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Immunomodulatory effectiveness of tacrolimus in preventing epidural scar adhesion after laminectomy in rat model

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ABSTRACT

There was no previous study about topical application of tacrolimus (FK506) could inhibit fibroblast proliferation and prevent epidural scar adhesion after laminectomy. We intended to illustrate the effect of FK506 on inhibiting fibroblast proliferation and preventing epidural scar adhesion after laminectomy in rat model. In our study, seventy-two rats were randomly divided into four groups (0.1 mg/ml group, 0.05 mg/ml group, 0.01 mg/ml group and control group). Laminectomy was performed at Lumbar-1 level, and then different concentrations of FK506 and saline were applied to the laminectomy sites. Four weeks later the rats were killed and the epidural adhesion was evaluated. Macroscopic assessment, hydroxyproline content analysis, histological analysis and mRNA measurements were used to evaluate the effect of FK506 on reducing epidural scar adhesion. The results showed that FK506 could prevent epidural scar adhesion in a dose-dependent manner. Little epidural adhesions were seen in the laminectomy sites treated with 0.1 mg/ml FK506. The hydroxyproline content, the number of fibroblasts, the mRNA expression level of IL-2 and TGF-β1 in 0.1 mg/ml FK506 group were significantly less than those of 0.05 mg/ml FK506 group, 0.01 mg/ml FK506 group and control group. However, dense epidural adhesions were found in 0.01 mg/ml FK506 group and control group. The hydroxyproline content and the number of fibroblasts in 0.01 mg/ml group showed no significant difference compared with those of control group. In conclusion, topical application of 0.1 mg/ml FK506 could inhibit fibroblast proliferation and prevent epidural scar adhesion after laminectomy in rat model.

1. Introduction

Extensive scar adhesion between dural mater and surrounding muscles is a major cause of fail back surgery syndrome, which is characterized by chronic nerve radicular or low back pain in the people after laminectomy (Robertson, 1996; Skaf et al., 2005). Because more complications such as dural tears, nerve root injury and bleeding may occur in revision spine surgery due to epidural adhesion, the outcome of re-operation for fail back surgery syndrome is unsatisfactory (Benoist et al., 1980; Cruccu et al., 2007). Therefore, a good modulation which can reduce scar adhesion formation after laminectomy is needed.

Although the cause of failed back surgery syndrome is multifactorial, epidural adhesion is considered as one of the major etiologies for this condition by many clinical investigators. Plenty of therapeutic schedules are utilized to prevent epidural scar adhesion. Microsurgical technique and good hemostasis have been applied to reduce the scar formation after laminectomy. Moreover, many types of biological and non-biological materials have been implanted into laminectomy defects to act as a barrier to prevent epidural scar adhesion in clinical and experimental study (Kitano et al., 1991; Sun et al., 2008). Pharmaceutical agents such as mitomycin C and steroid hormone are also used to reduce epidural scar adhesion (Sandoval and Hernandez-Vaquero, 2008). All these therapeutic schedules have achieved limited success in preventing epidural adhesion, but none of them can prevent epidural scar adhesion completely (Su et al., 2010). Recently, it has been reported that immunomodulatory agents, such as pimecrolimus could prevent epidural scar adhesion in rat model (Cemil et al., 2009).

Tacrolimus (FK506), a famous immunosuppressive agent, is widely used to treat inflammatory skin diseases and atopic dermatitis by suppressing *T*-cell function in dermatology department (Assmann et al., 2000). Recently, it has been reported that intraperitoneal injection of FK506 could prevent peritoneal scar adhesion after intestinal transplantation in rats (Wasserberg et al., 2007).

In our present study, we intended to illustrate the immunosuppressive effect of topical application of FK506 on inhibiting

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fibroblast proliferation and preventing epidural scar adhesion based on these previous researches, which may be useful in future human trials before clinical application.

2. Materials and methods

2.1. Animals

Seventy-two healthy male Sprague-Dawley rats (mean weight of 280 g) were purchased from Shanghai Laboratory Animal Center. The animals received care in compliance with the principles of International Laboratory Animal Care and the experimental protocol was approved by the Animal Care and Research Committee of Yangzhou University. Seventy-two rats were randomly divided into four groups (eighteen rats in each group): 0.1 mg/ml FK506 group, 0.05 mg/ml FK506 group, 0.01 mg/ml FK506 group and control (saline) group. Before the experiment, the rats were housed for a week to adapt to the condition of laboratory.

2.2. Reagents

FK506 was purchased from Astellas Ireland Co., Ltd. (Killorhlin, Co. Kerry, Ireland), β-dimethylaminobenzaldehyde was purchased from sigma.

2.3. Laminectomy model

All animals were anesthesized by intraperitoneal injection of 1% pentobarbital sodium solution (4 ml/kg body weight) and fixed on a special board in prone position. The hair around the L1 and L2 was shaved and the exposed skin was sterilized with iodophor for three times. Laminectomy model was performed according to the procedure in the previous study (Lee et al., 2004; Sun et al., 2007). A median incision of dorsal skin was made and the paraspinal muscles were separated on L1–L2 spine level. The dura mater of L1 level was exposed after removing the spinous process and vertebral plate with a rongeur. After laminectomy, satisfactory hemostasis was achieved by using gauze without using bone wax and cauterization. All these procedures were done carefully not to injure the neural tissues.

2.4. Topical application of drugs

FK506 in various concentrations of 0.1, 0.05, and 0.01 mg/ml or saline were applied to the laminectomy sites with cotton pads (application volume is about 0.7 ml) for 5 min separately. The surrounding tissues were covered by wet gauzes to avoid getting in touch with the agent. After the cotton pads were removed, the laminectomy sites were irrigated with saline solution for 3 times right away in order to eliminate any remaining agent. The muscles and skin were sewed in layers.

2.5. Macroscopic assessment of epidural scar adhesion

Macroscopic assessment was performed after four weeks. Six rats were randomly selected from each group and anesthesia by intraperitoneal administration of 1% pentobarbital sodium. The surgical sites were reopened carefully and the epidural scar adhesion was evaluated according to the Rydell classification by three professional pathologists who were blinded to the treatment group (Rydell, 1970). (Grade 0=epidural scar tissue was not adherent to the dura mater; Grade 1=epidural scar tissue was adherent to the dura mater, but easily dissected; Grade 2=epidural scar tissue was adherent to the dura mater, and difficultly dissected without disrupting the dura matter; Grade 3=epidural scar tissue was firmly adherent to the dura mater, and could not be dissected).

2.6. Hydroxyproline content (HPC) analysis

Hydroxyproline content (HPC) analysis was performed after four weeks. Six rats were sacrificed with an overdose of pentobarbital sodium after macroscopic observation and the scar tissue approximately 5 mg wet weight was obtained from the laminectomy sites. The content of hydroxyproline of different groups in scar tissue was determined according to the method of previous study (Woessner, 1961; Fukui et al., 2000).The samples were lyophilized, ground and hydrolyzed with 6 mol/l HCl at 130 °C for 12 h separately. Then they were neutralized with 2.5 N NaOH on the indication of methyl red. 1 ml chloramine T was added to the hydrolyzed samples and hydroxyproline standards of four known concentrations. After incubation for 20 min at room temperature, 1 ml hydroxyproline developer (β-dimethylaminobenzaldehyde solution) was added to the samples and the standards. The absorbance of the solution was measured at 558 nm with a spectrophotometer and the hydroxyproline content (HPC) per milligram of scar tissue was calculated according to the standard curve constructed by the serial concentration of commercial hydroxyproline.

2.7. Histological analysis

Histological analysis was performed postoperatively after four weeks. Six rats selected randomly were anesthesized by intraperitoneal injection of pentobarbital sodium and following intracardial perfusion with 4% paraformaldehyde. The whole L1 spine column including the paraspinal muscles and epidural scar tissue was removed en bloc and immersed into 10% buffered formalin for five days. The specimens were embedded in paraffin after decalcified for ten days. Successive transverse sections of 4 mm were made through the L-1 vertebra and stained with hematoxylin–eosin. The epidural scar adhesion was evaluated under the light microscope with the magnification $\times 40$. Three counting areas were selected at the middle and at the margins of the laminectomy sites and each was approximately 100×100 μm. The number of fibroblasts was calculated.

2.8. The mRNA measurements of IL-2 and TGF-β1

The mRNA analysis of IL-2 and TGF-β1 was performed after four weeks. Six rats were sacrificed with an overdose of pentobarbital sodium and the scar tissue was obtained from the laminectomy sites. Total RNA was extracted from the scar tissue and the RNA (2 μg) was transcribed into cDNA by use of AMV Reverse Transcriptase (Promega, Madison, Wisconsin, USA). Quantitative real-time PCR was performed using the Applied Biosystems 7300HT machine and Maxima TM SYBR Green/ROX qPCR Master Mix (Fermentas, USA). Primers were designed and synthesized by Sangong Company, Shanghai, China. Primers used are as follows: TGF-β1 (298 bp): forward, 5′gaaccaaggagacggaatacag3′; reverse, 5′aacccaggtccttccta aagtc3′; IL-2 (190 bp) forward, 5′agcgtgtgttggatttgactc3′; reverse, 5′at gatgctttgacagatggcta3′; GAPDH (252 bp) forward, 5′acagcaacagggt ggtggac3′; reverse, 5′ttgagggtgcagcgaactt3′. The PCR reaction was evaluated by melting curve analysis and by checking the PCR products on 2% agarose gels. GAPDH amplification was used as an internal control.

2.9. Statistical analysis

The statistical analysis was performed by SPSS software (version 13.0). The results of the data were expressed as mean ± standard