Impaired TGF-β signalling enhances peritoneal inflammation induced by E. Coli in rats

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Abstract

**Background.** Peritonitis is a common and severe complication of peritoneal dialysis (PD). Although TGF-β is a key mediator in peritoneal fibrosis with chronic PD, its role in acute peritoneal inflammation remains unclear. **Methods.** Potential role of TGF-β signalling in acute peritonitis was investigated in a rat model by infecting peritoneum with E. coli and in primary culture of peritoneal mesothelial cells (PMC) by LPS. **Results.** We found that a single infection of E. coli caused an acute, but transient peritonitis by a significant increase in ascites white blood cells (WBC), peritoneal CD45+ leukocytes, upregulation of TNFα, activation of NF-κB/p65 and impaired peritoneal function (all \( P < 0.01 \)). Interestingly, spontaneous recovery of acute peritonitis occurred with upregulation of TGF-β1 and activation of Smad2/3, suggesting a protective role of TGF-β signalling in acute peritonitis. This was demonstrated by the finding that blockade of the TGF-β signalling pathway with gene transfer of Smad7 inactivated peritoneal Smad2/3 but worsened E. coli-induced, NF-κB-dependent peritoneal inflammation and peritoneal dysfunction (all \( P < 0.01 \)). Furthermore, studies in vitro also found that impaired TGF-β signalling by overexpression of Smad7 in PMC were able to overcome the inhibitory effect of TGF-β on LPS-induced, NF-κB-mediated peritoneal inflammation. **Conclusion.** Results from this study demonstrate that TGF-β signalling is essential in protection against acute peritoneal inflammation induced by bacterial infection. **Keywords:** NF-κB/p65; peritonitis; Smads; TGF-β

Introduction

Recurrent peritonitis and the subsequent development of peritoneal fibrosis are the major factors associated with peritoneal failure during a long-term peritoneal dialysis (PD) [1,2]. Recurrent peritonitis, including inflammatory cell infiltration and upregulation of pro-inflammatory cytokines, often results in peritoneal fibrosis, ultimately damaging peritoneal function [1–4]. Increasing evidence shows that TGF-β is a key mediator in dialysis-related peritoneal fibrosis [3–6]. The functional importance of TGF-β in peritoneal fibrosis is well documented by the findings that overexpression of TGF-β leads to fibrosis with loss of peritoneal ultrafiltration [7,8], which is prevented by anti-TGF-β...
treatment with decorin or octreotide in animal models with chronic PD [9, 10]. Most recently, we and other investigators have also shown that specific blockade of the downstream TGF-β pathway by overexpressing an inhibitor Smad7 is able to attenuate peritoneal fibrosis and inflammation in a rat model of chronic PD induced in either uremic or non-uremic rats [11–13]. All these studies demonstrate a pathogenic role of TGF-β1 in peritoneal fibrosis associated with chronic PD.

TGF-β is a multi-functional cytokine and growth factor with a diverse role in fibrosis and inflammation [14]. While current studies in the fibrogenic role of TGF-β in dialysis-associated peritoneal fibrosis are overwhelming, little attention has been paid to anti-inflammatory activities of TGF-β in peritoneal inflammation, an initial step in the development of peritoneal fibrosis, which was examined in the present study in a rat model of acute peritonitis induced by a single infection of E. coli. The role of TGF-β signalling in acute peritoneal inflammation was examined by blocking the TGF-β signalling pathway with overexpression of Smad7 in vivo and in vitro.

Subjects and methods

Animal model of acute peritonitis

Male Sprague-Dawley rats weighing 180–200 g were obtained from the Sun Yat-Sen Experimental Animals Facility. Acute peritonitis was induced by intraperitoneal injection of 10 mL sterile phosphate-buffered saline (PBS) containing 3.3 × 10⁷ colony forming units of E. coli (ATCC 25922, American Type Culture Collection, Virginia) per animal. The dose used for this study was determined by a dose-dependent test (3.3 × 10⁶, 3.3 × 10⁷, 3.3 × 10⁸ and 3.3 × 10⁹) in groups of four animals in which the dose (3.3 × 10⁷) with development of significant peritonitis without lethality was defined. Groups of eight rats were euthanized at 3, 6, 12 h and 1, 3, 7 days after E. coli infection, respectively. Control animals received PBS, instead of E. coli, following the same protocol as E. coli treated animals.

Ultrasonic-mediated gene transfer of Smad7 into the peritoneum

To block activation of TGF-β signalling, an inhibitory Smad7 gene was transferred into the peritoneum at 3 days prior to E. coli infection using the ultrasonic-microbubble-mediated technique as previously described [11, 13]. Briefly, after mixing pTRE–m2 Smad7 and pEFpurop-Tet-on with Sonovue (Milano company, Italy) at a ratio of 1:1 (vol/vol), the mixed solution containing 100 µg of designated plasmids in total volume of 4 mL was injected into the peritoneal cavity and immediately received ultrasound treatment (FYSIOMED Sonic 15C, Belgium) in a disconnected-wave (1/10) at power output of 2.8 W/cm², 1–3 MHz with the continuous movement of the probe on the entire abdominal surface at the 10-min interval with the face-up and down position. The same procedure was applied to the control animals with empty plasmids and Sonovue. The Smad7 transfection rate and transgene (m2Smad7) expression were determined by an anti-Flag-m2 antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) as previously described [11, 13].

Histology and confocal imaging analysis

Both parietal and visceral peritoneal tissues were collected at various time-periods after intra peritoneal E. coli injection. Tissues were fixed with 10% formalin for 24 h, and 4-µm paraffin sections were examined by Hematoxylin and Eosin (H&E) and Masson Trichrome as previously described [11, 13]. Leukocytes within the peritoneal exudates were quantitatively analysed by automatic Haematology Analyzer, while leukocytes infiltrating the peritoneum were examined by confocal immunofluorescence. Briefly, 6-µm paraffin-embedded sections were cut and stained for goat anti-rat antibodies to CD45, TNF-α, Smad7 (Santa Cruz); a mouse

Subject: Peritoneal inflammation and fibrosis

Methods: Animal model

Results: Treatment with decorin or octreotide in animal models with chronic PD is effective. Studies demonstrate a pathogenic role of TGF-β1 in peritoneal fibrosis associated with chronic PD. TGF-β is a multi-functional cytokine and growth factor.

Ultrasound-mediated gene transfer of Smad7 into the peritoneum

Histology and confocal imaging analysis

Fig. 1. Peritoneum functional changes during peritonitis. (A) Total number of white blood cells (WBC) in ascites; (B) polymorph nuclear cells (PMN) in ascites; (C) mononuclear cells in ascites; (D) WBC in blood. Results are expected as mean ± SD for a group of eight animals. ∗P < 0.05, **P < 0.01 as compared to the time-matched PBS control.
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Fig. 2. Peritoneal histology and functional changes during peritonitis. (A) Representative pictures for Masson Trichrome staining of the visceral peritoneum from groups of eight animals. Results show that compared to animals treated with PBS control, a transient acute peritonitis is developed in E. coli infected rats, peaking at Day 3 but being recovered at Day 7. (B) Changes in peritoneal functions: (B-i) Ultrafiltration rate (UF); (B-ii) Protein transportation rate (D/S TP); (B-iii) Glucose transportation rate (G4/G0); (B-iv) Creatinine transportation rate (D/S Cr). Results are expected as mean ± SD for a group of eight animals. *P < 0.05, **P < 0.01 as compared to the time-matched PBS control. Magnification: × 200 (A).

monoclonal antibody to phosphorylated NF-κB/p65 (p-NF-κB/p65) (CST Co., Danvers, MA, USA); and rabbit antibodies to TGF-β1 and phosphorylated Smad2/3 (p-Smad2/3) (Santa Cruz) in 4°C for overnight, followed by a FITC-conjugated goat anti-mouse or rabbit antibody or rabbit anti-goat IgG (Santa Cruz) at 37°C for 45 min. To detect TGF-β1 production by infiltrating leukocytes, two-colour immunofluorescence was performed with FITC-anti-TGF-β1 and PE-CD45 antibodies. After being counterstained with DAPI, the sections were viewed under Zeiss LSM 510 Confocal Imaging System (Zeiss, Oberkochen, Germany) and analysed by the Zeiss Image Processing Software as described previously [11,13].

Enzyme-linked immunosorbent detection of cytokines TGF-β1 and TNF-α
Concentrations of TGF-β1 and TNF-α within the ascites, blood serum and culture supernatants were measured by ELISA following the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA).

Primary culture of rat peritoneal mesothelial cells
Rat peritoneal mesothelial cells were isolated from male Sprague-Dawley rats and cells at the second passage were cultured in serum-free DMEM medium with or without LPS (1 µg/mL, Sigma, from Escherichia coli serotype 055:85) for 24 h in the presence or absence of TGF-β1 (5 ng/mL,
Fig. 3. Confocal immunofluorescence shows peritoneal inflammation after a single *E. coli* infection. (A) Representative pictures for immunofluorescent staining of CD45 (i), TNFα (ii), and phospho-NF-κB/p65 subunit from groups of eight rats during peritonitis. A transient CD45+ leukocytic infiltration, TNFα expression and NF-κB/p65 activation are evident, peaking at Day 3 and returning to normal at Day 7. Green-yellow indicates positive signals for CD45, TNFα or phospho-NF-κB/p65, while red indicates nuclear staining with DAPI. (B–D) Quantitation of CD45+, TNFα+ and nucleated phospho-NF-κB/p65+ cells in the peritoneum during peritonitis. Results are expected as mean ± SD for a group of eight animals. *P < 0.05, **P < 0.01 as compared to normal animals. Magnification: × 400 (A).

R&D Systems, Minneapolis, MN, USA) as previously described [11]. The dose of LPS or TGF-β1 used in this study was determined by a dose-dependent assay in which a lower dose of LPS (1 µg/mL) that caused a significant inflammatory response and a lower dose of TGF-β1 (5 ng/mL) which was able to completely inhibit LPS (1 µg/mL)-induced inflammation was defined. To overexpress Smad7, cells were transiently transfected with the pcDNA3 plasmid containing Flag-m2-Smad7 for 24 h by a liposomal transfection kit (Lipofectamine TM2000, Invitrogen) and cultured in serum-free medium with or without LPS (1 µg/mL) for 24 h as described above. Cells transfected with empty pcDNA3 plasmid were used as control.

**RNA isolation and RT-PCR**

Total peritoneal RNA was isolated from visceral peritoneum using Trizol Reagent (Invitrogen life technologies, USA) and complementary DNA (cDNA) was synthesized and analysed as previously described [11,13]. All samples were subjected to RT-PCR for housekeeping gene GAPDH for densitometry. The PCR primers used were GAPDH: forward 5′-GGC AAG TTC AAT GGC ACA GT-3′, reverse 5′-AAG GTG GAG GAA TGG GAG TT-3′; TNFα: forward 5′-CGA GTG ACA AGC CCY TAG C-3′, and reverse 5′—CGG ACT CCG TGA TGT CTA AGT AC-3′.
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Fig. 4. Confocal microscope shows a change in peritoneal TGF-β signalling after a single E. coli infection. (A) Representative pictures for immunofluorescence staining of TGF-β1, phospho-Smad2/3 (pS2/3), and Smad7 from groups of eight animals. Green-yellow indicates positive signals for TGF-β1, phospho-Smad2/3 or Smad7, while red indicates nuclear staining with DAPI. (B–D) Quantitation of TGF-β1+, nucleated phospho-Smad2/3+ and Smad7+ cells in the peritoneum during peritonitis. Results show a significant, but transient peritoneal TGF-β1 expression, Smad2/3 activation and Smad7 expression with the peak at Day 3 and recovery at Day 7. Data are expected as mean ± SD for a group of eight animals. **P < 0.01 as compared to normal. Magnification: × 400.

Western blot analysis
Visceral peritoneum tissues or cultured peritoneal mesothelial cells were lysed and proteins were extracted, denatured and then transferred to a nitrocellulose membrane for Western blot analysis of phospho-Smad2/3, Smad7, TGF-β1, GAPDH (Santa Cruz, CA), phospho-Smad2, phospho-Smad3, NF-κB/p65 and phospho-NF-κB/p65 (CST Co., Danvers, MA, USA) as previously described [11]. The signals were visualized by an enhanced chemiluminescence system (GE, Piscataway, NJ, USA), and the ratio was subjected to the GAPDH.

Statistical analyses
Data were presented as the mean ± standard deviation (SD). The comparisons among groups were performed by ANOVA, with the post hoc tests of Fisher PLSD, using the SPSS version 11.0 software (SPSS, Chicago, IL, USA).

Results
Development of acute bacterial peritonitis
Following a single intra-peritoneal injection of E. coli, acute peritonitis was developed within 12 h. As shown in Figure 1(A–C), both PBS and E. coli injection caused a rapid influx of white blood cells (neutrophils and monocytes) into the ascites at equal levels before 12 h. While WBC in
Fig. 5. Western blot and ELISA analyses show a change in peritoneal TGF-β signalling during peritonitis. (A) Western blot analysis of peritoneal TGF-β1, phospho-Smad2/3 and expression of Smad7. (B,C) semi-quantitation of the ratio of phospho-Smad2/3 and Smad7. (D) ELISA analysis of TGF-β1 levels in both ascites and plasma. Each lane represents for one rat and data are expected as mean ± SD for a group of eight animals. *P < 0.05, **P < 0.01 as compared to normal rats.

PBS control was returned to normal levels after Day 1, a significant increase in total number of white blood cells (WBC), neutrophils, and monocytes within ascites was evident at 24 h post-\textit{E. coli} injection, declining to the normal level at Day 7 (Figure 1A–C). This was inversely associated with a change in blood WBC counts (Figure 1D). Histologically, although PBS control animals showed no evident of peritoneal histology changes throughout the 7-day periods (Figure 2Av–vii), \textit{E. coli} injection caused peritonitis such as inflammatory cell infiltration and oedema evident at 12 h, peaking at Day 3 (Figure 2Aiii), and returning to a normal level at Day 7 (Figure 2Aiv). This was further demonstrated by immunofluorescence with the findings that a significant upregulation of TNF-α occurred at 3 h, which preceded a significant peritoneal CD45+ leukocyte infiltration at 12 h (Figure 3A–C). A marked peritoneal CD45+ infiltration and TNFα expression were developed at Day 3 and then reduced to normal levels at Day 7 (Figure 3A–C). All these findings indicated that a single bacterial infection induced a transient and reversible peritonitis.
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Impaired peritoneum function during acute peritonitis

The development of peritoneal inflammation was associated with impaired peritoneal functions. As shown in Figure 2(B), the peritoneal ultrafiltration rate was significantly reduced over 24–48 h after *E. coli* injection (Figure 2Bi). This was associated with a significant increase in the transportation rate of total protein (Figure 2Biit) and glucose (Figure 2Biii), although there was no significant alteration in creatinine transportation when compared to PBS control (Figure 2Biv). Interestingly, all impaired peritoneal functions returned to normal levels at Day 7 post-bacterial infection (Figure 2B), again suggesting a transient peritoneal functional damage caused by a single bacterial infection.

Signalling mechanisms associated with a transient acute peritonitis

First, we studied activation of NF-κB signalling because it is a key pathway in response to bacterial infection [15]. As shown in Figure 3(Aiii and D), NF-κB/p65 was rapidly activated as demonstrated by nuclear translocation of the phosphorylated p65 subunit in peritoneal tissues at 3 h after *E. coli* infection, which remained high over the first 3 days, but returned to the normal level at Day 7 (Figure 3Aiii and D).

We then studied TGF-β/Smad signalling since TGF-β1 is a major anti-inflammatory cytokine and is capable of inhibiting bacterial-induced NF-κB-dependent inflammation [16]. Surprisingly, TGF-β signalling was transiently activated and followed the same pattern of NF-κB signalling. As shown in Figure 4, expression of peritoneal TGF-β1 and Smad7, and activation of Smad2/3 were significantly increased at 3 h, which remained high over the first 3 days, and then declined to the basal level at Day 7. This was further confirmed by Western blot analysis (Figure 5A–C). Interestingly, ELISA analysis revealed that an increase in concentrations of TGF-β1 in ascites was inversely associated with plasma levels of TGF-β1 before Day 3 and was associated with the recovery of peritoneal inflammation at Day 7 (Figure 5D). This indicated that although exudation of plasma TGF-β1 into the inflamed peritoneal cavity might contribute to an early increase in TGF-β1 concentrations within the ascites, local production of peritoneal TGF-β1 may be a major source of ascites TGF-β1 in response to bacterial infection. This was confirmed by two-colour immunofluorescence that the major cell type for TGF-β1 production was CD45+ leukocytes within the flamed peritoneal tissues at Day 3 (Figure 6).

Overexpression of Smad7 blocks TGF-β signalling and enhances acute bacterial peritonitis in rats

To explore the possible role of TGF-β signalling in resolution of peritoneal inflammation induced by *E. coli*, we...
performed a study by blocking the TGF-β signalling pathway with gene transfer of Smad7 using an ultrasound-microbubble-mediated gene transfer technique as previously described [11]. As shown in Figure 7, both confocal microscope and Western blot analysis detected a significant increase in peritoneal Smad7 at 1 day following ultrasound-mediated Smad7 gene transfer, which was associated with inhibition of phosphorylated Smad2/3 but without effect on TGF-β1 expression. Unexpectedly, gene transfer of Smad7 resulted in worsening of peritoneal inflammation (Figure 8A), including a further increase in infiltration of peritoneal CD45+ leukocytes, upregulation of TNF-α and activation of NF-κB/p65 (Figure 8B–E). This was also associated with a further increase in WBC counts in ascites (Figure 9A), but without effect on peripheral blood WBC (Figure 9B). Furthermore, Smad7 treatment also significantly enhanced peritoneal functional injuries, resulting in a further reduction in peritoneal ultrafiltration and an increase in the transportation rate of total protein, glucose and creatinine (Figure 9C–F).

**Overexpression of Smad7 blocks TGF-β signalling and sustains LPS-induced inflammatory response in cultured peritoneal mesothelial cells (PMC)**

Lipopolysaccharide (LPS) is a major endotoxin produced by *E. coli*. We next tested the essential role of TGF-β signalling
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Fig. 8. Overexpression of Smad7 sustains peritoneal histological damage and inflammatory response induced by E. coli infection. (A) Representative pictures for H&E staining of the visceral peritoneum, and (B) representative pictures for immunofluorescent staining of CD45, TNFα, and phospho-NF-κB/p65. Green-yellow indicates positive signals for CD45, TNFα, and phospho-NF-kB/p65, while red indicates nuclear staining with DAPI. Results show that compared to control animals, Smad7 gene transfer enhances peritonitis induced by E. coli. (C–E) Quantitation of CD45+, TNFα+ and nucleated phospho-NF-κB/p65+ cells infiltrating the peritoneum. Results are expected as mean ± SD for a group of eight animals. **P < 0.01 compared to normal; #P < 0.05 compared to empty vector control (CTL). Magnifications: × 200 (A), × 400 (B).

in protection against LPS-induced peritoneal inflammation in cultured PMC. As shown in Figure 10, both RT-PCR and ELISA detected that addition of TGF-β1 was able to block LPS-induced TNF-α mRNA and protein expression in PMC. In contrast, overexpression of Smad7 was capable of inhibiting activation of Smad3, but not Smad2, in PMC in response to LPS (Figure 11A) and significantly sustained LPS-induced NF-κB/p65 phosphorylation (Figure 11B), thereby enhancing LPS-induced TNFα mRNA and protein expression (Figure 11C and D).
Discussion

In the present study, we found that endogenous peritoneal TGF-β1 was temporally upregulated in response to a single *E. coli* infection. This resulted in a transient activation of the downstream TGF-β signalling pathway. Interestingly, this transient activation of TGF-β signalling did not cause peritoneal fibrosis. In contrast, it was associated with the resolution of peritonitis and the improvement of peritoneal dysfunction. This suggests that, unlike the prolonged activation of TGF-β1, which is pathogenic in peritoneal fibrosis associated with a long-term peritoneal dialysis, a transient upregulation of endogenous peritoneal TGF-β1 in response to a single bacterial infection may function to inhibit acute peritoneal inflammation because blockade of TGF-β signalling prior to peritoneal infection by overexpressing Smad7 enhanced acute peritoneal inflammatory response. Thus, transient activation of TGF-β signalling may be beneficial in bacterial-induced peritonitis. Findings from this study supported the notion that a single peritonitis episode in patients with peritoneal dialysis does not permanently affect peritoneal function, although recurrent peritonitis can cause peritoneal fibrosis [17]. Furthermore, results from this study also indicated that impaired TGF-β signalling may be harmful in the initial process of peritonitis, although it is beneficial by blocking TGF-β signalling when it has been overactive in a chronic peritoneal disorder [11–13]. Thus, the balance in TGF-β signalling...
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It has been well recognized that TGF-β is a key mediator of peritoneal fibrosis associated with chronic peritoneal dialysis [3–9]. Thus, blockade of TGF-β signalling under disease conditions by overexpressing Smad7 inhibits peritoneal fibrosis in both uraemic and non-uraemic rats [11–13]. In the present study, we found that a significant increase in concentrations of TGF-β1 within the peritoneal exudates may be associated with the early influx of plasma TGF-β1 or the temporal upregulation of endogenous peritoneal TGF-β1 by infiltrating leukocytes. However, this transient upregulation of peritoneal TGF-β1 did not cause peritoneal fibrosis, but exhibited a protective effect on *E. coli*-induced acute peritonitis. This suggests that inhibition of initial peritoneal inflammation and therefore termination of the inflammatory process may be a mechanism by which transient activation of TGF-β/Smad signalling did not cause peritoneal inflammation because prolonged peritoneal inflammation is a major cause of overexpression of TGF-β1 and activation of TGF-β signalling [11–14]. This was further confirmed by the finding that TGF-β signalling pathways impaired by overexpressing Smad7 under normal physiological conditions overcome the protective role of TGF-β on acute peritoneal inflammation. These contradictory results may be associated with a duel role of TGF-β1 in fibrosis and inflammation. It is known that TGF-β1 is a major anti-inflammatory cytokine and immunomodulator [14]. Thus, mice deficient for TGF-β1 gene develop autoimmune disease with uncontrollable systemic inflammation spontaneously [21,22], whereas overexpression of a latent form of TGF-β1 prevents renal inflammation in obstructive kidney disease and crescentic glomerulonephritis [23,24]. Indeed, in the present study, inflammatory cells within the ascites or infiltrating the peritoneal tissues were the major source of

is critical in the development of peritoneal fibrosis and inflammation.

Inhibition of NF-κB activation may be a key mechanism by which peritoneal TGF-β protects against peritonitis. It is known that activation of NF-κB is a major pathway of inflammation in response to LPS and bacterial infection [15] and that TGF-β1 negatively regulates NF-κB activation [18]. This may explain the finding that high levels of Smad7 maintain high NF-κB activity in gut inflammation, another disease associated with bacterial infection [18,19]. Results from this study also supported this notion. Indeed, an early activation of NF-κB/p65 and upregulation of TNFα at 3 h after *E. coli* infection may be related to LPS released from *E. coli*, while the late activation at Day 3 may attribute to pro-inflammatory cytokines such as TNFα produced locally in response to the *E. coli* infection. In contrast, upregulation of endogenous peritoneal TGF-β1 and activation of TGF-β signalling were closely associated with a marked inhibition of NF-κB activity and resolution of acute peritoneal inflammation after *E. coli* infection, which was reversed by impairing the TGF-β signalling pathway with gene transfer of Smad7. These results revealed a protective role of TGF-β signalling in NF-κB-mediated acute peritoneal inflammation. This was also demonstrated in vitro that addition of TGF-β1 was able to inhibit LPS-induced TNFα expression on PMC by blocking the NF-κB-dependent mechanism, whereas overexpression of Smad7 inhibited TGF-β signalling, thereby enhancing LPS-induced, NF-κB-driven peritoneal inflammation. Indeed, TGF-β is able to potentely inhibit LPS-induced NF-κB activation and TNF-α release from RAW 264.7 cells [16], while adenoviral delivery of Smad7 and dominant negative Smad5 reverses the TGF-β1-mediated inhibition of NF-κB-dependent gut inflammation [18]. Thus, targeting Smad7 restores the anti-inflammatory activities of TGF-β signalling in chronic inflammatory bowel disease [19]. Although the exact mode as to how TGF-β1 signals to inhibit NF-κB activation remains largely unclear, it may be associated with the known role of TGF-β in suppressing the proinflammatory response instigated by toll-like receptors by inducing a proteasomal degradation of MyD88 (myeloid differentiation factor 88) that leads to activate NF-κB by phosphorylation of IkBα [20]. This may also account for the mechanism by which blockade of TGF-β signalling with overexpression of Smad7 enhanced NF-κB activation and the severity of peritonitis in vivo and in vitro as seen in the present study.

**Fig. 10.** RT-PCR and ELISA show that addition of TGF-β1 inhibits LPS-induced TNF-α mRNA and protein production by peritoneal mesothelial cells in vitro. (A) RT-PCR. (B) ELISA. Results show that addition of TGF-β1 (5 ng/mL) blocks LPS (1 µg/mL)-induced TNFα expression by mesothelial cells. Results are expected as mean ± SD for at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to medium control;  †P < 0.05, ‡P < 0.01 as compared to empty vector control (pcDNA3).
peritoneal TGF-β1 production, which may, in turn, inhibit peritoneal inflammation via an autocrine or paracrine manner. Thus, in the context of inflammation, a temporal upregulation of TGF-β1 and activation of TGF-β signalling are essential for protection against bacterial infection as seen in this and also inflammatory bowel disease [18,19]. In contrast, long-term activation of TGF-β1 signalling in response to prolonged chronic inflammation may be pathogenic in fibrosis as seen in conditions associated with recurrent peritoneal infection and chronic peritoneal dialysis [7-10]. In such disease conditions, TGF-β1 signalling is already over-reactive; therefore, overexpression of Smad7 inhibits, rather than terminates, overactivation of TGF-β/Smad2/3, thereby restoring the balance of TGF-β signalling and inhibiting peritoneal fibrosis [11-13]. Thus, TGF-β1 may work in a complex manner to differentially regulate peritoneal inflammation and fibrosis under the complexity of pathological conditions.

It should be pointed out that transfection of Smad7 itself using the ultrasound-microbubble mediated system does not cause peritoneal inflammation or injury to peritoneal function in the normal rats as previously described [11]. A finding that blockade of TGF-β signalling by overexpressing Smad7 sustained NF-κB signalling and enhanced peritoneal inflammation in vivo and in vitro was contradictory to previous findings in a rat model of chronic peritoneal dialysis and in other disease conditions in which overexpression of Smad7 inhibits both fibrosis and inflammation [11-13,25]. Although the precise mechanisms remain largely unclear, this discrepancy may be associated with the disease nature, the signalling pathway inducing NF-κB activation and, importantly, the status of TGF-β signalling in the disease conditions. In the present study, Smad7 was transferred into the peritoneal tissues for 3 days prior to E. coli infection. This indicated that TGF-β signalling was inactive or became unresponsive at the time when peritonitis was
induced, thereby losing the anti-inflammatory properties of TGF-β1 and enhancing peritoneal inflammation in response to E. coli infection. This mimics, in some degree, the results seen in TGF-β knockout mice in which loss of TGF-β signalling results in autoimmune disease [21,22]. In contrast, when Smad7 is transferred at the same time as the disease is induced [13,25], overexpression of Smad7 reduces, rather than terminates, the levels of activated TGF-β/Smad2/3, thereby inhibiting fibrosis while maintaining TGF-β anti-inflammatory properties [11–13,25,27,28]. This is because Smad2/3 is rapidly activated by phosphorylation within a few minutes, but Smad7 is transcriptionally induced over 12–24 h after induction of the disease [26]. In addition, TGF-β may also work differentially to influence a ligand-dependent NF-κB signalling in a cell type-dependent manner. For example, IL-1 or TNF-α binds their individual receptors to activate the NF-κB signalling pathway, while LPS induces NF-κB signalling via the toll-like receptor-4 [29]. This may be also associated with the finding that Smad7 inhibits NF-κB activation in kidney tubular epithelial cells in response to IL-1β and TNF-α by inducing IkBα, an inhibitor of NF-κB in our previous study [23], but sustains NF-κB signalling in PMC in response to LPS by inhibiting TGF-β-induced degradation of MyD88, a LPS-induced NF-κB activator [20].

It should be pointed out that there were a number of limitations in the present study. First, it is possible that an increase in peritoneal TGF-β1 may be also associated with a transient neangiogenesis since TGF-β1 is capable of inducing VEGF expression [30]. This may also attribute to the decreased peritoneal ultrafiltration rate and higher peritoneal transport as reported previously [12]. Second, the transient nature of the E. coli infection, the lack of uraemia and the intact of peritoneal membrane without under PD conditions have limited the relevance of the current study to bacterial peritonitis in patients with peritoneal dialysis. Nevertheless, results from this study revealed that TGF-β signalling is essential in protection against acute peritoneal inflammation induced by bacterial infection and suggested that it should be caused when attempting to treat peritoneal fibrosis in PD patients with anti-TGF-β strategies.

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