Investigation for TGF-β1 expression in type 2 diabetes and protective effects of TGF-β1 on osteoblasts under high glucose environment

W.-D. WANG, B.-Z. CHENG, W.-B. KANG, R.-W. ZHENG, Y.-L. CAI, X.-J. WANG

[1]Department of Spine and Joint, The 2nd Hospital, Medical College, Shantou University, Shantou, Guangdong, China
[2]Department of Laboratory, The 1st Hospital, Medical College, Shantou University, Shantou, Guangdong, China
[3]Department of Otolaryngology, The 2nd Hospital, Medical College, Shantou University, Shantou University, Shantou, Guangdong, China

Abstract. – **OBJECTIVE:** The occurrence rate of delayed fracture healing in diabetes mellitus patients is high. Transforming growth factor β1 (TGF-β1) is an important regulatory factor in bone tissue repair and regeneration. However, TGF-β1 expression and its function in diabetic patient fracture have not been fully elucidated.

PATIENTS AND METHODS: Type 2 diabetes fracture patients (T2DM group), fracture patients without diabetes (non-T2DM group), and healthy volunteers (Control group) were selected for the research. TGF-β1 expression in peripheral blood was detected by using enzyme-linked immunosorbent assay (ELISA). Osteoblast cell line, MG-63 cells, were randomly divided into Control, high glucose group, and TGF-β1 group. TGF-β1 expression was evaluated by using Real Time-PCR (RT-PCR). Cell proliferation was evaluated by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell apoptosis activity was determined with caspase-3 activity and flow cytometry assay. The effect of TGF-β1 on NF-κB was detected by using Western blot.

RESULTS: TGF-β1 was significantly reduced in patients of T2DM and non-T2DM groups compared with Control ($p<0.05$), while it was lower in T2DM group ($p<0.05$). TGF-β1 expression was declined, cell proliferation was inhibited, caspase-3 activity was enhanced, cell apoptosis was elevated, and NF-κB expression was reduced in MG-63 cells of high glucose group compared to Control group ($p<0.05$). TGF-β1 significantly promoted cell proliferation, suppressed caspase-3 activity, alleviated cell apoptosis, and elevated NF-κB expression in MG-63 cells compared with high glucose group ($p<0.05$).

CONCLUSIONS: TGF-β1 decreased in diabetes fracture patients. Up-regulation of TGF-β1 regulates cell apoptosis and caspase-3 activity, and it facilitates osteoblasts proliferation.

Introduction

The incidence rate of diabetes mellitus (DM) increases following the changes of social lifestyle, the dietary habit, and the elevation of economic level. It ranks in the leading position in China and in the world[1,2]. DM could be divided into type 1 (T1DM) and type 2 (T2DM); T2DM shows a higher occurrence rate in our country[3,4]. DM is a type of autoimmune disease and chronic inflammatory disease[5,6]. A T2DM fracture may induce endocrine, bone metabolism, and mineral substance metabolic disorder because of high glucose environment. It may lead to multiple tissue and organs injury, resulting in muscle weakness, bone mineral substance decrease, and osteoporosis. Therefore, the occurrence rate of delayed fracture healing or bone non-union is relative high[7,8]. Delayed fracture healing may cause neural paralysis, urinary system infection, bedsore, gangrene, muscular atrophy, and even local or systemic infection[9,10]. Transforming growth factor-β (TGF-β) is a newly discovered factor that regulates cell growth and differentiation[11]. TGF-β superfamily contains at least four subtypes, including TGF-β1, TGF-β2, TGF-β3, and TGF-β1β2 in mammals[12]. Endothelial cells, osteoblasts, and even tumor cells can secrete inactivated TGF-β. Protein cracking makes the TGF-β complex to active TGF-β[13,14]. TGF-β expression is relatively high in cells with active differentiation, such as

Corresponding Author: Wei-Dong Wang, MD; e-mail: wmckkmmmigck@sina.com

osteoblast, kidney, bone marrow, and hematopoietic cell in fetal liver. TGF-β1 content is highest in human platelet and mammal bone. TGF-β2 content is highest in porcine platelet and mammal bone. TGF-β3 is mainly produced by cells derived from mesenchyme[15,16]. TGF-β1, TGF-β2, and TGF-β3 locate in chromosome 19q3, 1q41, and 14q24, respectively. They all contain 7 exons and 9 conserved Cys with high homology[17]. TGF-β1 may express in the healing wound near fracture[18]. However, TGF-β1 expression in T2DM fracture patients and the related mechanism have not been fully elucidated.

Patients and Methods

Object of Study

A total of 35 cases of T2DM complicated fracture patients between Jan 2016 and Dec 2017 were enrolled in the 2nd Hospital, Medical College, Shantou University (Shantou, China) with mean age at 42.1 ± 13.7 (range from 32 to 67) years old and body mass index (BMI) at 23.2 kg/m^2, including 18 males and 17 females. Another 30 cases of fracture patients without T2DM were selected as non-T2DM group with mean age at 40.1 ± 15.2 (35-65) years old and BMI at 22.1 kg/m^2, including 16 males and 14 females. All the enrolled T2DM subjects agreed with the diagnostic criteria of T2DM and T2DM complicated diabetic retinopathy published by the World Health Organization (WHO) on 2014[19]. The exclusion criteria included other endocrine disease complication and other autoimmune diseases. A total of 25 healthy volunteers received a physical examination in our hospital with mean age at 41.3 ± 11.5 (range from 31 to 66) years old, and BMI at 22.6 kg/m^2 were enrolled as normal control, including 13 males and 12 females. All the subjects in control exhibited normal OGTT result and were excluded from other related immune diseases. No statistical difference was observed among the three groups on gender, age, and BMI ($p>0.05$). This study was approved by the Ethics Committee of the 2nd Hospital, Medical College, Shantou University (Shantou, China). All of the enrolled objects had signed informed consents and approved this study.

Reagents and Instruments

Human osteoblast-like cell line MG-63 was purchased from Angio-Proteomie (Boston, MA, USA). penicillin-streptomycin and EDTA were bought from Hyclone (USA). DMSO and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Gibco (Grand Island, NY, USA). Trypsin-ethylene diamine tetraacetic acid (EDTA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3 activity detection kit and Western blot related reagents were provided by Beyotime (Shanghai, China). Polyvinylidene difluoride (PVDF) membrane was derived from Pall Life Sciences (Covina, CA, USA). RNA extraction kit and reverse transcription kit were purchased from Invitrogen/ Life Technologies (Carlsbad, CA, USA). Superoxide dismutase (SOD) activity detection kit was bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Rabbit anti-human Nuclear Factor-κB (NF-κB) monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were provided by Cell Signaling Technology (Danvers, MA, USA). Annexin V-FITC apoptosis detection kit and FACS Calibur flow cytometry were got from BD Biosciences (San Jose, CA, USA). DNA amplifier was obtained from PE Gene Amp PCR System 2400 (PE Applied Biosystems, Foster City, CA, USA). ABI 7500 Real Time-PCR amplifier was purchased from ABI (Foster City, CA, USA). Other reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China).

Materials and Methods

Sample Collection

A total of 10 ml blood was extracted from a median cubital vein in fasting status, including 5 ml non-anti-coagulative blood, were used for biochemical index detection. The blood was centrifuged at 3000 r/min for 15 min and stored at -80°C.

MG-63 Cell Culture And Grouping

MG-63 cells in 3rd-8th generation were seeded in the dish at 1×10^6 cells/cm^2 and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5.5 mmol/l glucose. MG-63 cells were divided into three groups, including normal control, high-glucose group that treaded by mi-

crovascular endothelial cell medium containing 33 mmol/l glucose for 72 h, and TGF-β1 group that in high-glucose environment treated by 1.0 mmol/l TGF-β1 for 72 h[20].

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to test TGF-β1 content in the serum. A total of 50 µl diluted standard substance were added to each well to establish a standard curve. Next, the plate was added with 50 µl sample and washed for five times. Then, the plate was incubated in 50 µl conjugate reagent at 37°C for 30 min. After washed for five times, the plate was treated with 50 µl color agent A and B at 37°C avoid of light for 30 min. At last, the plate was added with 50 µl stop buffer to stop the reaction and tested at 450 nm to obtain the optical density (OD) value. The OD value of the standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

Real Time-PCR

Total RNA was extracted from MG-63 cells by TRIzol and reverse transcribed to complementary DNA (cDNA). The primers were designed using PrimerPremier 6.0 software (Palo Alto, CA, USA) (Table I) and synthetized by Sangon Biotechnology Co. Ltd. (Shanghai, China). Real Time-PCR was performed at 52°C for 1 min, followed by 35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as internal reference. The relative expression of mRNA was calculated by $2^{-\Delta Ct}$ method.

Caspase 3 Activity Detection

Caspase 3 activity was tested according to the manual. The cells were digested by trypsin and centrifuged at 600 ×g and 4°C for 5 min. Next, the cells were added with 2 mM Ac-DEVD-pNA and detected at 405 nm to calculate caspase 3 activity.

Western Blot Assay

The cells were added with radioimmuno-precipitation assay (RIPA) containing protease inhibitor and cracked on ice for 15-30 min. Next, the cells were treated by ultrasound at 5 s for 4 times and centrifuged at 10000 ×g for 15 min. The protein was transferred to a new Eppendorf (EP) tube and stored at -20°C. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane at 100 mA for 1.5 h. After blocked by 5% skim milk for 2 h, the membrane was incubated in NF-κB monoclonal antibody (1: 2000) at 4°C overnight. Then, the membrane was incubated in goat anti-rabbit secondary antibody at room temperature for 30 min. Next, the membrane was treated by a developer for 1 min and exposed to observe the result. The film was scanned by Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed by the protein image processing system. Each experiment was repeated for four times.

MTT Assay

MG-63 cells in logarithmic phase were seeded in 96 well plate at 3000/well. The cells were randomly divided into three groups. After 48 h, the plate was incubated in 20 µl MTT solution at 5 g/l for 4 h. Next, the plate was added with 150 µl dimethyl sulfoxide (DMSO) for 10 min and tested at 570 nm to calculate cell proliferation. Each experiment was repeated for three times.

Flow Cytometry

The cells were digested and seeded in a 50 ml culture flask at 5×10^5/ml. After treated by TGF-β1 for 48 h, the cells were counted and washed by phosphate-buffered saline (PBS). Then, the cells were fixed by 75% ethanol and washed by PBS. The re-suspended cells were blocked in PBS containing 1% bovine serum albumin (BSA) and stained by 100 µg/ml propidium iodide (PI) solution (3.8% sodium citrate, pH 7.0). At last, the cells were added with 100 µl RnaseA at 10 mg/ml and incubated at 37°C avoid of light for 30 min. The data of flow cytometry was analyzed by using FCSExpress software (version: 3.0, De Novo Software, Los Angeles, CA, USA).

Table I. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
TGF-β1	GATCTACGCAGCGAAGAACTT	CTCTGGGACATCTCCGTCA
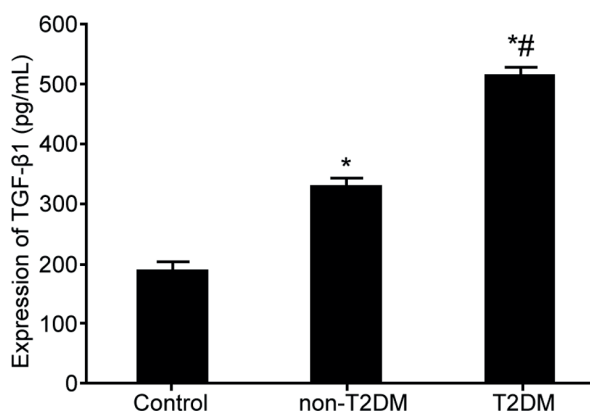
Figure 1. TGF-β1 expression in peripheral blood from T2DM fracture patients. *$p<0.05$, compared with control, #$p<0.05$, compared with non-T2DM group.
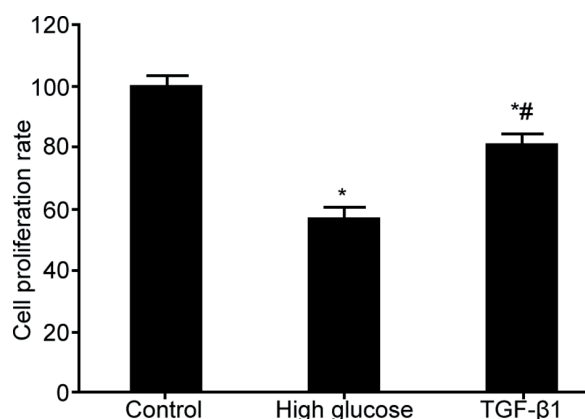

Figure 3. The impact of TGF-β1 on MG-63 cell prolife-ration under high-glucose environment. *$p<0.05$, compared with control, #$p<0.05$, compared with high-glucose group.

Statistical Analysis

All data were presented as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post hoc test was used to validate the ANOVA for comparing measurement data for the pair-wise comparisons. All data analyses were performed on SPSS11.5 software (SPSS Inc., Chicago, IL, USA). $p<0.05$ was depicted as statistical significance.

Results

TGF-β1 Expression Changed in T2DM Fracture Patients

TGF-β1 expression in the peripheral blood from T2DM group, non-T2DM group, and control was analyzed. TGF-β1 significantly reduced
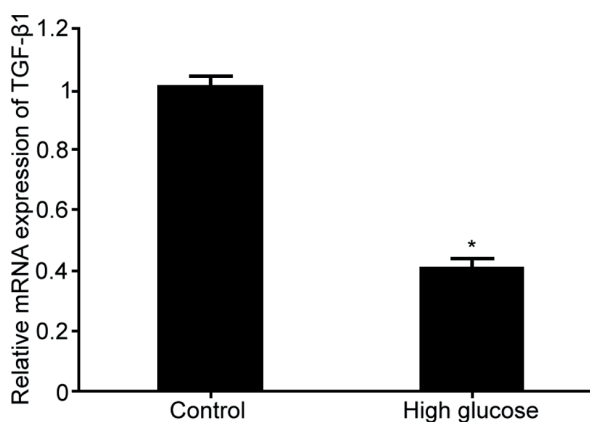

Figure 2. TGF-β1 expression changes in MG-63 cells under high-glucose environment. *$p<0.05$, compared with control.

in patients from T2DM and non-T2DM groups compared with control ($p<0.05$), while it was lower in T2DM group ($p<0.05$) (Figure 1).

TGF-β1 Expression Changed in MG-63 Cells Under High-Glucose Environment

Real Time-PCR was used to test TGF-β1 expression in MG-63 cells under high-glucose environment. TGF-β1 expression significantly decreased in MG-63 cells from high-glucose group compared with control ($p<0.05$) (Figure 2).

Impact of TGF-β1 on MG-63 Cell Proliferation Under High-Glucose Environment

MG-63 cell proliferation was inhibited under high-glucose environment compared with control (*$p<0.05$). TGF-β1 treatment significantly promoted MG-63 cell proliferation compared with high-glucose group (*$p<0.05$) (Figure 3). It suggested that TGF-β1 promoted osteoblast proliferation under high-glucose environment.

Influence of TGF-β1 on Caspase 3 Activity in MG-63 Cells Under High-Glucose Environment

Caspase 3 activity markedly enhanced in MG-63 cells under high-glucose environment compared with control ($p<0.05$). TGF-β1 significantly inhibited caspase 3 activity in MG-63 cells under high-glucose environment ($p<0.05$) (Figure 4).

Effect of TGF-β1 on MG-63 Cell Apoptosis Under High-Glucose Environment

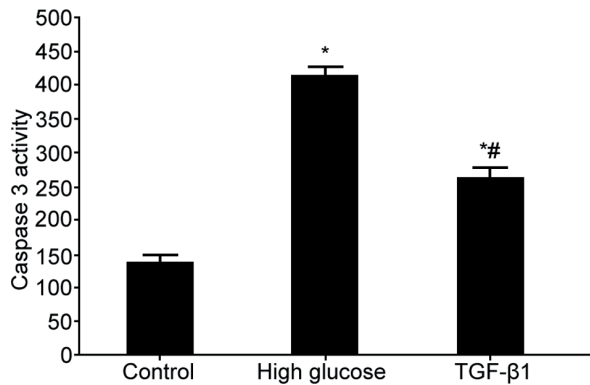MG-63 cell apoptosis significantly elevated in high-glucose group compared with control

Figure 4. The influence of TGF-β1 on caspase 3 activity in MG-63 cells under high-glucose environment. *$p<0.05$, compared with control, #$p<0.05$, compared with high-glucose group.

($p<0.05$). TGF-β1 markedly suppressed MG-63 cell apoptosis under high-glucose environment compared with control ($p<0.05$) (Figure 5).

Impact of TGF-β1 on NF-κB Expression in MG-63 Cells Under High-Glucose Environment

NF-κB expression significantly downregulated in MG-63 cells in high-glucose group compared
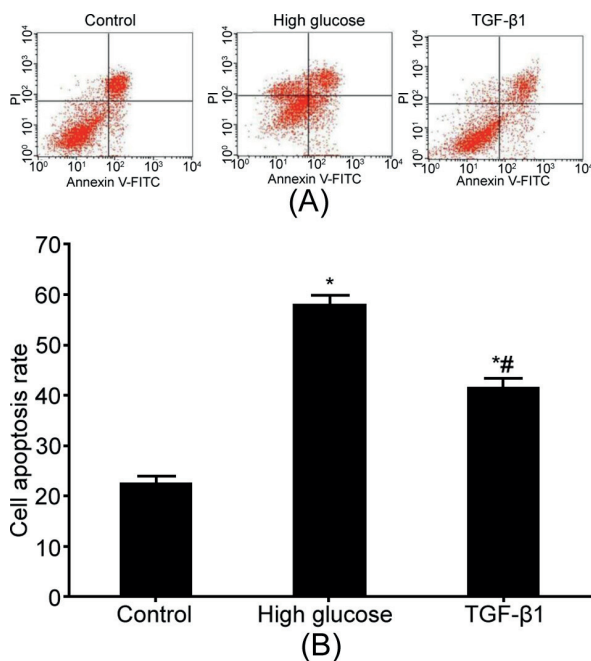
with control ($p<0.05$). TGF-β1 significantly facilitated NF-κB expression in MG-63 cells under high-glucose environment compared with control ($p<0.05$) (Figure 6).

Discussion

T2DM may cause blood glucose metabolism disorder, resulting in vasculopathy, neuropathy, and bone metabolism. Most DM fracture patients are affected by multiple factors, leading to healing slow, delay, and even nonunion[21,22]. Fracture healing is affected by multiple physical, physiological, and molecular biological factors, including age, bone mineral density, health, and local blood supply[23,24]. High glucose may block callus formation in DM patients after fracture. TGF-β1 can stimulate osteoblast, cartilage cell, and mesenchymal stem cell proliferation, and promote secretion of bone morphogenetic protein and insulin-like growth factor[17,18]. However, TGF-β1 expression and related mechanism in T2DM fracture are still unclear.

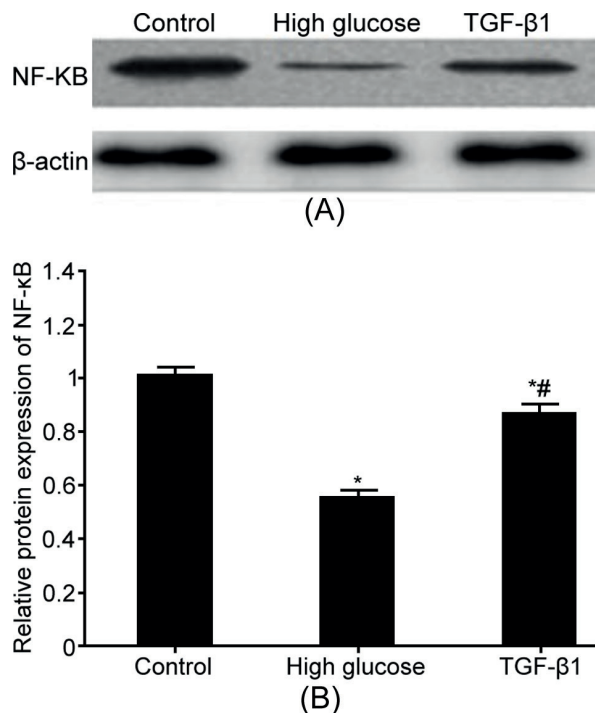This study compared TGF-β1 expression in serum from T2DM fracture patients, non-T2DM

Figure 5. The effect of TGF-β1 on MG-63 cell apoptosis under high-glucose environment. **A,** flow cytometry detection of cell apoptosis. **B,** MG-63 cell apoptotic rate analysis. *$p<0.05$, compared with control, #$p<0.05$, compared with high-glucose group.

Figure 6. The impact of TGF-β1 on NF-κB expression in MG-63 cells under high-glucose environment. **A,** Western blot detection of NF-κB expression. **B,** NF-κB expression analysis. *$p<0.05$, compared with control, #$p<0.05$, compared with high-glucose group.

fracture patients, and healthy control. It was shown that TGF-β1 significantly reduced in patients from T2DM and non-T2DM groups compared with control, while it was lower in T2DM group, suggesting its role in T2DM fracture delayed healing. It was reported[20] that TGF-β1 level significantly declined in osteoblasts from T2DM patients and may lead to poor fracture healing. Further analysis revealed that cell proliferation was suppressed, caspase 3 activity increased, cell apoptotic rate elevated, and NF-κB expression downregulated in MG-63 under high-glucose environment. As a member of transactional factor family, NF-κB activation promotes cell transcription and proliferation. Caspase 3 enhancement may induce cell apoptosis[25,26]. TGF-β1 significantly promoted cell proliferation, suppressed caspase-3 activity, alleviated cell apoptosis, and elevated NF-κB expression in MG-63 cells under high glucose environment.

Conclusions

We showed that TGF-β1 decreased in T2DM fracture patients. Up-regulation of TGF-β1 regulates cell apoptosis and Caspase-3 activity, and it facilitates osteoblasts proliferation.

Conflict of Interest
The Authors declare that they have no conflict of interest.

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