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Exogenous IL-19 mediates downregulation of TGF-β through Erk and p38 pathway to inhibit epidural fibrosis

Q.-J. WANG, A.-L. ZHANG, Z.-Q. KANG, Z.-T. ZHANG, Y.-S. WANG

Department of Orthopedics, No. 89 Hospital of Chinese People's Liberation Army, Weifang, China

Abstract. – OBJECTIVE: To evaluate the effect of interleukin-19 (IL-19) treatment on epidural fibrosis and its mechanism of action with transforming growth factor β (TGF-β).

MATERIALS AND METHODS: Initially, IL-19 (10, 20, 50 and 100 ng/L) was used to pretreat rat fibroblasts. TGF-β (10 μg/L) was then applied to activate fibroblasts. The protein expression levels of TGF-β receptor, extracellular-signal-regulated kinase (Erk) and p-38 were measured by Western blotting. In addition, we performed laminectomy at T10 vertebral plate in rats, followed by injection of IL-19 in caudal vein one week after injury. Furthermore, IL-19, TGF-β and fibrosis indexes were measured by quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blotting at 7 and 28 days after injury, respectively.

RESULTS: Concentration-dependent IL-19 significantly down-regulated TGF-β receptor expression and inhibited phosphorylated Erk (p-Erk) and phosphorylated p38 (p-p38). *In vivo*, IL-19 reduced the expressions of TGF-β and connective tissue growth factor (CTGF) at 7 days. Furthermore, IL-19 significantly suppressed extracellular matrix productions formation, including α smooth muscle actin (α-SMA) and collagen-1 (COL-1), and fibronectin at 28 days.

CONCLUSIONS: IL-19 inhibited TGF-β expression via Erk and p38 pathway. Moreover, it decreased CTGF expression to suppress α-SMA, COL-1 and fibronectin in scar tissues, thereby preventing spinal cord from compression of scar tissues.

Key Words:

Interleukin-19 (IL-19), Transforming growth factor Ð (TGF-β), Extracellular matrix production, Epidural fibrosis.

Introduction

Laminectomy, commonly used in spinal surgery, is the main solution for spinal canal decompression[1,2]. Due to nerve compression, postoperative epidural scar hyperplasia leads to neurogenic chronic or back pain, seriously affecting the life quality of patients for a long time[3]. Therefore, it is necessary to reduce the degree of epidural scar formation and symptoms of nerve compression after laminectomy. Currently, the pathogenesis of epidural fibrosis (EF) remains unclear. It is reported that the regulation of fibroblast proliferation and extracellular matrix (ECM) production play a leading role in the development of EF after laminectomy[4]. TGF-β has been reported as a key promoter of EF. Meanwhile, excessive transforming growth factor β (TGF-β) signaling leads to phenotypes of many diseases, including cell proliferation and ECM overproduction[5,6]. Therefore, TGF-β or TGF-β-related pathways can reduce the proliferation of fibroblasts and excessive production of extracellular matrix. This may be an effective way to attenuate the growth of epidural scar[7,8]. TGF-β induced fibroblast proliferation and ECM overproduction are mainly regulated by Smad 2/3 pathway[9,10]. The stimulation of TGF-β can induce the phosphorylation of receptor activated transcription factor Smad 2/3, eventually forming a heteromeric complex with Smad 4 and transferring to the nucleus[11-13]. Mitogen-activated protein kinase (MAPK) is a large class of proteins. Previous studies[14,15] have shown that MAPK is involved in a variety of cellular processes, including regulation of TGF-β stimulation, which is an important mechanism independent of Smad signaling. Interleukin-19 (IL-19) was first discovered in 2000. It was cloned into IL-10 homologue by searching expressed sequence tag database[16]. IL-19 acts through a receptor complex composed of IL-20R α and IL-20R β, which is also used by IL-20 and IL-24[17,18]. Like IL-10, IL-19

Corresponding Author: Yingsheng Wang, MM; e-mail: 215328430@qq.com

has been considered as an anti-inflammatory interleukin promoting anti-inflammatory Th2 rather than Th1 response[19]. Due to the anti-inflammatory effect of IL-19, we speculated that IL-19 might affect the activation of TGF-β in fibroblasts. Therefore, the aim of this study was to investigate the effect of exogenous IL-19 on TGF-β1 and its mechanism in rats with EF. Furthermore, the effect of IL-19 on ECM in fibrotic scar was explored as well.

Materials and Methods

Primary Fibroblast Culture

Primary fibroblasts were isolated from epidural scar tissue of rats one week after laminectomy. Obtained extracted cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin and streptomycin. All cells were maintained in a cell incubator with 5% CO_2 at 37°C. The cells were then incubated for 24 hours, with a confluence of 70-80%. After that, the cells were starved in serum-free DMEM for 24 hours, followed by treatment with 10 ng/mL TGF-β alone or in pretreatment with IL-19 at various concentrations (10, 20, 50 and 100 ng/L).

Surgery Procedure of Modeling

This study was approved by the Animal Ethics Committee of No. 89 Hospital of Chinese People's Liberation Army Animal Center. Forty 8-week-old male Sprague-Dawley (SD) rats weighed 180-200 g were randomly divided into three groups, including: control group (n = 8), laminectomy group (n = 16), and IL-19 group (n = 16). All rats were first anaesthetized by intraperitoneal injection with 10% chloral hydrate (0.5 mL/100 g) and then fixed on an operation board in the prone position. The surgical procedure of laminectomy in rats was described as follows: skin preparation was accomplished on the back of T10 location. The skin was disinfected with iodophor, and a 2.5 cm incision was made. Subsequently, blunt dissection was performed to separate muscle and fascia, followed by exposure of the T10 vertebral plate. Next, we carried out laminectomy to remove vertebral plate and expose the spinal cord. After hemostasis and saline flushing, the incision was sutured layer by layer and the skin was disinfected. The rats were maintained under constant temperature of 20-25°C and relative humidity of 55-65%, with free assess to normal food and water. After dissolved with 0.9% normal saline, 20 mg/kg exogenous IL-19 was injected *via* tail vein for one week every day. After the rats were sacrificed, epidural scar and surrounding tissues were collected for subsequent analysis.

Western Blot Analysis

Total protein in epidural scar tissue or treated fibroblasts was extracted on ice using a total protein extraction kit containing protease inhibitors and phosphatase inhibitors. Protein containing mixture was centrifuged by high-speed centrifuge (13000 rpm, 15 minutes) at low temperature (4°C), followed by collection of the supernatant fluid. The concentration of extracted protein was determined by double Bicinchoninic Acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at 4°C for 2 h. 5% non-fatty milk was prepared with Tris-buffered saline with Tween-20 (TBST) to block a specific antigen for 1 h. After washing with TBST for 3 times, the membranes were incubated with primary antibodies (p-Erk, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; t-Erk, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; p-p38, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; t-p38, Cell Signaling Technology, Danvers, MA, USA, Rabbit, 1:1000; Collagen I, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; α-SMA, Abcam, Cambridge, MA, USA, Mouse, 1:1000; connective tissue growth factor, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; TGF-β, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; TGF-β receptor, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; fibronectin, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Glyceraldheyde 3-phosphate dehydrogenase (GAPDH), Proteintech, Rosemont, IL, USA, 1:10000) at 4°C overnight. On the next day, the membranes were washed with TBST for 3 times and incubated with corresponding secondary antibodies (Goat Anti-Mouse IgG, Goat Anti-Rabbit IgG, YiFeiXue Biotechnology, Nanjing, China, 1:10000) at room temperature for 2 h. Enhanced chemical luminescence (ECL) method was used to visualize target proteins on the exposure machine.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

1 mL TRIzol (Invitrogen, Carlsbad, CA, USA) was added to epidural scar tissues and homogenized after shearing. The nucleic acid protein complex was completely separated after 5 minutes of incubation at room temperature. For cells, 0.5 mL TRIzol was added to each of six orifice plates, followed by shaking on ice shaker for 10 minutes. 0.2 mL chloroform was added into every 1 mL TRIzol, and the tubes were violently shaken for 15 s and incubated at room temperature for 3 min. After centrifugation for 15 min (10000 RPM, 4°C), the upper water phase of the mixture was sucked out, and isopropyl alcohol was added. The mixture was vibrated and placed at room temperature for 10 minutes. After centrifugation for 10 minutes (10000 rpm, 4°C), RNA precipitation was obtained and the supernatant was discarded. After washing RNA precipitation with 75% ethanol, the mixture was centrifuged at 10000 RPM and 4°C for 5 minutes. The supernatant was discarded, and 30 μL RNase free water was added to dissolve it. The concentration of extracted RNA was measured on Nano Drop to determine absorbance at 260 nm, 230 nm and 280 nm. A260/A280 between 1.8 and 2.0 indicated standard quality of extracted RNA, which could be used in subsequent experiments. The mRNA quantitative analysis was achieved using Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A 25 μL reaction system was used, including: 12.5 μL of SYBR Green, 10 Mm of primers (0.5 mL each from the stock), 10.5 μL of water and 0.5 μL of template. Specific PCR conditions were as follows: denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s; annealing at 60°C for 30 s and extension at 72°C for 30 s. Experimental data was analyzed by SDS software, and the results were output to EXCEL for further analysis. Endogenous GAPDH was used as an internal reference. Comparative threshold cycle (Ct) method, namely the $2^{-\Delta\Delta Ct}$ method was used to calculate fold amplification. Primer sequences used in this study were as follows: IL-20R, F: 5'-CACCCGGAACCTCCTTACGG-3', R: 5'-GGATGTGTCCCTAGGAAGGCC-3'; IL-20R, F: 5'-GTAACATCCTGAGGGTGCTG-3', R: 5'-GCGTGTCGAGTATTCG-3'; TGF-β, F: 5'-GCAGCTTGGCATGGACA-3', R: 5'-CTGTGAATAGCAGTTCCGT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Measurement data was expressed as $\chi \pm s$. *t*-test was used for comparison between two groups. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc Test. Least Significant Difference (LSD) test or Student-Newman-Keuls (SNK) test was used for pairwise comparison under the condition of homogeneity of variance. $p<0.05$ was considered statistically significant.

Results

Concentration-Dependent IL-19 Suppressed TGF-β Receptor and Down-Regulated p-Erk and p-38 Expression

Fibroblasts treated with different concentrations of IL-19 were isolated into proteins for Western blotting. The results displayed that 100 ng/L IL-19 significantly inhibited TGF-β receptor expression. Meanwhile, the protein expressions of p-Erk and p-p38 in MAPK pathway were also remarkably declined in each IL-19 treatment group (Figure 1A-1D). The results concluded that concentration-dependent IL-19 reduced the role of TGF-β in fibroblasts activation *via* down-regulation of TGF-β receptor and decrease of Erk and p38 in MAPK pathway.

Administration of IL-19 Activated the Transcription of its Receptor and Reduced the Transcription of TGF-β in Vivo

We established EF model in rats after laminectomy and performed IL-19 treatment one week after surgery. All rats were sacrificed at 7 days, and epidural scar tissues were extracted into RNA for qRT-PCR analysis. The results indicated that the expressions of IL-20R α and IL-20R β were significantly up-regulated, while TGF-β was significantly declined (Figure 2A and 2B). Injection of IL-19 played a role in the epidural scar site and suppressed the transcription level of TGF-β.

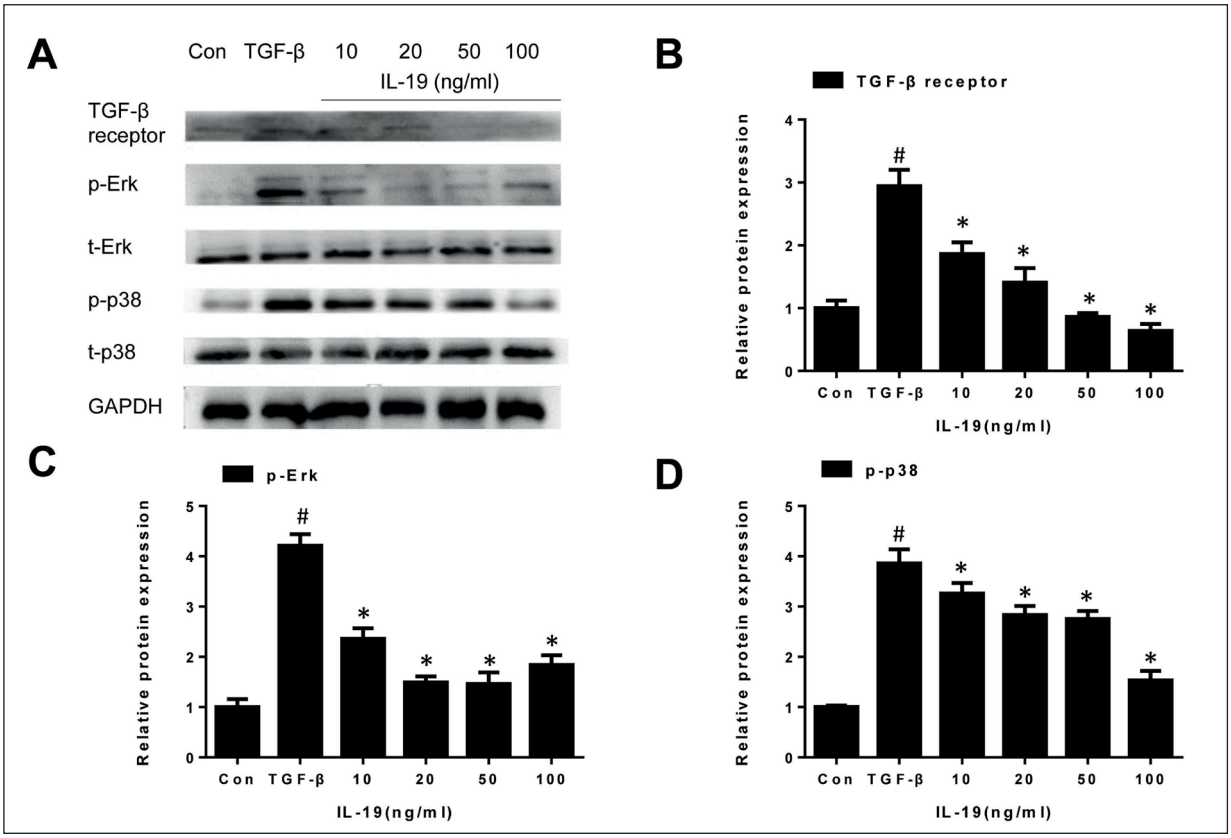

Figure 1. Concentration-dependent IL-19 suppressed TGF-β receptor and down-regulated p-Erk and p-38 expression. ***A,*** The protein bands of TGF-β receptor, p-Erk, p-p38, t-Erk and t-p38. ***B,*** The grey level analysis on the protein band of TGF-β receptor. ***C,*** The grey level analysis on the protein band of p-Erk. ***D,*** The grey level analysis on the protein band of p-p38.

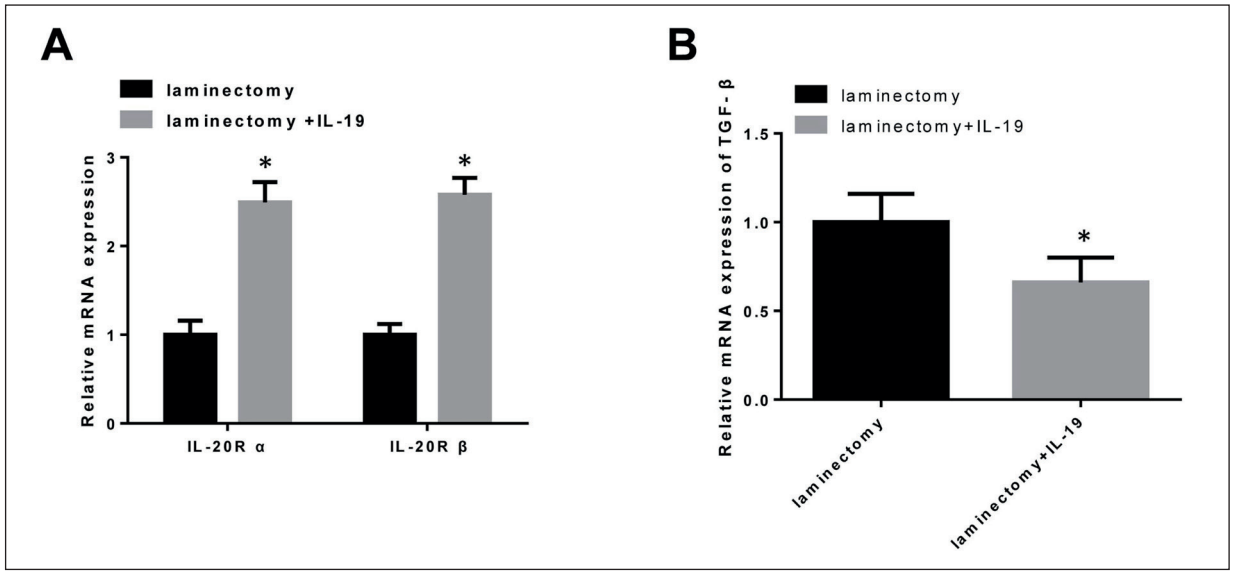

Figure 2. Administration of IL-19 activated the transcription of its receptor and reduced the transcription of TGF-β *in vivo*. ***A,*** The mRNA levels in IL-20R α and IL-20R β at 7 days after operation. ***B,*** The mRNA levels of TGF-β at 7 days after laminectomy.

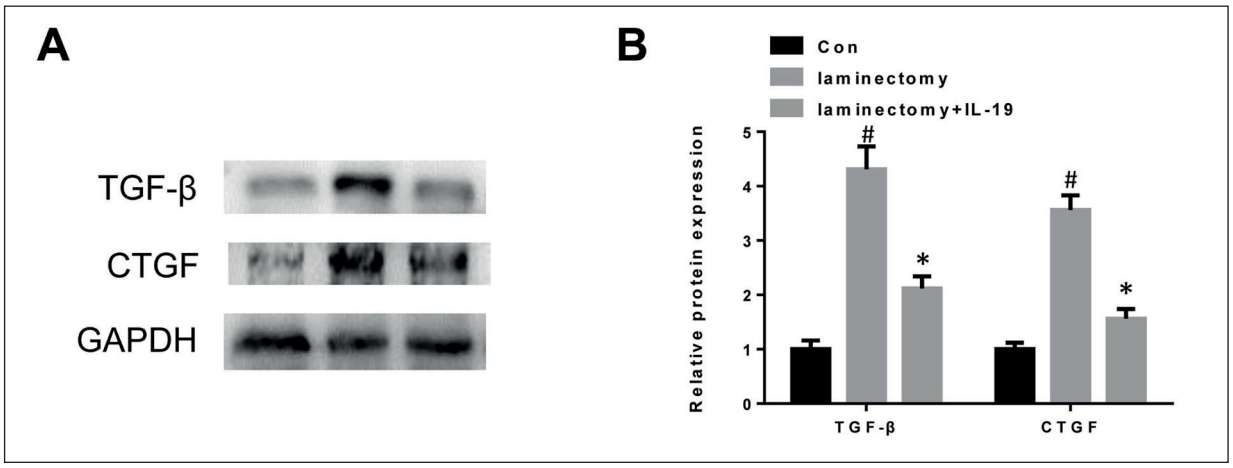

Figure 3. Exogenous IL-19 reduced TGF-β expression and inhibited CTGF production *in vivo*. ***A,*** The protein bands of TGF-β and CTGF at 7 days after laminectomy. ***B,*** The grey level analysis on the protein bands of TGF-β and CTGF.

Exogenous IL-19 Reduced TGF-β Expression and Inhibited CTGF Production In Vivo

Total protein was extracted from epidural scar tissues for Western blotting. We then hypothesized that IL-19 reduced the levels of fibroblast stimulating factors. TGF-β and CTGF are the key factor in stimulating fibroblast activation, thereby increasing ECM in the proliferation of wound scar tissues. Their expression levels determine the degree of EF. Therefore, we detected their proteins levels in scar tissues at 7days after injury. The results showed that TGF-β and CTGF were lowly expressed in IL-19 group, whereas highly expressed in laminectomy group (Figure 3A and 3B). The results suggested that IL-19 suppressed TGF-β and CTGF expressions after laminectomy.

Expression of ECMs were Significantly Inhibited by Injection of Exogenous IL-19

We measured the protein expression of ECMs at 28 days after operation. Meanwhile, the proliferative intensity of EF after IL-19 administration was evaluated as well. α-SMA, COL-1 and fibronectin are the most typical extracellular matrix components secreted by fibroblasts after activation. Their contents determine the thickness and stiffness of the scar. In the present study, the results demonstrated that the production of fibroblasts in IL-19 group was significantly less than that of laminectomy group (Figure 4A and 4B). Our findings suggested that exogenous IL-19 micro-modulated fibroblast secretion and weakened ECM accumulation, eventually reducing the degree of EF.

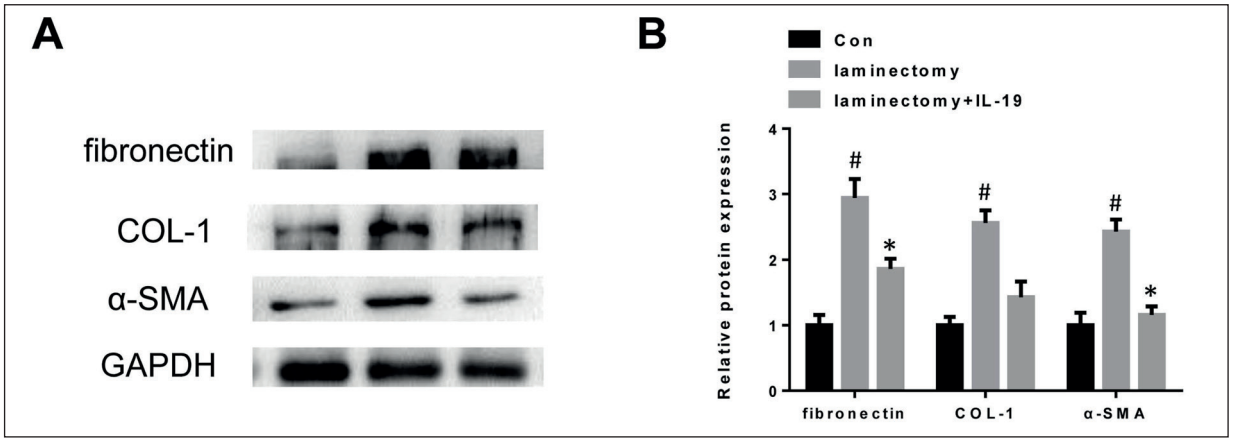

Figure 4. Expressions of ECMs were significantly inhibited by injection of exogenous IL-19. ***A,*** The protein bands of α-SMA, COL-1 and fibronectin at 28 days after laminectomy. ***B,*** The grey level analysis on the protein bands of α-SMA, COL-1 and fibronectin.

Discussion

Epidural fibrosis (EF) is a common postoperative complication after spinal surgery. Mild fibrosis does not affect the space between the spinal canal and foramina[20]. However, stiff and thick fibrotic epidural scarring can compress the spinal cord or nerve root pathways. This may eventually result in a series of neurological symptoms, such as chronic pain in the lower back and numbness in the lower extremities[21,22]. The degree of EF depends on fibroblasts proliferation and cell activity after activation[23,24]. In these processes, TGF-beta is the most important factor. It acts as a guide in regulating the proliferation and secretion of extracellular matrix of fibroblasts[25,26]. IL-19 is a major member of the il-10 family, showing typical anti-inflammatory properties of IL-10[27]. Therefore, we hypothesized that IL-19 might regulate the activation of fibroblasts activated by TGF-β. In the present study, we found that IL-19 significantly inhibited excessive activation of fibroblasts by down-regulating the expressions of p-Erk and p-p38. Meanwhile, it inhibited the secretion process of extracellular matrix by down-regulating CTGF simultaneously. Eventually, this might alleviate EF. Although we have demonstrated the role of IL-19 mediated TGF-β, the specific mechanism of CTGF has not been explored, which needs to be confirmed by further studies. This study proposed a new treatment idea in fibrotic scar, especially in EF. This could effectively inhibit the activation level of fibroblasts, attenuate the secretion of extra-cellular matrix, and effectively reduce the thickness and hardness of fibrotic scar. Furthermore, our findings provided supplementary support for the exploration of emerging fibrosis treatment strategies.

Conclusions

IL-19 down-regulated the expression of TGF-β by inhibiting p-Erk and p-p38 pathways. In addition, it simultaneously reduced the level of CTGF and inhibited the secretion of extracellular matrix by fibroblasts and alleviating EF.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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