Transforming Growth Factor-β1 Potentiates Renal Tubular Epithelial Cell Death by a Mechanism Independent of Smad Signaling

Chunsun Dai‡, Junwei Yang‡, and Youhua Liu§

From the Division of Cellular and Molecular Pathology, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Tubular atrophy resulting from epithelial cell loss is one of the characteristic features in the development of chronic renal interstitial fibrosis. Although the trigger(s) and mechanism for tubular cell loss remain undefined, the hyperactive transforming growth factor (TGF)-β1 signaling has long been suspected to play an active role. Here we demonstrate that although TGF-β1 did not induce cell death per se, it dramatically potentiated renal tubular cell apoptosis initiated by other death cues in vitro. Pre-incubation of human kidney epithelial cells (HKC) with TGF-β1 markedly promoted staurosporine-induced cell death in a time- and dose-dependent manner. TGF-β1 dramatically accelerated the cleavage and activation of pro-caspase-9, but not pro-caspase-8, in HKC cells. This event was followed by an accelerated activation of pro-caspase-3. To elucidate the mechanism underlying TGF-β1 promotion of tubular cell death, we investigated the signaling pathways activated by TGF-β1. Both Smad-2 and p38 mitogen-activated protein (MAP) kinase were rapidly activated by TGF-β1, as demonstrated by the early induction of phosphorylated Smad-2 and p38 MAP kinase, respectively. We found that overexpression of inhibitory Smad-7 completely abolished Smad-2 phosphorylation and activation induced by TGF-β1 but did not inhibit TGF-β1-induced apoptosis. However, suppression of p38 MAP kinase with chemical inhibitor SC68376 not only abolished p38 MAP kinase phosphorylation but also obliterated apoptosis induced by TGF-β1. These results suggest that hyperactive TGF-β1 signaling potentiates renal tubular epithelial cell apoptosis by a Smad-independent, p38 MAP kinase-dependent mechanism.

Epithelial cell loss characterized as tubular atrophy is considered to be a hallmark in the development of chronic renal interstitial fibrosis (1–3). This pathologic process not only directly contributes to the progressive loss of renal function but also exacerbates the accumulation and deposition of extracellular matrix components leading to tissue fibrosis, probably because of the collapse and concentration of extracellular matrix surrounding the lost cells. In this regard, tubular atrophy and interstitial fibrosis are often inter-dependent, mutually stimulating events that ultimately lead to end stage renal failure (4). Although the mechanism of epithelial cell loss remains uncertain, it is conceivable to assume that tubular atrophy primarily results from apoptotic cell death under pathologic conditions (5–7), because there is little or no evidence for the presence of necrosis in chronically diseased kidneys. However, the trigger(s) and underlying mechanism responsible for tubular cell apoptosis in vivo are largely unknown.

Transforming growth factor-β1 (TGF-β1) is a pleiotropic protein that plays a central role in tissue fibrogenesis after injury (8–11). Many lines of evidence implicate hyperactive TGF-β1 as a major pathologic factor in the initiation and progression of chronic renal interstitial fibrosis. Overexpression of TGF-β1 axis is found in virtually every type of chronic renal diseases in experimental animal models and in patients (12–14). Inhibition of TGF-β1 signaling by various strategies prevents renal fibrotic lesions and tubular atrophy and attenuates renal dysfunction (15–18). Conversely, transgenic mice overexpressing TGF-β1 develop chronic renal disease with increased expression of fibrotic matrix proteins (19, 20). However, despite a causal relationship between hyperactive TGF-β1 signaling and chronic renal fibrosis in which tubular atrophy is a characteristic feature, there is little direct, convincing evidence demonstrating that TGF-β1 per se induces tubular epithelial cell apoptosis.

Numerous studies indicate that TGF-β1 is an important regulator of cell survival and apoptosis under diverse circumstances (21–23). However, the fate of the cells after TGF-β1 treatment is often determined by cellular context and experimental conditions. For instance, TGF-β1 acts as death stimulus inducing apoptotic death in fetal hepatocytes, podocytes, and certain neuronal cells (22, 23), whereas it also elicits prosurvival activity to protect macrophages against apoptosis (24, 25). As to renal tubular epithelial cells, preliminary studies in our laboratory failed to demonstrate that TGF-β1 by itself significantly affects their survival in cultured conditions (data not shown). Such disparity between TGF-β1 signaling and cell apoptosis implies that hyperactive TGF-β1 signaling alone may not be sufficient for causing tubular epithelial cells to die. This observation led us to propose a “two-hit model” in which both TGF-β1 signaling and a second death cue work in concert to lead to tubular epithelial cells undergoing apoptosis.

This work was supported in part by National Institutes of Health Grants DK-02611, DK-54922, and DK-61408 (to Y.L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ‡ Supported by postdoctoral fellowships from the American Heart Association Pennsylvania-Delaware Affiliate. § To whom correspondence should be addressed: Dept. of Pathology, University of Pittsburgh School of Medicine, S-405 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261. Tel.: 412-648-8253; Fax: 412-648-1916; E-mail: liuy@msx.upmc.edu.

† The abbreviations used are: TGF-β1, transforming growth factor-β1; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

Received for publication, January 23, 2003
Published, JBC Papers in Press, January 30, 2003, DOI 10.1074/jbc.M300777200

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
In the present study, we demonstrate that although TGF-β1 by itself did not induce tubular epithelial cells to die, it markedly potentiated cell apoptosis initiated by other death cues by accelerating the activation of procaspase-9 and -3. This action of TGF-β1 is likely mediated by a mechanism independent of Smad signaling. Our results suggest that TGF-β1 promotes tubular epithelial cell death by increasing their susceptibility to secondary death stimuli.

MATERIALS AND METHODS

Antibodies and Reagents—The antibodies for cleaved caspase-9, full-length and cleaved caspase-8, cleaved caspase-3, phospho-specific p38 MAP kinase, phospho-specific ERK1/2, phospho-specific JNK, and total JNK were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The antibody against total ERK1/2 was obtained from Sigma. Affinity-purified secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Recombinant human TGF-β1 was purchased from R & D Systems (Minneapolis, MN). Sc68356 (p38 MAP kinase inhibitor) and fluorescent dye H-33258 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against total ERK1/2 was obtained from Sigma. Affinity-purified secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Recombinant human TGF-β1 was purchased from R & D Systems (Minneapolis, MN).

Cell Culture and Treatment—Human proximal tubular epithelial cells (HKC) were kindly provided by Dr. L. Racusen of The Johns Hopkins University (28). Cells were cultured in Dulbecco’s modified Eagle’s medium-F-12 medium supplemented with 10% fetal bovine serum (27). HKC cells were seeded at ~70% confluence in complete medium containing 10% fetal bovine serum. Twenty-four hours, the cells were changed to serum-free medium and incubated for 16 h. Cells were then treated with recombinant TGF-β1 for various periods of time as indicated. Staurosporine was added to the cultures at the final concentration of 1 μM followed by incubating for additional periods of time ranging from 1 to 24 h. The cells were then collected at different time points for apoptosis detection and caspases activation analysis.

Apoptosis Detection by Fluorescent Dye H-33258 Staining—Nuclear chromatin morphology was examined by staining with the fluorescent dye H-33258 (5 μg/ml) as described previously (28). Briefly, adherent and detached cells were pooled, washed with phosphate-buffered saline, fixed with 3% paraformaldehyde, and then treated with 0.2% Triton X-100 for 10 min. After washing, the slides were mounted and observed on a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a digital camera (Melville, NY). Apoptotic cells with characteristic nuclear condensation and fragmentation were counted in at least ten random fields and expressed as a percentage of the total cell number (apoptotic index).

TUNEL Staining—In situ detection of DNA fragmentation was performed using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining. A fluorescein-based TUNEL staining protocol was employed by using an apoptosis detection system (29). Cells were washed with phosphate-buffered saline, fixed with 3% paraformaldehyde, and then treated with 0.2% Triton X-100 for 10 min. After pre-equilibration in 100 μl of buffer containing 200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride, strands of DNA were end-labeled by incubation at 37 °C for 1 h in 50 μM fluorescein-12-dUTP, 100 μM dATP, 10 μM Tris-HCl, pH 7.5, 1 mM EDTA, and terminal deoxynucleotidyltransferase. The reaction was stopped by adding 2× sodium chloride/sodium citrate hybridization buffer for 15 min. After washing, the slides were mounted and observed on a Nikon Eclipse E600 Epi-fluorescence microscope.

DNA Laddering—DNA laddering was assessed essentially according to the procedure described previously (28). Briefly, adherent and detached cells were combined and lysed in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% Triton X-100 on ice for 20 min. After a spin at 13,000 × g at 4 °C for 20 min, the supernatant (rich in low molecular weight DNA) was incubated with protease K (200 μg/ml) at 55 °C for 16 h and 20
Western Immunoblot Analysis—Detection of pro- and cleaved caspases was performed by immunoblotting using specific antibodies. HKC cells following different treatments were washed with phosphate-buffered saline and lysed in Chaps cell extract buffer containing 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.5, 2 mM EDTA, 0.1% Chaps, 5 mM dithiothreitol, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After freezing and thawing three times, cell lysates were centrifuged at 13,000 × g at 4 °C for 10 min; the supernatants were added with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). Samples were heated at 100 °C for ~10 min before loading and separated on pre-cast 10 or 15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins were electrotransferred to a nitrocellulose membrane (Amersham Biosciences) in transfer buffer containing 48 mM Tris-HCl, 0.037% SDS, and 20% methanol at 4 °C for 1 h. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes were incubated for 16 h at 4 °C with various primary antibodies in TBS buffer containing 5% milk at the dilutions specified by the manufacturers. The binding of primary antibodies was followed by incubation for 1 h at room temperature with the secondary horse-radish peroxidase-conjugated IgG in 1% nonfat milk. The signals were visualized by enhanced chemiluminescence reagent (ECL; Amersham Biosciences).

**Establishment of Stable Cell Line Overexpressing Smad-7**—HKC cells were transfected with the pSmad-7 expression vector (kindly provided by Dr. P. ten Dijke of Ludwig Institute for Cancer Research, Uppsala, Sweden) (30) using the LipofectAMINETM 2000 according to the instructions specified by the manufacturer (Invitrogen). Twenty-four h after transfection and every 3–4 days thereafter, the cells were re-fed with fresh selective medium containing G418 (Invitrogen) at a final concentration of 800 μg/ml. Neomycin-resistant clones were first visible after 7 days and continuously cultured in selective medium for about 14 days. The clones were then individually transferred into 6-well plates for expansion using a cloning cylinder (Sigma). After two further passages in selective medium, expanded independent clones were cul-

**FIG. 2.** TGF-β1 potentiates renal tubular epithelial cell death induced by staurosporine. HKC cells were pretreated with or without 2 ng/ml of TGF-β1 for 16 h and followed by incubating with 1 μM staurosporine for 24 h. A–D, TUNEL staining. A–C, representative micrographs show TUNEL-positive cells. A, control HKC cells; B, staurosporine alone; C, TGF-β1 plus staurosporine. Arrowheads indicate apoptotic cells. D, graphic presentation of HKC cell apoptosis after various treatments. Data are expressed as -fold induction over control HKC cells. *, p < 0.05 versus control; **, p < 0.05 versus staurosporine alone. E, DNA laddering analysis shows DNA fragmentation in HKC cells after various treatments with TGF-β1 or/and staurosporine. Lane 1, control HKC cells; lane 2, TGF-β1 alone; lane 3, staurosporine alone; lane 4, TGF-β1 plus staurosporine.

**FIG. 3.** TGF-β1 accelerates the activation of pro-caspase-9, but not caspase-8, following staurosporine treatment. HKC cells were pretreated with 2 ng/ml of TGF-β1 for 16 h and followed by incubating with 1 μM staurosporine. At different time points as indicated, cells were collected, and cell lysates were subjected to Western blot analysis with specific antibodies. A, Western blot demonstrates the kinetics of pro-caspase-9 activation. Samples were immunoblotted with antibodies against cleaved caspase-9 and actin, respectively. B, graphical presentation of cleaved caspase-9 levels after normalization to actin in tubular epithelial cells following various treatments. Solid circle, staurosporine alone; open circle, TGF-β1 plus staurosporine. Western blot (C) and graphical presentation (D) of the kinetics of pro-caspase-8 activation in HKC cells are shown. An identical pattern of pro-caspase-8 activation is observed in HKC cells pretreated either with or without TGF-β1. Solid circle, staurosporine alone; open circle, TGF-β1 plus staurosporine.
HKC cells with TGF-β undergoing apoptotic cell death. However, pre-incubation of HKC cells with 2 ng/ml of TGF-β dramatically accelerated this activation process in HKC cells. A Western blot demonstrates the kinetics of pro-caspase-3 activation. Cell lysates were blotted with specific antibodies against either full-length or cleaved caspase-3, respectively. B, graphical presentation of the relative abundance of cleaved caspase-3 (fold induction relative to control cells) after normalization to pro-caspase-3 in HKC cells following various treatments. C, effects of different concentrations of TGF-β1 on accelerating pro-caspase-3 activation. HKC cells were pretreated for 16 h with various concentrations of TGF-β1 for 16 h followed by incubation with 1 μM staurosporine for 2 days did not significantly affect cell viability and apoptosis as evidenced by nuclear condensation and fragmentation, as well as caspases activation (data not shown), suggesting that exogenous TGF-β1 per se does not induce tubular epithelial cells undergoing apoptotic cell death. However, pre-incubation of HKC cells with TGF-β1 dramatically potentiated tubular epithelial cell apoptosis induced by a well known death stimulus, staurosporine. As shown in Fig. 1A, staurosporine induced tubular epithelial cell apoptosis in a time- and dose-dependent manner. After treatment with 1 μM staurosporine for 24 h, about 20% of HKC cells underwent nuclear condensation and fragmentation as visualized by H-33258 staining (Fig. 1E). Pretreatment of HKC cells with 2 ng/ml of TGF-β1 for 16 h drastically increased the percentage of cells undergoing apoptosis to ~40%, as assessed by H-33258 staining (Fig. 1F). TGF-β1 potentiation of HKC cell apoptosis induced by staurosporine was apparently dependent on the duration of pre-incubation; simultaneous incubation of HKC cells with TGF-β1 and staurosporine did not significantly increase cell death compared with staurosporine treatment alone (Fig. 1G). The ability of TGF-β1 to enhance staurosporine-induced cell death was also dose-dependent and was effective at a concentration as low as 0.5 ng/ml (Fig. 1H).

To further confirm TGF-β1 potentiation of tubular epithelial cell death, we employed two additional approaches to detect cell apoptosis after various treatments of HKC cells, namely TUNEL staining and DNA laddering analysis. As shown in Fig. 2, TGF-β1 pre-incubation for 16 h markedly enhanced HKC cell apoptosis induced by staurosporine. TUNEL staining revealed more than 2-fold induction of apoptosis in the TGF-β1-pre-treated group as compared with that with staurosporine treatment alone (Fig. 2A). Similarly, DNA laddering analysis also exhibited a greater DNA fragmentation observed in TGF-β1-pre-treated cells as compared with that in the cells treated with staurosporine alone (Fig. 2E, lanes 3 and 4).

TGF-β1 Promotes Renal Epithelial Cell Apoptosis—Incubation of human kidney proximal tubular epithelial HKC cells with various concentrations of TGF-β1 for 2 days did not significantly affect cell viability and apoptosis as evidenced by nuclear condensation and fragmentation, as well as caspases activation (data not shown), suggesting that exogenous TGF-β1 per se does not induce tubular epithelial cells undergoing apoptotic cell death. However, pre-incubation of HKC cells with TGF-β1 dramatically potentiated tubular epithelial cell apoptosis induced by a well known death stimulus, staurosporine. As shown in Fig. 1A, staurosporine induced tubular epithelial cell apoptosis in a time- and dose-dependent manner. After treatment with 1 μM staurosporine for 24 h, about 20% of HKC cells underwent nuclear condensation and fragmentation as visualized by H-33258 staining (Fig. 1E). Pretreatment of HKC cells with 2 ng/ml of TGF-β1 for 16 h drastically increased the percentage of cells undergoing apoptosis to ~40%, as assessed by H-33258 staining (Fig. 1F). TGF-β1 potentiation of HKC cell apoptosis induced by staurosporine was apparently dependent on the duration of pre-incubation; simultaneous incubation of HKC cells with TGF-β1 and staurosporine did not significantly increase cell death compared with staurosporine treatment alone (Fig. 1G). The ability of TGF-β1 to enhance staurosporine-induced cell death was also dose-dependent and was effective at a concentration as low as 0.5 ng/ml (Fig. 1H).

To further confirm TGF-β1 potentiation of tubular epithelial cell death, we employed two additional approaches to detect cell apoptosis after various treatments of HKC cells, namely TUNEL staining and DNA laddering analysis. As shown in Fig. 2, TGF-β1 pre-incubation for 16 h markedly enhanced HKC cell apoptosis induced by staurosporine. TUNEL staining revealed more than 2-fold induction of apoptosis in the TGF-β1-pre-treated group as compared with that with staurosporine treatment alone (Fig. 2A). Similarly, DNA laddering analysis also exhibited a greater DNA fragmentation observed in TGF-β1-pre-treated cells as compared with that in the cells treated with staurosporine alone (Fig. 2E, lanes 3 and 4).
caspase-9 peaked at 3 h (about 60-fold) following addition of death stimulus in TGF-β1-pretreated cells, whereas it reached the highest level at 6 h in non-pretreated HKC cells. These results suggest that TGF-β1 potentiates tubular epithelial cell apoptosis by accelerating pro-caspase-9 activation after death challenge.

Staurosporine also induced pro-caspase-8 activation in tubular epithelial cells in a time-dependent manner. However, preincubation with TGF-β1 did not change the kinetics of pro-caspase-8 cleavage and its subsequent activation. The pattern of pro-caspase-8 activation after treatment with death stimulus was essentially identical in the tubular epithelial cells pre-treated with or without TGF-β1 (Fig. 3, C and D).

**TGF-β1 Activates the Activation of Downstream Effector Pro-caspase-3**—We further investigated the effect of TGF-β1 on the activation of downstream effector pro-caspase in tubular epithelial cells after death challenge. As shown in Fig. 4, pro-caspase-3 activation following staurosporine treatment in HKC cells was also significantly accelerated by TGF-β1 pretreatment. At 3 h after addition of death stimulus, active caspase-3 level in TGF-β1-pretreated HKC cells was more than 11-fold of that in untreated cells (Fig. 4, A and B). Significant acceleration on pro-caspase-3 activation by TGF-β1 pretreatment was also found at other time points in HKC cells (Fig. 4). Dose dependence studies revealed that TGF-β1, at a concentration of 0.5 ng/ml, was sufficient for enhancing pro-caspase-3 activation (Fig. 4, C and D). A higher dose of TGF-β1 did not result in a further significant increase in the levels of active caspase-3 in HKC cells (Fig. 4).

**TGF-β1 Activates Both Smad-2 Signaling and p38 MAP Kinase in Renal Tubular Cells**—To decipher the signal pathway(s) responsible for TGF-β1 potentiation of renal tubular cell apoptosis, we first investigated the potential signaling events initiated by TGF-β1 in HKC cells. As shown in Fig. 5, incubation of HKC cells with TGF-β1 induced rapid activation of Smad signaling. The phosphorylated state of Smad-2 increased as early as 10 min, peaked at 30 min, and gradually returned toward basal level at 3 h after TGF-β1 stimulation (Fig. 5, A and C). As Smad-2 is an effector intermediate signaling molecule for TGF-β1, its phosphorylation and activation manifests that the Smad signaling is probably one of the major signal transduction pathways that is readily activated and potentially mediates the cellular actions of TGF-β1 in renal tubular epithelial cells.

Besides Smad signaling, we also examined the effect of TGF-β1 on other signal pathways in renal tubular epithelial cells. Fig. 5B shows the activation of p38 MAP kinase upon TGF-β1 stimulation in HKC cells. Treatment of HKC cells with TGF-β1 induced p38 MAP kinase phosphorylation at 1 h, and this induction was largely sustained throughout the entire experimental scheme of 48 h (Fig. 5, B and C). Under the same conditions, TGF-β1 did not significantly activate the ERK1/2 and JNK, the other two subfamilies of the MAP kinases (see Fig. 8). These results suggest that both Smad signaling and p38 MAP kinase are activated in renal tubular epithelial cells upon stimulation by TGF-β1.

**Overexpression of Inhibitory Smad-7 Abrogates Smad-2 Activation but Does Not Block TGF-β1 Potentiation of Cell Death**—To investigate the potential role of Smad signaling in mediating TGF-β1 potentiation of renal tubular cell death, we
studied the effects of blocking Smad signaling on cell apoptosis by overexpressing inhibitory Smad-7. To this end, stable cell lines were established after transfecting with either pSmad-7 expression plasmid or pcDNA3 empty vector. As shown in Fig. 6, HK-pSmad-7 cell line robustly expressed Smad-7, as demonstrated by Western blot analysis (Fig. 6A) and immunofluorescence staining (Fig. 6B). Interestingly, overexpression of Smad-7 completely abolished Smad-2 activation following TGF-β1 incubation in the HK-pSmad-7 cells (Fig. 6C). There was no significant increase in the levels of phosphorylated Smad-2 in the HK-pSmad-7 cells after TGF-β1 treatment, in contrast to the HK-pcDNA3 cells (Fig. 6C), suggesting that inhibitory Smad-7 can effectively blunt TGF-β1-initiated Smad signaling. Of note, overexpression of Smad-7 did not affect p38 MAP kinase activation induced by TGF-β1 in renal epithelial cells (Fig. 6D).

We examined and compared the effects of TGF-β1 on staurosporine-induced apoptosis in HK-pSmad-7 and control HK-pcDNA3 cell lines. As shown in Fig. 7, no significant difference in apoptotic index was found between HK-pcDNA3 and HK-pSmad-7 cells. Likewise, it was virtually identical in the abundance of the cleaved, active caspase-3 in the HK-pcDNA3 and HK-pSmad-7 cells after incubation with staurosporine for various periods of time (Fig. 6, C and D). Thus, overexpression of inhibitory Smad-7 in renal tubular cells abrogates Smad-2 activation but does not abolish TGF-β1 potentiation of cell death, indicating that TGF-β1 promotes cell apoptosis by a mechanism independent of Smad signaling.

**TGF-β1 Promotion of Renal Tubular Cell Apoptosis Is Dependent on p38 MAP Kinase Activation**—We also investigated the possible role of p38 MAP kinase in renal tubular cell apoptosis. Treatment of HK cells with SC68376, a specific inhibitor of p38 MAP kinase, abolished TGF-β1-induced p38 MAP kinase phosphorylation and activation in a dose-dependent manner (Fig. 8A). SC68376 at a concentration of 20 μM completely abrogated p38 MAP kinase activation but did not influence Smad-2 phosphorylation induced by TGF-β1. Of note, both TGF-β1 and p38 MAP kinase inhibitor SC68376 failed to significantly affect the phosphorylation status of the ERK1/2 and JNK in renal epithelial HKC cells (Fig. 8, C and D).

We next examined the effects of blockade of p38 MAP kinase activation on cell death and caspase-3 activation. As shown in Fig. 9, A and B, SC68376 markedly abolished TGF-β1-induced caspase-3 activation in renal tubular epithelial cells but did not affect basal caspase-3 activation induced by staurosporine alone. Consistently, inhibition of p38 MAP kinase activation by SC68376 also obliterated TGF-β1 potentiation of cell apoptosis in a time- and dose-dependent fashion (Fig. 9, C and D). These results indicate that TGF-β1 promotes renal tubular epithelial cell apoptosis by a p38 MAP kinase-dependent mechanism.

**DISCUSSION**

Although TGF-β1 has long been alleged to play a critical role in the tubular atrophy characterized by epithelial cell loss (3, 9), there is little direct evidence demonstrating that TGF-β1 actually causes tubular epithelial cells to die in vitro. Incubation of cultured tubular epithelial cells with TGF-β1 at the concentration comparable with physiologic or pathophysiologic circumstances resulted in little or no appreciable cell death (see Figs. 1 and 2). This observation suggests that TGF-β1 signaling is certainly not sufficient to trigger tubular epithelial cells to commit suicide. In this study, we have demonstrated that TGF-β1 plays an imperative role in promoting tubular epithelial cell death, not by directly inducing cell apoptosis but rather by sensitizing these cells to a hit from secondary death cues. These findings are consistent with a two-hit model of tubular epithelial cell death, in which the primary role of hyperactive TGF-β1 signaling perhaps is to render tubular cells readily susceptible to a second hit from other death stimuli.

The necessity for two hits to induce tubular epithelial cell
death may potentially have its biologic advantages, which could provide an effective means for preventing renal tubules from unwanted, accidental cell loss under normal physiologic conditions. It is reasonable to speculate that such two-hit scenario will retain double checkpoints to ensure the death/survival of tubular cells under tight control in vivo. Transient TGF-β1 up-regulation without secondary hit may eventually leave tubular epithelial cells to survive. However, sustained activation of TGF-β1 signaling followed by secondary death challenges, a situation that presumably occurs under chronically injured conditions (32), could have lethal consequence to the tubular cells. Our two-hit working model is supported by the in vivo observation that the expression of both TGF-β1 and its type I receptor is specifically up-regulated in renal tubules of the obstructed kidneys (12, 32). Furthermore, this tubule-specific induction of TGF-β1 axis is an early event that takes place at 1 day after ureteral obstruction (32), a timing that precedes significant cell apoptosis in the kidneys. These spatial and temporal correlations between the activation of TGF-β1 signaling and tubular epithelial cell apoptosis imply that hyperactive TGF-β1 may play a critical role in promoting tubular cell death in the diseased kidneys, probably by priming these

**Fig. 8.** SC68376 specifically inhibits p38 MAP kinase activation by TGF-β1 in renal tubular epithelial cells. A, SC68376 inhibits p38 MAP kinase activation by TGF-β1 in a dose-dependent manner. HKC cells were pretreated with different doses of SC68376 as indicated for 30 min prior to incubation with 2 ng/ml of TGF-β1. Cell lysates were immunoblotted with either phospho-specific or total p38 MAP kinase, respectively. B, SC68376 at a high concentration (20 μM) does not influence Smad-2 activation by TGF-β1 in renal epithelial cells. C and D, neither TGF-β1 nor p38 MAP kinase inhibitor SC68376 affects ERK-1/2 (C) and JNK (D) phosphorylation in renal epithelial cells.

**Fig. 9.** Blockade of p38 MAP kinase activation by SC68376 abolishes TGF-β1 potentiation of caspase-3 activation and cell apoptosis. SC68376 inhibits TGF-β1 potentiation of caspase-3 activation in renal epithelial HKC cells, as demonstrated by Western blot analysis (A) and graphical presentation (B). C, SC68376 abolishes TGF-β1 potentiation of renal tubular cell apoptosis in a time-dependent manner. HKC cells were treated without or with 2 ng/ml of TGF-β1 in the absence or presence of 20 μM SC68376, followed by treated with staurosporine for various periods of time as indicated. Solid circle, staurosporine alone; solid triangle, TGF-β1 and staurosporine; open circle, SC68376 plus TGF-β1 and staurosporine. D, SC68376 abolishes TGF-β1 promotion of cell apoptosis in a dose-dependent way. HKC cells were treated with 2 ng/ml of TGF-β1, 1 μM staurosporine, and different doses of SC68376 as indicated. Data are presented as mean ± S.E. (n = 3). *, p < 0.05 versus staurosporine alone; **, p < 0.05 versus TGF-β1 plus staurosporine.
cells to commit suicide upon secondary death challenges.

Although the exact death cues responsible for tubular epithelial cell apoptosis in the obstructed kidneys in vivo remain to be identified, there are a plenty of candidates that could serve as the "second hit" to trigger apoptotic program in the tubular cells that once were primed by hyperactive TGF-β1 signaling. For example, in kidneys with persistent and complete ureteral obstruction, chronic hypoxia induced by a compromised interstitial blood flow could result in cellular ATP deprivation that might serve as an apoptosis trigger (33, 34). Likewise, infiltration of inflammatory cells as seen in the obstructed kidneys may contribute to cell death by producing pro-death ligand or cytokines (35–37). Furthermore, disruption of normal cell-to-cell and cell-to-matrix interactions in the diseased kidneys provides a hostile environment for tubular cell survival that ultimately leads cells to die. In this context, the suppression of E-cadherin expression in the obstructed kidneys, as reported previously (31), may act as a secondary hit that results in tubular cell death. E-cadherin is an epithelial cell adhesion receptor found within adherens-type junctions. Although it is widely recognized that E-cadherin plays an essential role in the maintenance of structural integrity of renal tubular epithilia (38), recent studies also implicate it as a major survival factor for tubular epithelial cells (39). In addition, the progressive deposition of extracellular matrix as seen in the chronically diseased kidneys will eventually disrupt normal cell-matrix interactions of renal tubules (40–42). Such disturbance of the microenvironment that surrounds the tubular cells could potentially function as a second hit for renal epithelial cell apoptosis. Collectively, there are a wide variety of factors present in the diseased kidneys that could conceivably work in concert with TGF-β1 to lead tubular epithelial cells to commit to die in vivo.

The finding that TGF-β1 only accelerates the activation of pro-caspase-9, but not pro-caspase-8, suggests that its action is probably mediated via a pathway involving mitochondria (43, 44). Both caspase-9 and caspase-8 are recognized as initial caspases whose activation triggers a cascade of activation process of downstream effector caspsases such as caspase-3 (45, 46). However, the upstream pathways leading to activation of caspase-9 and caspase-8 are believed to be different. Although caspase-8 activation is involved in death receptor pathway such as FasL and Fas, pro-caspase-9 cleavage and subsequent activation is coupled with the events associated with the release of cytochrome C from mitochondria and activation of Apaf-1 (47–49). The specific acceleration of caspase-9 activation by TGF-β1 suggests that its pro-apoptotic signaling is implicated in a pathway involved in mitochondria, although the details remain to be unraveled. Of note, TGF-β1 apparently only accelerates pro-caspase-9 activation but not increases the magnitude of active caspase-9 peak level (Fig. 3). For instance, TGF-β1 pre-incubation followed by treatment with death stimulus produced the peak of active caspase-9 3 h earlier over the control cultures. This phenomenon could be attributable to the assumption that endogenous pro-caspase-9 may be fully activated upon stimulation with death challenges in tubular epithelial cells.

The present study attempted to dissect the signaling pathways that lead to TGF-β1 potentiation of renal tubular cell apoptosis. It has been well documented that TGF-β1, through binding to its type II and type I receptors, elicits a wide range of cellular responses that modulate cell proliferation, differentiation, and apoptosis (10, 25, 50). Many of the signaling responses initiated by TGF-β1 are primarily mediated by intermediate signaling molecules Smad proteins (51–53). It appears clear that TGF-β1 activates at least two separate intracellular signal transduction pathways that result in both Smad and p38 MAP kinase activation in renal tubular cells (Fig. 5). Of interest, despite early and marked activation of Smad-2, Smad signaling appears not to mediate TGF-β1 promotion of cell death (see Figs. 5 and 7). This is in contrast to the TGF-β1-induced apoptosis in podocytes, in which Smad-7 is demonstrated to play a critical role (29). Of note, Smad-7 is an inhibitory Smad that can functionally antagonize receptor-regulated Smad (such as Smad-2 and -3) signaling by competitively binding to TGF-β receptors (10). Although not determined yet, it is reasonable to speculate that overexpression of inhibitory Smad-7 may also block Smad-3 activation in renal epithelial cells, in view of the fact that Smad-2 and Smad-3 are highly homologous and often activated simultaneously by TGF-β1 (51). Of interest, neither endogenous Smad-7 in renal tubular epithelial cells is induced by TGF-β1 (data not shown), nor does overexpression of exogenous Smad-7 affect cell death/survival under basal conditions. Furthermore, although overexpression of Smad-7 abolished Smad-2 phosphorylation and activation in response to TGF-β1 stimulation (Fig. 6), it failed to obliterate renal tubular cell apoptosis (Fig. 7). Therefore, unlike in podocytes, it appears obvious that TGF-β1 promotes renal tubular cell apoptosis via a mechanism independent of Smad signaling.

Our results provide a mechanistic link between TGF-β1 promotion of cell apoptosis and p38 MAP kinase activation (see Figs. 8 and 9). The p38 MAP kinase belongs to a subfamily of the MAP kinases and is activated primarily in response to stress, as well as cytokine stimulation (54, 55). Earlier studies suggest that different subfamilies of MAP kinases play a distinct role in regulating cell apoptosis, with ERK-MAP kinase as pro-survival and p38 MAP kinase and JNK as pro-death signals (56–58). Thus, the ratio among different MAP kinases may dictate cell fate in certain conditions. In this regard, we found no significant alterations in ERK-MAP kinase and JNK, suggesting that the p38 MAP kinase activation likely plays a predominant role in mediating TGF-β1-induced cell death. The requirement of p38 MAP kinase activation for mediating TGF-β1-induced apoptosis is illustrated by abrogating cell death after blockade of its activation (Fig. 9). Of note, a sustained, but not transient, p38 MAP kinase activation may also be of importance in inducing cell apoptosis, because a long period of pre-incubation with TGF-β1, which induces sustained p38 MAP kinase activation (Fig. 5B), is required for optimal potentiation of staurosporine-induced cell death (Fig. 1G). Thus it is concluded that a p38 MAP kinase-dependent, Smad-independent signaling mediates TGF-β1-induced renal tubular cell apoptosis.

In summary, we have shown in this report that TGF-β1 dramatically potentiates cell apoptosis triggered by other death cues by accelerating the activation of caspase-9 and caspase-3 in tubular epithelial cells. Thus, hyperactive TGF-β1 signaling promotes renal interstitial fibrogenesis not only by its well documented profibrotic actions but also by its potentiation of tubular epithelial cell apoptosis. Moreover, our results unravel a novel mode of TGF-β1 action in the regulation of cell death/survival, in which it primarily renders the tubular epithelial cells readily susceptible to secondary death cues rather than directly inducing them to die. This action of TGF-β1 in promoting cell death appears to be mediated by a mechanism dependent of p38 MAP kinase but independent of Smad signaling.

Acknowledgment—We thank Dr. P. ten Dijke for generously providing Smad-7 expression vector.

REFERENCES

1. Klahr, S., and Morrissey, J. (2002) Am. J. Physiol. Renal Physiol. 283, F861–F875
2. Remuzzi, G., and Bertani, T. (1998) N. Engl. J. Med. 339, 1448–1456
3. Chevalier, R. L. (1999) Pediatr. Nephrol. 13, 612–619
