Effect of transforming growth factor-beta on activity of connective tissue growth factor gene promoter in mouse NIH/3T3 fibroblasts[1]

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ABSTRACT

AIM: To investigate the regulatory mechanism of transforming growth factor-beta on activity of connective tissue growth factor promoter in mouse NIH/3T3 fibroblasts. **METHODS:** The regulation fragment of the 5' flanking region of the human CTGF gene was linked to pGL3-Basic vector, a firefly luciferase reporter construct without promoter. The recombinant plasmid p*CTGF-luc* was transiently transfected to NIH/3T3 fibroblasts. The activity of CTGF promoter after treatment of TGF-β_1 and MAPK pathway inhibitors were assayed with luciferase reporter gene assay system. **RESULTS:** TGF-β_1-induced increase of CTGF promoter activity was concentration-dependent, with a plateau at 5 μg/L by 2.67-fold *vs* control (P<0.05). The TGF-β_1 stimulation of CTGF promoter activity was time-dependent, too. After exposure to TGF-β_1 (5 μg/L), the maximal level of luciferase activity was reached at 12 h and maintained to 24 h by 2.76- and 2.20-fold *vs* control, respectively (P<0.05). Blockade of mitogen-activated protein kinases (MAPK) pathway with PD98059 (10 μmol/L), the MAP kinase kinase 1 inhibitor, and SB203580 (10 μmol/L), the p38 MAP kinase inhibitor, decreased basal and TGF-β_1-induced activation of CTGF promoter. However, inhibition of c-Jun-N-terminal kinase/stress-activated protein kinase by SP600125 (20 μmol/L) was without effect. **CONCLUSION:** TGF-β_1 stimulated the transcriptional activity of CTGF gene promoter in NIH/3T3 fibroblasts in a dose- and time-dependent manner. MAPK pathway may play a role in the regulation of TGF-β_1-induced CTGF expression.

INTRODUCTION

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine that regulates growth and differentiation of diverse types of cells. Secretion of TGF-β by platelets, macrophages, and neutrophils serves to induce a cascade of events leading to connective tissue formation that is essential for normal wound repair and leads to the initiation and progression of many fibrotic disorders[1]. Many of the effects of TGF-β on fibroblast proliferation and extracellular matrix (ECM) deposition are mediated by connective tissue growth factor (CTGF)[2,3]. CTGF is a 38 kDa cysteine-rich peptide

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and involves in the regulation of wound repair and fibrosis[4]. Overexpression of TGF-β and CTGF have been observed in fibrotic disorders of the skin, kidney, liver, and heart[5-8]. CTGF gene expression is selectively induced by TGF-β and this regulation appears to be controlled primarily at the level of transcription[9]. Identification of the mechanism regulating the increased expression of CTGF by TGF-β is of considerable importance, since blocking CTGF production may ameliorate the fibrotic response in a variety of disease situations.

TGF-β induction of CTGF is mediated through sequences located in the CTGF gene promoter. The motifs shown to be likely involved in the regulation of CTGF gene expression are a unique TGF-β response element ($T_{\beta}RE$) and a Smad response element (SRE)[9,10]. However, several general transcription factor binding sites have been predicted in the promoter region of the CTGF gene, such as AP-1, SP-1, and TATA box binding sites[11]. Although Smad proteins appear to be the major mediators for TGF-β-induced CTGF expression, other signaling molecules, such as mitogen-activated protein kinase (MAPK), are also activated by TGF-β in various cell systems[12]. To better understand the regulatory mechanism of TGF-β on CTGF gene expression and to test whether other signal transduction pathways were involved in this process, the activity of CTGF promoter in NIH/3T3 fibroblasts after treatments of TGF-β_1 and MAPK pathway inhibitors was observed in the present study.

MATERIALS AND METHODS

Reagents Human recombinant TGF-β_1 was purchased from Pepro Tech EC Ltd (UK). PD98059 were from Biolabs (USA), SB203580 and SP600125 were from BIOMOL (USA). pGL3-Basic vector, pRL-SV40 vector, and Dual-Luciferase® Reporter Assay System were from Promega (USA). Lipofectamine™ 2000 Reagent was from Invitrogen (USA). Nucleic acid amplification, restricted digestion, and ligation agents were from TaKaRa (Dalan, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin solution were from Gibco (USA).

Cell culture NIH/3T3 murine fibroblasts were purchased from ATCC (USA). Cells were cultured in DMEM containing 10 % FBS at 37 ºC in a 5 % CO_2 humidified atmosphere.

Polymerase chain reaction (PCR) amplification of human CTGF promoter region Human genomic DNA was extracted from 5 mL venous blood by a common salting-out protocol. Using this genomic DNA as a template, a 875-bp fragment of the CTGF promoter containing nucleotides –742 to +133 (with the transcription initiation site as +1) was amplified by Pyrobest™ DNA polymerase. The primers used for amplification were designed according to the published CTGF promoter sequence (GenBank accession number AF316368): forward primer: 5'-AGGGCTAGCCA-CTCGTCCCTTGTCCTTGC -3', with a *Nhe* I restriction site under-lined, reverse primer: 5'-CTGAAGCTT-TGGCGGTGGTCGGAGCTG-3', with a *Hind* III restriction site under-lined.

PCR was performed as follows: initial denaturation at 95 ºC for 5 min; followed by 35 cycles denaturation at 94 ºC for 1 min, annealing at 62.5 ºC for 35 s, and extending at 72 ºC for 90 s; elongation at 72 ºC for 10 min in the end. The PCR products were visualized by 1 % (w/v) agarose gel electrophoresis and ethidium bromide staining.

Plasmid construction The resulting PCR product was ligated into the pGL3-Basic vector, using *Nhe* I and *Hind* III clonging sites. The constructed plasmid was termed p*CTGF-luc*. This recombinant plasmid with CTGF promoter fragment was confirmed by dual-orientation DNA sequencing on an ABI PRISM™ 377 DNA Sequencer with common RVprimer3 and GLprimer2.

Transient transfection and luciferase assay system NIH/3T3 cells were seeded onto 24-well plates and grown to 80 % confluence. Then transfection was performed with Lipofectamine™ 2000 Reagent according to the manufacturer's protocol. pRL-SV40 vector containing the *Renilla* luciferase gene to provide an internal standard for transfection efficiency was transiently cotransfected with p*CTGF-luc*. Each transfection included 0.9 μg pCTGF-luc and 0.1 μg pRL-SV40 vector. Cells were incubated in serum-free DMEM for 24 h after transfection, followed by incubation with TGF-β_1 and other test reagents in DMEM with 0.2 % FBS. A dual luciferase assay system was applied for sequential measurement of firefly and *Renilla* luciferase activities with specific substrates, ie, beetle luciferin and coelenterazine, respectively. Quantification of luciferase activities and calculation of relative ratios were carried out with a luminometer (Lumat LB 9507).

Statistical analysis All data were expressed as mean±SD. Statistical analyses were performed using

the one-way ANOVA. $P<0.05$ was statistically significant. Each experiment was performed at least twice. The analysis results were obtained using SAS®, version 6.12.

RESULTS

Expression of firefly luciferase in NIH/3T3 cells transient transfected with p*CTGF-luc* NIH/3T3 cells were cultured for 48 h after transfection with 1 μg p*CTGF-luc* and pGL3-Basic vector per well and then harvested. The luciferase activity was found to be remarkably high in cells with p*CTGF-luc*, compared with cells with pGL3-Basic vector (Fig 1B).

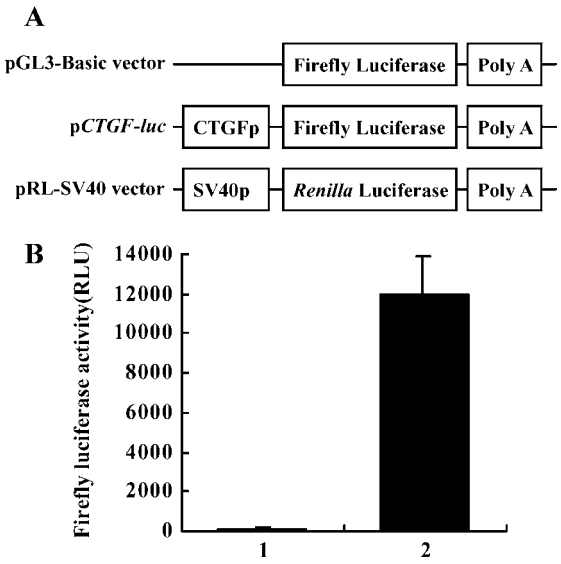

Fig 1. Construction and expression of p*CTGF-luc*. A) Schemas of the plasmids used for the transient transfection experiments. CTGFp, SV40p, and Poly A denote the CTGF promoter, SV40 promoter, and polyadenylation, respectively. B) Expression of firefly luciferase in NIH/3T3 cells. Lane 1: pGL3-Basic vector; Lane 2: p*CTGF-luc*. All the values are represented as relative luminescent units (RLU), as measured with a luminometer. *n*=3. Mean±SD.

Effect of TGF-β_1 on the activity of CTGF gene promoter in NIH/3T3 cells TGF-β_1 stimulated the activity of CTGF gene promoter in a dose-dependent manner, with the maximal levels at 5 μg/L to 10 μg/L by 2.67-fold and 2.33-fold *vs* control, respectively ($P<0.05$) (Fig 2A). Then cells were treated with TGF-β_1 (5 μg/L) for various length of time (15 min to 24 h) (Fig 2B). There was an elevation of luciferase activity at the earliest time-point tested (15 min) by 1.45-fold *vs* control ($P>0.05$). It reached its peak at 12 h and maintained to 24 h by 2.76-fold and 2.20-fold *vs* control, respectively ($P<0.05$).

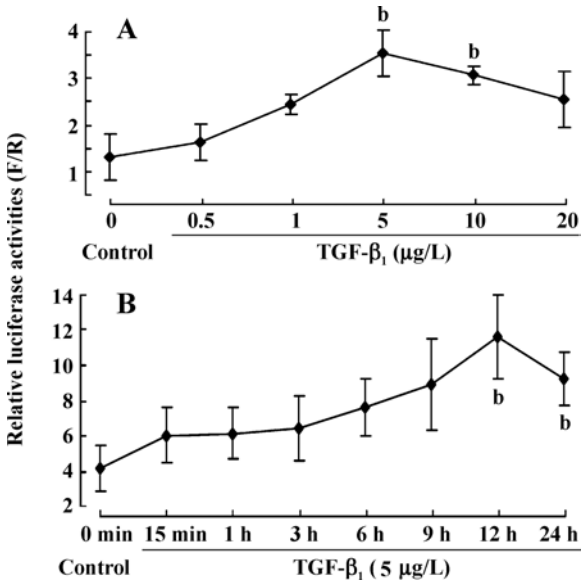

Fig 2. Effect of TGF-β_1 on the activity of CTGF gene promoter in NIH/3T3 cells. A) Concentration-dependent induction of CTGF gene promoter activation by TGF-β_1. B) Time course of CTGF gene promoter activation by TGF-β_1. Expression levels are represented as relative luminescence values of firefly luciferase *vs* *Renilla* luciferase. *n*=3. Mean±SD. $^bP<0.05$ *vs* control group.

Effects of MAPK pathway inhibitors on TGF-β_1-induced activity of CTGF gene promoter in NIH/3T3 cells NIH/3T3 cells were transiently transfected with reporter genes and serum starved for 24 h. After preincubation with PD98059, the inhibitor of extracellular signal-regulated protein kinase (ERK) upstream effector MEK1, SB203580, the p38 MAP kinase (p38 MAPK) inhibitor, and SP600125, the specific inhibitor of c-Jun-N-terminal kinase/stress-activated protein kinase (JNK) for 1 h, cells were cultured in the presence or absence of TGF-β_1 (5 μg/L) for another 24 h. PD98059 and SB203580 tended to block TGF-β_1-induced activity of CTGF promoter in a concentration dependent manner. PD98059, at 10 μmol/L, partially decreased basal and TGF-β_1-induced activity of CTGF promoter by 43.3 % *vs* control group ($P<0.05$) and 46.8 % *vs* TGF-β_1 treatment group ($P<0.05$) (Fig 3A). Similarly, incubation with SB203580 (10 μmol/L) led to a partial reduction on basal and TGF-β_1-induced activity of CTGF promoter by 63.1 % *vs* control group ($P<0.05$) and 42.4 % *vs* TGF-β_1 treatment group ($P<0.05$) (Fig 3B). However, SP600125 (20 μmol/L)

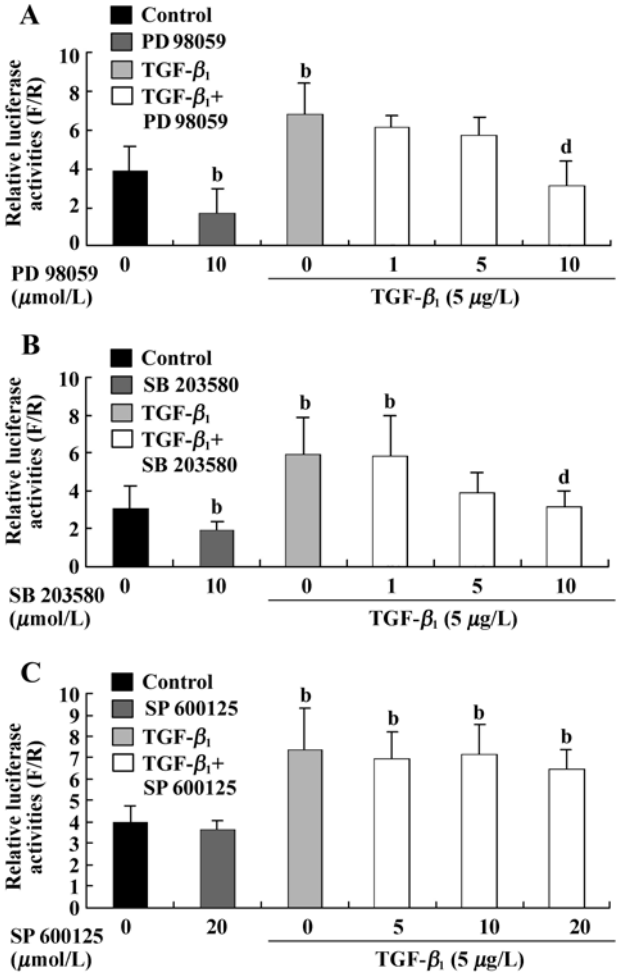

Fig 3. Effects of MAPK pathway inhibitors on TGF-β_1 induced the activation of CTGF gene promoter in NIH/3T3 cells. A) PD98059 treatment groups. B) SB203580 treatment groups. C) SP600125 treatment groups. Expression levels are represented as relative luminescence values of firefly luciferase *versus Renilla* luciferase. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* control group. $^{d}P<0.05$ *vs* TGF-β_1 treatment group.

had no effect on the activation of CTGF promoter (Fig 3C).

DISCUSSION

In the present study, we investigated the effect of TGF-β_1 on the activity of CTGF promoter in mouse NIH/3T3 cells. Our data demonstrated that TGF-β_1 stimulated the transcriptional activity of CTGF gene promoter in a dose- and time-dependent manner. The rapid induction of CTGF promoter activation by TGF-β_1 is consistent with the role of CTGF as an immediate downstream effector of TGF-β.

Because TGF-β can stimulate all three members of MAPK pathway (ERK, p38 MAPK, and JNK), we analyzed the effect of three specific MAPK inhibitors on the activity of CTGF gene promoter induced by TGF-β_1. The results showed that blockade of ERK with MEK1 inhibitor PD98059 and p38 MAPK with SB203580 partially decreased the basal and TGF-β_1-induced activation of CTGF promoter. However, inhibition of JNK with SP600125 had no effect. These data indicated that MAPK pathway might play a role in the regulation of CTGF expression. At present, we can not completely rule out the mechanisms of MAPK regulation in TGF-β_1-induced activation of CTGF promoter. One possible way may involve with the transcription factor AP-1, as the components in AP-1 complex serve as substrates for MAPK and AP-1 binding site exists in the promoter region of the CTGF gene. Additionally, cross-talk between MAPK and Smad pathways may also contribute to the regulation effect of MAPK, since ERK and p38 MAPK pathway can directly interact with activated Smad proteins in the nucleus to prevent their binding to target genes[14]. Chen *et al* found that PD98059 stimulated CTGF mRNA expression induced by TGF-β in human cardiac fibroblasts and upregulated TGF-β-induced increase of CTGF promoter activity in neonatal rat cardiac myocytes. Whereas Heusinger-Ribeiro *et al* found that PD98059 and SB203580 had little effect on the basal CTGF expression in human renal fibroblasts[7,15]. We had no ready explanation for the differences between our results and those of the other laboratories, but they might be due to cell-specific or species variation.

In summary, our results demonstrated that TGF-β stimulated the transcriptional activity of CTGF gene promoter in NIH/3T3 cells in a dose- and time-dependent manner. MAPK pathway played a role in the regulation of TGF-β_1-induced CTGF expression. Thus, the MAPK pathway may provide a target for developing therapeutic agents, which would be potentially useful in the treatment of fibrotic conditions by blocking TGF-β-induced CTGF expression.

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