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**Interleukin-1 beta regulates proximal tubular cell transforming growth factor beta-1 signalling**

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**Abstract**

**Background.** Increased transforming growth factor beta-1 (TGF beta) expression in the kidney is central not only to the pathogenesis of tubulointerstitial fibrosis but also in repair following acute injury. Recent work suggests that pro-inflammatory cytokines may determine epithelial cell responses to TGF beta in the contexts of acute injury and chronic wounding.

**Methods.** In this study, we examined the effects of interleukin-1 beta (IL-1) on proximal tubular cell (PTC) response to TGF beta.

**Results.** IL-1 induced the rapid activation of NF-κB in PTC. This was associated with inhibition of Smad2 and Smad3 signalling. NF-κB activation by IL-1 was transient, with a change from p65/p50 heterodimer to p50/p50 homodimer formation by 24 h and a switch to enhanced Smad signalling response to TGF beta. This was associated with IL-6 generation and prevented by IL-6 receptor blockade.

**Conclusion.** In summary, IL-1 has a biphasic effect on PTC TGF beta signalling, with early NF-κB-mediated inhibition and delayed sensitization via an autocrine IL-6 loop. These results provide mechanistic insight into how
acute and chronic inflammation help define epithelial cell response to TGF beta, and hence how TGF beta can have apparently contradictory roles, being involved in controlled healing following acute injury on one hand, yet the principal promoter of scarring in chronic disease on the other.

**Keywords:** fibrosis; inflammation; IL-1; Smad; TGF beta

**Introduction**

Chronic tissue injury leads to fibrosis in many organs, including the kidney. Transforming growth factor beta-1 (TGF beta) is not only a key profibrotic cytokine [1] but also plays an important role in repair following acute injury [2]. Acute inflammation is also associated with repair following injury, but more prolonged inflammation is associated with renal fibrosis [3,4]. The mechanisms by which inflammation may modulate profibrotic responses to TGF beta in the kidney are largely obscure. In other contexts, pro-inflammatory cytokines such as interferon gamma are important negative regulators of TGF beta signalling in acute inflammation [5]. In contrast, recent work suggests that chronic inflammation may promote fibrosis by enhancing epithelial cell signalling responses to TGF beta [6,7], and we have previously shown that the pro-inflammatory cytokine interleukin-6 (IL-6) increases signalling response to TGF beta [24].

Smads 2 and 3 transduce the TGF beta signal to the nucleus. Deletion of Smad3 prevents TGF beta-induced epithelial from mesenchymal transition (EMT) and attenuates fibrotic sequelae [8]. Positive and negative effects of interleukin-1 beta (IL-1) on Smad signalling have been reported. IL-1 induces local Smad3-dependent tissue fibrosis when over-expressed in the murine lung [9] or peritoneum [10], and chronic IL-1 administration induces EMT [11], a key step in fibrogenesis [12]. Conversely, IL-1 inhibits renal mesangial cell TGF beta generation [13] and delays TGF beta-induced fibroblast for myofibroblast differentiation [14]. Furthermore, interleukin receptor antagonist-deficient mice, in whom IL-1 signalling is enhanced, exhibit suppressed TGF beta signalling [15].

Therefore, IL-1 is reported to both stimulate and inhibit TGF beta signalling via unknown mechanisms, and modulation of TGF beta signalling by IL-1 may play a significant role in determining proximal tubular cell fate following renal injury. In this paper, we investigate the effects of IL-1 on PTC TGF beta signalling. We show that IL-1 induces rapid but transient inhibition of PTC TGF beta signalling, with a subsequent switch to an enhanced signalling response to TGF beta following prolonged IL-1 stimulation. We delineate the independent mechanisms by which these effects occur. These results provide mechanistic insight into the differential effects of acute and chronic inflammation on TGF beta-dependent fibrosis.

**Materials and methods**

Antibodies used were as follows:

- Rabbit polyclonal anti-phospho-Smad3 (Cell Signalling Technology, CA, USA, Catalog Number 9514s), dilution 1:1000
- Anti-Rabbit IgG-HRP conjugated (Santa Cruz, CA, USA, SC2004), dilution 1:10 000
- Rabbit anti-Smad3 (Zymed Laboratories, CA, USA, 51-1500)
- Pan-specific anti-TGF beta (R and D Systems, Abingdon, UK)
- Rabbit Anti-IκB a (Santa Cruz, sc-371), dilution 1:500
- Rabbit Anti-IκB b (Santa Cruz, sc-945), dilution 1:500
- Rabbit Anti-IκK a/b (Santa Cruz, sc-7607), dilution 1:500
- Rabbit Anti-P-IκK a/b (Cell Signalling Technology, MA, USA, 2687), dilution 1:1000
- Rabbit Anti-p65 (Santa Cruz, sc-109), dilution 1:5
- Rabbit Anti-p50 (Santa Cruz, sc-7178), dilution 1:5
- Rabbit Anti-Smad7 (abCam, Camb, UK, ab5825), dilution 1:500

Other reagents were used as follows:

- Recombinant human IL-1 beta (R and D Systems) and TGF beta (R and D Systems)
- Human IL-6 Sandwich ELISA (DY206, R and D Systems)
- Human TGF beta Sandwich ELISA (DY240, R and D Systems)
- Mouse monoclonal anti-IL-6 receptor blocking antibody (R & D, MAB227), dilution 1:500

**Cell culture**

HK-2 cells [human renal proximal tubular epithelial cells immortalized by transduction with human papilloma virus (HPV) 16 E6/E7 genes] were cultured in DMEM/Ham’s F12 medium (Gibco BRL, Paisley, UK) supplemented with 10% FCS (Biological Industries Ltd, Cumbernauld, UK), 2 μM t-glutamine (Gibco BRL), 20 mM HEPES buffer (Gibco BRL), 5 μg/ml insulin, 5 μg/ml transferrin (Sigma, Poole, UK), 40 ng/ml hydrocortisone (Sigma) and 5 ng/ml sodium selenite (Sigma). The cells were grown at 37°C in 5% CO2 and 95% air. A fresh growth medium was added to the cells every 3–4 days until confluent. With the exception of the cells used for transfection, the remaining cells were growth-arrested in the serum-free medium for 48 h before use in experiments. All experiments were performed under serum-free conditions.

**Immunoblotting**

The cell extracts were prepared in the sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 min at 95°C. The proteins were visualized using enhanced chemiluminescence (Amersham, UK) according to the manufacturer’s instructions. Densitometry was performed using the Quantity One Image Analysis Software on a ChemiDoc film scanner (BioRad, Hertfordshire, UK).

**ELISA**

The concentration of TGF beta and IL-6 in cell culture supernatant was assayed with commercial sandwich ELISA kits (R and D Systems) used according to the manufacturer’s instructions.

**Transient transfection and reporter gene analysis**

The CAGA(4) Smad3 responsive reporter construct was a gift from Aristidis Moustakas [18]. The ARE and MF1 Smad2 responsive reporter constructs were a gift from Lalage Wakefield [19]. The pRL-CMV renilla control plasmid was purchased from Promega. Transient transfection and reporter gene analysis using HK-2 cells were performed as previously described [17]. For Smad3 experiments, 0.9 μg of the Smad 3/4-specific reporter SBE-Luc was transfected with 0.1 μg of pRL-CMV renilla (Promega, WI, USA) to control for transfection efficiency. For Smad2 responsive experiments, 0.45 μg of the Smad 2/4-specific promoter ARE-Luc was transfected together with 0.45 μg of its co-plasmid MF1 and 0.1 μg pRL-CMV renilla. Following lysis of the cells in the reporter lysis buffer (Promega), firefly and renilla luciferase contents were quantified using the Dual-Glo assay (Promega).

**Electrophoretic mobility shift assay**

Nuclear protein extraction and electrophoretic mobility shift assays for nuclear factor-kappaB (NF-κB) were performed as previously described.
IL-1 modulates TGF beta signalling

Fig. 1. IL-1 inhibits Smad signalling response to TGF beta. (A–C) HK-2 cells were treated with 1 ng/ml TGF beta ± 1 ng/ml IL-1 for time points to 60 min, before protein extraction and immunoblotting for phospho-Smad3 (A and B) or phospho-Smad2 (C). Blots were stripped and reprobed for total Smad3 or GAPDH to confirm equal protein loading. Representative immunoblots for three independent experiments are shown. (D and E) HK-2 cells were transfected with the Smad3 reporter construct CAGA(4) (D) or the Smad2 reporters ARE/MF1 (E) together with pRL-CMV renilla before incubation with 1 ng/ml TGF beta ± 1 ng/ml IL-1 for 6 h. Subsequently, firefly and renilla luciferase activities were assayed. Ratio of firefly to renilla luciferase is displayed and normalized to control. n = 3, mean ± SD is plotted.

Statistical analysis

Unless otherwise specified, statistical analysis between groups was performed using the unpaired Student’s t-test and for multiple groups by ANOVA, with a value of P < 0.05 considered to represent a significant difference. The data are presented as means ± SD of n experiments as indicated in figure legends. For each individual experiment, the mean of duplicate determinations was calculated.

Results

IL-1 rapidly inhibits Smad signalling response to TGF-beta 1

Initially, we evaluated the effect of co-administration of IL-1 with TGF beta on Smad signalling response. HK-2 cells were cultured for 1 h in the presence of 1 ng/ml TGF beta ± 1 ng/ml IL-1 before detection of Phospho-Smad3 by immunoblotting. Co-administration of IL-1 caused a minor reduction in Smad3 phosphorylation, compared to TGF beta alone (Figure 1A). Quantification by densitometry showed a reduction in the phospho-Smad3/total Smad3 ratio of 17–33% in the cells incubated with IL-1
Inhibition of TGF beta signalling by IL-1 occurs via NF-kB

NF-kB is an important downstream effector for IL-1 and has been linked to inhibition of TGF beta signalling [14,21]. The role of NF-kB in inhibition of Smad signalling was investigated using co-transfection of p65 and p50 over-expression vectors with the Smad3 reporter construct CAGA(4). Over-expression of p65 and p50 or p65 alone inhibited Smad2-dependent luciferase signal (Figure 1C). In related experiments, IL-1 inhibited the Smad2-dependent signal from the Smad2-responsive construct ARE-luc (Figure 1D). Taken together, these data show that in short-term co-administration, IL-1 inhibits Smad2 and Smad3 response to TGF beta.

Mechanism of sensitization of Smad Signalling by IL-1

Possible mechanisms for the observed increases in Smad2 and Smad3 signalling following chronic IL-1 exposure included increased TGF beta generation or activation, changes in TGF beta receptor sensitivity or changes in Smad degradation or dephosphorylation. NF-kB responsive elements are present in the TGF beta promoter, and IL-1 increases TGF beta generation in human alveolar epithelial cells [23]. Therefore, we quantified the release of TGF beta into the cell culture supernatant by PTC in response to IL-1. HK-2 cells were cultured with IL-1 (dose range 0–1 ng/ml) for 24 h before the assay of TGF beta generation by ELISA. No increase in TGF beta release was seen in response to IL-1 (Figure 5A).

To test whether the sensitizing effect of IL-1 on TGF beta required TGF beta receptor–ligand interaction, Smad3 reporter gene experiments were performed in the presence of TGF beta receptor blockade. Addition of a blocking antibody to the TGF beta type II receptor inhibited IL-1-mediated changes in Smad signalling in a dose-dependent fashion (Figure 5B), suggesting that the effect of IL-1 on Smad signalling was due to increased response to TGF-beta 1, rather than the activation of Smad signalling independent of TGF-beta receptors.

Role of IL-6 in sensitization of PTC to TGF beta by chronic IL-1

The above data, showing that sensitization to TGF beta by IL-1 was dependent on TGF beta receptors, but not
IL-1 modulates TGF beta signalling

Fig. 2. Inhibition of TGF beta signalling by IL-1 occurs via NF-κB. (A) HK-2 cells were transfected with an empty vector alone, empty vector and p65 over-expression vector, empty vector and p50 over-expression vector or p65 and p50 over-expression vectors for 24 h before incubation with TGF beta for 6 h. Control cells were incubated in the TGF beta-free medium. Equal amounts of DNA were transfected into all wells. N = 3, mean ± SD is plotted. The empty vector plus TGF beta versus p65 plus TGF beta P < 0.001, versus p65 and p50 plus TGF beta P < 0.001, versus p50 plus TGF beta P = not significant. (B) HK-2 cells were incubated with TGF beta (lane 1), TGF beta and SN50 (lane 2) TGF beta and IL-1 (lane 3) or TGF beta, IL-1 and SN50 (lane 4) for 1 h before lysis and analysis by immunoblotting for phospho-Smad3. Subsequently, blots were stripped and reprobed for total Smad3. phospho-Smad3 was not detectable in the absence of TGF beta (not shown). One representative experiment of three experiments giving similar results is shown. (C) HK-2 cells were transfected with CAGA(4) and pRL-CMV renilla before incubation for 6 h with the control medium, 1 ng/ml TGF beta ± IL-1 1 ng/ml or TGF beta + IL-1 + SN50. Ratio of firefly to renilla luciferase activity is shown, normalized to control cells. N = 3, mean ± SD is plotted.

Involving alterations in TGF beta generation and Smad dephosphorylation, suggested that IL-1 altered TGF beta signalling at the receptor level. No changes in TGF beta receptor expression were detected by immunoblotting in IL-1-treated cells (data not shown). We have previously shown that IL-6 sensitizes PTC to TGF beta signalling via a shift of receptors from the lipid raft to the non-raft component of the plasma membrane [24]. IL-6 may also be an important downstream mediator for IL-1 in chronic inflammation. Therefore, IL-6 generation in PTC in response to IL-1 was examined. Twenty-four hours of IL-1 stimulation with doses as low as 10 pg/ml led to significant IL-6 generation (Figure 6A). The time course of IL-6 generation was examined, using stimulation with 1 ng/ml IL-1. Increased IL-6 generation was seen after 3 h or more IL-1 stimulation, but not at earlier time points (Figure 6B).

Incubation with IL-1 at concentrations as low as 10–100 pg/ml led to significant IL-6 generation in PTC. To test whether this IL-6 was causally related to enhanced TGF
beta sensitivity, the cells were transfected with the Smad3 reporter CAGA(4) and incubated with IL-1 10 or 100 pg/ml or the control medium, for 24 h. Subsequently, luciferase response to 6 h TGF beta was quantified. Enhanced signal was seen in IL-1-treated cells (Figure 6C). Subsequently, the effect of a blocking antibody to the IL-6 receptor on TGF beta responsiveness in the cells stimulated with IL-1 for 24 h was determined. Phospho-Smad3 immunoblotting showed that IL-6 receptor blockade abrogated sensitization of Smad signalling by IL-1, without inhibiting baseline TGF beta response (Figure 6D and E).

Early inhibition and late enhancement of Smad signalling are independent effects

To test whether the early inhibitory and late stimulatory effects of IL-1 on TGF beta signalling were independent, we examined whether IL-1-mediated IL-6 generation required NF-kB signalling. Blockade of NF-kB with SN50 had no effect on IL-1-stimulated IL-6 generation (Figure 7A). Subsequently, the effect of NF-kB blockade on sensitization to TGF beta by IL-1 was examined. Addition of SN50 had no effect on sensitization to TGF beta by IL-1 (Figure 7B).
IL-1 modulates TGF beta signalling

These data are consistent with the early and late effects of IL-1 being independent of one another.

Discussion

Increased expression of TGF beta is widely recognized as a key stimulus to the progression of renal fibrosis after renal injury. However, TGF beta is not the sole cause of renal fibrosis, and the mechanisms by which other environmental cues alter response to TGF beta are of interest. Renal inflammation and macrophage influx adversely affect prognosis in chronic kidney disease, whereas acute inflammation in conditions such as acute tubular necrosis, acute tubulointerstitial nephritis and acute rejection following renal transplantation does not appear to have the same consequences.

Pro-inflammatory cytokines such as interferon gamma [5] and tumour necrosis factor alpha [21] have previously been shown to be important negative regulators of TGF beta signalling and exhibit anti-fibrotic activity (reviewed in [25]). More recently, sensitization to TGF beta, and hence profibrotic effects, has been described for pro-inflammatory stimuli. Specifically, in the liver, bacterial lipopolysaccharide-mediated toll like receptor 4 (TLR4) activation sensitizes hepatic stellate cells to TGF beta and leads to hepatic fibrogenesis [6]. Similar TGF beta-dependent effects are observed in PTC in which TLR2 is activated by leptospiral membrane proteins in vitro [7], but the differential effects of acute and chronic inflammation on TGF beta signalling are otherwise largely unstudied.

Here, we have shown that short-term IL-1 exposure inhibits PTC TGF beta signalling and that this inhibitory effect of IL-1 is mediated via NF-kB activation, which occurs rapidly and transiently following exposure of PTC to IL-1. After 6 h or more of IL-1 treatment, there is a switch from NF-kB p65/p50 heterodimer to p50/p50 homodimer formation, and IL-1 no longer inhibits TGF beta signalling.
Our data show clear inhibition of Smad signalling at early time points (Figure 1D and E), but the effect of IL-1 on Smad phosphorylation by TGF beta at these time points is relatively modest (Figure 1A–C) suggesting that NF-κB acts on Smad signalling predominantly beyond the level of Smad phosphorylation by the activated receptor complex. NF-κB has previously been shown to inhibit TGF beta-induced gene expression via sequestration of the transcriptional coactivator p300 by p65 NF-κB [26], and we speculate that this mechanism underlies our findings.

Independent of the early inhibition of TGF beta signalling by IL-1, we have shown that in PTC, longer term IL-1 stimulation leads to augmentation of Smad2 and Smad3 response to TGF beta, and to detectable Smad3 phosphorylation in the absence of exogenous TGF beta. Canonical TGF beta signalling involves binding of TGF beta to its type II
IL-1 modulates TGF beta signalling

Fig. 6. Sensitization to TGF beta by chronic IL-1 stimulation is mediated via an autocrine IL-6 loop. (A) Dose-dependent IL-6 generation following IL-1 stimulation. HK-2 cells were cultured in the medium containing IL-1 (dose range 0–1000 pg/ml) for 24 h before the assay of IL-6 in the supernatant by ELISA. N = 3, mean ± SD is plotted. P < 0.001 for IL-1 10, 100 and 1000 pg/ml compared to control. (B) Time course of IL-6 generation following IL-1. HK-2 cells were cultured in the medium containing IL-1 1 ng/ml for time points to 24 h before assay of IL-6 in the supernatant by ELISA. N = 3, mean ± SD is plotted. P < 0.0001 for time points of 3, 6, 12, 24 h compared to control, P = NS for earlier time points. (C) Sensitization to TGF beta by low-dose IL-1. HK-2 cells were transfected with the Smad3 reporter construct CAGA(4) together with pRL-CMV renilla before incubation with the control medium or IL-1 at doses of 10 and 100 pg/ml for 24 h. Subsequently, cells were PBS-washed, and incubated with the control medium or 1 ng/ml TGF beta for 6 h (IL-1 free). Firefly and renilla luciferase activities were assayed. N = 3, mean ± SD is plotted. (D) IL-6 blockade prevents sensitization of TGF beta signalling by IL-1. HK-2 cells were cultured in the medium containing IL-1 1 ng/ml and 1 ng/ml TGF beta ± IL-6 receptor-blocking antibody for 24 h. Control cells were cultured in the medium containing TGF beta alone. Whole cell lysates were immunoblotted for phospho-Smad3 and reprobed for total Smad3. One representative blot of three experiments giving similar results is shown. (E) Combined densitometry results of the blots from three experiments described in D. Mean phospho-Smad3/total Smad3 is blotted. TGF+IL-1 versus TGF alone, P < 0.05. TGF+IL-1 versus TGF+IL-1+IL-6 receptor antibody, P < 0.01. TGF+IL-1+IL-6 receptor antibody versus TGF alone, P = NS.

receptor and subsequent formation of a heteromeric complex of type I and II receptors, leading to type I receptor autophosphorylation, and subsequent R-Smad phosphorylation. Binding of hyaluronan to CD44v3 has previously been shown to lead to the activation of type I TGF beta receptors and to R-Smad phosphorylation without TGF beta ligand-receptor interaction or formation of the conventional activated receptor complex [32]. However, the augmentation of TGF beta signalling by IL-1 was prevented by a blocking antibody to the type II receptor, suggesting that it was dependent on TGF beta receptor/ligand interaction. Additionally, IL-1 did not alter TGF beta synthesis or Smad3
dephosphorylation. These results suggested that IL-1 enhanced response to endogenous/exogenous TGF beta via alteration in TGF beta receptor expression or distribution. Following TGF-beta stimulation of epithelial cells, TGF beta receptors remain active for at least 3–4 h, and continuous receptor activity is required to maintain active Smads in the nucleus and for TGF-beta-induced transcription [27]. Continuous nucleocytoplasmic shuttling of Smads means that receptor activity is continuously monitored, and that the levels of activated receptors in the cytoplasm directly dictate the concentration of active Smads in the nucleus.

TGF-beta receptors internalize into both caveolin- and EEA1-positive vesicles. Clathrin-dependent internalization into the EEA1-positive endosome promotes TGF-beta signalling. In contrast, the lipid raft-caveolar internalization pathway contains the Smad7-Smurf2-bound receptor and is required for a rapid receptor turnover [28]. We have previously shown that in PTC, TGF-β1 receptor distribution in the plasma membrane is regulated by stimuli including hyaluronic acid (via CD44) and IL-6, leading to changes in receptor degradation and signalling response to TGF beta [24,29].

IL-6 is a multifunctional cytokine produced by a variety of cells in response to IL-1 during infection, trauma and immunological challenge [30]. We have previously shown that IL-6 increases signalling response to TGF beta via a shift of receptors from lipid rafts to non-raft associated plasma membrane [24]. In our current work, IL-1 treatment led to increased PTC IL-6 generation, and IL-1 doses and times required to stimulate IL-6 generation mirrored those required to sensitize PTC signalling response to TGF beta.

A blockade of IL-6 signalling abrogated sensitization of TGF beta signalling by IL-1, confirming that in PTC, IL-1 augments Smad signalling response to TGF beta via an autocrine IL-6 loop.

The relative simplicity of TGF beta signalling pathways contrasts with the complexity and diversity of epithelial cell responses to this cytokine. Changes in epithelial cell response to TGF beta secondary to important environmental cues such as inflammation provide an explanation for the apparently contradictory roles of TGF beta in the kidney, suggesting how this cytokine can be involved in controlled healing following acute injury on the one hand, yet be the principal promoter of scarring in chronic disease on the other.

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Endoplasmic reticulum stress induces autophagy in renal proximal tubular cells

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Abstract

Background. Autophagy, an intracellular self-degradation system conserved throughout eukaryotes, plays an important role in a variety of biological processes, including cell death, development, cancer, defence against infection and neurodegeneration. However, little information about autophagy in renal tubular cells is available. We investigated the relationship of autophagy with endoplasmic reticulum (ER) stress in proximal tubular cells.

Methods. Immortalized rat proximal tubular cells were exposed to the classic ER stress inducers tunicamycin or brefeldin A. Autophagy was detected mainly by immunoblot analysis of LC3, a widely used marker of autophagy, and also by immunofluorescent cytochemistry of LC3 and electron microscopy. Biological significance of the phenomenon was studied using bafilomycin A1, an inhibitor of autophagosome degradation. Signal transduction pathways following ER stress were also investigated using inhibitors of the MAPK pathway.

Results. Both ER stress inducers significantly increased LC3-II as a marker of autophagy in immunoblot analysis. Immunocytochemistry of LC3 and electron microscopy also showed activation of autophagy by ER stress. Inhibition by bafilomycin A1 showed that autophagy following

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