Proteasome inhibitor MG132 inhibits the process of renal interstitial fibrosis

LIN HAN[1], BINGBING ZHU[2], HUI CHEN[2], YUANMENG JIN[2], JIAN LIU[2] and WEIMING WANG[2]

[1]Department of Nephrology, Yangpu Hospital, Tong Ji University, School of Medicine, Shanghai 200090;
[2]Department of Nephrology, Ruijin Hospital, Shanghai Jiao Tong University,
School of Medicine, Shanghai 200025, P.R. China

Abstract. The proteasome inhibitor pathway serves a crucial role in cell cycle progression and apoptosis, and in the activation of transcription factors and cytokines in tumor cells. The aim of the current study was to investigate the effect of the proteasome inhibitor, MG132, on transforming growth factor (TGF)-β1-induced expression of extracellular matrix proteins in rat renal interstitial fibroblasts (NRK-49F cells) and to better elucidate the mechanism by which MG132 functions. The level of connective tissue growth factor (CTGF), α-smooth muscle actin (SMA), fibronectin (FN) and collagen type III (Col III) in the MG132-pretreated groups was significantly decreased compared with groups treated with TGF-β1 alone. MG132 significantly decreased mRNA and the protein levels of fibrosis-associated factors induced by TGF-β1 treatment. The MG132-pretreated groups exhibited lower phosphorylated-mothers against decapentaplegic homolog (p-Smad)2, p-Smad3 and FN protein expression compared with the groups treated with TGF-β1 alone. In conclusion, MG132 reduced mRNA and protein expression of fibrosis-associated factors. It can successfully inhibit the inflammatory reaction induced by TGF-β via the Smad signaling pathway. These results indicate that MG132 appears to have a potent effect in counteracting renal fibrosis. MG132 may be applied in the treatment of patients with chronic kidney disease.

Introduction

Renal interstitial fibrosis is the final common pathway of end stage renal disease (1). It is characterized by the increased deposition of extracellular matrix (ECM) materials, including collagen type I (Col I) and type III (Col III), fibronectin (FN), and laminin, as well as activated renal interstitial fibroblasts (2). Transforming growth factor (TGF)-β1 is one of the main factors that can induce fibrosis (3). TGF-β1 has been identified as a central mediator in renal fibrosis (4). TGF-β initiates canonical and non-canonical pathways to exert multiple biological effects (5). Among them, the mothers against decapentaplegic homolog (Smad) signaling pathway has been recognized as a major pathway of TGF-β signaling in progressive renal fibrosis (6). Connective tissue growth factor (CTGF) is one of the downstream factors that induce fibrosis (7). In a normal physiological environment, CTGF has been demonstrated to be mainly involved in angiogenesis and cell differentiation (8). It is worth noting that CTGF could mediate the process of tissue repair and fibrosis under pathological conditions (9). In the process of myocardial injury, repair and fibrosis, CTGF has been revealed to be a molecule that activates fibroblasts (10). The central link of renal interstitial fibrosis is the activation of fibroblasts with the expression of α-smooth muscle actin (SMA) serving as the main biomarker (11). α-SMA is a hallmark of a variety of renal phenotypic transformations and has been commonly used to detect the phenotypic transformation of fibroblasts into myofibroblasts (12). α-SMA-positive myofibroblasts have been demonstrated to be the main synthetic cells that deposit ECM materials (12).

The amount of collagen secreted by myofibroblasts has been determined four to five times greater than that of fibroblasts; collagen has been revealed to increase the deposition of ECM (13). Furthermore, myofibroblasts have a strong contractile capacity, which has been demonstrated to cause the remodeling of the kidney structure, resulting in fibrosis (14). Smads are involved in the TGF-β1 signal transduction pathway (15). It has been consistently demonstrated that Smad2 and Smad3 are extensively activated in the fibrotic kidney in patients with and animal models of chronic kidney disease (6). The phosphorylated (p-)Smad2 and p-Smad3 form an oligomeric complex with a common Smad, Smad4, which has been revealed to translocate into the nucleus to regulate the transcription of target genes in collaboration with various co-activators and co-repressors, ultimately inducing fibrosis (16). Proteasome inhibitors can interfere with and influence cellular functions by inhibiting the activity of the proteasome (17). Therefore, using a proteasome inhibitor

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Correspondence to: Professor Weiming Wang, Department of Nephrology, Ruijin Hospital, Shanghai Jiao Tong University, School of Medicine, 197 Ruijin Second Road, Shanghai 200025, P.R. China
E-mail: wwm11120@rjh.com.cn

to change the activity of the proteasome cleavage site is the focal point of studies on inflammation by our group (18). A previous study demonstrated that a proteasome inhibitor, MG132, inhibited proliferation and induced apoptosis in renal interstitial fibroblasts that had been stimulated to differentiate into myofibroblasts by TGF-β1 (19). In the current study, the authors examined the influence of MG132 on TGF-β1-induced renal interstitial fibrosis to investigate the potential application of MG132 in slowing renal interstitial fibrosis, as well as the potential mechanism by which it functions.

Materials and methods

Chemicals. TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) cell culture medium and trypsin-EDTA were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal calf serum (FCS) was purchased from PAA Laboratories (GE Healthcare, Chicago, IL, USA). The proteasome inhibitor, MG132, was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA) and dissolved in dimethyl sulfoxide as a 40 μM stock solution stored at -20˚C. Reverse transcription associated reagents were purchased from Promega Corporation (Madison, WI, USA). Quantitative polymerase chain reaction (qPCR) primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Rabbit anti-rat Smad2/3 (cat. no. 5678), p-Smad2 (cat. no. 3104), p-Smad3 (cat. no. 9520), α-SMA (cat. no. #19245) and GAPDH (cat. no. #8884) monoclonal antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit anti-rat CTGF (cat. no. 555SR-100) monoclonal antibodies were obtained from BioVision, Inc. (Milpitas, CA, USA). Mouse anti-rat FN monoclonal antibodies (cat. no. AB2051) were purchased from EMD Millipore. Mouse anti-rat β-actin (cat. no. A1978) and rabbit anti-rat Col III (cat. no. SAB4200749) monoclonal antibodies were bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated immunoglobulin (Ig)G secondary antibodies (goat anti-rabbit, cat. no. 5220-0337; goat anti-mouse, cat. no. 5450-0011) were obtained from Kirkegaard & Perry Laboratories (SeraCare Life Sciences Inc., Milford, MA, USA). Enhanced chemiluminescence reagent (Western lightning Plus-ECL; cat. no. NEL105001EA) was purchased from Edo Biotech AB (Lindome, Sweden) and polyvinylidene fluoride (PVDF) membranes were purchased from EMD Millipore.

Cell culture. Rat renal fibroblast NRK-49F cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F12 medium supplemented with 10% FCS in a humidified incubator containing 5% CO_2 at 37˚C.

Reverse transcription (RT)-qPCR analysis. Cells plated in 6-well plates (2x10⁶/well) were treated with TGF-β1 (5 ng/ml) with or without MG132 at specific concentrations (0, 0.5, 1, 2.5 and 5 μM) for 24 h. The control group was defined as untreated cells cultured for 24 h. Another group of cells plated in 6-well plates (2x10⁶/well) were treated the cells with TGF-β1 (5 ng/ml) with or without MG132 (2.5 μM) for defined lengths of time (0, 6, 12 and 24 h). In this group, the

control group was defined as cells cultured for 0 h. RNA was purified using an RNA extraction kit (cat. no. Z3100; Promega Corporation), converted to cDNA using TaqMan™ microRNA RT kit (cat. no. 4366596; Thermo Fisher Scientific, Inc.) and the following genes were amplified: CTGF, α-SMA, FN, Col III and GAPDH using the primers listed in Table I. SYBR Green reagent (Tokobo Ltd., London, UK) was used for PCR amplification and detection of transcripts. The reaction conditions were as follows: Denaturation at 94˚C for 10 min, and 40 cycles of denaturation at 94˚C for 15 sec, and annealing and extension at 60˚C for 1 min. Each experiment was repeated three times in triplicate. Relative mRNA expression was calculated using the relative quantification method with GAPDH as an internal control (20). Data are expressed as n times the untreated group.

Treatment groups. The first collection of cells (8x10⁶) were treated with TGF-β1 (5 ng/ml) for defined lengths of time (0, 6, 12 and 24 h) with or without MG132 (2.5 μM) pretreatment for 0.5 h; the control group was defined as cells cultured for 0 h. The second collection of cells were treated with TGF-β1 (5 ng/ml) for 24 h with or without 0.5 h pretreatment with MG132 at 0.5, 1, 2.5 and 5 μM. In these two collections, CTGF, α-SMA, FN, Col III and GAPDH protein expression levels were assessed. The third collection of cells were treated with TGF-β1 (5 ng/ml) for different times (0, 15, 30 min, 1 and 2 h); the control group was defined as cells cultured for 0 min. The fourth collection of cells were treated with or without 0.5 h MG132 pretreatment at 0, 0.5, 1, 2.5 and 5 μM and TGF-β1 (5 ng/ml) treatment for 1 h; the control group was defined as untreated cells cultured for 1 h. In these two collections, p-Smad2, p-Smad3, Smad2/3 and β-actin protein expression levels were assessed. The fifth collection of cells were treated with TGF-β1 (5 ng/ml) with or without MG132 at 0.5, 1, 2.5 and 5 μM for 24 h; the control group was defined as untreated cells cultured for 24 h. FN and β-actin protein expression levels were assessed in this collection.

Western blotting. Following all treatments, cells were lysed in cell lysis solution (phenylmethylsulfonyl fluoride: Radio-immunoprecipitation assay, 1:100; cat. no. P0013B; Shanghai Biyuntian Bio-Technology Co., Ltd., Shanghai, China) at 4˚C and centrifuged at 4,024 x g for 30 min at 4˚C. Protein concentrations in the supernatant were determined using the Bradford method. Protein and loading buffer were mixed at a ratio of 4:1, boiled at 100˚C for 5 min and then samples (100 μg/lane) were separated on 10% SDS-PAGE gels. Bands from the gels were transferred to PVDF membranes using 0.2 A and then blocked for 2 h at room temperature with 5% skim milk in TBS/T buffer solution. Mouse anti-rat FN (1:1,000), mouse anti-rat β-actin (1:500), mouse anti-rat Col III (1:1,000) and rabbit anti-rat GPADH (1:1,000), CTGF (1:2,000), Smad2/3 (1:1,500), p-Smad2 and p-Smad3 monoclonal antibodies (1:5,000) were incubated overnight with the membranes at 4˚C. Membranes were then washed, and HRP-conjugated goat anti-mouse IgG (1:2,000) or goat anti-rabbit (1:1,500) secondary antibodies were incubated with the membranes for 2 h at 4˚C. Membranes were then covered with ECL reagent in a darkroom for 5 min and exposed to X-ray film; the film was then developed and fixed. ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA) was used for the densitometric analysis of the blots.

Table I. Primer sequences.

Gene	Type	Primer sequence (5'-3')	Product length (bp)
GAPDH	Forward	AGTATGACTCCACTCACGGCAA	100
	Reverse	TCTCGCTCCTGGAAGATGGT	
α-Smooth muscle actin	Forward	CATCCGACCTTGCTAACG	168
	Reverse	TCCAGAGTCCAGCACAATAC	
Connective tissue growth factor	Forward	ATCCCTGCGACCCACACAAG	145
	Reverse	CAACTGCTTTGGAAGGACTCGC	
Fibronectin	Forward	CCAGGCACTGACTACAAGAT	146
	Reverse	CATGATACCAGCAAGGAGT	
Collagen type III	Forward	TGATGGGATCCAATGAGGGAGA	143
	Reverse	GAGTCTCATGGCCTTGCGTGTTT	

Statistical analysis. All the data are expressed as mean ± standard deviation, representative of three repeats and were analyzed by the SPSS 11.0 software package (SPSS, Inc., Chicago, IL, USA). Comparisons were made among groups using one-way analysis of variance followed by Fisher's Least Significant Difference test. $P<0.05$ indicated that the difference between groups was statistically significant.

Results

MG132 downregulates the mRNA level of fibrosis-associated factors. NRK-49F cells have been demonstrated to express CTGF, α-SMA, FN and Col III (6-9). Following treatment with different concentrations of MG132, the mRNA expression of CTGF, α-SMA, FN and Col III decreased compared with the control group, and exhibited a potential dose-dependent effect (Fig. 1).

MG132 pretreatment decreases the mRNA level of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in a potential time-dependent manner. It was found that 2.5 µM MG132 was effective in reducing the TGF-β1-induced expression of fibrosis-associated genes (CTGF, α-SMA, FN and Col III) at each time point assessed (6, 12 and 24 h; Fig. 2). Compared with the control group, CTGF mRNA levels in the TGF-β1 group were 3.9-fold more greatly expressed at 6 h, 3.6-fold higher at 12 h, and 2.3-fold higher at 24 h, indicating that the difference in expression gradually declined with increasing time following initial treatment. Following pretreatment with 2.5 µM MG132, fold-changes decreased to 2.1, 1.8 and 1.4-fold for 6, 12 and 24 h, respectively. The mRNA level of α-SMA was also elevated after 6 h of TGF-β1 stimulation. α-SMA expression reached a peak at 12 h and was 26.2-fold more highly expressed compared the control group, but was only 10.9-fold higher at 24 h. Compared with the control group, α-SMA expression was significantly increased in after 6 and 12 h of TGF-β1 stimulation (both $P<0.05$). Following pretreatment with 2.5 µM MG132, these fold-changes in α-SMA expression decreased to 4.2, 1.2 and 1.1 at 6, 12 and 24 h, respectively, of which 6 h of treatment increased expression significantly compared with the control group ($P<0.05$).
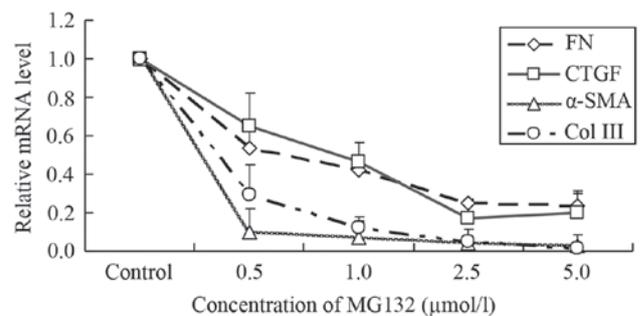

Figure 1. MG132 downregulates the mRNA level of fibrosis-associated factors. The mRNA levels were normalized to the control group. NRK-49F cells were treated with the proteasome inhibitor, MG132, at specific concentrations (0-5 µM) for 24 h. $P<0.05$. Col III, collagen type III; FN, fibronectin; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin.

The mRNA level of FN was 2.8-, 3.3- and 7.4-fold more highly expressed in TGF-β1-treated cells at 6, 12 and 24 h, respectively, of which the 24 h stimulation was significantly increased compared with the control group ($P<0.05$). Following pretreatment with 2.5 µM MG132, these values decreased to 1.9, 1.8 and 1.1-fold at 6, 12 and 24 h, respectively. FN expression after 6 h of MG132 and TGF-β1 treatment was significantly increased compared with the control group ($P<0.05$). The mRNA levels of Col III were 2.0, 2.5 and 2.8-fold higher in TGF-β1-treated cells prior to pretreatment with MG132 and were reduced to 0.7, 0.1 and 0.1-fold at 6, 12 and 24 h after pretreatment, respectively. Col III expression was significantly increased 24 h after TGF-β1 treatment and significantly decreased 6, 12 and 24 h after MG132 and TGF-β1 treatment compared with the control group (all $P<0.05$). The results indicated that 2.5 µM MG132 decreased the mRNA levels of fibrosis-associated factors following stimulation with 5 ng/ml TGF-β1, which causes the fibroblasts to differentiate into myofibroblasts associated with fibrotic processes (21).

MG132 pre-treatment decreases on the mRNA level of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in a potential concentration-dependent manner. Cells pretreated with MG132 exhibited significant decreases in α-SMA, CTGF, FN and Col III mRNA levels compared
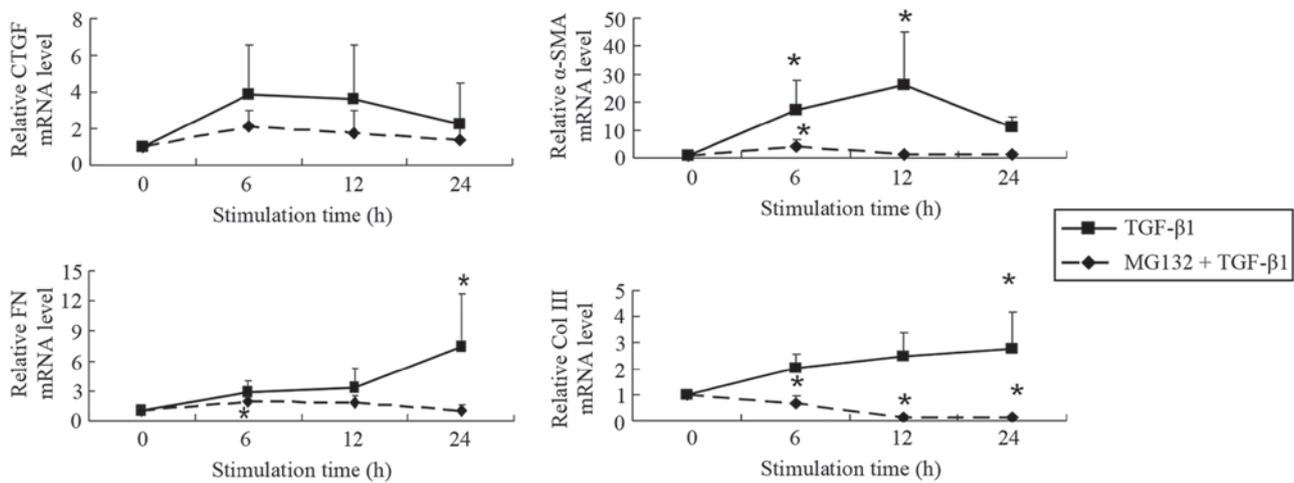
Figure 2. MG132 pretreatment decreases the mRNA level of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in what appears to be a time-dependent manner. The mRNA levels were normalized to the control group. NRK-49F cells were treated with TGF-β1 (5 ng/ml) with or without MG132 (2.5 μM) for designated lengths of time (0-24 h). *P<0.05 vs. the control group. Col III, collagen type III; FN, fibronectin; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin; TGF, transforming growth factor.

with the TGF-β1 group (P<0.05; Fig. 3). TGF-β1 treatment significantly increased α-SMA, CTGF, FN and Col III mRNA levels compared with the control group (P<0.05). α-SMA mRNA levels significantly decreased in the presence of MG132 compared with the control group (P<0.05). TGF-β1 mRNA levels significantly decreased with increasing MG132 concentration compared with the TGF-β1 group (P<0.05); no significant changes were observed compared with the control group (P>0.05). FN mRNA levels significantly decreased compared with the control group at 2.5 and 5 μM MG132 (both P<0.05). Col III mRNA levels significantly decreased with in the presence of MG132 compared with the control group (P<0.05). All mRNA levels exhibited a potential concentration-dependent manner.

MG132 pretreatment decreases the protein expression of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in a potential time-dependent manner. Compared with the control group set to 1, CTGF expression was increased to 1.5-fold at 6 h, 1.7-fold at 12 h and 1.4-fold at 24 h in the TGF-β1 group; indicating that expression may declined over time following an initial increase (Fig. 4). In the group pretreated with MG132, CTGF expression of 1.4, 1.6 and 1.3-fold were measured at 6, 12 and 24 h, respectively. CTGF expression did not change significantly over time or between the TGF-β1 and the MG132 pretreatment groups (P>0.05). Compared with the control group set to 1, α-SMA protein levels increased to 2.6-fold at 6 h, 2.8-fold at 12 h and 3.1-fold at 24 h. Following pretreatment with 2.5 μM MG132, these values were 1.3, 1.7 and 2.0 at 6, 12 and 24 h, respectively; differences between the TGF-β1 and the pretreatment groups or over time were not significant (P>0.05). Protein levels of FN were determined at 2.3, 3.5 and 5.8-fold TGF-β1-treated cells at 6, 12 and 24 h, respectively, compared with the control group. The values determined at 12 and 24 h were significantly increased in the TGF-β1 group compared with the 0 h control (P<0.05). After pre-treatment with MG132, FN protein expression values decreased to 1.8, 1.8 and 2.2-fold at 6, 12 and 24 h, respectively; differences over time or between

the groups were not significant. Expression of Col III was 2.1, 2.0 and 2.8-fold in the TGF-β1 group at 6, 12 and 24 h, respectively and for the MG132 pretreatment group these values were 1.7, 1.8 and 1.8, respectively, compared with the 0 h control; differences between the groups were not significant. Col III protein expression was significantly increased at 24 h in the TGF-β1 group compared with the 0 h control (P<0.05).

MG132 decreases the protein level of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in a potential concentration-dependent manner. Following pretreatment with 5 μM MG132 and stimulation with 5 ng/ml TGF-β1, the protein levels of CTGF, α-SMA, FN and Col III were significantly decreased compared with the TGF-β1 group (P<0.05; Fig. 5). Compared with the TGF-β1 group, the expression of CTGF decreased to 0.6-, 0.4-, 0.3- and 0.3-fold as the MG132 concentration increased to 0.5, 1, 2.5 and 5 μM, respectively; of which changes at 1, 2.5 and 5 μM MG132 were significant (all P<0.05). The expression of α-SMA decreased to 0.7-, 0.6-, 0.6- and 0.5-fold as the MG132 concentration increased to 0.5, 1, 2.5 and 5 μM, respectively, compared with the TGF-β1 group; only the 5 μM MG132 concentration significantly decreased α-SMA expression (P<0.05). The expression of FN decreased to 0.5-, 0.5-, 0.3- and 0.2-fold, and Col III expression stagnated at 1.0, and decreased to 0.8-, 0.4- and 0.3-fold as the MG132 concentration increased to 0.5, 1, 2.5 and 5 μM, respectively, compared with the TGF-β1 group. Compared with the TGF-β1 group, Col III expression was significantly decreased with 2.5 and 5 μM MG132 (both P<0.05).

TGF-β1 increases on the phosphorylation of Smads. The results indicated that NRK-49F cells express the p-Smad2, and p-Smad3 β proteins (Fig. 6). After the cells were stimulated with TGF-β1 for 15 min, the expression of p-Smad2 and p-Smad3 increased, reaching their peak expression after 1 h of treatment, but decreasing after 2 h. Compared with the control group, p-Smad3 levels were 1.8-fold more greatly expressed at 15 min, 3.6-fold higher at 30 min, 4.6-fold higher at 1 h and 2.7-fold
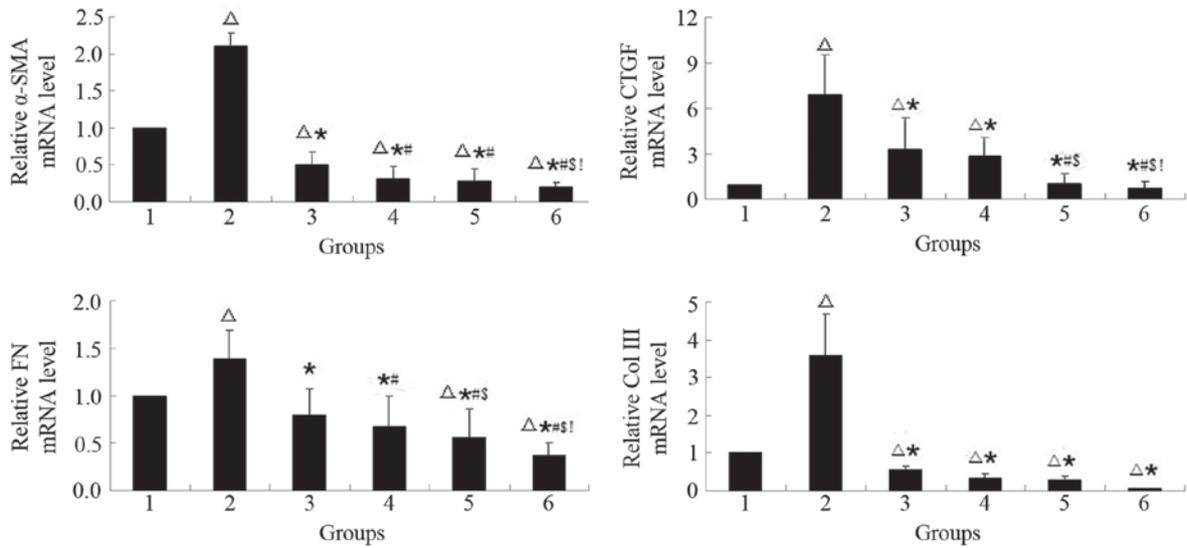
Figure 3. MG132 pre-treatment decreases on the mRNA level of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in a concentration-dependent manner. The mRNA levels were normalized to the control group. The cells were treated with the proteasome inhibitor, MG132, at specific concentrations (0-5 μM) with or without TGF-β1 (5 ng/ml) for 24 h. $^{\Delta}P<0.05$ vs. 1; $^{*}P<0.05$ vs. 2; $^{#}P<0.05$ vs. 3; $^{\$}P<0.05$ vs. 4; and $^{!}P<0.05$ vs. 5. 1, control; 2, 5 ng/ml TGF-β1; 3, 0.5 μM MG132 + 5 ng/ml TGF-β1; 4, 1 μM MG132 + 5 ng/ml TGF-β1; 5, 2.5 μM MG132 + 5 ng/ml TGF-β1; 6, 5 μM MG132 + 5 ng/ml TGF-β1; Col III, collagen type III; FN, fibronectin; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin; TGF, transforming growth factor.
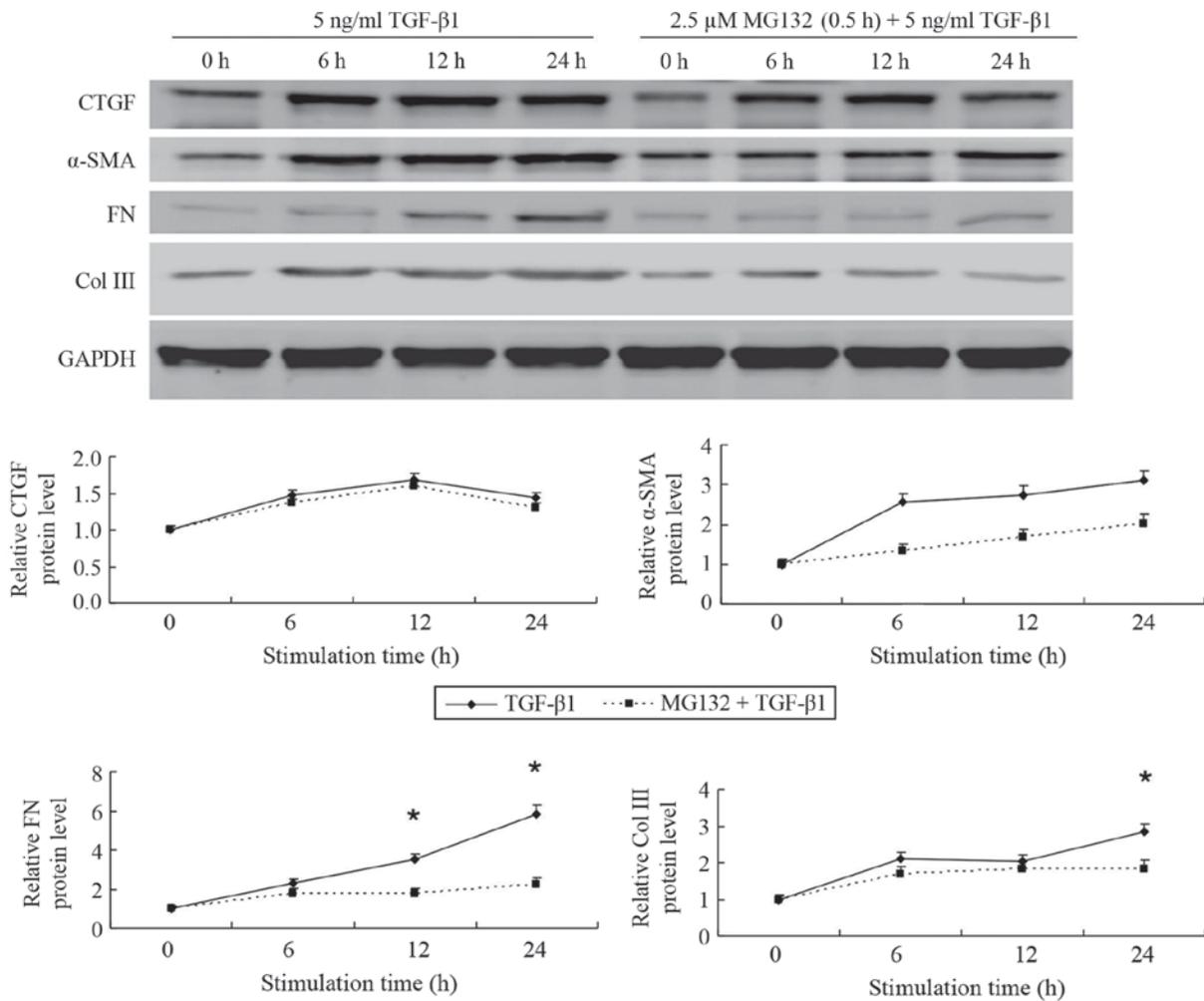

Figure 4. MG132 pretreatment decreases the protein expression of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in what appears to be a time-dependent manner. The protein levels were normalized to the control group. NRK-49F cells were treated with TGF-β1 (5 ng/ml) with or without MG132 (2.5 μM) for 0, 6, 12 or 24 h. $^{*}P<0.05$ vs. the control group. Col III, collagen type III; FN, fibronectin; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin; TGF, transforming growth factor.
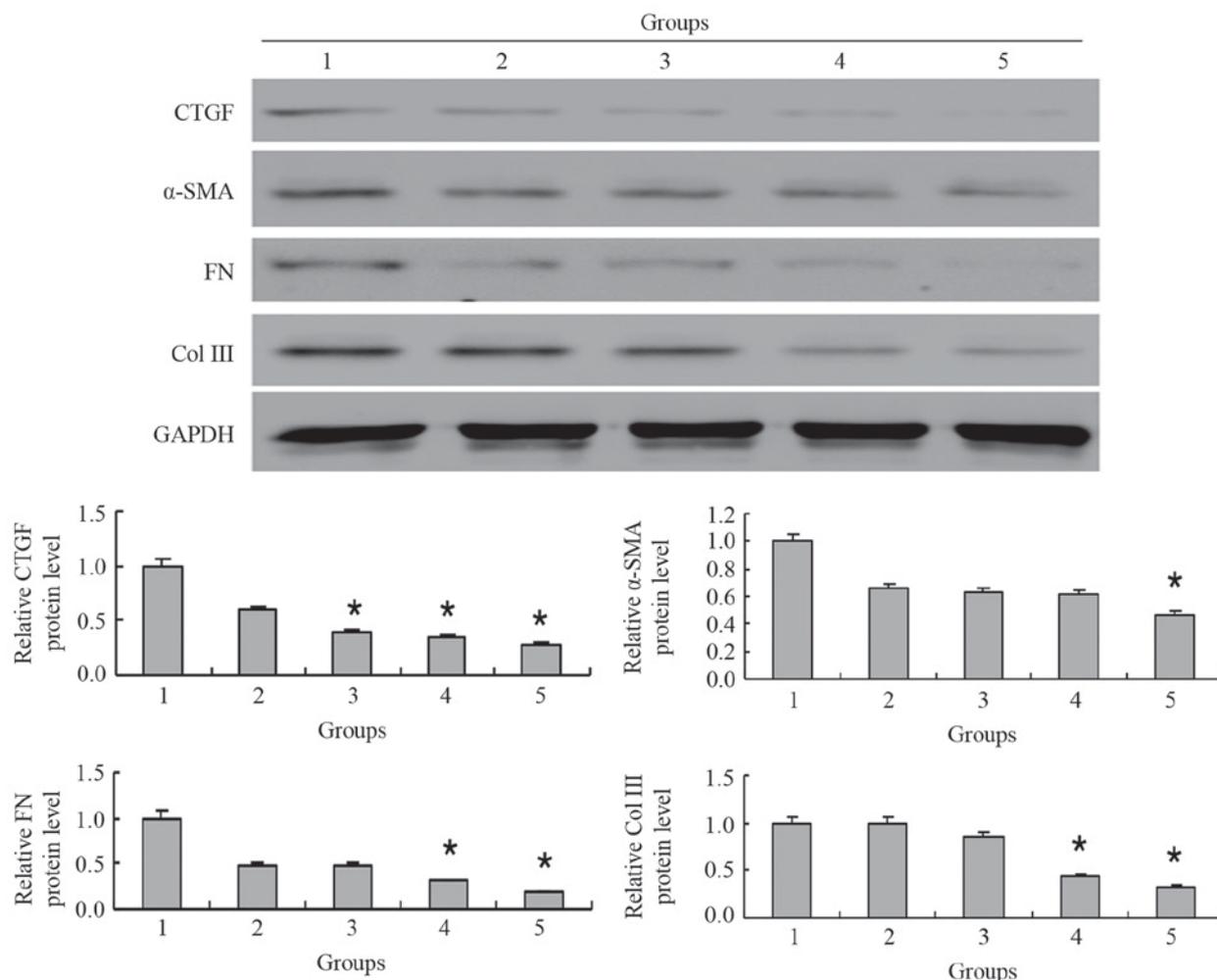
Figure 5. MG132 decreases the protein level of fibrosis-associated factors in NRK-49F cells simulated by TGF-β1 in what appears to be a concentration-dependent manner. The protein levels were normalized to the control group. The cells were treated with the proteasome inhibitor, MG132, at specific concentrations (0-5 μM) with TGF-β1 (5 ng/ml) for 24 h. *P<0.05 vs. 2. 1, 5 ng/ml TGF-β1; 2, 0.5 μM MG132 + 5 ng/ml TGF-β1; 3, 1 μM MG132 + 5 ng/ml TGF-β1; 4, 2.5 μM MG132 + 5 ng/ml TGF-β1; 5, 5 μM MG132 + 5 ng/ml TGF-β1; Col III, collagen type III; FN, fibronectin; CTGF, connective tissue growth factor; α-SMA, α-smooth muscle actin; TGF, transforming growth factor.

higher at 2 h. p-Smad3 levels were significantly increased after 30 min and 1 h of TGF-β1 treatment compared with the control group (both P<0.05). The levels of p-Smad2 expression were 1.4-, 3.9-, 5.5- and 2.5-fold more highly expressed at 15, 30 min, 1 and 2 h, respectively. p-Smad2 levels were significantly increased after 15, 30 min and 1 h of TGF-β1 treatment compared with the control group (all P<0.05).

MG132 decreases the phosphorylation of Smads in NRK-49F cells simulated by TGF-β1. The results indicated that 5 ng/ml TGF-β1 significantly increased the expression of p-Smad2 and p-Smad3 compared with the control group (P<0.05; Fig. 7). After pretreatment with different concentrations of MG132 and then stimulation with 5 ng/ml TGF-β1, the expression levels of p-Smad2 and p-Smad3 decreased. TGF-β1 increased the p-Smad3 protein expression levels to 5.9-fold compared with the control group. This change was decreased to 4.6-, 4.2-, 3.0- and 2.6-fold as the MG132 concentration increased to 0.5, 1, 2.5 and 5 μM, respectively, compared with the control group. p-Smad3 levels were significantly decreased with 1, 2.5 and 5 μM MG132 pretreatments compared with the TGF-β1

group (all P<0.05). TGF-β1 increased the p-Smad2 levels to 1.2-fold compared with the control group. Compared with the control group, fold-changes decreased to 1.1-, 1.0-, 1.0- and 0.9-fold, as the MG132 concentration increased to 0.5, 1, 2.5 and 5 μM, respectively. p-Smad2 levels were significantly decreased with 2.5 and 5 μM MG132 pretreatments compared with the TGF-β1 group (both P<0.05).

MG132 decreases the expression of FN in NRK-49F cells simulated by TGF-β1. TGF-β1 (5 ng/ml) induced a significant, 11.9-fold increase in the FN protein expression levels compared with control group (P<0.05; Fig. 8). Fold-changes decreased to 8.0, 6.5, 3.1 and 2.1-fold as the MG132 concentration increased to 0.5, 1, 2.5 and 5 μM, respectively. FN levels decreased significantly compared with the control and TGF-β1 groups, respectively (both P<0.05).

Discussion

Renal interstitial fibroblasts are a subtype of renal interstitium intrinsic cells, which constitute the main ECM-secreting cell
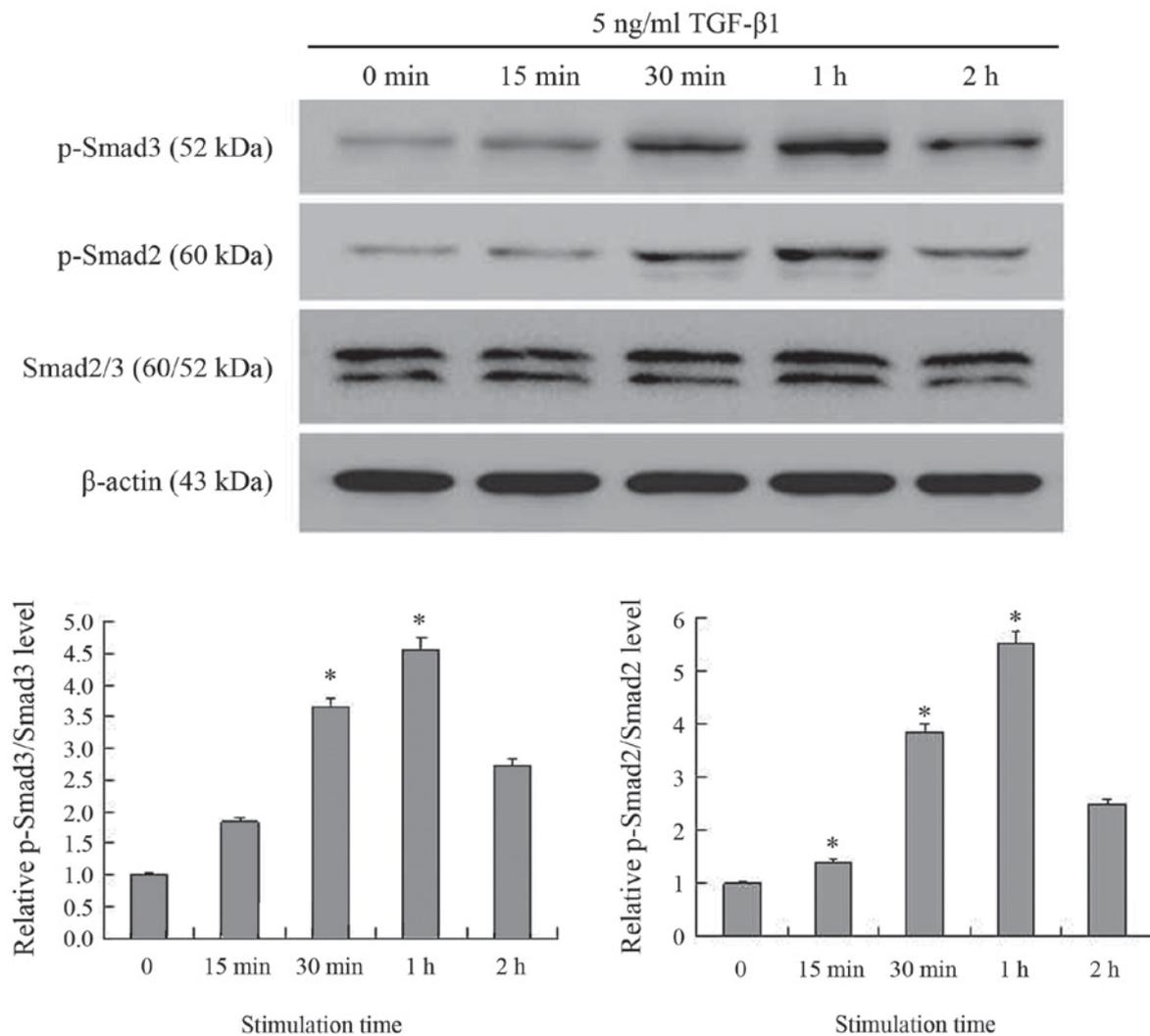
Figure 6. TGF-β1 increases on the phosphorylation of Smads. The protein levels were normalized to the control group. NRK-49F cells were treated with 5 ng/ml TGF-β1 for 0-120 min. *P<0.05 vs. the control group. The experiments were repeated three times. TGF, transforming growth factor; p-, phosphorylated; Smad, mothers against decapentaplegic homolog.

type during fibrosis (22). Due to their ability to proliferate quickly, interstitial fibroblasts can produce copious amounts of FN and collagen, specifically Col I and Col III (23). These cells serve a very important role in renal interstitial fibrosis, overproducing ECM materials that lead to scarring and fibrosis (24). Therefore, the authors of the current study monitored the expression of ECM materials and the signal transduction pathways regulating their production when renal interstitial fibroblasts were stimulated with pro-inflammatory signals (e.g. TGF-β1). TGF-β1 is closely connected with renal interstitial fibrosis, as it promotes the production of ECM materials (Col I, III and IV, and FN), while decreasing the expression of matrix metalloproteinases, thereby increasing the generation of plasminogen activator inhibitor and tissue inhibitor of metalloproteinases, which altogether slow ECM degradation (25). When the TGF-β1 signal is transduced, it connects with TGF-β1 receptor I and II, causing phosphorylation of the signal transduction factors, Smad2 and Smad3; these factors translocate to the cell nucleus with Smad4 to initiate the transcription of genes involved in fibrosis, cell proliferation and inflammation (26). The Smad signaling pathway has been

implicated in several renal diseases and pathophysiologic reactions, including diabetic nephropathy, glomerular nephritis and glomerular sclerosis (27).

CTGF is an important downstream effector of TGF-β1 (28). It has been demonstrated to promote TGF-β1-induced cell proliferation and ECM deposition, inducing conglutination and chemotactic effects, while promoting epithelial to mesenchymal transition (29). Several *in vitro* studies have determined that CTGF stimulates the proliferation of cardiac fibroblasts and increase the production of the ECM (30,31). Resulting myofibroblasts and tubular epithelial cells have been revealed to produce ECM materials to induce interstitial fibrosis (32). α-SMA is a phenotypic transformation marker that is highly expressed in myofibroblasts, which are widely used as a marker of cell differentiation, while its production simultaneously contributes significantly to fibrosis (33).

Activation of the ubiquitin-proteasome pathway has been demonstrated to lead to the selective degradation of intracellular proteins and to the regulation of their degradation (34). By controlling the concentration of intracellular proteins, cells can maintain their internal environment (35). Key
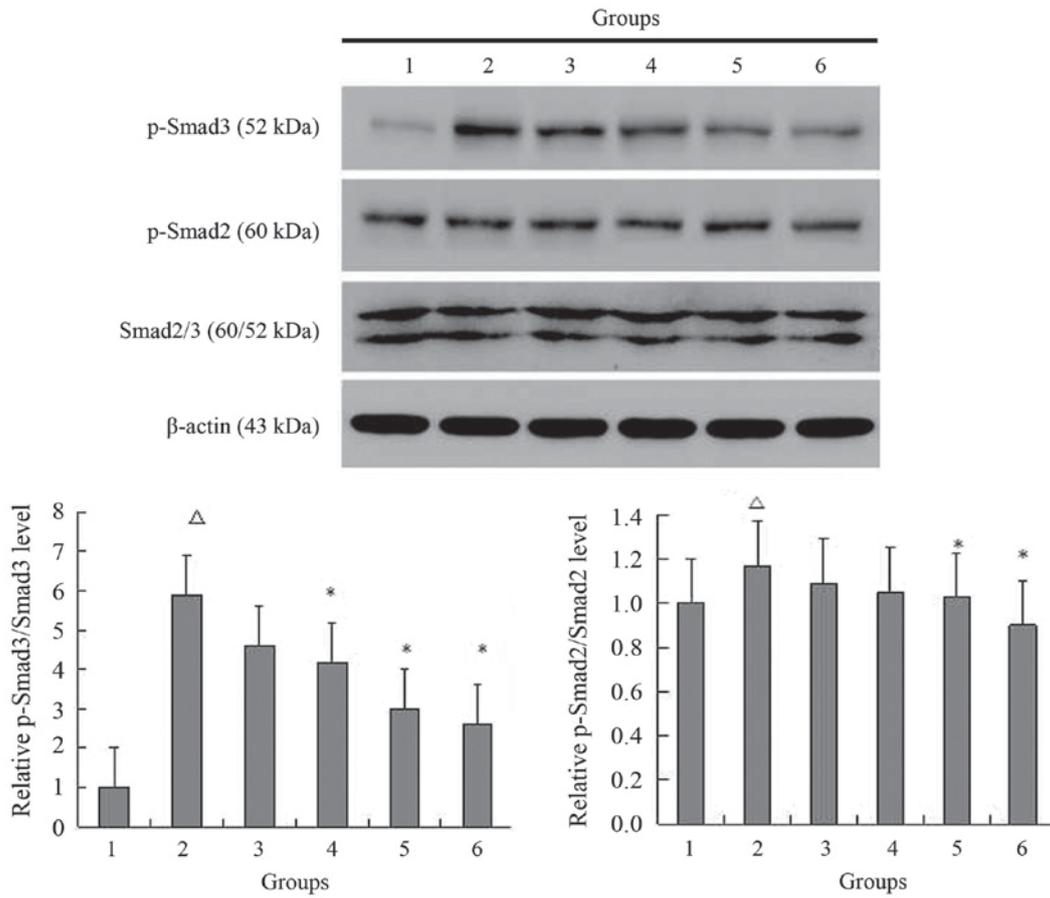
Figure 7. MG132 decreases the phosphorylation of Smads in NRK-49F cells simulated by TGF-β1. The protein levels were normalized to the control group. The cells were treated with the proteasome inhibitor MG132 at specific concentrations (0-5 μM) with or without TGF-β1 (5 ng/ml) for 1 h. *P<0.05 vs. 2; ᴬP<0.05 vs. 1. The experiments were repeated three times. 1, control; 2, 5 ng/ml TGF-β1; 3, 0.5 μM MG132 + 5 ng/ml TGF-β1; 4, 1 μM MG132 + 5 ng/ml TGF-β1; 5, 2.5 μM MG132 + 5 ng/ml TGF-β1; 6, 5 μM MG132 + 5 ng/ml TGF-β1; TGF, transforming growth factor; p-, phosphorylated; Smad, mothers against decapentaplegic homolog.
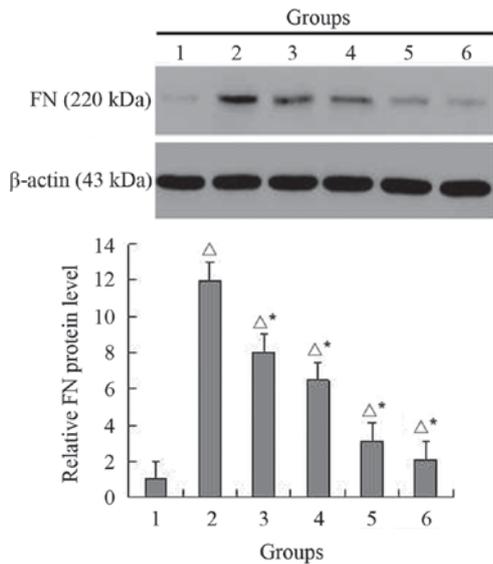

Figure 8. MG132 decreases the expression of FN in NRK-49F cells simulated by TGF-β1. The protein levels were normalized to the control group. The cells were treated with the proteasome inhibitor MG132 at specific concentrations (0-5 μM) with or without TGF-β1 (5 ng/ml) for 24 h. *P<0.05 vs. 2; ᴬP<0.05 vs. 1. The experiments were repeated three times. 1, control; 2, 5 ng/ml TGF-β1; 3, 0.5 μM MG132 + 5 ng/ml TGF-β1; 4, 1 μM MG132 + 5 ng/ml TGF-β1; 5, 2.5 μM MG132 + 5 ng/ml TGF-β1; 6, 5 μM MG132 + 5 ng/ml TGF-β1. TGF, transforming growth factor; FN, fibronectin.

proteins in this pathway include those that control inflammation and the cell cycle (36). Therefore, proteasome inhibitors have potential therapeutic applications in limiting inflammation and tumor growth (37). Clinical have demonstrated that Bortezomib (the first proteasome inhibitor drug) can induce the apoptosis of several haemal and solid tumors, including multiple myeloma, mantle cell lymphoma, non-small cell lung carcinoma, oophoroma, carcinoma of the pancreas, carcinoma of the prostate, and head and neck neoplasms (38). Proteasome inhibitors have been adopted in pilot studies involving antibody-mediated renal rejection in amyloid light chain amyloidosis with increasing scientific interest in their possible applications in lupus, IgA nephropathy, idiopathic nephrotic syndrome and renal fibrosis therapies (39,40). The ubiquitin-proteasome inhibitor, MG132, is a specific inhibitor that directly affects uridine phosphorylase (UPP) (41). When UPP is inhibited, the degradation of intracellular abnormal proteins, such as caspase 3, reduces (42). Activated caspase 3 decomposes substrates in the cytoplasm and nucleus, resulting in chromosome condensation, mitochondrial swelling and ultimately apoptosis (43). Caspase 3 can thereby reduce extracellular matrix secretion through the lysis of cells involved in its generation (44). Studies have revealed that MG132 can inhibit alimentary canal neoplasms and leukemia (45-47).

The authors of the current study used TGF-β1 to induce myofibroblast transformation in NRK-49F cells and observed that p-Smad2 and p-Smad3 protein expression increased; these proteins have been known to promote the signal transduction pathways involved in fibrosis. It was demonstrated that MG132 can decrease the effects of TGF-β1 by reducing the transcription of key factors involved in fibrosis, including CTGF, α-SMA, FN and Col III.

During the TGF-β1 signal transduction process, there are no known proteins that readily switch off transcription (48). Therefore, inhibiting proteins involved in the TGF-β1 signaling pathway (e.g. Smad2, 3 and 4) is a plausible approach to limiting fibrosis (49). Another possible target would be the down-regulation of the Smad7 protein, which can lead to the inhibition of receptor-activated Smad-Smad4-complex activity, preventing the signal from progressing, thereby also decreasing or slowing the fibrotic process (50).

Ultimately, proteasome inhibitors possess some efficacy in delaying or impeding the process of renal interstitial fibrosis. They can promote cell apoptosis while down-regulating cytokine production, inflammation and the deposition of ECM materials, which has been determined to contribute to fibrosis (51). Therefore, the application of proteasome inhibitors in the treatment of fibrosis may be widely beneficial.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH served an important role in interpreting the results, and drafting and writing the manuscript. LH, HC and JL performed experiments. BZ and YJ performed the statistical analyses of the data. WW was involved in drafting and reviewing the manuscript and contributed to the analysis and interpretation of data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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