Expression change of stem cell-derived neural stem/progenitor cell supporting factor gene in injured spinal cord of rats

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Abstract: **Objective** To explore the expression change of stem cell-derived neural stem/progenitor cell supporting factor (SDNSF) gene in the injured spinal cord tissues of rats, and the relation between the expressions of SDNSF and nestin. **Methods** The spinal cord contusion model of rat was established according to Allen's falling strike method. The expression of SDNSF was studied by RT-PCR and *in situ* hybridization (ISH), and the expression of nestin was detected by immunohistochemistry. **Results** RT-PCR revealed that SDNSF mRNA was upregulated on day 4 after injury, peaked on day 8–12, and decreased to the sham operation level on day 16. ISH revealed that SDNSF mRNA was mainly expressed in the gray matter cells, probably neurons, of spinal cord. The immunohistochemistry showed that accompanied with SDNSF mRNA upregulation, the nestin-positive cells showed erupted roots, migrated peripherad and proliferation on the 8-day slice. However, the distribution pattern of these new cells was different from that of SDNSF-positive cells. **Conclusion** (1) SDNSF is expressed in the gray matter of spinal cord. The expression of SDNSF mRNA in the spinal cord varies with injured time. (2) The nestin-positive cells proliferate accompanied with spinal cord injury repair, but do not secrete SDNSF.

Keywords: stem cell-derived neural stem/progenitor cell supporting factor; nestin; spinal cord injury; rat

1 Introduction

Adult neural stem/progenitor cells can secrete autocrine or paracrine factors, including fibroblast growth factor (FGF) 9^[1], erythropoietin (EPO)^[2], insulin-like growth factor (IGF-I)^[3] and glycosylated cystatin C (CCg)^[4], to participate in regulation of adult neurogenesis. Toda *et al.*^[5] have identified a novel secretory molecule, stem cell-derived neural stem/progenitor cell supporting factor (SDNSF), from adult hippocampal neural stem/progenitor cells. They found that the expression of SDNSF in adult central nervous system was localized to the hippocampus, including the dentate gyrus (where neurogenesis persists throughout life), CA3, and subiculum. SDNSF was up-regulated in ischemic hippocampus when neurogenesis was induced

and improved the survival of adult neural stem/progenitor cell in a dose-dependent manner after basic FGF-2 withdrawals. Their research suggested an important role of SDNSF as an autocrine/paracrine factor in maintaining stem cell potential and lifelong neurogenesis in adult central nervous system.

SDNSF has been found express in the brain, heart, lung, liver, spleen, kidney, testis, and skeletal muscle of adult rat. But whether it is expressed in adult spinal cord during spinal cord injury (SCI) is still unknown. In this study, we attempted to investigate the SDNSF expression pattern after SCI by using RT-PCR, *in situ* hybridization (ISH), and immunohistochemistry (IHC) techniques, and to analyze the relation between the expressions of SDNSF and neural stem cell marker nestin.

2 Materials and methods

2.1 Animals treatment and grouping Twenty-one adult Sprague-Dawley female rats (Special Pathogen Free) [weighing (220±15) g] used in this study were provided by Experiment Animal Center, Nantong University. The rats

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were randomly divided into sham operation group ($n = 6$) and injury group ($n = 15$), and the injury group were subdivided into 4-day ($n = 3$), 8-day ($n = 6$), 12-day ($n = 3$) and 16-day ($n = 3$) post-injury groups. The rats in the sham operation group and the 8-day injury group ($n = 3$ each) were also used for ISH and IHC.

2.2 Establishment of animal model Animals of injury groups were anesthetized by sodium pentobarbital (40 mg/kg, i.p.) and then fixed in a prone position on a board. The animal back was shaved and a longitudinal incision was made in the middle skin. The deep tissues were incised and the vertebral laminae from T₈ to T₁₂ were resected to expose the spinal duramater. Injury of the spinal cord was created according to Allen's falling strike method^[6] (10 g $\times$ 10 cm) after putting a plastic clout (0.3 cm $\times$ 1 cm) covered gelatin sponge on both sides of the lesion site. After the injury, the muscles and skin were closed in layers, and the rats were placed in a temperature- and humidity-controlled chamber. Manual bladder emptying was performed at least three times daily until reflex bladder emptying was recovered. The sham operation group received the same treatment except without the strike-injury. Soon after the operation (sham operation group), or on day 4, day 8, day 12 and day 16 after the spinal injury (injury group), the exposed section of the spinal cord was resected and then stored in liquid nitrogen.

2.3 RT-PCR Total RNA was extracted using TRIzol solution according to the manufacturer's protocol (Qiagen, USA). Two microgram total RNA was used to prepare cDNA with the reverse transcriptase in the kit. The house-keeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control. The synthetic oligonucleotides (Fermentas Inc.) were used for amplifying SDNSF (with a 385-bp product) and GAPDH (with a 561-bp product). The primers were designed by Premier Primer 5.0 software (Premier Biosoft International, USA) and synthesized by Invitrogen, Shanghai: SDNSF: forward 5'-TTCAGT GAC GGTGGAGGGA-3', reverse 5'-TAAGCCAAGAAG GGAGCC-3'; and GAPDH: forward 5'-TCG TGGAGT CTA CTGGCGTCT-3', reverse 5'-CAACCTGGTCCTCAGTGT AG-3'. The PCR reaction system (25 μ L) contained cDNA fragment 1.0 μ L, 10 $\times$ PCR Buffer 2.5 μ L, Taq polymerase 0.3 μ L, MgCl₂ (25 mmol/L) 2.0 μ L, dNTP mix (2 mmol/L) 2 μ L, primers (10 μ mol/L) for SDNSF or GAPDH 1 μ L, and ddH₂O 16.2 μ L. The reaction mixture was incubated for 5 min at 94 °C, and then subjected to amplification (30 cycles for SDNSF and 26 cycles for GAPDH): melting at 94 °C for 30 s, anneal-

ing at 72 °C for 30 s, and elongation at 72 °C for 40 s; finally extending at 72 °C for 7 min. Then the aliquot were run on 1.5 g/L agarose gels containing ethidium bromide, and the isolated PCR products were visualized under the ultraviolet illumination.

2.4 Probe preparation A 385-bp SDNSF cDNA fragment generated by PCR amplification was subcloned into the pGEM-T vector (Promega, USA) and the authenticity was confirmed by sequencing. The probes were labeled with digoxigenin-UTP by SP6 or T7 RNA polymerase using the DIG RNA labeling kit (1175025, Roche, USA). The preliminary experiment had proved that the T7 probe was plus sense and the SP6 probe was antisense.

2.5 Perfusion and tissue preparation Rats both in the sham operation group and the 8-day injury group were sacrificed at day 8 ($n = 3$ each) after the injury by administration of overdose sodium pentobarbital (60 mg/kg) and then intracardiacally perfused with 4% paraformaldehyde in cold PBS. After perfusion, a 10-mm long spinal segment covering the injury area was removed carefully, post-fixed in the same fixative overnight at 4 °C, and dehydrated sequentially with diethylpyrocarbonate (DEPC) treated 10%, 20%, and 30% buffered sucrose. The transverse sections were cut consecutively at 16 μ m on a cryostat, then mounted on slides and stored frozen at -80 °C prior to ISH and IHC.

2.6 ISH ISH was performed as previously described^[7]. Tissue sections (16 μ m thick) were mounted on superfrosted glass slides (Superfrost Plus, Erie Scientific Company, Germany) and incubated sequentially with 0.2 mol/L of HCl for 10 min, 0.3% Triton X-100 for 15 min, 0.2% glycine in PBS for 15 min at room temperature, and with 10 μ g/mL of Proteinase K for 10 min at 37 °C. The sections were then postfixed in PBS containing 4% PFA for 15 min, and incubated twice in 0.1 mol/L of PBS for 15 min each. The prehybridization solution buffer contained 50% formamide, 5 $\times$ SSC, 10% dextran sulfate, 5 $\times$ Denhardt's solution and 0.5% SDS. Denatured salmon sperm DNA (100 μ g/mL) were added before use. Hybridization was performed in a moist chamber by adding 5 μ g/mL of the indicated digoxigenin-labeled probe for 16 h at 42 °C.

The sections were washed sequentially at 42 °C with 2 $\times$ SSC (20 min), 2 $\times$ SSC/ 0.1% SDS (three times, 10 min each), 1 $\times$ SSC (two times, 15 min each), 0.1% SDS (two times, 15 min each), and 0.1 $\times$ SSC/ 0.1% SDS (two times, 30 min each) after hybridization. Next, the sections were washed briefly with Buffer I solution at room temperature

and Buffer II at 37 °C, for 30 min each. Thereafter, they were incubated with an alkalinephosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragment solution (1:2 000 dilution) for 2 h at 37 °C. The sections were then washed twice with Buffer I solution for 15 min, equilibrated with buffer III solution (100 mmol/L Tris-HCl, 100 mmol/L NaCl, 50 mmol/L MgCl₂, pH 9.5) for 3 min, and incubated with the staining solution containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in a dark box for 2 h, at room temperature. After dehydrated with alcohol, the sections were mounted in an aqueous mounting medium, and photographed using a Leica microscope.

2.7 IHC IHC was performed as previously described^[8]. IHC for nestin staining was performed using mouse anti-nestin monoclonal antibody (MAB353; Chemicon, USA) to detect the presence of nestin protein. The slides adjacent to the layer used in ISH were placed in 0.01 mol/L PBS with 1 g/L of Triton X-100 for three times (5 min each), then washed in 0.01 mol/L PBS for three times (5 min each), and exposed to 3% BSA at 37 °C for 30 min followed by washing again. Add the anti-nestin monoclonal antibody (1:200 diluted in 3% BSA) and incubate the slides at 4 °C for 72 h. Subsequently, the slides were washed and then incubated with FITC labeled goat anti-mouse antibody (1:200 diluted in 3% BSA) (10362-010; Roche, USA) at 4 °C overnight. Finally, reactions were photographed by using a Leica fluorescence microscope. The control slides were treated similarly except for substitution of the primary antibody by BSA.

2.8 Statistical analysis Statistical analysis was performed employing one-way ANOVA. $P < 0.05$ was considered statistically significant.

3 Results

3.1 RT-PCR analysis RT-PCR revealed that SDNSF mRNA was upregulated on day 4 after the injury, peaked on day 8 and day 12, and decreased to the sham operation level on day 16 (Fig. 1). Differences were found in groups 4-day, 8-day, and 12-day compared with the sham operation group ($P < 0.05$, Fig. 2).

3.2 ISH ISH revealed that the SDNSF mRNA was mainly localized in the gray matter cells, probably neurons, in rat spinal cord (Fig. 3). The number of SDNSF-positive cells in the 8-day injury group was more than that in the sham operation group.

3.3 IHC IHC showed that there were nestin-positive cells

Fig. 1 RT-PCR of SDNSF mRNA expression after spinal cord injury. SDNSF mRNA upregulated on day 4 post-injury, peaked on day 8 and day 12, and then decreased to the sham operation level on day 16. M: DL-2000 Marker; N: Sham operation group.

Fig. 2 By statistical analysis of the SDNSF/GAPDH ratio, differences were found in groups 4-day, 8-day, and 12-day compared with the sham operation group. * $P < 0.05$, ** $P < 0.01$ vs sham operation group.

around the central canal of adult rat's spinal cord. Accompanied with SDNSF mRNA upregulation, the nestin-positive cells erupted roots, migrated peripherad and proliferated on the 8-day slice, but these new cells showed different distribution patterns from SDNSF-positive cells.

4 Discussion

SDNSF, a novel secretory molecule, was supposed to be involved in maintenance of adult neurogenesis, so whether SDNSF play a role in SCI and repair arouse our interest. In this study, we cloned the fragment of SDNSF gene (GenBank AF475282), and for the first time, analyzed the distribution of SDNSF in the spinal cord of rat by ISH. The results proved the presence and revealed its location of SDNSF mRNA in adult rat spinal cord. Also, we found nestin-positive cells that erupted roots, migrated peripherad and proliferated around central canal of spinal cord on day 8 after injury. These nestin-positive cells are considered as the reactive astrocyte for inflammatory response^[9], and seems having no relation with the SDNSF expression. In the lower vertebrate such as newt, nestin cells act as neural stem/progenitor cells contributing to nerve regeneration^[10], and they proliferate and differentiate into

Fig. 3 SDNSF mRNA was mainly localized in the gray matter cells, probably neurons, of rat spinal cord. There were more SDNSF-positive cells in 8-day group than in sham operation group. A, B: Sham operation group; C, D: 8-day post-injury group. Arrows in B and D point the SDNSF-positive cells; E: negative control using the sense probe. Scale bar, 200 μ m in A and C, 40 μ m in B and D, 400 μ m in E.

Fig. 4 There were nestin-positive cells around the central canal of adult rat's spinal cord. Accompanied with the upregulation of SDNSF mRNA, the nestin-positive cells erupted roots, migrated peripherad and proliferated on the 8-day slice. A: Sham operation group; B: 8-day post-injury group. Red arrows point the nestin-positive cells and white arrows point the central canal. Scale bar, 40 μ m.

some cell types to repair the damaged tissue. In rats, this regenerative ability is weaker, but it is reported that the rat spinal cord suffering a minor SCI still can regenerate, which is coincident with what we found in this study. Recent investigation showed that reactive astrocytes were crucial for the tissue repair and the initial functional recovery after SCI in mice^[11]. But in this study, we found SDNSF was not secreted by these reactive astrocytes.

Toda *et al.*^[5] found that SDNSF was localized in the zones of central nervous system where the active adult neurogenesis existed. We found in this research that the adult rat spinal cord was also such a zone where neurogenesis existed and SDNSF was upregulated if suf-

fering a minor injury, as what happened in the brain. What puzzled us is, in spinal cord, the expression of SDNSF seems having no relation with nestin, which were considered to contribute to regeneration. We supposed that SDNSF was most probably a molecular component in neurogenic micro-environment in adult central nervous system.

SDNSF is also a component of MCD2-LMAN1 complex that functions as a specific cargo receptor for transporting proteins from ER to Golgi^[12]. Mutations in MCD2 causes factor 5 and factor 8 combined deficiently. As an autocrine/paracrine factor, the role of SDNSF played in the course of central nervous system injury is still unknown, further investigation should be done in this area, including

techniques of eukaryotic expression and gene knock-out, to provide more theory basis for mechanism of SCI and regeneration.

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神经干细胞生存因子在大鼠脊髓损伤前后的表达变化

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摘要 目的 观察大鼠脊髓损伤后干细胞来源的神经干细胞生存因子(SDNSF) mRNA在大鼠正常和损伤脊髓的表达变化, 以及SDNSF的表达与VI类中间丝蛋白的表达之间的关系。**方法** 按改良的Allen重物打击法制备大鼠脊髓损伤模型, 采用RT-PCR、原位杂交方法, 观察SDNSF mRNA在大鼠脊髓中的表达位置及在损伤脊髓中的表达变化。应用免疫组化的方法, 显示脊髓中nestin的表达。**结果** RT-PCR检测SDNSF mRNA在正常大鼠脊髓中的表达, 损伤后4天SDNSF的mRNA表达上升, 损伤8天到达高峰, 此后SDNSF的mRNA表达逐渐减少, 到16天恢复到正常水平; 脊髓切片原位杂交结果发现SDNSF的mRNA阳性细胞主要分布于脊髓灰质细胞中, 可能是神经元细胞, 结果表明正常脊髓可表达SDNSF; 脊髓损伤后8天, 原位杂交显示SDNSF阳性细胞明显增多。同时与此切片相邻层面的切片免疫组化证实nestin阳性细胞增殖、变大、向周围发出突起, 但这些阳性细胞在分布上与SDNSF无关。**结论** (1) SDNSF在脊髓中表达于灰质, 脊髓损伤后SDNSF的mRNA表达随时间发生变化。(2)随着脊髓损伤的修复, nestin阳性细胞增殖, 但是这些细胞并不表达SDNSF。

关键词: 干细胞来源的神经干细胞生存因子; VI类中间丝蛋白; 脊髓损伤; 大鼠