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Synergistic effects of c-Jun and SP1 in the promotion of TGFβ1-mediated diabetic nephropathy progression

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ABSTRACT

Diabetic nephropathy (DN) is a major complication of diabetes mellitus. Transforming growth factor beta 1 (TGFβ1) is a well-distinguished mediator of progressive renal fibrosis in DN. However, the molecular mechanisms contributing to enhanced TGFβ1 expression in the progression of DN are not fully understood. Herein, we reported that c-Jun and specificity protein 1 (SP1) were critical upstream regulators of TGFβ1 expression in DN. The increase in c-Jun and SP1 expressions was positively correlated with TGFβ1 in both high glucose-treated human renal mesangial cells (HRMCs) and diabetic kidneys. Furthermore, c-Jun dose-dependently promoted SP1-mediated TGFβ1 transcription and *vice versa*. The synergistic effects of c-Jun and SP1 were attributed to their auto-regulation and cross-activation. Moreover, enhanced phosphorylation levels of c-Jun and SP1 were accompanied with increased TGFβ1 expression in diabetic kidneys. Accordingly, dephosphorylation of c-Jun and SP1 by the specific c-Jun N-terminal kinase (JNK) inhibitor SP600125 prevented the increase in TGFβ1 expression. These results suggested that c-Jun and SP1 synergistically activated profibrotic TGFβ1 expression in the development of DN by auto-regulation, cross-activation and phospho-modification.

1. Introduction

Diabetes mellitus (DM) is a well-recognized endocrine metabolic disorder fundamentally characterized by hyperglycemia disturbing the metabolism of carbohydrate, fat and protein (Pal et al., 2014). Of the various DM complications, diabetic nephropathy (DN) is the most common one, which is the leading cause of end-stage renal disease (ESRD) worldwide (Alsaad and Herzenberg, 2007). DN is histologically featured by extracellular matrix (ECM) expansion and loss of glomerular architecture (Jiang et al., 2010). Multiple profibrotic factors are critically involved in ECM deposition, and collaboratively lead to eventual development of renal fibrosis (Lam et al., 2003; Fraser et al., 2002; Tu et al., 2011).

During late stage of DN, transforming growth factor beta 1 (TGFβ1) overexpression plays a crucial role in the development of glomerulosclerosis (Jiang et al., 2010; Koya et al., 2003). As a well-known profibrotic factor, TGFβ1 enhances synthesis of ECM (*i.e.* FN and Collagen IV) and inhibits their degradations, thereby promoting glomerular sclerosis (Weigert et al., 2000). TGFβ1 could be activated by continuous hyperglycemia-induced production of ROS in DN. Activated TGFβ1 in turn elevates ROS levels, leading to excessive production of ECM and eventual severe renal fibrosis (Bakin et al., 2005). The major biological effects of TGFβ1 operate mainly through TGFβ1 type I and type II receptors. Meanwhile, TGFβ1 also acts directly on fibroblasts to induce expression of α-smooth muscle actin (α-SMA) and transform them into collagen I-producing myofibroblasts (Shi-wen et al., 2009). All these mechanisms contribute to TGFβ1-mediated sclerosis, whereas treatments against TGFβ1 were reported to be renoprotective. For instance, repression of TGFβ1 transcription by anti-oxidant Nrf2 or its activator Sulforaphane (SFN) was protective of kidneys from glomerulosclerosis (Jiang et al., 2010; Cui et al., 2012). Administration

Abbreviations: DM, diabetes mellitus; DN, diabetic nephropathy; ESRD, end-stage renal disease; ECM, extracellular matrix; TGFβ1, transforming growth factor-β1; HRMCs, human renal mesangial cells; ROS, reactive oxygen species; α-SMA, α-smooth muscle actin; Nrf2, nuclear factor erythroid 2-related factor 2; SFN, sulforaphane; FN, fibronectin; AP-1, activating protein-1; SP1, specificity protein 1; PAI-1, plasminogen activatorinhibitor-1; IHC, immunohistochemistry; Scr, serum creatinine; BUN, blood urea nitrogen; Ua, uric acid; HE, hematoxylin–eosin; PAS, periodic acid-Schiff stain; PBS, phosphate buffer saline; DAB, diaminobenzidine; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; TBST, Tris-buffered saline with Tween-20; HRP, horseradish peroxidase; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; SD, standard deviation.

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of TGFβ1-neutralizing antibody attenuated renal fibrosis in an experimental glomerulonephritis and DN models (Border et al., 1990; Sharma et al., 1996). Therefore, chronically elevated TGFβ1 expression is the major mediator of progressive fibrosis in DN (Weigert et al., 2000), while therapeutics against TGFβ1 may be promising in DN treatments.

However, the upstream signaling contributing to TGFβ1 expression during DN progression has not been fully characterized. A recent study suggested that ROS-induced c-Jun might be responsible for triggering downstream TGFβ1 activation in DN. Mutation of c-Jun binding sites in *TGFβ1* promoter or treatments with curcumin (a c-Jun inhibitor) abolished high glucose-induced TGFβ1 promoter activity in mesangial cells (Weigert et al., 2000). It was also shown that specificity protein 1 (SP1) was involved in the regulation of high glucose-induced gene expression in the bovine aortic endothelial cells (Du et al., 2000). These studies implied that expression of TGFβ1 might be transcriptionally regulated during renal fibrosis in DN.

c-Jun is one of the components of AP-1 and activates transcription after binding to TRE elements in target gene promoters (Jochum et al., 2001). SP1 is a ubiquitous transcription factor that activates a broad spectrum of mammalian genes (Li and Davie, 2010). Our previous study revealed that both c-Jun and SP1 were activators of TGFβ1 transcript (Gao et al., 2014). However, expression patterns of c-Jun and SP1 in clinical DN cases, especially in late stages, remain unclear. Even less is known about the detailed mechanisms by which c-Jun and SP1 regulate expression of the profibrotic factor TGFβ1 during progression of renal fibrosis in DN. Herein, the aim of this study was to investigate the regulatory mechanisms of TGFβ1 activation by c-Jun and SP1 *in vitro* and *in vivo*. Given that phosphorylation is critical for both transcription factors (Ventura et al., 2003; Chuang et al., 2008), it was also explored whether phosphorylation functioned in c-Jun- and SP1-regulated TGFβ1 expression.

2. Materials and methods

2.1. Human specimens, mouse models and ethical statement

In total, 13 human DN cases for renal biopsy were collected. Another 13 cases that underwent renal biopsies and were diagnosed with minimal lesion were included for control. For each case, 10 consecutive slides were provided for histology and immunohistochemistry (IHC) analysis. Permission to use human biopsy sections was approved by an institutional review board at School of Basic Medical Sciences, Fudan University. All patients provided their full consent to participate in our study. Mogensen et al. (1983) introduced a five-stage category of DN staging in 1983. This staging method has been widely applied in clinical practices and preclinical researches. However, due to the scarcity of clinical tissues (only 13 clinical DN tissues), this study combined stages I, II and III as early stage of DN, while the latter two stages were combined as late stage. Each case was diagnosed and graded as early stage or late stage DN by two experienced pathologists. Mouse kidney tissues from our previously established type II DM model were also used. Ten C57BLKS/J-Lepr (db) (db/db) mice, which genetically developed type II DM shortly after birth and presented with DN after a 24-week feeding period (Gao et al., 2014), were set as DM group. Another ten C57BLKS/J mice, which grew normally were synchronously fed and set as the control group.

2.2. Histology and IHC

Initially, the paraffin-embedded clinical tissues were subjected to hematoxylin and eosin (H&E) staining, periodic acid-Schiff (PAS) staining and Masson's trichrome staining respectively for histological examinations. Subsequently, IHC was performed as described in our previous study (Gao et al., 2014). For IHC quantification, 3 glomerular fields for each case were randomly chosen for automatic counting of positive staining using Image-pro Plus 4.5 (Media Cybernetics, Silver Spring, USA).

2.3. Cells and reagents

Human renal mesangial cells (HRMCs) were purchased from ScienCell (San Diego, CA, USA) and maintained in Dulbecco's modified eagle medium (DMEM) (Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). For some experiments, culture media was modified with low glucose (LG, 5.6 mM) or high glucose (HG, 30 mM) according to the experimental design.

Primary antibodies against c-Jun and SP1 were purchased from Cell Signaling (Boston, MA, USA). Primary antibodies against TGFβ1 and FN were purchased from Abcam (Cambridge, UK) and Calbiochem (San Diego, CA, USA), respectively. Anti-GAPDH antibody and secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibodies against p-c-Jun (Ser73) and p-SP1 (Thr739) were purchased from Signalway Antibody Biotech (College Park, Maryland, USA).

Table 1
Oligonucleotides used in siRNA, qRT-PCR, ChIP, and deletion mutagenesis assays.

Assays	Forward sequences (5′-…-3′)	Reverse sequences (5′-…-3′)
siRNA		
sic-Jun	aacgaccuucuaugacgaugc	gaucgucauagaaggucguu
siSP1	gacaggucaguuggcagacucuaca	uguagagucugccaacugaccuguc
Negative	uucuccgaacgugucacgutt	acgugacacguucggagaatt
qRT-PCR		
c-Jun	tccaagtgccgaaaaaggaag	cgagttctgagctttcaaggt
SP1	agttccagaccgttgatggg	gtttgcacctggtatgatctgt
TGFβ1	tgcacattgcctgttctgct	tgcatcttggttggctgcat
FN	tgcacattgcctgttctgct	tgcatcttggttggctgcat
GAPDH	ctgacttcaacagcgacacc	tgctgtagccaaattcgttgt
ChIP: primers for the four distinct *TGFβ1* promoter regions, respectively		
−2853/−2269	agaacagagtctagcacagg	tccccaggtaaccatcatg
−2268/−1660	atccctcctttcccctctc	caactgttctcgccaactg
−1659/−1159	agactgtcagagctgaccc	ttactgagcacctcccatg
−1158/−504	ctttggtggcgcttg	accgtcctcatctcgcgtg
Site-directed mutagenesis of *TGFβ1* promoter (−2853/−2269)*		
Δc-Jun	gatctccaagggctttatctaccagactg	ataaagcccttggagatccagccccatct
ΔSP1 mut1	gatatcagcttcacgggttcaggaatttttg	aacccgtgaagctgatatcctagacaacgaat
ΔSP1 mut2	agcatgagatgagatggttctggatctcca	aaccatctcatctcatgctgatcccttct
ΔSP1 mut3	gggcccaaagagagcagttcagggacatga	aactgctctctttgggcccagggccttctt

* Indicates the primers used to mutate the one c-Jun binding site and three SP1 binding sites in *TGFβ1* promoter (−2853 to −2269), respectively. Mutated bases are italicized, bold and underlined.