Pharmacological Reports
2011, 63, 1137–1144
ISSN 1734-1140

IFN-γ suppresses the high glucose-induced increase in TGF-β1 and CTGF synthesis in mesangial cells

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Abstract:
Mesangial cells are the main source of renal interstitial fibrosis in diabetic nephropathy (DN). Interferon-γ (IFN-γ) is a key cytokine that may play a potential therapeutic role in reducing fibrosis. Here, we focus on the effects of IFN-γ on human mesangial cells (HMCs) treated with high glucose. This study shows that IFN-γ phosphorylates STAT1, suppresses HMC proliferation, and down-regulates mRNA and protein levels of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) in HMCs treated with high glucose. The regulation of P-STAT1 could change HMC proliferation and the expression of fibrotic cytokines TGF-β1 and CTGF in HMCs. These data indicate that IFN-γ could activate STAT1 to suppress the increase in TGF-β1 and CTGF synthesis in HMCs induced by high glucose. This paper may lead to new therapeutic treatments of DN.

Key words:
CTGF, TGF-β1, STAT1, mesangial cell

Abbreviations: CTGF – connective tissue growth factor, DN – diabetic nephropathy, ECM – extracellular matrix, HG – high glucose (25 mmol/l glucose), HMC – human mesangial cells, IFN-γ –interferon-γ, JAK2/STAT – Janus kinase 2/signal transducers and activators of transcription, NG – normal glucose (5.5 mmol/l glucose), mRNA – messenger ribonucleic acid, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PVDF – polyvinylidene fluoride, RT-PCR – reverse transcription polymerase chain reaction, SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TGF-β1 – transforming growth factor-β1

Introduction

Increased interstitial mesangial fibrosis correlates closely with declining renal function in diabetic nephropathy (DN) [17, 19]. IFN-γ have been reported to play a potential therapeutic role in reducing fibrosis [20, 21]. However, it is unclear how IFN-γ affects mesangial cells that have been treated with high levels of glucose.

Mesangial cells can affect renal injury in several ways. Cell proliferation and extracellular matrix (ECM) synthesis, which are characteristics of mesangial cell activation, occur in DN and cause interstitial fibrosis [7, 12]. We have previously reported that human mesangial cells (HMCs) treated with high glucose demonstrate two features: they grow faster and produce fibrotic cytokines [8]. Fibrotic cytokines transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) are important in the glomerular accumulation of ECM and can induce persistent fibrosis [15, 27, 28]. These functions are important in treating DN with renal interstitial fibrosis.

We have shown that the inhibition of Janus kinase 2/signal transducers and activators of the transcription (JAK2/STAT) pathway is helpful in treating DN [8], but the affects of regulating STAT separately from this pathway remain unclear. The interaction of IFN-γ with its cell surface receptor leads to the phosphorylation of JAK2, which then phosphorylates the transcription factor STAT1. Phosphorylated STAT1 forms a homodimer and translocates to the nucleus where it binds to the interferon-γ activation site [5, 6, 25]. Fludarabine is a nucleoside analog used in the treatment of hematologic malignancies that can also inhibit cytokine-induced STAT1 phosphorylation. Fludarabine can specifically deplete STAT1 without depleting other STAT proteins [11, 13].

In this study, we investigated the effects of IFN-γ on HMCs treated with high glucose as well as the molecular mechanisms underlying these effects.

Materials and Methods

Cell line and reagents

Primary human mesangial cells (HMCs) and mesangial cell medium were purchased from ScienCell Research Laboratories (San Diego, CA, USA). The antibodies against JAK2, P-JAK2, STAT1, P-STAT1 and β-actin were all purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies against CTGF and TGF-β1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The RT-PCR system was purchased from Takara Biotechnology (Dalian, China – subsidiary of Japan TaKaRa Bio Inc.). IFN-γ was purchased from Roche Diagnostics (Nutley, NJ, USA). D-(+)-glucose, and the STAT-1 inhibitor fludarabine were purchased from Sigma Aldrich (St. Louis, MO, USA). Other chemicals were of analytical reagent grade and were purchased locally from commercial suppliers.

Cell culture

Primary HMCs were seeded in 25-cm^2 tissue culture flasks in mesangial cell medium under either normal glucose (NG, 5.5 mmol/l glucose) or high glucose (HG, 25 mmol/l glucose) conditions. The culture medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere. The cell medium was changed every other day until the cells became confluent. Cells in passages 3–6 were used. HMCs at approximately 70–80% confluence were cultured in serum-free 1640 medium with NG for 24 h to synchronize the cell growth. Then, the medium was replaced with fresh serum-free medium containing NG, HG or HG in the presence of IFN-γ or fludarabine.

Cell proliferation measurements

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in 96-well microplates. Nine groups were given different levels of glucose with or without IFN-γ or fludarabine. Medium (180 μl) was added to every well. After 44 h, 20 μl of 5 mg/ml MTT was added to each well. Four hours later, the medium was replaced with 200 μl of dimethyl sulfoxide, and the 96-well microplate was shaken gently. Next, the absorbance was measured at 570 nm, and these data were transformed into a variable representing the number of cells in each well by using a curve that correlated the absorbance to the number of HMCs.

RNA extraction and RT-PCR analyses for TGF-β1 mRNA and CTGF mRNA

Two micrograms of template RNA was reverse transcribed using oligo (dT)18 primers in a final volume of 20 μl. Human TGF-β1 and β-actin were amplified using the following primers: TGF-β1 (161 bp), forward 5'-GCCCTGGACACCAACTATTGC-3', reverse 5'-AGGCTCCAAATGTAGGGGCAGG-3'; β-actin (539 bp), forward 5'-GTGGGGCGCCCCAGGCACCA-3', reverse 5'-CTCCTTAATGTCACGCACGATTTC-3'.

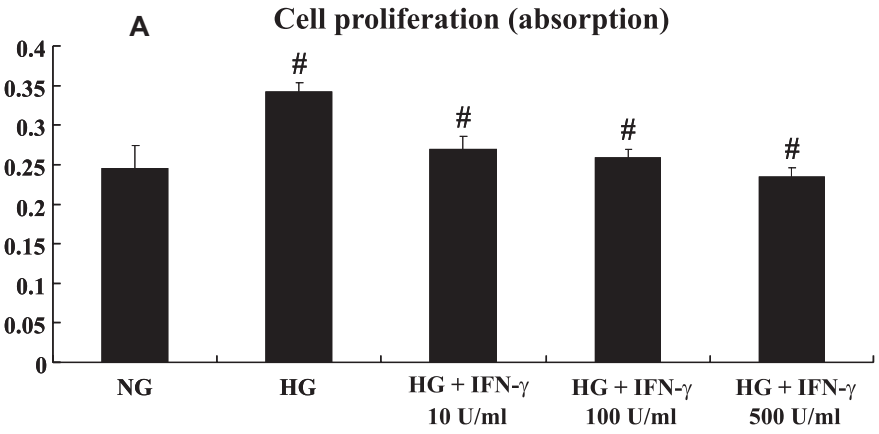
The PCR conditions were 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 60.5°C for 20 s, and 72°C for 50 s. Human CTGF (379 bp) was amplified using the following primers: forward 5'- CTAAGACCTGTG-GGATGGGC-3', reverse 5'-CTC AAAGATGTCATT-GTCCCC-3'. The PCR conditions were 94°C for 2 min, 35 cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 1.5 min, followed by final extension for 10 min at 72°C. The PCR products were subjected to 2% agarose gel electrophoresis, and the resulting gel was analyzed with a GDS-8000 Bioimaging system (UVP, Upland, CA, USA) and GelWorks 1D Grab It software. RNA expression was quantified by comparison with the internal-control β-actin.

Western blot analyses of JAK2, P-JAK2, STAT1, P-STAT1, TGF-β1 and CTGF proteins

To extract the total protein, each dish was treated for 60 min with ice-cold lysis buffer. The lysates were then centrifuged at 10,000 × g for 5 min at 4°C. The protein concentration was assessed by a Bradford protein assay (Bio-Rad, Richmond, CA, USA). Subsequently, samples (50 μg of protein/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7%), transferred to polyvinylidene fluoride (PVDF) membranes, and blocked by a 60-min incubation at room temperature (22°C) in TTBS (TBS with 0.05% Tween 20, pH 7.4) plus 5% BSA. PVDF membranes were then incubated overnight at 4°C with CTGF, TGF-β1, JAK2, P-JAK2, STAT1, P-STAT1 or β-actin antibodies. After thoroughly washing, membranes were incubated for 100 min at room temperature with goat antirabbit IgG horseradish peroxidase conjugate. The proteins were detected using an ECL detection system. The intensities of the bands were measured using Image J.

Data analysis

All results are expressed as the mean ± SD. Differences were evaluated using unpaired *t*-tests and an ANOVA; $p < 0.05$ was considered statistically significant.



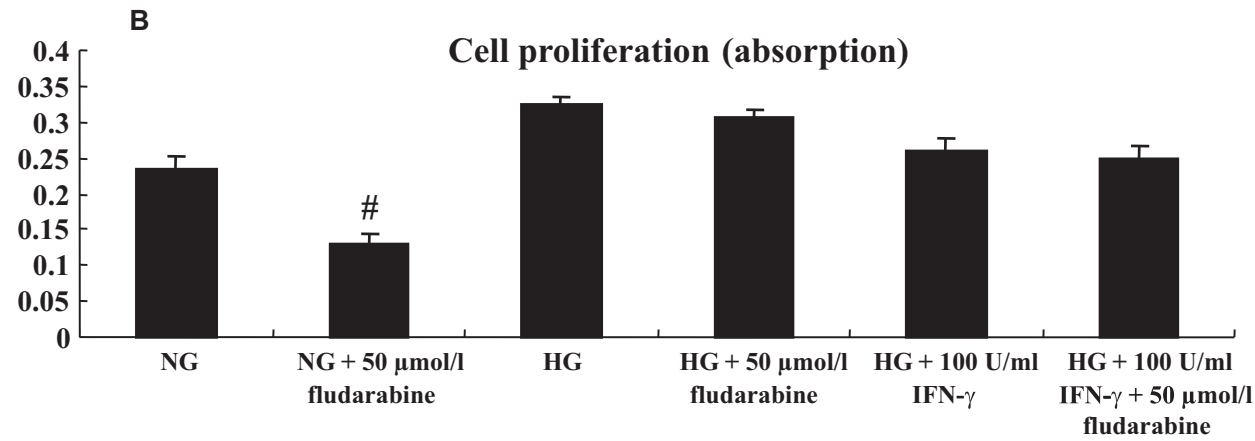


Fig. 1. Effects of IFN-γ on overproliferation induced by HG. HMC proliferation was measured with an MTT assay. Values are expressed as the means ± SD for five independent experiments. (**A**) HG induced a significant increase in HMC proliferation compared with the NG group; (# $p < 0.01$) IFN-γ inhibited HMC proliferation significantly and dose dependently (# $p < 0.01$). (**B**) Fludarabine inhibited HMC proliferation in NG conditions (# $p < 0.01$) but failed to inhibit HMC proliferation treated with HG or IFN-γ

Results

IFN-γ inhibits the HG-induced HMC overproliferation

To examine the antiproliferative effects of IFN-γ on HMCs treated with high glucose, we analyzed HMC proliferation with MTT assays. As shown in Figure 1A, the proliferation of HMCs peaked in the HG group (NG: 0.244 ± 0.030 *vs.* HG: 0.342 ± 0.012, $p < 0.01$, $n = 5$). The addition of 10 U/ml IFN-γ and 100 U/ml IFN-γ decreased HMC proliferation significantly (HG: 0.342 ± 0.012 *vs.* HG + 10 U/ml IFN-γ: 0.269 ± 0.017 HG + 100 U/ml IFN-γ: 0.258 ± 0.011 $p < 0.01$ $n = 5$) while adding 500 U/ml IFN-γ overinhibited HMC proliferation (HG + 500 U/ml IFN-γ: 0.235 ± 0.011 *vs.* NG: 0.244 ± 0.030). These results suggest that IFN-γ inhibits the overproliferation of HMCs induced by HG in a dose-dependent manner. Figure 1B shows that 50 μmol/l fludarabine decreases HMC proliferation when the cells are cultured in NG (NG: 0.233 ± 0.019 *vs.* NG + 50 μmol/l fludarabine: 0.130 ± 0.015 $p < 0.01$ $n = 5$) but that it has no significant effect when the cells are treated with HG and IFN-γ.

IFN-γ decreases the upregulated mRNA levels of TGF-β1 and CTGF induced by high glucose

To examine the antifibrotic effects of IFN-γ on HMCs treated with high glucose, we focused on TGF-β1 mRNA and CTGF mRNA. As shown in Figure 2, HMCs in the HG group showed a significantly higher mRNA level of TGF-β1 than the NG group (NG: 0.575 ± 0.034 *vs.* HG: 0.845 ± 0.088, $p < 0.01$ $n = 3$). Both 10 U/ml and 100 U/ml of IFN-γ significantly suppressed the upregulation of TGF-β1 mRNA (HG: 0.845 ± 0.088 *vs.* HG +10 U/ml IFN-γ: 0.733 ± 0.028 $p < 0.05$ $n = 3$; HG: 0.845 ± 0.088 *vs.* HG +100 U/ml IFN-γ: 0.664 ± 0.009 $p < 0.01$ $n = 3$). The HG-induced upregulation of CTGF mRNA was attenuated by 100 U/ml IFN-γ (HG: 0.608 ± 0.100 *vs.* HG + 100 U/ml IFN-γ: 0.393 ± 0.080; $p < 0.05$ $n = 3$). IFN-γ decreased the upregulated mRNA levels of TGF-β1 and CTGF induced by high glucose in a dose-dependent manner.

IFN-γ reduces the syntheses of TGF-β1 and CTGF proteins induced by high glucose

To further confirm the antifibrotic effects of IFN-γ on HMCs, we investigated the changes of TGF-β1 and CTGF proteins by western blot analysis. As shown in Figure 3, HG induced the overexpression of the TGF-β1 and CTGF proteins (TGF-β1, NG: 1.243 ± 0.279 *vs.* HG: 1.825 ± 0.252, $p < 0.01$ $n = 3$) (CTGF, NG: 0.999 ± 0.127 *vs.* HG: 1.815 ± 0.166, $p < 0.01$ $n = 3$). The overexpression of TGF-β1 and CTGF were attenuated by 10 U/ml IFN-γ (TGF-β1, HG: 1.825 ± 0.252 *vs.* HG + 10 U/ml IFN-γ: 1.203 ± 0.220 $p < 0.05$ $n = 3$) (CTGF, HG: 1.815 ± 0.166 *vs.*

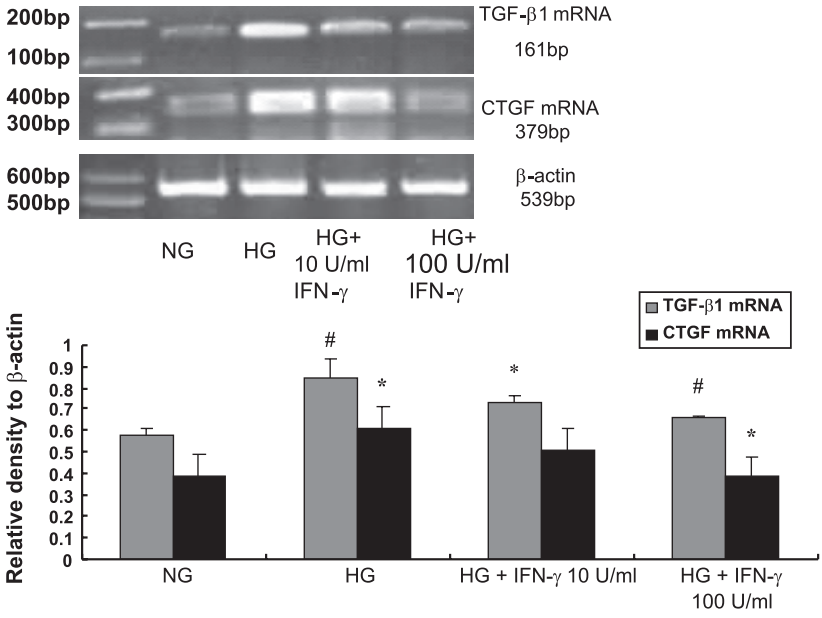


Fig. 2. IFN-γ inhibited the upregulated mRNA levels of TGF-β1 and CTGF induced by high glucose. HMC were pretreated with NG, HG, HG + 10 U/ml IFN-γ, or HG + 100 U/ml IFN-γ for 48 h. Densitometry results were based on three experiments. Representative RT-PCR results show HG upregulated mRNA levels of TGF-β1 and CTGF in HMCs compared with the NG group (* $p < 0.05$, # $p < 0.01$). IFN-γ inhibited the upregulation of mRNA levels of TGF-β1 and CTGF induced by HG in a dose-dependent manner (* $p < 0.05$, # $p < 0.01$)

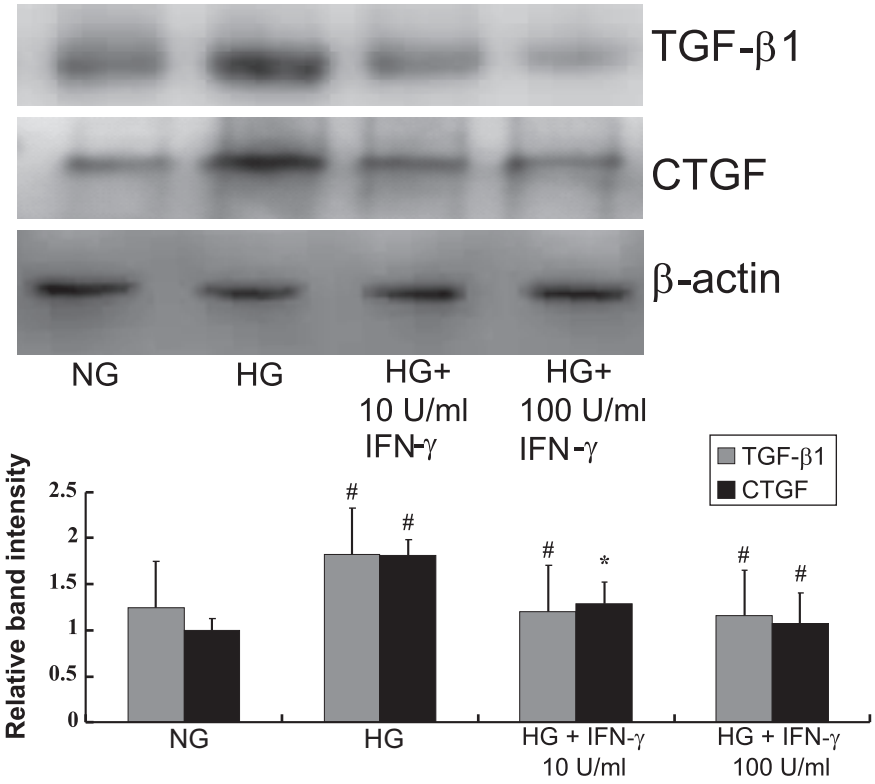


Fig. 3. Suppression of HG induced productions of TGF-β1 and CTGF proteins by IFN-γ. HMCs were pretreated with NG, HG, HG + 10 U/ml IFN-γ or HG + 100 U/ml IFN-γ for 48 h. Immunoblotting of cell extracts was then performed for TGF-β1 and CTGF as described. Values are expressed as the means ± SD for three independent experiments. HG induced the overexpression of both TGF-β1 and CTGF proteins compared with the NG group (# $p < 0.01$). IFN-γ decreased the overexpression of the fibrotic cytokines TGF-β1 and CTGF in a dose-dependent manner (* $p < 0.05$, # $p < 0.01$)

HG + 10 U/ml IFN-γ: 1.290 ± 0.240; $p < 0.01$ n = 3). The addition of 100 U/ml IFN-γ was more effective than 10 U/ml IFN-γ (TGF-β1, HG: 1.825 ± 0.252 *vs.* HG + 100 U/ml IFN-γ: 1.151 ± 0.037 $p < 0.01$ n = 3) (CTGF, HG: 1.815 ± 0.166 *vs.* HG + 100 U/ml IFN-γ: 1.075 ± 0.330; $p < 0.01$ n = 3). These results confirm that IFN-γ decreases the syntheses of the fibrotic cytokines TGF-β1 and CTGF in HMCs treated with high glucose in a dose-dependent manner.

Effects of the regulation of P-STAT1 on TGF-β1 and CTGF

To study the molecular mechanism for the protective effect of IFN-γ on HMC, the JAK2-STAT1 pathway was regulated by IFN-γ and fludarabine. In Figure 4, P-STAT1 was significantly higher in the HG + 100 U/ml IFN-γ group than in the HG group (HG + 100 U/ml IFN-γ: 2.545 ± 0.323 *vs.* HG: 1.929 ± 0.268, $p < 0.05$ n = 3) while the upregulation of P-STAT1 was decreased by 50 μmol/l fludarabine (HG + 100 U/ml IFN-γ: 2.545 ± 0.323 *vs.* HG + 100 U/ml IFN-γ + 50 μmol/l fludarabine: 1.937 ± 0.119, $p < 0.05$ n = 3). The protein levels of JAK2, P-JAK2, and STAT1 remained unchanged. Moreover, as shown in Figure 4, the overexpression of TGF-β1 and CTGF proteins induced by high glucose was decreased by 100 U/ml IFN-γ (TGF-β1 HG: 2.788 ± 0.466 *vs.* HG + 100 U/ml IFN-γ: 1.600 ± 0.248 $p < 0.05$ n = 3) (CTGF HG: 2.159 ± 0.157 *vs.* HG + 100 U/ml IFN-γ: 1.368 ± 0.386 $p < 0.05$ n = 3). However, the levels of both proteins were increased by the addition of 50 μmol/l fludarabine (TGF-β1 HG + 100 U/ml IFN-γ: 1.600 ± 0.248 *vs.* HG + 100 U/ml IFN-γ + 50 μmol/l fludarabine: 2.393 ± 0.437 $p < 0.05$ n = 3) (CTGF HG + 100 U/ml IFN-γ: 1.368 ± 0.386 *vs.* HG + 100 U/ml IFN-γ + 50 μmol/l fludarabine: 2.054 ± 0.212 $p < 0.05$ n = 3). When IFN-γ phosphorylated STAT1, the levels of TGF-β1 and CTGF decreased, and when fludarabine inhibited the activation of STAT1, the levels of TGF-β1 and CTGF increased. These results indicate that IFN-γ suppresses the increase of TGF-β1 and CTGF proteins induced by high glucose through the activation of STAT1.

Discussion

Given the central role of IFN-γ in reducing fibrosis, the effect of IFN-γ on the declining renal function in

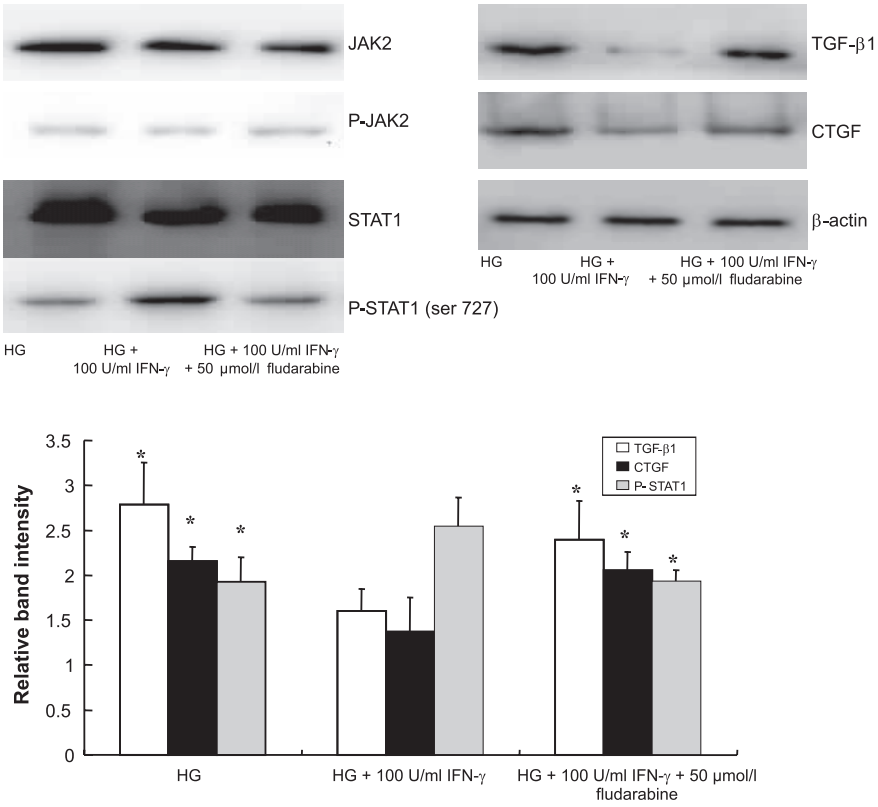


Fig. 4. Effects of the regulation of P-STAT1 on TGF-β1 and CTGF. HMCs were pretreated with HG for 48 h, and then, 100 U/ml IFN-γ and 50 μmol/l fludarabine were added to the corresponding groups. After 30 min of stimulation, immunoblotting of cell extracts was performed for STAT1, P-STAT1, JAK2, and P-JAK2, as previously described. After another 48 h, TGF-β1 and CTGF proteins were examined. Values are expressed as the means ± SD for three independent experiments. The western blots that are shown are representative of three independent experiments. The overexpression of TGF-β1 and CTGF proteins induced by high glucose were decreased by the addition of 100 U/ml IFN-γ (* $p < 0.05$), while the synthesis of TGF-β1 and CTGF proteins increased when HMCs were pretreated with 50 μmol/l fludarabine (* $p < 0.05$). However, STAT1 was activated by 100 U/ml IFN-γ (* $p < 0.05$), and 50 μmol/l fludarabine inhibited the activation of STAT1 (* $p < 0.05$)

DN is a subject of considerable interest. The antiproliferative effect of IFN-γ has been investigated in many cell types, such as mouse embryo fibroblast cells, pancreatic stellate cells and rat mesangial cells [9, 14, 20]. Because of the vital role of HMCs in DN, we examined the effect of IFN-γ on the HMCs treated with high glucose. We found that IFN-γ inhibited the overproliferation of HMCs induced by HG in a dose-dependent manner. The appropriate concentrations were 10 U/ml and 100 U/ml while 500 U/ml IFN-γ was excluded from our following experiments because it was harmful to the normal growth of HMCs. TGF-β1 and CTGF secreted by HMCs have paracrine actions in renal interstitial fibrosis [16, 26]. Our results showed that addition of either 10 U/ml or 100 U/ml IFN-γ decreased the mRNA and protein levels of TGF-β1 and CTGF. HMC overproliferation and secretions of fibrotic cytokines are important in interstitial fibrosis, which leads to the declining renal function in DN [7, 27]. Treatment with IFN-γ could possibly improve the renal interstitial fibrosis in DN effectively.

STAT1 is implicated in the inhibition of cell proliferation in several cell systems. STAT1 activation has an important role in fibroblast growth arrest, and mice deficient in STAT-1 have increased susceptibility to bleomycin-induced lung fibrosis, indicating that STAT-1 plays a protective role during fibrogenesis [1, 2, 25]. We have shown that the inhibition of the JAK2/STAT pathway is helpful in treating DN [8]. However, the STATs are a family of seven latent cytoplasmic proteins that differentially regulate the expression of numerous genes involved in several cell functions. For example, STAT1 and STAT3 have opposite functions [3, 22, 23]. We have shown that IFN-γ activates STAT1, inhibits the overproliferation of HMCs, and decreases the high levels of TGF-β1 and CTGF proteins induced by HG. The levels of the fibrotic cytokines TGF-β1 and CTGF changed with the regulation of P-STAT1: they decreased when IFN-γ activated STAT1 and increased when the activation of STAT1 was inhibited by fludarabine. Interestingly, 50 μmol/l fludarabine inhibited the activation of STAT1 and decreased the excretion of cytokine TGF-β1 and CTGF in HMCs treated with high glucose. Fludarabine reduced HMC proliferation in NG but failed to inhibit cell proliferation when HMCs were treated by HG or IFN-γ. However, fludarabine is

a specific inhibitor of STAT1 and has cell cytotoxicity [10, 11]. Our studies indicate that its cytotoxicity was neutralized by the antiproliferative effect of the activated STAT1. Previous studies have shown that IFN-γ did not inhibit the growth of U3A cells, which are deficient in STAT1 [3], and the cytotoxicity of fludarabine is associated with a specific depletion of STAT1 in B-CLL cells [11]. These reports indicate that STAT1 is a protective factor. As the activator of STAT1, IFN-γ could activate STAT1 to suppress cell proliferation and decrease the production of the fibrotic cytokines TGF-β1 and CTGF in HMCs treated with high glucose.

Cooker observed that IFN-γ had no effect on either CTGF or collagen activity and produced a mild inhibition of TGF-β1-induced production of collagen only at a high concentration (500 U/ml) [4]. However, we have shown that IFN-γ activates STAT1 to decrease the excessive excretion of TGF-β1 induced by high glucose. Additionally, there is also a decrease in the levels of CTGF, which is a downstream cytokine of TGF-β1. A different effect of IFN-γ on fibrosis in other cell type has been reported. Nareika showed that high glucose and IFN-γ had a synergistic effect on MMP-1 expression by enhancing STAT1 phosphorylation and STAT1 transcriptional activity in U937 macrophages [18].

We think that appropriate concentrations of IFN-γ may improve renal interstitial fibrosis, which can be difficult to treat in DN therapy. The regulation of STAT1 can possibly provide a new path in the investigation of renal interstitial fibrosis in DN. However, it is crucial to investigate the safety and feasibility of such a therapy with further experiments in clinically relevant animal model.

Acknowledgments:
This study was supported by the Major Basic Project of China (973)(2007CB507400), the National Natural Science Foundation (30700369) and the Scientific Research Foundation of the First Clinical Hospital of China Medical University. We thank the research institute of First Hospital of China Medical University for valuable suggestions and help. We thank Yanhui Song (Department of Neonatology, Medical College of Wisconsin, Milwaukee, Wisconsin, USA) for the help on language.

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Received: November 25, 2010; **in the revised form:** March 25, 2011; **accepted:** April 14, 2011.