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Review Article

Aggravation of spinal cord injury by TGF-β via activating NF-κB

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Abstract: The incidence of spinal cord injury (SCI) is gradually increased by years. Nuclear factor kappa B (NF-κB) has multiple biological activities including regulating inflammatory response, oxidative stress and further participating in SCI pathogenesis and progression. Transforming growth factor beta (TGF-β) plays a role in inflammation and tissue repair. Its role and mechanism if SCI, however, have not been illustrated. Wistar rats were randomly divided into control group, SCI group which was prepared by improved ALLEN's weight-drop method, and TGF-β group, which received tail vein injection of TGF-β anti-sense oligonucleotide TGF-β total phosphoric acid AODN. Basso, Beattie Bresnahan (BBB) locomotor rating scale was compared, plus SCI sensory function Reuter score. Real-time PCR and Western blot were used to detect TGF-β and NF-κB expression. Caspase 3 activity was further measured, with TNF-α and IL-2 levels measured by ELISA. Compared to control group, SCI group had enhanced mRNA and protein expressions of NF-κB and TGF-β, lower BBB score, higher Reuter score, higher caspase 3 activity, and more secretion of TNF-α and IL-2 ($P<0.05$). TGF-β treatment significantly depressed NF-κB and TGF-β expression, increased BBB score, lowered Reuter score, inhibited caspase 3 activity, and decreased TNF-α and IL-2 secretion ($P<0.05$ compared to SCI group). SCI group had elevated TGF-β expression, further activating NF-κB and facilitating inflammation, enhancing apoptosis and aggravating SCI. Using TGF-β as the target inhibited NF-κB expression, suppressing inflammation and inhibiting apoptosis, thus alleviating SCI.

Keywords: Spina cord injury, TGF-β, cytokines, NF-κB, inflammation, caspase 3

Introduction

Severe spinal cord injury (SCI) may be caused after car accidents or falling, and is one common traumatic syndrome [1, 2]. With economic development, industrial progression and mineralization, transportation and infrastructure, the incidence of SCI is gradually increasing by years [3, 4]. It is estimated that hundreds of thousands of SCI cases occur each year worldwide, with most patients in middle aged people over 40 years [5, 6]. SCI has high morbidity [7]. Due to its high incidence and morbidity, SCI frequently caused severe body limb dysfunction under injury segment. Moreover, high medical cost and major surgery trauma also lead to severe mental and physical damage to patients, plus heavy economic burdens for the society as a whole [8, 9].

Nuclear transcription factor kappa B (NF-κB) is widely distributed in body tissues including vascular endothelial cells, spinal cord, neurons and glial cells [10]. SCI can leads to nerve injury, plus cell apoptosis or necrosis caused by secondary inflammation and infection, further aggravating spinal cord injury, and eventually developing into irreversible pathology change [11, 12]. Under static state of cells, NF-κB is at inactive status. After SCI-caused focal tissue ischemia, hypoxia, trauma or cell damage, DNA injury can further lead to NF-κB activation in endothelial cells and neuron, further modulating inflammation, further causing spinal cord damage [13, 14]. Macrophage and lymphocytes can produce transforming growth factor beta (TGF-β) with relatively smaller molecular weight and dimers formed by disulfide bond connection [15]. With pluripotent pharmaceutical activity, TGF-β plays a role in multiple pathology and physiology processes including tissue injury repair, inflammation modulation, and organ fibrosis [16]. Its role and functional mechanism in SCI, however, has not been fully illustrated.

This study thus established a rat SCI model to analyze the regulatory mechanism of TGF-β in SCI. This study thus established a rat SCI model, on which the regulatory mechanism of TGF-β in SCI was investigated.

Materials and methods

Experimental animal

Healthy male Wistar rats (2 month of age, SPF grade, body weight 250±20 g) were purchased from Soochow University Laboratory Animal Center and were kept in an SPF grade facility with fixed temperature (21±1°C) and relative humidity (50~70%) with 12 h/12 h light-dark cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Second Affiliated Hospital of Soochow University.

Materials and equipment

Pentobarbital sodium and lidocaine were purchased from Zhaohui (China). PVDF membrane was purchased from Pall Life Sciences (US). Western blotting reagents were purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-mouse TGF-β, anti-mouse NF-κB monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP)-labelled IgG secondary antibody were purchased from Cell Signaling (US). TNF-α and IL-2 ELISA kits were purchased from R&D (US). RNA extraction kit and reverse transcription kit were purchased from ABI (US). TGF-β total phosphoric acid AODN was synthesized by Sangon (China). Caspase 3 activity assay kit was purchased from Cell signaling (US). Microscopic surgical instruments were purchased from Suzhou Medical Instrument (China). Multi-Parameter Monitor for physiology in small animals was purchased from Yuyan Instrument (China). Amp PCR System 2400 DAN amplifier was purchased from PE Gene (US). Imark microplate reader was purchased from BD (US).

Animal grouping and treatment

Wistar rats were randomly divided into three groups: control group; SCI group, which was prepared for SCI model using improved ALLEN's weight-drop method; and TGF-β treatment group, which received 10 μg TGF-β total phosphoric acid AODN via tail vein 30 min after SCI modeling.

Rat SCI model preparation

Improved ALLEN's weight-drop method [17] was used to generate rat SCI model. After general anesthesia by 30 mg/kg pentobarbital sodium via intraperitoneal injection, rats were fixed on the table to remove vertebral disc and spines at T9-T11 segments. Using T10 spinal cord segment as the center, a round area (4 mm diameter) was exposed as the injury site. A plastic spacer with 3 mm length, 2 mm width a 1 mm thickness with pre-curved processing based on physiological angle of rat spinal cord was placed outside the dura of T10 segment. A sleeve was placed vertically on the center of spacer. Using improved ALLEN's weight-drop apparatus, a pouching rod was made to drop on the spacer in a free-falling manner. Successful generation of SCI model was identified when retraction movement occurred in body and bilateral forelimbs, spastic swings of the tail. Surgical wound was closed by layers, with antibiotics for anti-inflammation.

BBB score and Reuter score in rats

BBB rating was performed 20 d after surgery to evaluate motor recovery of joints and hindlimbs. Higher BBB score indicated better recovery. Reuter score was evaluated to reflect pain retraction reflex, stretch reflex, muscle strength, muscle tension and back sensation, as higher score indicates worse functional recovery [17, 18].

Sample collection

20 d after surgery, 5 ml blood samples were collected from the tail vein, in parallel with control sample. Blood was centrifuged at 3000 rpm for 15 min to collect serum from the supernatant and was stored at -80°C fridge. Rats were then sacrificed to collect spinal cord tissues, which were stored at -80°C fridge for further use.

ELISA for serum TNF-α and IL-2

Serum samples were collected from all groups of rats to detect the level of TNF-α and IL-2 following the manual instruction of test kits. In brief, 96-well plate was added with 50 μl serially diluted samples, which were used to plot standard curves. 50 μl test samples were then

Table 1. Primer sequence

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
TGF-β	TCTCGACTCCACACAGT	GCCGGGTCATTAGCTATATT
NF-κB	CAGGTATCCACACGCC	TCATTAGCCATGTCTAACC

added into test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 sec vortex. The rinsing procedure was repeated for 5 times. 50 μl enzyme labelling reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 μl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 μl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentration of standard samples and respective OD values. Sample concentration was further deduced based on OD values and regression function.

Caspase 3 activity assay

Caspase 3 activity in myocardial tissues was evaluated using test kit from all groups. In brief, cells were digested by trypsin, and were centrifuged at 600 g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20,000 g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. Absorbance (A) values at 450 nm wavelength was measured to reflect caspase 3 activity.

Real-time PCR for TGF-β and NF-κB mRNA expression

Tissues were homogenized in liquid nitrogen and were extracted for mRNA using Trizol reagents. cDNA was synthesized reverse transcription. PrimerPremier 6.0 was used to design PCR specific primer (**Table 1**), which was synthesized by Invitrogen (China). Real-time PCR was used to test target gene expression under the following conditions: 92°C 30 s, followed by 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s. Fluorescent quantitative PCR was used to collect data. CT values of standard samples were calculated based on internal reference gene GAPDH for plotting standard curve. Semi-quantitative analysis was performed by $2^{-\Delta Ct}$ method.

Western blot for TGF-β and NF-κB protein expression

Total proteins were extracted from rat spinal cord tissues. In brief, tissues were mixed with lysis buffer for 15~30 min iced incubation. Using ultrasonic rupture (5 s, 4 times) and centrifugation (10,000 g, 15 min), proteins were quantified from the supernatant and were kept at -20°C for Western blotting. Proteins were separated in 10% SDS-PAGE (20 KD to 67 KD range), and were transferred to PVDF membrane by semi-dry method (160 mA, 1.5 h). Non-specific binding sites were blocked by 5% defatted milk powders for 2 hours. Anti-TGF-β monoclonal antibody (1:1,000) or anti-NF-κB antibody (1:500) was applied for 4°C overnight incubation. Goat anti-rabbit IgG (1:2,000) was then added for 30-min incubation. After PBST washing and ECL development for 1 min, the membrane was exposed under X-ray. An imaging analyzing system and Quantity one software were then used to scan X-ray films and to detect the density of bands with repeated measures (N=4).

Statistical processing

SPSS 19.0 software was used to collect all data, of which measurement data were expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across groups. A statistical significance was defined when $P<0.05$.

Results

TGF-β mRNA and protein expression in rat spinal cord tissues

Real-time PCR and Western blot were used to test mRNA and protein expression of TGF-β in rat spinal cord tissues. Results showed that compared to control group, SCI rats had elevated TGF-β mRNA and protein expressions ($P<0.05$). TGF-β treatment significantly depressed mRNA and protein expressions of TGF-β

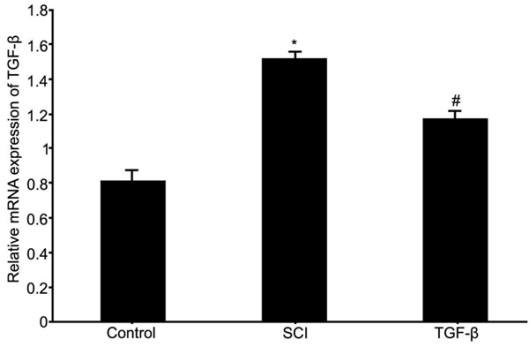

Figure 1. TGF-β mRNA expression in rat spinal cord tissues. *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

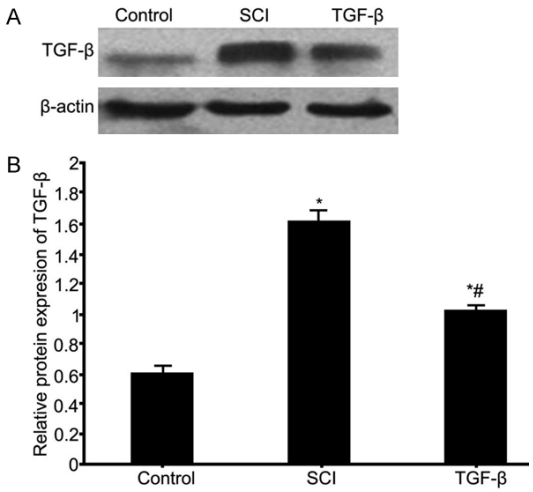

Figure 2. TGF-β protein expression in rat spinal cord tissues. A. TGF-β protein expression in rat spinal tissues. B. Quantitative analysis of TGF-β protein expression. *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

(P<0.05 compared to SCI group, **Figures 1** and **2**).

Effects of TGF-β on SCI rats

We evaluated the effect of TGF-β on SCI rats via BBB score and Reuter score. Results showed elevated TGF-β expression in SCI group, which also had lower BBB score and higher Reuter score (P<0.05 compared to control group). TGF-β treatment significantly enhanced BBB score and suppressed Reuter score (P<0.05 compared to SCI group, **Table 2**). These results suggested that TGF-β treatment significantly improved pathological process of SCI and facilitated injury recovery.

Table 2. Observation of the effect of TGF-β on SCI rats

Group	BBB score	Reuter score
Control	22.1±1.5	2.3±0.1
SCI group	13.2±1.6*	5.2±0.6*
TGF-β group	17.1±1.7*,#	3.2±0.7*,#

Note: *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

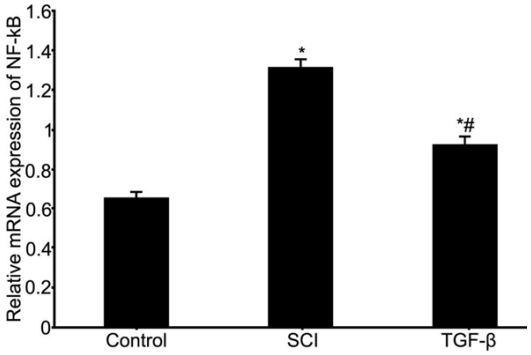

Figure 3. NF-κB mRNA expression in rat spinal cord tissues. *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

NF-κB mRNA and protein expression in rat spinal cord tissues

Real-time PCR and Western blot were used to test mRNA and protein expression of NF-κB in rat spinal cord tissues. Results showed that compared to control group. SCI rats had elevated NF-κB mRNA and protein expressions (P<0.05). TGF-β treatment significantly depressed mRNA and protein expressions of NF-κB (P<0.05 compared to SCI group, **Figures 3** and **4**). These results suggested that TGF-β could aggravate SCI via activating NF-κB. The inhibition of TGF-β expression thus suppresses NF-κB and alleviates SCI.

Effects of TGF-β on caspase 3 activity in SCI rats

20 d after surgery, rats were sacrificed to quantify the activity of caspase 3 in spinal cord tissues by ELISA kit. Results showed enhanced caspase 3 activity in SCI group (P<0.05 compared to control group). TGF-β treatment significantly depressed caspase 3 activity (P<0.05 compared to SCI group, **Figure 5**). These results suggested that the inhibition of TGF-β could

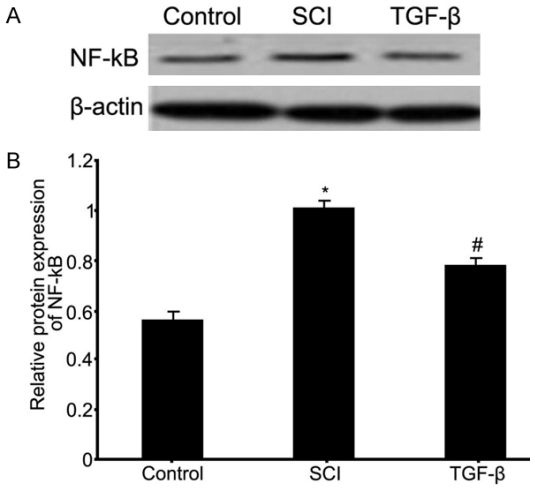

Figure 4. NF-κB protein expression in rat spinal cord tissues. A. NF-κB protein expression in rat spinal tissues. B. Quantitative analysis of NF-κB protein expression. *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

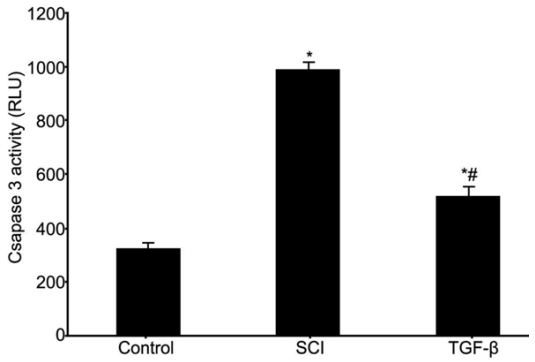

Figure 5. Effect of TGF-β on caspase 3 activity in SCI rats. *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

suppress caspase 3 activity, further regulating apoptosis and alleviating spinal cord injury.

Effects of TGF-β on serum TNF-α and IL-2 expressions in SCI rats

ELISA was used to test the effect of TGF-β on serum TNF-α and IL-2 expressions in SCI rats. Results showed elevated serum TNF-α and IL-2 levels in SCI rats (P<0.05 compared to control group). TGF-β treatment significantly decreased serum TNF-α and IL-2 levels (P<0.05 compared to SCI group, **Figure 6**). These results suggested that the modulation of TGF-β could inhibit secretion of inflammatory factors, further alleviating inflammatory injury on SCI rats.

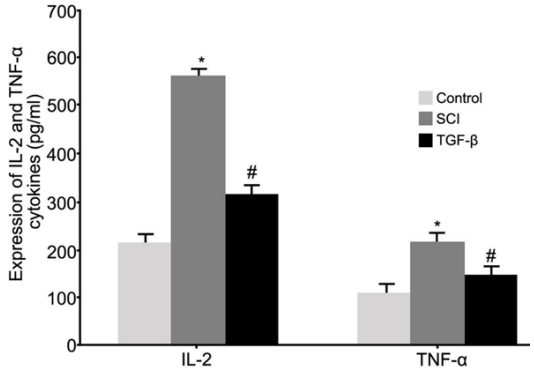

Figure 6. Effects of TGF-β on serum TNF-α and IL-2 expressions in SCI rats. *, P<0.05 compared to control group; #, P<0.05 compared to SCI group.

Discussion

SCI is one complicated pathology and physiology process involving multiple factors such as inflammation and oxidative stress. Due to the accompanied neuronal damage, glial scar tissue proliferation, and loss of neurotrophic factors in SCI injury site, it is difficult for repairing spinal cord nerve or axonal regeneration [19]. Therefore the illustration of SCI pathogenesis mechanism can help in further treatment and improving prognosis.

TGF-β plays a critical role in regulating cell growth, differentiation and immune function. It can inhibit the proliferation of immune active cells, inhibit lymphocyte differentiation, facilitates synthesis of extracellular matrix, and can modulate progression of fibrosis via decreasing the synthesis of extracellular matrix degrading enzymes such as matrix metalloproteinase (MMP), plus enhancing expression of proteinase inhibitor [20, 21]. Previous study showed that the induction of TGF-β anti-sense oligonucleotide by vectors such as cation liposome, i.e. TGF-β total phosphoric acid AODN, could inhibit TGF-β expression and tissue fibrosis [22]. The effect of TGF-β on SCI, however, has not been illustrated. This study firstly established a rat SCI model, on which TGF-β expression was compared to normal rats. Our results showed elevated TGF-β mRNA and protein levels in SCI model. Further experiments inhibited TGF-β expression in SCI model via anti-sense oligonucleotide TGF-β total phosphoric acid AODN. Results demonstrated significantly higher BBB score and lower Reuter score by inhibiting TGF-β expression, thus improving pathology of

SCI and facilitated recovery from injury with better efficacy.

Further mechanism study about the role of TGF-β in SCI found elevated TGF-β expression in SCI model could activate NF-κB and further aggravate SCI. Whilst the inhibition of TGF-β expression significantly suppressed NF-κB to alleviate SCI. The activation of NF-κB by TGF-β in SCI model facilitated TNF-α and IL-2 secretion, activated inflammatory response and benefited leukocyte adhesion. The modulation of TGF-β could inhibit secretion of inflammatory factors, further alleviating inflammatory injury on SCI rats. The activation of NF-κB in SCI model aggravated recruitment and adhesion of neutrophil and macrophage to release abundant free oxygen species, and to release secretion of inflammatory factors including TNF-α and IL-2 secretion, thus destructing integrity of spinal cord tissues, damaging vascular endothelial structures, aggravating edema and necrosis of spinal cord tissues, eventually leading to secondary SCI injury [23]. During cell apoptosis, caspase-3 is the most important proteinase and the common downstream effector shared by all apoptotic pathways [24, 25]. Further study also revealed that the inhibiting of TGF-β also suppressed caspase-3 activity, further modulating apoptosis and decreasing SCI.

Conclusion

TGF-β was up-regulated in SCI for further activation of NF-κB, facilitation of inflammation and increase of apoptosis, eventually aggravating SCI. Using TGF-β as the target, NF-κB can be inhibited to decrease inflammation to suppress apoptosis and effectively alleviate SCI.

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Disclosure of conflict of interest

None.

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