Inhibition of Proinflammatory RANTES Expression by TGF-β1 Is Mediated by Glycogen Synthase Kinase-3β-dependent β-Catenin Signaling*

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TGF-β1 is a pleiotropic cytokine with potent anti-inflammatory property. However, the mechanisms underlying TGF-β1 suppression of inflammation remain largely unexplored. In this study, we demonstrated that TGF-β1 inhibited TNF-α or IL-1-induced RANTES expression in human kidney tubular epithelial cells (HKC-8). To delineate the mechanism by which TGF-β1 inhibits RANTES expression, we examined the potential signal pathway activated by TGF-β1 in suppressing NF-κB signaling. TGF-β1 affected neither TNF-α-induced IκBα phosphorylation and subsequent degradation, nor p65 NF-κB phosphorylation and its nuclear translocation. However, TGF-β1 could inhibit p65 and p50 binding to the κB site in human RANTES promoter as revealed by chromatin immunoprecipitation assay and protein-DNA binding assay. We found that TGF-β1 induced glycogen synthase kinase-3β (GSK-3β) phosphorylation on Ser-9 in HKC-8 cells, leading to its inactivation. Knockdown of GSK-3β mimicked TGF-β1 and inhibited RANTES induction, whereas overexpression of GSK-3β abolished the inhibitory effect of TGF-β1 and completely restored RANTES expression. Furthermore, TGF-β1 induced the dephosphorylation and activation of β-catenin, a major downstream target of GSK-3β. Ectopic expression of constitutively active β-catenin mimicked the TGF-β1 effect and completely suppressed RANTES expression induced by TNF-α. Interestingly, TGF-β1 induced a physical interaction between β-catenin and p65 NF-κB, which prevented p65 binding to the κB site, sequestered its trans-activating activity, and repressed p65-mediated gene transcription. We conclude that TGF-β1 inhibition of proinflammatory RANTES expression is mediated by β-catenin-triggered blockade of NF-κB signaling.

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine that plays an important role in diverse biological processes such as cell proliferation, cell survival/apoptosis, immune modulation, epithelial-mesenchymal transition, and extracellular matrix production (1, 2). In chronic kidney diseases, the role of TGF-β1 in mediating epithelial-mesenchymal transition, excessive extracellular matrix deposition, and scar tissue formation is extensively documented and well established (3–6). Much less, however, is known about its role in regulating renal inflammation. Although there is evidence indicating that inhibition of TGF-β1 is associated with a reduced renal infiltration of inflammatory cells after injury (7–9), a large body of the literature demonstrates that TGF-β1 possesses potent anti-inflammatory property that specifically inhibits the expression of proinflammatory mediators in various circumstances (10–14). The anti-inflammatory actions of TGF-β1 are also substantiated in the genetic knock-out studies in which mice lacking it develop a rapid wasting syndrome and die of a multifocal inflammatory disease in many tissues (15, 16). Thus far, the mechanism underlying TGF-β1 inhibition of inflammation, however, remains poorly understood.

Inflammatory cell infiltration is an early event of tissue response after renal injury and is guided primarily by the chemokine gradients built around the injured kidney tubules (17–19). Of many chemokines, RANTES (regulated upon activation, normal T cell expressed and secreted), also known as CC-chemokine ligand 5 (CCL5), is one of the best characterized and is closely relevant to the pathogenesis of chronic kidney diseases (20). Increased RANTES expression has been reported in a variety of kidney disorders such as acute kidney injury, renal transplant rejection, and chronic kidney insufficiency (21–24). RANTES is a broad chemoattractant that is capable of recruiting many types of immune cells, including T lymphocytes, monocytes/macrophages, and natural killer cells, and it promotes the chemotaxis of these cells along its gradient (20). Earlier studies indicate that RANTES is induced primarily in kidney tubular cells after obstructive injury by a mechanism dependent of NF-κB signaling, which in turn provokes inflammatory cell recruitment, infiltration, and activation (21, 25). In this context, RANTES regulation in kidney tubular epithelium not only is instrumental for establishing the peritubular influx of T cells and monocytes/macrophages in pathological conditions, but also could serve as a valuable model system for studying the regulation of NF-κB signaling, chemokine production, and inflammatory responses.

In this study, we have investigated the effect of TGF-β1 on RANTES expression in kidney tubular epithelial cells. We show that TGF-β1 inhibits the stimulus-dependent RANTES

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expression through a glycogen synthase kinase-3β (GSK-3β)-dependent β-catenin pathway. Our data point to a critical role of β-catenin in mediating the anti-inflammatory action of TGF-β1.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokine Treatment—Human proximal tubular epithelial cells (HKC, clone-8) were provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, MD). Cell culture and cytokine treatments were carried out according to the procedures described previously (21). Briefly, HKC-8 cells were seeded in complete medium that contained 5% calf serum at ∼70% confluence. After an overnight incubation, cells were serum-starved in serum-free medium for 24 h before addition of cytokines. Recombinant human TNF-α and TGF-β1 were purchased from R & D Systems (Minneapolis, MN). HKC-8 cells were pretreated with different concentrations of TGF-β1 for 16 h, followed by incubation without or with TNF-α (2 ng/ml) for 24 h, unless otherwise indicated. Whole cell lysates were prepared and then subjected to various analyses.

Western Blot Analysis—Cell lysates were prepared as described previously (26, 27). Samples were heated at 100 °C for 5–10 min before loading and separated on 10% or 15% SDS-polyacrylamide gels. Western blot analysis of protein expression was carried out by using routine procedures as described elsewhere (28). The primary antibodies were obtained from following sources: anti-RANTES (sc-1410) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p65 NF-κB, anti-phospho-p65 NF-κB (Ser-536), anti-IκBα, anti-phospho-IκBα (Ser-32/36), anti-GSK-3B, anti-β-catenin (Santa Cruz Biotechnology), and anti-α-tubulin (Sigma).

Nuclear Protein Preparation—For preparation of nuclear protein, HKC-8 cells after various treatments as indicated were washed twice with cold phosphate-buffered saline (PBS) and scraped off the plate with a rubber policeman. After centrifugation, cell pellets were resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5% Nonidet P-40, and 1% protease inhibitor mixture (Sigma)) and lysed with homogenizer. Cell nuclei were collected by centrifugation at 5,000 rpm for 15 min. After washing with buffer B (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, and 1% protease inhibitor mixture), nuclei were lysed in SDS sample buffer and subjected to Western blot analysis as described previously (29).

Plasmid Constructs and Transfection—The green fluorescent protein (GFP)-tagged p65 NF-κB expression vector (pGFP-p65) was kindly provided by Dr. Johannes Schmid (University of Vienna, Vienna, Austria) (30). The HA-tagged GSK-3B expression vector (pHA-GSK-3B) was provided by Dr. James Woodgett (Toronto, ON, Canada). The FLAG-tagged N-terminally truncated, constitutively active β-catenin expression vector (pDEL-β-cat) and HA-tagged Wnt1 expression vector (pHA-Wnt1) were described previously (31). HKC-8 cells were transiently transfected with various expression vectors by using Lipofectamine 2000 reagent (Invitrogen). Cell lysates were subjected to subsequent immunoblotting and immunoprecipitation, respectively. To establish the stable cell line with overexpression of constitutively active β-catenin or Wnt1, HKC-8 cells were transfected with pDEL-β-cat or pHA-Wnt1 expression vectors using the Lipofectamine 2000, respectively. The empty vector pcDNA3 was used as a mock transfection control. Neomycin-resistant clones were individually selected and expanded, as described previously (32), and ectopic expression of β-catenin was confirmed by Western blot analysis.

Immunoprecipitation—Immunoprecipitation was carried out by using an established method (33). Briefly, HKC-8 cells were incubated with or without TGF-β1 and/or TNF-α as indicated. For some experiments, cells were transfected with GFP-tagged p65 NF-κB expression vector (pGFP-p65) by using Lipofectamine 2000 reagent. Cells were lysed on ice in 1 ml of non-denaturing lysis buffer that contained 1% Triton X-100, 0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 0.025% NaN3, 1% protease inhibitors mixture, and 1% phosphatase inhibitor mixtures I and II (Sigma). After preclearing with normal IgG, cell lysates (1 mg of protein) were incubated overnight at 4 °C with 4 μg of anti-β-catenin (Santa Cruz Biotechnology), followed by precipitation with 30 μl of protein A/G Plus-agarose for 1 h at 4 °C. The precipitated complexes were separated on SDS-polyacrylamide gels and immunoblotted with anti-p65 antibody. In some experiments, cell lysates were immunoprecipitated with anti-GFP followed by immunoblotted with anti-β-catenin.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed to analyze in vivo interactions of NF-κB and its cognate cis-acting element in RANTES promoter (21). This assay was carried out essentially according to the protocols specified by the manufacturer (ChIP assay kit; Upstate). Briefly, HKC-8 cells after various treatments as indicated were cross-linked with 1% formaldehyde and then resuspended in SDS lysis buffer containing protease inhibitors. The chromatin solution was sonicated, and the supernatant was diluted 10-fold. An aliquot of total diluted lysate was used for total genomic DNA as input DNA control. The anti-p65 NF-κB antibody was added and incubated at 4 °C overnight, followed by incubation with protein A/G-agarose for 1 h. The precipitates were washed, and chromatin complexes were eluted. After reversal of the cross-linking at 65 °C for 4 h, the DNA was purified, and ChIP samples were used as a template for PCR using the primer sets for human RANTES promoter regions containing NF-κB response element (34). The sequences of primers used for ChIP assay were as follows: forward, 5′-GGGAAAAGATGTTGCTTAAAC-3′; and reverse, 5′-TGTGGAAAATCAAGGGACAG-3′.

NF-κB-DNA Binding Assay—NF-κB-DNA binding assay was used to quantify the active form of NF-κB in the nuclear extracts. This assay was carried out essentially according to the protocols specified by the manufacturer (NF-κB family transcriptional factors assay; Active Motif, Carlsbad, CA). Briefly, HKC-8 cells after various treatments as indicated were harvested, and nuclear protein was extracted. Five micrograms of nuclear protein in 20 μl of complete lysis buffer was
added to a 96-well plate precoated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′), and incubated for 1 h at room temperature. After extensive washing, NF-κB antibody was added and continued to incubate for 1 h, followed by adding horseradish peroxidase-labeled secondary antibody. After further incubation in developing buffer for 30 min, DNA-bound NF-κB protein abundance was quantified by reading the absorbance at 450 nm.

Knockdown of GSK-3β by siRNA—For small interfering RNA (siRNA) inhibition studies, HKC-8 cells were transiently transfected with either control siRNA or GSK-3β-specific siRNA (SignalSilence® GSK-3β siRNA; Cell Signaling Technology) by using Oligofectamine reagent according to the instructions specified by the manufacturer (Invitrogen), as described previously (35). At 24 h after transfection, cells were treated with or without TNF-α and IL-1 for another 24 h. Whole cell lysates were prepared for Western blot analysis using the various antibodies as indicated.

DNA Affinity Precipitation Assay—DNA-protein interaction was examined by a simple DNA affinity precipitation assay (32). Briefly, the 5′-biotinylated, double-stranded oligonucleotide (0.2 nM) containing the NF-κB binding site (see Fig. 6D) was mixed with 500 μg of whole cell extracts for 3 h at 4 °C. After streptavidin-agarose (30 μl of packed beads) was added, the incubation was continued for an additional 3 h at 4 °C. The beads were washed, and the eluted proteins were analyzed by Western blotting with anti-p65 antibody.

Statistical Analyses—All data examined were expressed as mean ± S.E. Statistical analyses of the data were performed using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA, followed by a Student-Newman-Keuls test. p < 0.05 was considered significant.

RESULTS

TGF-β1 Inhibits RANTES Expression in Kidney Epithelial Cells—To investigate the effect of TGF-β1 on the inflammatory response, we examined its ability to regulate RANTES expression in HKC-8 cells. As shown in Fig. 1, both TNF-α and IL-1 markedly induced RANTES expression. However, preincubation with TGF-β1 substantially inhibited the RANTES expression induced by TNF-α or IL-1. The inhibitory effect of TGF-β1 apparently required its preincubation because simultaneous incubation with TNF-α or IL-1 was less effective in inhibiting RANTES induction (Fig. 1A). It appeared that TGF-β1 at a concentration as low as 0.5 ng/ml was sufficient to exert its inhibitory effect. Further increase of its doses did not result in additional inhibition (Fig. 1, B–E).
is mediated by NF-κB signaling in tubular epithelial cells (25). In this context, we next examined the effects of TGF-β1 on the early events of NF-κB activation, including IκBα phosphorylation and its subsequent degradation as well as p65 NF-κB phosphorylation. As shown in Fig. 2A, IκBα was phosphorylated as early as 5 min after TNF-α stimulation in HKC-8 cells, and it underwent rapid degradation. Meanwhile, p65 NF-κB was rapidly phosphorylated and activated after TNF-α treatment as well (Fig. 2B). However, preincubation with TGF-β1 did not significantly affect the TNF-α-induced IκBα phosphorylation, its subsequent degradation, and p65 phosphorylation (Fig. 2, A and B). We further examined whether TGF-β1 affects p65 nuclear translocation. Subcellular fractionation and immunofluorescence staining revealed that preincubation with TGF-β1 also failed to affect p65 nuclear translocation (Fig. 2, C and D). These results suggest that TGF-β1 does not affect the early events of NF-κB signaling, such as IκBα phosphorylation and its subsequent degradation, as well as p65 phosphorylation and its nuclear translocation.

TGF-β1 Inhibits NF-κB Binding to the cis-Acting κB Site in the RANTES Promoter—Once in the nuclei, NF-κB regulates the transcription of its target genes via binding to the cis-acting κB element. Therefore, we next examined whether TGF-β1 affects p65 binding to its cognate DNA element by employing an in situ ChIP assay. As shown in Fig. 3A, TNF-α stimulation markedly increased p65 binding to κB elements in human RANTES promoter. However, preincubation with TGF-β1 inhibited p65 binding to κB elements induced by TNF-α (Fig. 3A). Notably, TNF-α also induced p50 binding to the κB element as shown by ChIP assay, and TGF-β1 blocked p50 interaction with the κB element in the RANTES promoter (Fig. 3B). Similar results were obtained when p65 and p50 binding to the κB element was detected by a quantitative transcription factor-DNA binding assay (Fig. 3, C and D). Together, these data suggest that preincubation with TGF-β1 disrupts p65 and p50 NF-κB binding to its cognate DNA element in the RANTES promoter, thereby inhibiting NF-κB-mediated gene expression.

FIGURE 2. TGF-β1 does not affect the early events of NF-κB signaling. A and B, HKC-8 cells were pretreated with TGF-β1 for 16 h followed by incubation with TNF-α (5 ng/ml) for different periods of time as indicated. Cell lysates were immunoblotted with specific antibodies against phospho-IκBα, total IκBα, phospho-p65, or total p65, respectively. C and D, TGF-β1 does not block p65 NF-κB nuclear translocation induced by TNF-α. HKC-8 cells were treated with TGF-β1 and/or TNF-α for various periods of time as indicated. C, nuclear proteins were prepared and immunoblotted for p65 NF-κB and histone H3, respectively. D, immunofluorescent staining showed p65 NF-κB localization in HKC-8 cells at 1 h after various treatments as indicated.

FIGURE 3. TGF-β1 inhibits NF-κB binding to the cis-acting κB site in the RANTES promoter. A and B, ChIP assay (IP) revealed that TNF-α induced p65 (A) and p50 (B) binding to the RANTES promoter, which was inhibited by TGF-β1. C and D, quantitative NF-κB-DNA binding assay also revealed that TGF-β1 inhibited p65 (C) and p50 (D) binding to the κB site. Error bars, S.E. *, p < 0.05 versus controls; †, p < 0.05 versus TNF-α alone (n = 3).
GSK-3β Inactivation Mediates RANTES Suppression—To elucidate the mechanism underlying TGF-β1 blockade of NF-κB signaling, we explored the potential signal pathway leading to inhibition of RANTES expression in tubular epithelial cells. As shown in Fig. 4A, TGF-β1 rapidly induced GSK-3β phosphorylation on Ser-9, whereas it had no effect on GSK-3α. It is well known that phosphorylation of GSK-3β leads to its inactivation. To test whether GSK-3β inactivation mediates RANTES suppression, HKC-8 cells were treated with LiCl, a well known GSK-3 inhibitor. We found that LiCl treatment induced GSK-3β phosphorylation on Ser-9 (Fig. 4B); and similar to TGF-β1 treatment, LiCl also inhibited TNF-α-induced RANTES expression (Fig. 4C). To confirm the relevance of GSK-3β inactivation by TGF-β1 to RANTES inhibition, we sought to knock down GSK-3β expression by siRNA strategy. As shown in Fig. 4D, knockdown of GSK-3β dramatically reduced RANTES expression after TNF-α treatment. We also employed an opposite strategy to test the role of GSK-3β in regulating RANTES expression. HKC-8 cells were transfected with GSK-3β expression vector. Ectopic expression of GSK-3β was confirmed after transfection (Fig. 4E). We found that overexpression of exogenous GSK-3β completely negated the inhibitory effect of TGF-β1 on the TNF-α-induced RANTES expression (Fig. 4E). These results suggest that GSK-3β phosphorylation and inactivation could play a critical role in mediating TGF-β1 inhibition of RANTES expression in tubular epithelial cells.

β-Catenin Activation Represses RANTES Expression—Because the major target of GSK-3β is β-catenin, we next examined its activation status following TGF-β1 treatment in kidney epithelial cells. As shown in Fig. 5A, TGF-β1 rapidly induced β-catenin activation, as the dephosphorylated, active β-catenin was increased in HKC-8 cells after TGF-β1 incubation. Kinetic studies revealed that substantial β-catenin activation occurred at 2 h, a time point significantly lagged behind the GSK-3β phosphorylation after TGF-β1 stimulation (Fig. 4A). This is consistent with the notion that GSK-3β is the upstream kinase that phosphorylates β-catenin and induces its ubiquitin-mediated degradation. To investigate the relevance of β-catenin activation to RANTES suppression, we examined the RANTES expression in the HKC-8 cells stably transfected with expression vector for N-terminally truncated, constitutively active β-catenin (pDel-β-cat). As shown in Fig. 5B, overexpression of constitutively active β-catenin completely abolished RANTES expression induced by TNF-α, suggesting an essential role of β-catenin activation in mediating TGF-β1 inhibition of RANTES expression. To confirm this observation further, we sought to activate β-catenin by another strategy via overexpressing Wnt1, the canonical Wnt/β-catenin signaling. To this end, HKC-8 cell lines with stable transfection of HA-tagged Wnt1 expression vector (pHA-Wnt1) were established and incubated with TNF-α. As showed in Fig. 5C, overexpression of Wnt1 completely suppressed TNF-α-induced RANTES expression as well, supporting a critical role for β-catenin signaling in inhibiting proinflammatory RANTES production.

β-Catenin Physically Interacts with p65 and Sequesters Its trans-Activating Activity—To understand how activated β-catenin blocks RANTES expression, we sought to explore whether β-catenin represses NF-κB signaling through physical interaction with p65. To test this, HKC-8 cells were treated with TGF-β1 and/or TNF-α, respectively. Cell lysates were immunoprecipitated with anti-β-catenin antibody, followed by immunoblotting with anti-p65. As shown in Fig. 6A, p65 was detected in the immunocomplexes precipitated by anti-β-catenin antibody. p65/β-catenin complex formation was maximal in the HKC-8 cells treated with both TGF-β1 and TNF-α (Fig. 6A, lane 4), suggesting that activation of β-catenin (by TGF-β1) and p65 (by TNF-α) facilitates their interaction. Of note, a weak band of p65/β-catenin complex was also observable in HKC-8 cells treated with TGF-β1 alone, implying that activated β-catenin (by TGF-β1) can in-
teract with endogenous p65 in the absence of TNF-α (Fig. 6A, lane 2). In the reciprocal experiments, β-catenin was also detected in the immunocomplexes precipitated by anti-GFP-p65 antibody (Fig. 6B).

To study the functional consequence of this p65/β-catenin interaction, we investigated the p65-DNA binding as well as the transcriptional activity of NF-κB luciferase reporter gene. As shown in Fig. 6C, p65/β-catenin complex formation induced by TGF-β1 apparently sequestered p65 and disrupted its binding to the κB site in human RANTES promoter in a DNA affinity precipitation assay. Furthermore, ectopic expression of constitutively active β-catenin effectively blocked p65-mediated gene trans-activation (Fig. 6D). Consistent with p65/β-catenin interaction data, over-expression of β-catenin alone also repressed the luciferase reporter activity in the unstimulated conditions, suggesting a role for β-catenin in controlling the endogenous, basal NF-κB transcriptional activity.

**DISCUSSION**

Despite some conflicting data in the literature regarding the role of TGF-β1 in regulating inflammatory responses (7–9, 12, 14), the results presented in this study clearly demonstrate that TGF-β1 is able to inhibit the stimulus-dependent RANTES expression in HKC-8, consistent with its anti-inflammatory potential. This inhibitory action of TGF-β1 is apparently mediated by a GSK-3β-dependent β-catenin pathway. Through its physical interaction with p65, β-catenin effectively sequesters its trans-activating activity, thereby inhibiting the NF-κB-mediated proinflammatory chemokine expression. Our results provide molecular explanations for the anti-inflammatory action of TGF-β1 and point to a critical role of β-catenin in mediating this process.

TGF-β1 is a well-characterized fibrogenic cytokine that plays a crucial role in the initiation and progression of tissue fibrosis in many organs, including kidney (2, 5). However, given its anti-inflammatory potential, it remains uncertain whether a long term inhibition of TGF-β1 is an ideal approach for the therapeutic treatment of chronic kidney fibrotic disorders. In that regard, previous studies indicate that overexpression of latent TGF-β1 in transgenic mice actually prevents renal fibrotic lesions primarily by inhibiting renal inflammation in a model of obstructive nephropathy (36). Notably, this reduction in renal inflammation in transgenic mice is closely associated with an increase in TGF-β1 in circulation and kidney tissues, suggesting a direct role of TGF-β1 in inhibiting T cell and macrophage infiltration and the up-regulation of IL-1β, TNF-α, and intercellular adhesion molecule-1 in this model (36). These results raise some legitimate concerns about the feasibility of TGF-β1 inhibition as a therapeutic strategy for chronic kidney diseases, in which renal inflammation is a major pathologic feature. It should be stressed that in the advanced stage of chronic kidney diseases, an increased TGF-β1 expression is often accompanied by renal inflammation, a phenomenon that seems contradictory to its role as an anti-inflammatory factor. This is possible because oxidants accumulated in chronically injured tissues selectively reverse TGF-β1 suppression of the NF-κB-driven production of proinflammatory mediators (12).

The mechanism underlying TGF-β1 inhibition of inflammation remains enigmatic, although it is most likely related to its ability to block NF-κB signaling. Several studies suggest that inhibition of inflammation by TGF-β1 is associated with an up-regulation of IκBα (10, 14, 36). Because IκBα is the major inhibitory protein that binds to and subsequently sequesters p65 NF-κB activity in the cytoplasm, its induction would lead to an inhibition of NF-κB activation and NF-κB-driven inflammatory response. Smad7 has also been shown to participate in mediating TGF-β1-driven inhibition of NF-κB signaling, but the molecular details are elusive and controversial (14, 36). Although overexpression of Smad7 is able to up-regulate IκBα directly in one study (36), Smad7 blocks the TGF-β1-stimulated IκBα promoter transcriptional activity, leading to an increased NF-κB activity in another (14). Regardless the mechanism of IκBα regulation by Smad7, however, we found that TGF-β1 inhibition of RANTES expression in HKC-8 cells...
has little to do with IκBα expression because pretreatment with TGF-β1 does not significantly affect IκBα abundance (Fig. 2). This conclusion is further supported by the observation that TGF-β1 fails to affect the TNF-α-triggered IκBα phosphorylation and subsequent degradation, as well as p65 phosphorylation and its nuclear accumulation (Fig. 2). It appears, therefore, conceivable that TGF-β1 inhibits NF-κB activity primarily by negatively modulating the postnuclear events of its signaling.

The results presented in this study, indeed, provide compelling evidence that TGF-β1 inhibits proinflammatory RANTES production in kidney tubular cells by disrupting the postnuclear signaling of NF-κB. Such an inhibitory action of TGF-β1 is predominantly mediated by GSK-3β-dependent β-catenin signaling. This conclusion is substantiated by several lines of evidence. First, TGF-β1 rapidly induces GSK-3β phosphorylation and inactivation, which leads to β-catenin dephosphorylation and activation (Figs. 4 and 5). Second, forced expression of GSK-3β abolishes the inhibitory effect by TGF-β1 and restores RANTES expression, whereas knockdown of GSK-3β mimics TGF-β1 and inhibits RANTES induction by TNF-α (Fig. 4). Third, ectopic expression of constitutively active β-catenin or Wnt1 completely prevents RANTES expression induced by TNF-α (Fig. 5). Finally, β-catenin physically interacts with p65 and sequesters its ability to bind to the κB site and trans-activate its target gene transcription (Figs. 3 and 6). In view of the fact that β-catenin activation by TGF-β1 inhibits p65 binding to DNA as shown by multiple approaches (Figs. 3 and 6C), it is highly unlikely that the p65–β-catenin complex can bind to the κB site in the RANTES promoter. Altogether, our present study has established a fundamental role for GSK-3β-dependent β-catenin pathway in mediating TGF-β1 inhibition of NF-κB activation.

The findings of this study are also supported by earlier studies demonstrating a critical role of GSK-3β as well as β-catenin in regulating NF-κB activation and its target gene expression (25, 37–40). GSK-3β is positioned on the crossroads of several signal pathways downstream of TGF-β1. On one hand, GSK-3β can directly phosphorylate p65 on Ser-468, which is indispensable for NF-κB activation and RANTES gene transcription (25, 38); and as a result, TGF-β1-mediated GSK-3β inactivation would block NF-κB signaling. On the other hand, GSK-3β inactivation leads to β-catenin dephosphorylation, which stabilizes it by preventing from the ubiquitin-mediated protein degradation. Activated β-catenin binds to p65, blocks its binding to the κB site, and sequesters its ability to activate the transcription of its target genes (39, 40). It is of interest to point out that TGF-β1 also induces Wnt expression, which could lead to β-catenin activation via GSK-3β-dependent pathway as well. Taken together, TGF-β1, as a potent anti-inflammatory cytokine, is able to block NF-κB signaling and proinflammatory mediator production by targeting multiple sites in Wnt/GSK-3β/β-catenin signaling.

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