition of renal fibroblasts and the loss of renal blood vessels following recovery from ARF. These factors may underlie important alterations in the physiology of the postischemic kidney that predispose the kidney to develop chronic renal disease; these possibilities remain to be explored in detail.

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