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Original Article

Study on the expression of nerve growth associated protein-43 in rat model of intervertebral disc degeneration

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Abstract

Objective: In the present work we studied the expression of nerve growth associated protein (GAP-43) in a rat model of intervertebral disc degeneration. **Methods:** 16 healthy adult SD rats, male or female, with an average weight 220g were selected. FluoroGold was injected in L5-L6 disc as the tracer. After 7 days, Freund's adjuvant was then injected to build model of intervertebral disc degeneration. After 1, 3, 7 and 14 days of modeling immune-histochemical method was used to detect the T13-L6 dorsal root ganglion and positive expression of GAP-43, TNF-α and IL-1 in L5-L6 intervertebral disc; RT-PCR method was used to detect GAP-43 mRNA and Western blot method was utilized to detect the expression levels of protein. **Results:** In the observation group, the dorsal root ganglion, positive expression rates of GAP-43, TNF-α and IL-1, expression levels of GAP-43 mRNA and protein in the intervertebral disc at each time point were significantly higher than those in the control group, and the differences were statistically significant (P<0.05); the positive expression rates of GAP-43, TNF-α and IL-1, expression levels of GAP-43 mRNA and protein of the observation group reached the peak at 3d, and dropped at 7d; dorsal root ganglion reached the peak at 7d and dropped at 14d. **Conclusion:** Degenerative changes might be mediated by the abnormal high expression of GAP-43 and intervertebral disc inflammation jointly.

Keywords: Nerve Growth Associated Protein-43, Intervertebral Disc, Inflammatory Reaction, TNF-α, IL-1

Introduction

The low back pain caused by intervertebral disc degeneration is a common clinical condition, and intervertebral disc inflammation reaction plays an important role in degenerative changes[1]. Nerve fibers only exist in the outer layer of normal annulus fibrosus fibrocartilaginis intervertebralis; inner layer and long nerve fibers in the nucleus pulposus might be important to the pathogenesis of low back pain[2]. Nerve growth associated protein 43 (GAP-43) is a neuron specific phosphoprotein that plays an important role in the process of nerve growth, development and regeneration[3]. In the absence of other nutritional factors GAP-43 could also cause new terminal of the neuron, which is believed to be the intrinsic factor in the growth of neurons as a sign of neural regeneration[4]. Previous studies have focused on the effect of inflammatory response in intervertebral disc degeneration[5]. The present study further illustrated the pathogenesis of nerve fiber growth in intervertebral disc inflammation and provided reference for the pathological process of low back pain.

Materials and methods

Experimental animals

16 healthy adult SD rats, (male or female), aged 6-8 months, with the average weight of 250 g were normally fed and adapted to the environment for 1 week previous the experiment. The rats were purchased from animal experimental center of Shanghai Sangon Animal Experiment Center.

Construction of the intervertebral disc degeneration model: 4% of chloral hydrate (Pharmaceutical Factory of Shanghai, China) was injected into the abdominal cavity as anesthesia (10 ml/kg). This was followed by the fixation of limbs, abdominal shaving, disinfection and drape were conducted. In the ventral midline incision, subcutaneous structure was exposed sequentially, abdominal organs were separated to expose the posterior peritoneum, the left psoas

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Table 1. The analysis of immunohistochemical staining results (%).

Group	Day	Dorsal root ganglion				Intervertebral disc			
		Control group	Observation group	t-Test	P-value	Control group	Observation group	t-Test	P-value
GAP-43	1	6.5±1.3	25.7±6.4	8.632	<0.0005	10.2±1.9	38.2±11.3	16.234	<0.0005
	3	6.6±1.5	36.9±7.3	15.426	<0.0005	10.5±1.8	62.1±15.7	34.527	<0.0005
	7	6.2±1.4	48.7±9.2	24.632	<0.0005	10.3±1.6	49.3±12.4	22.163	<0.0005
	14	6.3±1.2	32.3±8.4	18.628	<0.0005	9.8±1.5	41.7±13.3	23.524	<0.0005
TNF-α	1	12.6±5.3	41.2±21.3	7.624	<0.0005	18.6±5.6	55.6±24.6	8.629	<0.0005
	3	11.7±5.5	56.7±24.6	14.632	<0.0005	20.3±5.8	78.2±32.5	24.326	<0.0005
	7	12.5±5.6	72.3±28.7	18.625	<0.0005	18.9±5.9	69.3±26.7	16.854	<0.0005
	14	13.4±5.2	65.6±24.3	15.532	<0.0005	19.5±6.1	58.9±25.5	13.524	<0.0005
IL-1	1	8.2±2.3	36.4±14.2	7.235	<0.0005	11.4±2.7	49.7±19.2	7.629	<0.0005
	3	8.6±2.2	48.2±14.7	13.265	<0.0005	11.6±2.8	67.9±24.3	18.629	<0.0005
	7	8.3±2.4	57.9±15.3	17.524	<0.0005	12.2±2.9	57.3±21.2	15.432	<0.0005
	14	8.2±2.3	44.5±14.5	13.629	<0.0005	12.3±3.2	46.5±18.7	14.207	<0.0005

major muscle was separated, and the left ventrolateral side of the L5-L6 intervertebral disc was exposed. 22G intravenous puncture needle tip was used to puncture 1-2 mm with FluoroGold (F-G) crystal particles; puncture point was closed with cyanoacrylate and sutured layer by layer. The rats were divided into cages and moved freely. 7 days later anesthesia was conducted again, the surgical incision approach into the intervertebral disc was injected 50 μl Freund's adjuvant (Sigma, USA) respectively and then sutured layer by layer.

Experimental grouping

Random grab method was utilized to divide the rats into the control group (n=4) and the observation group (n=12). The control group was injected with 50 ul normal saline after 7 days of injection of the F-G. Further on, T13-L6 dorsal root ganglion and positive expression of GAP-43, TNF-α and IL-1 were detected by immunohistochemical method after 1, 3, 7 and 14 days of re-modeling; RT-PCR method was used to detect GAP-43 mRNA and Western blot method was used to detect the protein expression level.

Immunohistochemical method

Immunohistochemical staining of paraffin sections of T13-L6 dorsal root ganglion was done in accordance with standard protocols using antibodies anti- GAP-43, anti-TNF-α and anti-IL-1 (CST, USA). Three fields were randomly selected under fluorescence microscope (Applied Biosystems, USA) and the staining intensities were measured by Image J software.

RT-qPCR

The Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, USA) was used to extract total RNA following the manufacturer's instructions. Then the reverse transcription kit (Takara, Japan) was used for cDNA synthesis. GAP-43 and GAPDH expression were measured by RT-qPCR using SYBR Premix Ex Taq (Takara, Japan). The oligonucleotide primers were synthesized by BIOTNT Corporation (China). Expression values of GAP-43 were normalized to the geometric mean of GAPDH measurements. Primers used in the present study are presented below. GAP-43: (F): 5'- AGGAAAGGAGAGAAGGCAGG-3', (R) 5'-GCAGGAGAGACAGGGTTCAG-3'. GAPDH: (F): 5'-TGCTTCACCACCTTCTTGA-3', (R): 5'-TCACCATCTTCCAGGAGC-3'.

Western blot

Total proteins were extracted by RIPA purchased from Beyotime Institute of Biotechnology (China). Equal amounts of protein lysates were electrophoretically separated on SDS-PAGE gels and transferred to PVDF membranes. Afterwards, 5% nonfat dried milk was used to block the membranes for 2 hour at room temperature. Then the membranes were incubated with primary antibodies overnight in 4°C. After that, the membranes were washed three times with PBST buffer, incubated with secondary antibody for 1 hour at room temperature and they were finally washed three times with PBST buffer. The protein bands were detected using the ECL detection system (Pierce). The antibodies, anti-GAP-43 and anti-GAPDH, were purchased from Cell signaling technology. Gray value was measured by Quality One software (Bio-Rad, USA). The expression levels of GAP-43 protein were represented by gray value of GAP-43/GAPDH.

Statistical methods

SPSS 20.0 software was used for statistics analysis, and measurement data were expressed as mean±standard deviation. Comparisons between groups adopted independent samples t-test, and comparisons within group underwent ANOVA of repeated measurement data; $P<0.05$ suggested that the differences were statistically significant.

Table 2. The analysis of RT-PCR method results.

Group	Dorsal root ganglion				Intervertebral disc			
	Control group	Observation group	t-Test	P-value	Control group	Observation group	t-Test	P-value
1 day	0.0624±0.0063	0.1652±0.0524	7.624	<0.0005	0.0963±0.0058	0.2654±0.0865	9.632	<0.0005
3 days	0.0636±0.0059	0.2452±0.0462	12.305	<0.0005	0.0859±0.0054	0.4257±0.0923	18.624	<0.0005
7 days	0.0652±0.0072	0.3162±0.0421	16.524	<0.0005	0.0832±0.0063	0.3629±0.1201	16.235	<0.0005
14 days	0.0641±0.0069	0.2251±0.0396	14.425	<0.0005	0.0814±0.0057	0.3125±0.0854	13.254	<0.0005
F-value	0.123	8.632			0.162	9.234		
P-value	0.865	<0.0005			0.764	<0.0005		

Table 3. The analysis of Western blot method results.

Group	Dorsal root ganglion				Intervertebral disc			
	Control group	Observation group	t-Test	P-value	Control group	Observation group	t-Test	P-value
1 day	0.06±0.02	0.12±0.05	7.624	<0.0005	0.09±0.03	0.24±0.12	8.632	<0.0005
3 days	0.05±0.02	0.21±0.07	15.432	<0.0005	0.10±0.04	0.38±0.13	21.534	<0.0005
7 days	0.07±0.03	0.28±0.09	19.624	<0.0005	0.11±0.04	0.32±0.14	16.532	<0.0005
14 days	0.05±0.02	0.23±0.06	16.324	<0.0005	0.08±0.03	0.25±0.11	13.524	<0.0005
F-value	0.213	12.326			0.245	21.524		
P-value	0.658	<0.0005			0.624	<0.0005		

Results

The analysis of immune-histochemical staining results

The positive expression rates of GAP-43, TNF-α, IL-1 in the intervertebral disc as well as in the dorsal root ganglion of the observation group at each time point were significantly higher than those of the control group, (P<0.05); The positive expression rates of GAP-43, TNF-α, IL-1 in the intervertebral disc of the observation group reached a peak at 3 days, and dropped at 7 days after re-modeling ; dorsal root ganglion reached the peak at 7 days and dropped at 14 days after re-modeling (Table 1).

RT-PCR results

The dorsal root ganglion and expression levels of GAP-43 mRNA in the intervertebral disc as well as in the dorsal root ganglion of the observation group at each time point were significantly higher than those of the control group, (P<0.05). The expression levels of GAP-43 mRNA in the intervertebral disc of the observation group reached the peak at 3d and dropped at 7d; dorsal root ganglion reached the peak at 7d and dropped at 14d (Table 2).

Western blot method results

Similarly, expression levels of GAP-43 protein in the intervertebral disc of the observation group as well as in the dorsal root ganglion at each time point were significantly higher than those of the control group. The expression levels of GAP-43 protein in the intervertebral disc of the observation group reached the peak at 3d and dropped at 7d; dorsal root ganglion reached the peak at 7d and dropped at 14d (Table 3).

Discussion

Clinical discogenic low back pain often occurs at L4-L5 and L5-S1 intervertebral disc. L5-L6 intervertebral disc of rats corresponds to the L4-L5 intervertebral disc of human. The injury information from L4-L5 intervertebral disc of human mainly transmits through sympathetic trunk LI-L2 dorsal root ganglion of parallel vertebral. Dorsal root ganglion is the link of the internal as well as external environment and spinal cord. Further, the peripheral sensory information is transmitted to the spinal cord and other high-level centers[6].

Complete Freund's adjuvant is basically water with oil mixture of *mycobacterium tuberculosis*, which is responsible for the inflammatory reaction at the site of local injection. In the present study, positive expression rates of GAP-43, TNF-α and IL-1, expression levels of GAP-43 mRNA and proteins in the intervertebral disc of the observation group were significantly higher than those of the control group at each point. Further, our work yielded consistent results with an earlier study showing up regulation of TNF-α, IL-1, nerve growth fac-

tor (NGF), macrophages and other inflammatory mediators[7-8].

The present study also showed decline in the expression of GAP-43, TNF-α and IL-1, on 14 day. The above results could be related to the observation of a recent study suggesting proteoglycan in nucleus pulposus could prevent the nerve from entering the intervertebral disc, and the significant decrease of proteoglycan content in the intervertebral disc degeneration provides the premise for the nerve ingrowth[9]. The amount of nerve ingrowth in intervertebral disc with pain symptoms is significantly greater than that of intervertebral disc without pain symptoms[10], suggesting that nerve ingrowth might be closely related to the occurrence of pain. GAP-43 is widely distributed in the central nervous system, spinal cord, posterior root ganglion and the autonomic nervous system. The developing neurons express along the axon, and the expression in growth cone is especially rich, suggesting that GAP-43 plays an important role in the regulation of neural development and plasticity[11]. Moreover, high expression levels of foreign genes encoding GAP-43 in transgenic mice could cause spontaneous formation of abnormal connections[12]. Moreover, the above results also suggested that the inflammatory reaction might induce abnormal expression of GAP-43[13], and levels of TNF-α as well as IL-1 are closely related to the expression of GAP-43. Furthermore, nerve ingrowth in intervertebral disc is related to pain conduction of dorsal root ganglion, and degenerative changes might be mediated jointly by the abnormal high expression of GAP-43 and intervertebral disc inflammation[14-17].

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