Curcumin blocks multiple sites of the TGF-β signaling cascade in renal cells

JENS GAEDKE, NANCY A. NOBLE, and WAYNE A. BORDER

Fibrosis Research Laboratory, Division of Nephrology, University of Utah, Salt Lake City, Utah; and Med Klinik m.S. Nephrologie, Charité, Campus Mitte, Humboldt Universität, Berlin, Germany

Curcumin blocks multiple sites of the TGF-β signaling cascade in renal cells.

Background. Over-expression of transforming growth factor-β (TGF-β) contributes greatly to fibrotic kidney disease. The activator protein-1 (AP-1) inhibitor curcumin, a polyphenolic compound derived from Curcuma longa, has been shown to reduce collagen accumulation in experimental pulmonary fibrosis. Here, we investigate curcumin’s ability to modulate TGF-β’s profibrotic actions in vitro.

Methods. NRK49F rat renal fibroblasts were stimulated with TGF-β (5 ng/mL), and the effects of curcumin on TGF-β-regulated genes, TGF-β receptors, and phosphorylated SMAD isoforms were analyzed by Northern blotting, enzyme-linked immunosorbent assay (ELISA), and Western blotting. The effects of c-jun depletion on TGF-β–regulated gene and protein expression were analyzed with RNAi.

Results. When applied 30 minutes before TGF-β, curcumin dose dependently and dramatically reduced TGF-β–induced increases in plasminogen activator inhibitor-1 (PAI-1), TGF-β1, fibronectin (FN) and collagen I (Col I) mRNA, and in PAI-1 and fibronectin protein. Prolonged curcumin treatment (> 6 h) significantly reduced TGF-β receptor type II levels and SMAD2/3 phosphorylation in response to added TGF-β. Depletion of cellular c-jun levels with a RNAi method mimicked the effects of curcumin on expression of TGF-β1, FN, and Col I, but not PAI-1.

Conclusion. Curcumin blocks TGF-β’s profibrotic actions on renal fibroblasts through down-regulation of TGFRII, and through partial inhibition of c-jun activity. These in vitro data suggest that curcumin might be an effective antifibrotic drug in the treatment of chronic kidney disease.

The accumulation of extracellular matrix proteins is the key feature of chronic fibrotic kidney disease. Transforming growth factor-beta (TGF-β) is central to the development of fibrosis through its stimulating effect on matrix protein generation and its inhibitory effect on matrix protein removal [1]. Expression of TGF-β is elevated in multiple forms of experimental and human kidney disease, ranging from diabetic nephropathy and glomerulonephritis to tubulointerstitial nephritis. Experimental therapies aimed to reduce fibrosis have been shown to reduce TGF-β over-expression whether they directly target TGF-β or not. Thus, direct targeting of TGF-β may well be a valid therapeutic approach. Based upon significant recent progress elucidating TGF-β’s complex signaling cascade, it now appears possible to attempt therapeutic strategies aimed at disrupting TGF-β signaling [2, 3].

In this study we evaluated the antifibrotic effect of curcumin, a natural polyphenolic compound because it had been shown to limit the accumulation of collagen fibers in a TGF-β–driven model of fibrotic lung disease [4]. In addition, curcumin has been shown to be an inhibitor of the transcription factor c-jun/activator protein-1 (AP-1) through its ability to directly bind to c-jun and block its interaction with its DNA binding site [5–8].

Using cultured renal fibroblasts we tested the hypothesis that curcumin inhibits TGF-β–induced increases in fibrotic matrix components. We further hypothesized that curcumin acts by inhibiting TGF-β signaling by inhibiting the action of c-jun.

METHODS
Reagents

Cell culture reagents, curcumin, and all other reagents were from Sigma (St. Louis, MO, USA). TGF-β1 was purchased from R&D Systems (Minneapolis, MN, USA). Fetal calf serum (FCS) was from Hyclone (Logan, UT, USA). Curcumin was dissolved in 100% ethanol as a stock solution of 20 mmol/L, and was kept protected from light at 20°C.

Cell culture

Primary rat mesangial cells were derived from rat glomeruli and used between passages 5 to 8. Rat renal fibroblasts from American Type Culture Collection (NRK49F; Manassas, VA, USA) were propagated in complete RPMI 1640 with 10% FCS. Cells were seeded in 6-well plates, grown to confluency, and rested in medium.
without FCS overnight. The next morning, cells were treated with curcumin at the indicated doses and times, followed by TGF-β treatment at 5 ng/mL. Preliminary experiments showed that curcumin was nontoxic at doses of 0 to 10 μmol/L, and that toxicity occurred at doses of 20 μmol/L in NRK49F fibroblasts and rat mesangial cells (Table 1). Initial control experiments included solvent (ethanol) at doses of up to 1 μL/mL, which did not affect TGF-β effects or cell viability (data not shown).

Cells were harvested after six hours for determination of RNA levels, and after 24 hours for analysis of TGF-β-regulated protein expression.

### Northern blotting

After treatment, RNA was harvested with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Northern blotting was performed using Ambion’s NorthernMax Gly System (Austin, TX, USA). Probes were labeled with P-32 using the Strip-EZ Labeling kit (Ambion), and hybridized overnight in UltraHyb hybridization buffer (Ambion). After washing, blots were exposed to films that were scanned and analyzed by densitometry. Optical density (OD) values were standardized to 18s rRNA bands to correct for loading variations.

### Western blotting

For Western blotting, treated cells were harvested at the indicated times by the addition of ice-cold lysis buffer for 15 minutes. After that, an aliquot was removed for protein determination, and the remainder of the sample was boiled at 95°C after the addition of 4× Laemmli buffer. Samples were run on Novex 10% SDS-PAGE gels (Invitrogen), and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes. After blocking with 5% dry milk in Tris-buffered saline with 0.1% Tween (TBST), blots were incubated with antibodies in 1% bovine serum albumin (BSA) in TBST for 1 hour at room temperature or overnight at 4°C. The following antibodies were used: plasminogen activator protein-1 (PAI-1; American Diagnostica, Inc., Greenwich, CT, USA); TβRII (C16), TβRI (R20), AT1R (N10), c-jun (D), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p-SMAD2/3 (Calbiochem, San Diego, CA, USA), beta-actin (Sigma). All secondary antibodies were from Santa Cruz Biotechnology. Blots were developed with chemiluminescence with the ECL reagent from Amersham (Piscataway, NJ, USA).

Blots were also stained for beta-actin to control for loading variations. For PAI-1 Western blots, aliquots from the cell culture medium were removed from the dish, centrifuged shortly, and boiled for 5 minutes after the addition of 4× Laemmli buffer. The cell layer was harvested and analyzed for protein content.

### ELISA

Fibronectin enzyme-linked immunosorbent assay (ELISA) measurements were performed as previously described [9].

### siRNA experiments

RNA interference experiments were performed using a 21-mer siRNA molecule that targets bp 445 to 466 of the rat c-jun molecule (Genbank accession # X 17163, sequence of sense strand: 5′-GAUUCUGAGCAGAGCAUGt-3′, sequence of antisense strand: 3′-ttCUAAGACUCUCUGUCGUAC-5′). Both strands were synthesized at the University of Utah DNA Synthesis Core Facility, and contain at their 3′-ends two DNA thymidines. Annealing was performed as suggested by Elbashir et al [10]. Before transfection, fibroblasts were rested in 1% FCS medium overnight and then transfected with Oligofectamine (Invitrogen) and siRNA according to the manufacturer’s instructions, and as indicated in the figure legends. Forty-eight hours after transfection, cells were harvested as described above, and c-jun content was analyzed by Western blotting. To test the effects of c-jun depletion on TGF-β effects, cells were treated with TGF-β for 6 hours for RNA experiments, or 24 hours for protein experiments as described above after c-jun depletion.

### Statistical analysis

Results were analyzed for statistical significance by t test with Bonferroni correction. A P value of < 0.05 was regarded as significant.

### RESULTS

#### Dose-dependent curcumin effects on TGF-β–induced expression of profibrotic genes

TGF-β treatment up-regulates mRNA expression of several profibrotic genes in quiescent renal fibroblasts. As
Curcumin effects on TGF-β-induced gene expression over time

To determine whether the inhibition of TGF-β effects by curcumin is stable over time, we incubated fibroblasts with or without curcumin pretreatment (30 minutes) with TGF-β for up to 24 hours. Figure 2 shows that curcumin can block TGF-β induction of PAI-1 and FN for up to 8 hours. The effect of this short pretreatment is lost between 8 and 24 hours.

Time-dependent curcumin effects on TGF-β: Effect of pre- and post-treatment with curcumin relative to TGF-β

Although the inhibitory effect of curcumin on TGF-β–mediated gene transcription could be seen after a rather short pretreatment of 30 minutes, we wondered if curcumin was also effective if applied to cells after TGF-β. As Figure 3 shows, curcumin could be applied to cells as much as 2 hours after TGF-β and still significantly reduce TGF-β effects on PAI-1 and FN. However, maximal inhibitory effects were observed with curcumin pretreatment. TGF-β–induced PAI-1 was maximally reduced when curcumin was applied 1 hour before TGF-β, and was lost after 24 hours. FN expression remained significantly inhibited even after 24 hours of curcumin pretreatment (Fig. 3).

Curcumin effects on TGF-β–induced protein expression

The effect of curcumin treatment on TGF-β–induced PAI-1 and FN protein production was measured in cell culture supernatant that was derived from cells treated for 24 hours with TGF-β after a short (30 minutes) pretreatment with curcumin. As Figure 4 shows, TGF-β induces a strong increase in PAI-1 (Fig. 4A and B) and FN (Fig. 4C) released into the supernatant. These TGF-β effects are blocked dose dependently by curcumin (Fig. 4).
Curcumin reduces TGF-β receptor type II expression on renal fibroblasts

Binding of TGF-β to its type II receptor starts the interaction of a complex chain of signaling molecules, and finally leads to TGF-β–induced gene transcription. To determine which part of TGF-β signaling was changed by curcumin we investigated the expression of TGF-β receptors I and II after curcumin treatment. As Figure 5 shows, there is a dose-dependent decrease in TβRII protein expression after 8-hour treatment with curcumin. This effect is small and nonsignificant at low doses of curcumin, but becomes large and highly significant at doses of 5 to 10 µmol/L curcumin. In addition, as shown in Figure 6, cells must be treated for at least 6 hours with curcumin before any down-regulation of TβRII occurs. The protein expression of the type I TGF-β receptor (TβRI) and the unrelated angiotensin type I (AT1R) cell surface receptors remain unchanged by curcumin treatment (Fig. 6).

Prolonged curcumin treatment reduces TGF-β–induced phosphorylation of SMAD 2/3

To show that the down-regulation of TβRII by curcumin is of functional significance, we indirectly measured the kinase activity of the TGF-β receptor complex by analyzing SMAD2/3 phosphorylation after TGF-β treatment. SMAD proteins are phosphorylated by the activated TβR complex, and subsequently translocate to the nucleus, where they act as DNA-binding transcription factors. Treatment of quiescent cells with 5 ng/mL TGF-β profoundly increases phosphorylated SMAD 2/3, which is detectable as early as 1 minute after treatment, and reaches a plateau after 5 minutes of treatment (Fig. 7). Pretreatment with curcumin for 8 hours blocks SMAD phosphorylation by about 50%. Our conclusions, based on the data presented in Figures 5 to 7, are that prolonged treatment with curcumin at higher doses for several hours blocks the TGF-β signaling chain by interfering with TβRII expression and SMAD phosphorylation. While this may explain the inhibitory effects of curcumin on TGF-β–induced proteins after 24 hours of TGF-β treatment (shown in Fig. 4), it fails to explain the profound inhibitory effect seen after only 30 minutes of pretreatment shown in Figures 1 to 3.
Fig. 5. Curcumin dose dependently reduces TGF-β receptor type II (TβRII) expression. Fibroblasts were incubated with curcumin at the indicated dose for 8 hours. TβRII protein expression was analyzed by Western blot along with β-actin as a loading control. (A) Results of a typical experiment. (B) The mean ± SD of values obtained from densitometric analysis of all individual experiments. *P < 0.01 vs. control.

Effects of curcumin on mesangial cells stimulated by TGFβ

To exclude that the observed effects of curcumin are dependent on the specific genetic background of a single, transformed cell line, we also analyzed its effects in primary rat mesangial cells. While somewhat similar to renal fibroblasts, these mesangial cells are not transformed, and possibly represent the in vivo situation more closely than a cell line. Figure 8 shows the effects of curcumin treatment in mesangial cells (passage 5–8). As Figure 8A shows, treatment of mesangial cells with curcumin blocks the induction of PAI-1 and FN mRNA by TGFβ. Similar results were obtained with NRK52E cells, a renal epithelial cell line (data not shown). Similar to the results obtained in fibroblasts, we also found that prolonged curcumin treatment (8 hours) led to a reduction of TGFβ receptor type II levels on mesangial cells (Fig. 8B). Thus, the inhibitory effect of curcumin on TGFβ signals can be observed in multiple renal cell types.

Down-regulation of c-jun expression mimics curcumin effects on TGF-β–induced gene expression

Curcumin acts as an inhibitor of the transcription factor c-jun by directly inhibiting the binding of the c-jun/AP-1 complex to its cognate DNA sequence. Because activation of c-jun/AP-1 by TGF-β has been shown to be involved in TGF-β–induced fibronectin synthesis, we wondered if the AP-1 inhibitory effect of curcumin might be responsible for the inhibition of TGF-β effects after short treatment periods.

To address this issue, we measured the effect of reduced levels of cellular c-jun, the main component of the AP-1 complex, on TGF-β–regulated gene expression. To reduce cellular c-jun protein, we used an RNA interference (RNAi) technique. RNAi is induced in
mammalian cells by short interfering RNAs (siRNA), which are double-stranded, 21-base pair RNA molecules that are homologous to the targeted RNA, and induce selective destruction of target RNA through a multi-enzyme complex (for a recent review, see [11]). As Figure 9 shows, transfection of a siRNA molecule that targets bp 445 to 466 of c-jun mRNA. Total c-jun expression was measured by Western blot (A) and standardized for densitometric analysis to β-actin levels (B). *P < 0.01 vs. control.

As Figure 10 shows, c-jun–depleted renal fibroblasts produce slightly less TGF-β, FN, and COL I at baseline, and show a significantly reduced response to treatment with TGF-β. Interestingly, c-jun depletion slightly increases PAI-1 mRNA levels both in the absence of added TGF-β (second bar of Fig. 11), and when TGF-β is present (third and fourth bars, Fig. 11). These effects are also seen when TGF-β–induced protein expression is analyzed (Fig. 12). Baseline and TGF-β–induced PAI-1 protein in cell culture supernatant is increased after
c-jun depletion, and the TGF-β-induced increase in fibronectin levels is reduced to baseline. These data show that curcumin effects on TGF-β-induced gene transcription are mimicked to a great degree by inhibition of c-jun/AP-1.

**DISCUSSION**

In this study, we analyzed the antifibrotic properties of curcumin in renal cells. The main finding of our study is that curcumin blocks TGF-β-induced expression of several mediators of fibrosis. Furthermore, this antifibrotic effect of curcumin is mediated through down-regulation of TβRII expression, and probably through inhibition of the transcription factor c-jun/AP-1.

Curcumin is a naturally occurring polyphenolic compound derived from the root of *Curcuma longa*, and is consumed in high quantities in India, where it is used as a spice and as an anti-inflammatory compound in traditional medicine. Increasingly, its therapeutic effects are being investigated in well-defined models of disease, such as inflammatory hepatic and pancreatic disease [12–14] and pulmonary models of inflammation-triggered fibrosis [4, 15]. The antifibrotic effect of curcumin was first demonstrated in the bleomycin model of pulmonary fibrosis, where curcumin application prevented collagen accumulation [4]. No mechanistic explanation was proposed in that study. Here, we show that curcumin blocks the action of TGF-β, which drives fibrosis in the bleomycin model, and is the predominant fibrotic cytokine in renal disease.

Limiting TGF-β effects has been shown to be beneficial in a number of fibrotic renal disease models, and there is good preliminary data supporting this concept in human fibrotic diseases. TGF-β over-expression has been linked to angiotensin II [16], and as large clinical trials have shown, anti-angiotensin therapy slows the progression of renal disease in humans. However, clinical progression to end-stage renal disease (ESRD) is not halted, and experimental studies show that even maximal angiotensin blockade with ACEi or ARBs, or the combination of both, fails to normalize TGF-β levels in experimental fibrotic renal disease [9]. Therefore, therapies that directly target TGF-β or components of the TGF-β signal transduction cascade are of great interest. As our data show, curcumin inhibits the TGF-β-induced increase of several genes and proteins that either contribute directly (collagen I, fibronectin) or indirectly (TGF-β1, PAI-1) to the accumulation of matrix proteins in renal disease. The inhibitory effect of curcumin was quite profound because TGF-β effects were almost completely blocked for an extended time even when curcumin was given after TGF-β stimulation was initiated. While this manuscript was prepared, an inhibitory effect of...
Curcumin on TGFβ expression was reported in the model of amiodaron-induced lung fibrosis, in which curcumin reduced TGFβ expression and subsequent fibrosis [17]. In an attempt to elucidate mechanisms underlying the inhibitory effect of curcumin, we analyzed key components of TGFβ signal transduction. TGFβ signaling is initiated by TGFβ binding to its cell membrane receptor and formation of the activated receptor complex, which then phosphorylates intracellular messenger molecules like SMAD proteins [2]. Our data show that curcumin decreased TGFβ receptor II protein levels. This was associated with reduced SMAD2/3 phosphorylation after curcumin treatment, indicating reduced biological activity of the activated TGFβ receptor complex. The effect of curcumin on TβRII expression was quite specific; no down-regulation of either TβRI, which has high homology to TβRII, or the unrelated angiotensin receptor type I was observed. Because curcumin inhibits the activity of the transcription factor c-jun [5–8], and also has nuclear factor κB (NFκB) inhibitory activity [12, 18–21], it is possible that curcumin limits essential transcription factor availability, which subsequently leads to reduced TβRII mRNA expression. AP-1 sites have been reported in human, canine, and mouse TβRII promoters. AP-1 activity correlates with inducible TβRII promoter activity in renal epithelial cells stimulated with high glucose of ANG II [15, 22]. Whether AP-1 is also involved in maintaining unstimulated, constitutive TβRII expression, which was analyzed in this study, is presently unclear and demands further investigation.

Reducing endogenous TβRII activity indirectly by over-expressing soluble TβRII has been shown to block TGFβ action and fibrosis in fibrotic kidney disease in vivo [23]. Thus, down-regulation of TβRII by curcumin provides an attractive explanation for the antifibrotic curcumin effects observed in vivo in the bleomycin model.

The effects of curcumin we observed in vitro, however, suggest that curcumin acts in at least two ways. Dose and time experiments showed that high doses of curcumin (5 to 10 μmol/L) and prolonged incubation times (at least 6 hours) were necessary to induce down-regulation of TβRII. Thus, while decreased TβRII may explain the observed reduction in TGFβ–induced proteins with curcumin and TGFβ treatment for 24 hours (Fig. 4), decreased TβRII cannot explain the observed action of curcumin to markedly reduced TGFβ effects with only a 30-minute curcumin treatment (Figs. 1 to 3). We therefore investigated whether the inhibitory effect of curcumin on c-jun activity might explain these early effects. TGFβ induces c-jun transcriptional activity [24], and induction of fibronectin expression has been directly linked to c-jun transcriptional activity [25]. Our data show that down-regulation of c-jun protein by RNA interference was associated with reduced induction of FN, TGFβ, and COLI mRNA and FN protein after TGFβ treatment. Although indirect, these data suggest that c-jun may regulate TGFβ–induced genes. Consistent with this idea are data published by Hocevar et al [25], showing that TGFβ–induced fibronectin production, but not PAI-1 production, is dependent on c-jun signaling. Using dominant negative mutants of c-jun terminal kinase (JNK), which phosphorylates and activates c-jun, and of upstream activators of JNK, they were able to show that TGFβ induces FN synthesis almost exclusively through a c-jun–dependent pathway, with little or no contribution of the classic SMAD pathway. Our data suggest that regulation of TGFβ target genes, COLI and TGFβ, in addition to FN, may also be dependent on c-jun activity.

While c-jun depletion decreased expression of TGFβ, FN, and COLI, c-jun depletion increased PAI-1 mRNA and protein (Fig. 12), suggesting that c-jun negatively regulates PAI-1 expression in NRK49F cells. This conclusion is consistent with findings published by Verrecchia et al, who analyzed the interaction of c-jun with SMAD proteins in TGFβ signaling [26–28]. Using an artificial promoter-reporter construct derived from the PAI-1 promoter, they showed that in both unstimulated cells and TGFβ–stimulated cells, c-jun physically interacts with SMAD proteins, blocking their DNA-binding ability and thereby limiting basal and TGFβ–induced stimulation of the PAI-1 promoter. These data offer a mechanistic explanation for the effects of c-jun depletion on PAI-1 expression seen in our experiments. However, they fail to explain the inhibitory effect of curcumin on PAI-1 induction after TGFβ treatment seen in our study (Figs. 1 and 2).

The implications of our study are several. First, we have demonstrated that curcumin is a potent blocker of TGFβ effects in renal cells. Given the proven ability of curcumin to block the accumulation of extracellular matrix proteins in a pulmonary fibrosis model, and the known pivotal role of TGFβ in renal fibrosis, it is reasonable to assume that curcumin will be beneficial in chronic renal disease. Second, we have been able to show that the antifibrotic effect of curcumin in vitro is mediated by at least 2 mechanisms, down-regulation of TβRII and inhibition of c-jun activity. Third, we have successfully used RNA interference technology to study signaling pathways in renal cells. Given the growing concern over the specificity of small molecule inhibitors of signaling molecules, shutting down signaling pathways with RNAi at precisely targeted sites offers an attractive and valid technical approach.

One possible caveat of our study is the direct applicability of the doses used in our study to the situation in vivo. Curcumin is highly lipohilic, cannot be injected intravenously in long-term experiments, and has a poor oral bioavailability [29–31]. Although doses of up to 8 g per day have been reported to be both safe and efficacious in humans [32], it is unclear if oral dosing will provide the drug levels necessary to achieve the effects demonstrated...
in this study. The recent development of water-soluble curcumin analogs [33, 34], which could presumably be injected intravenously, might aid in the further in vivo testing of this compound.

CONCLUSION
Curcumin is a novel antifibrotic substance that reduces TGF-β effects and might be a potential add-on therapeutic to the standard anti-angiotensin therapies currently used in the treatment of chronic renal disease.

Reprint requests to Nancy A. Noble, Fibrosis Research Laboratory, 391 Chipeta Way, Suite E, Salt Lake City, UT 84108. E-mail: Nancy.Noble@hsc.utah.edu

REFERENCES