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. 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. 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. 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. Previous studies have focused on the effect of inflammatory response in intervertebral disc degeneration. 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...
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 by cyanoacrylate and sutured layer by layer. The rats were divided into cages and moved freely. 7 days later anesthesia was conducted again, then the surgical incision approach into the intervertebral disc was injected 50 μl Freund’s adjuvant (Sigma, USA) respectively and then sutured layer by layer. For the control group (n=4) and the observation group (n=12).

**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 μl 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 and positive expression of GAP-43, TNF-α and IL-1 were performed according to 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’- AGGAAAGGAGAGAAACAGAT-3’, (R): 5’-TCACCATCTTCACATGATG-3’. TNF-α: (F): 5’-TGCTTCACCACCTTCTTGA-3’, (R): 5’-TCACCATCTTCACATGATG-3’. IL-1: (F): 5’-TGCTTCACCACCTTCTTGA-3’, (R): 5’-TCACCATCTTCACATGATG-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. 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 1. The analysis of immunohistochemical staining results (%).

| Group  | Day | 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 |

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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 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).

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 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-

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   |       |        |

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 L1-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 centers6.

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.

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.

The amount of nerve ingrowth in intervertebral disc with pain symptoms is significantly greater than that of intervertebral disc without pain symptoms, 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, 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 nerve ingrowth might be closely related to the occurrence of pain.