Electro-acupuncture promotes differentiation of mesenchymal stem cells, regeneration of nerve fibers and partial functional recovery after spinal cord injury

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ABSTRACT

In order to improve the structure and function of acute spinal cord injury, the present study investigated the effect of electro-acupuncture (EA) on the differentiation of mesenchymal stem cells (MSCs) and the regeneration of nerve fibers in transected spinal cord of rats. The differentiation of MSCs into neuron-like cells and neuralglial cells and regeneration of 5-hydroxytryptamine (HT) nerve fibers in the injured site of spinal cord were assessed after treatment with EA, MSCs transplantation, and EA plus MSCs transplantation. Compared with the control and MSCs groups, the content of endogenous neurotrophin-3 (NT-3) in the injured site and nearby tissues was increased in EA and EA+MSCs group. The differentiation of MSCs into neuronal-like cells and oligodendrocyte-like cells and number of 5-HT positive nerve fibers in the injured site were enhanced in the EA+MSCs group. Basso, Beattie, Bresnahan score of the paralyzed hindlimbs was highest in the EA+MSCs group. The present study demonstrates that electro-acupuncture can promote the differentiation of MSCs and regeneration of nerve fibers in injured spinal cord through induction of endogenous NT-3, and that combination of EA and MSCs transplantation can improve partial function of paralyzed hindlimbs.

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Introduction

There are various experimental methods used for the treatment of spinal cord injury (SCI). However, no simple method can receive satisfactory curative effect. Cell transplantation has become a widely used experimental strategy these days. A variety of cells such as neural stem cells (NSCs) (Lu et al., 2003), Schwann cells (SCs) (Bunge and Kleitman, 1999; Porter et al., 1987), olfactory ensheathing cells (OECs) (Ramón-Cueto et al., 2000; Barnett and Chang, 2004) and mesenchymal stem cells (MSCs) (Chopp et al., 2000; Hofstetter et al., 2002) have been considered as candidates for transplantation. Among them, MSCs have received the most attention because MSCs are easily isolated and can be used for autotransplantation without ethical problems. In addition, MSCs have the potential to differentiate into other cells including osteoblasts, adipocytes and chondrocytes (Stanheworth and Newland, 2001; Caplan, 1991). More importantly, MSCs can differentiate into neural lineage under the specific induction both in vitro and in vivo. Recently, MSCs have achieved great success in animal models of central nervous system diseases such as Parkinson’s disease (PD) (Offen et al., 2007). Similarly, transplantation of MSCs has become increasingly attractive for reducing the magnitude of spinal cord injury and diseases (Cizkova et al., 2006). However, the differentiation rate of MSCs transplanted in injured spinal cord still needs to be improved. Therefore, increasing the differentiation rate of transplanted MSCs into neurons and oligodendrocytes is important to compensate for lost neurons and oligodendrocytes successfully.

Electro-acupuncture (EA) has long been used to treat spinal cord injury in traditional Chinese medicine. EA on Governor Vessel is used for the treatment of spinal cord injury because the impairment of Governor Vessel is regarded as the essence of the damage of spinal cord in Chinese medicine. Indeed, EA on Governor Vessel has been shown to alleviate the secondary damage after spinal cord injury in both patients and animal models. Interestingly, we found that EA on Governor Vessel could promote the secretion of NT-3 and enhance the differentiation rate of exogenous neural stem cells into neuron-like cells in the injured site in rats with complete spinal cord transection (Chen et al., 2008). Also, the combination of EA on Governor Vessel and NSCs transplantation may promote axonal regeneration in...
completely transected spinal cord of rats (Li et al., 2006) and improve the function of hindlimbs (Li et al., 2004). However, the application of NSCs in clinic is limited for ethical problems. In the present study, we investigated the effects of EA on the differentiation of MSCs and the regeneration of nerve fibers in transected spinal cord, and tested the hypothesis that combination of EA and MSCs transplantation would improve the structure and function of injured spinal cord. Our results suggest that EA can promote the differentiation of MSCs and regeneration of nerve fibers in injured spinal cord, and that combination of EA and MSCs transplantation can improve partial function of paralyzed hindlimbs, and these effects might be mediated through induction of endogenous NT-3.

Materials and methods

Isolation and culture of mesenchymal stem cells (MSCs) for transplantation

 Cultured MSCs were obtained from the femurs of young male Sprague-Dawley (SD) rats following the procedure described in details in our previous study (Zhang et al., 2006). Briefly, MSCs were isolated from bone marrow using full bone marrow method and further purified by constant passages. After purification, the differentiation of MSCs into osteoblast or adipogenesis was induced by culturing cells in osteoblast or adipogenesis inducing medium, respectively. The differentiation was examined by detecting the form of calcium nodules and accumulation of lipid droplet through the staining of alizarin red S and Red Oil O. The MSCs with the capability of multipotential differentiation were used as stable cell resources for new experiments. When cultured for passages 3–5, MSCs were observed to grow well. The cells of passages 3–5 were used for the experiment. To trace transplanted MSCs in vivo, MSCs were labeled with a nuclear fluorescence Hoechst33342 (10 μg/1 mL, Sigma) 2 h before transplantation. Labeled MSCs were washed several times with D-Hank’s balanced salt solution (HBSS, pH 7.4) and removed from the culture flasks using 0.25% trypsin, 0.02% EDTA (Sigma). MSCs were centrifuged and resuspended in Dulbecco’s modified Eagle medium (DMEM) for cell counting. The cell suspension was adjusted to a final concentration of 1 × 10^6 viable cells/μL in culture medium for transplantation.

Animals

 Female SD rats (N=36; 220–250 g) were randomly divided into four groups: control group, EA treatment group (EA group), MSCs transplantation group (MSCs group) and EA treatment plus MSCs transplantation group (EA+MSCs group). There were 9 rats in each group. Four of them were used to offer tissues for ELISA analysis and other five were used for behavior evaluation and then morphological analysis.

Spinal cord surgery and MSCs transplantation

 Rats were anesthetized with 1% sodium pentobarbital (30 mg/kg, i.p.). A laminectomy was carried out at the T8–T10 level to expose the T10 spinal segment. The dura was cut and the T10 spinal segment was transected completely. A piece of gelatin foam (2 × 2 × 2 mm³), which was pre-injected with cell suspension containing MSCs (1 × 10^6 cells/μL, 5 μL), was placed in the transected site of the spinal cord in the MSCs group and EA+MSCs group while gelatin foam pre-injected with culture medium only was placed in the transected site of the spinal cord in the control group and EA group. The muscle, subcutaneous tissue and skin were sutured in layers. After surgery, all animals received an intramuscular injection of penicillin (160,000 U/mL/d) and then were caged separately and placed on thick soft bedding in individual cage. Manual emptying of the bladders was performed twice or three times daily. All procedures were approved and in accordance with the public Health Service Guide for the Care and Use of Laboratory Animals.

Electro-acupuncture (EA) therapy

 Seven days after spinal cord surgery, EA therapy was administered to the rats in the EA group and EA+MSCs group every other day while no treatment was given to the rats in the control group and MSCs group. EA stimulation was performed at four acupoints in Governor Vessel, namely GV9 (Zhiyang), GV6 (Jizhong), GV2 (Yaoshu) and GV1 (Changqiang). Two pairs of needles were connected with the output terminals of an electro-acupuncture apparatus (Model G 6805-2, Shanghai Medical Electronic Apparatus Company, China). Alternating strings of dense–sparse frequencies (60 Hz for 1.05 s and 2 s for 2.85 s alternately) were used for EA. The intensity was adjusted to induce slight twitch of the hindlimbs (≤ 1 mA), with the intensity lasting for 20 min.

NT-3 detected by enzyme-linked immunosorbent assay (ELISA)

 ELISA was utilized to measure the content of neurotrophine-3 (NT-3) of spinal tissue in and around injured site 2 weeks post operation. 16 animals (N=4 for each group) were anesthetized with 1% sodium pentobarbital (40 mg/kg, i.p.), and were perfused with 0.9% NaCl solution (containing Heparin) and then 0.1 mol/L phosphate buffer (PB, pH 7.2). Spinal cords were quickly dissected from T6-L1 segments. Each spinal cord segment (150 mg) was measured and ground in ice bath to prepare tissue homogenate of which the supernatant liquid was extracted for ELISA analysis. All reagents and working standards were prepared following the manufacturer’s instructions (NT-3 Emax ImmunoAssay System; Boster).

Tissue processing for immunofluorescence

 Thirty days after spinal cord transected, 20 rats (N=5 for each group) were anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L PBS. T8–T12 spinal cord segments containing the injury sites were dissected, placed into 4% paraformaldehyde for 24 h and then placed into 0.1 mol/L PBS containing 30% buffered sucrose until the tissue sank down to the bottom of the container at 4°C. Longitudinal sections (25 μm) of the spinal cord (T8–T12) were cut on a freezing microtome. The sections were immunofluorescently stained as described in our previous publications (Guo et al., 2007). Primary antibodies were used as follows: monoclonal anti-MAP2 (microtubulin associated protein 2, 1:1000, Sigma), monoclonal anti-MOSP (mouse anti-oligodendrocyte special protein, 1:1000, Chemicon), mouse anti-neurofilament (NF, 1:200, Boster), rabbit polyclonal anti-serotonin (5-HT, 1:200, Sigma) and rabbit polyclonal anti-calcitonin gene-related peptide (CGRP, 1:8000, Chemicon).

Quantification of differentiation of transplanted MSCs

 A cell count was conducted in 8 longitudinal spinal cord sections including the injury site for each rat in the MSCs group (N=5) and EA+MSCs group (N=5). The surviving MSCs (labeled with nuclear fluorescence Hoechst33342) were counted in 3 unit
areas (0.09 mm² each) distributed in the injury site. The total number of labeled cells from these 3 areas was used as surviving cells. Differentiated neuron-like cells (both nuclear fluorescence and MAP2 positive) and oligodendrocyte-like cells (both nuclear fluorescence and MOSP positive) were also counted and expressed as percentage of total surviving MSCs. The percentages of differentiated cells were compared between the MSCs and EA+MSCs groups.

**Quantification of NF and 5-HT positive nerve fibers**

The spinal cords were cut in longitudinal sections and every fifth section was mounted on slide. Nerve fiber counting was conducted in 8 longitudinal spinal cord sections including injury site for 20 rats (N=5 for each group). A calibrated reticle eyepiece was used to delineate regions 300 μm rostral to the injury site, the injury site and 300 μm caudal to the injury site. 3 unit areas (0.09 mm² each) distributed in the rostral site, the injury site, and the caudal site were produced respectively. The regenerative nerve fibers positive in NF or 5-HT staining with a length over 50 μm were counted at 200× magnification in 3 unit areas (0.09 mm² each) respectively. For each rat, the average number of NF or 5-HT positive nerve fibers in each site was used for analysis.

**Behavioral evaluation**

Twenty (N=5 for each group) SD female rats without functional deficits, BBB score=21, were selected to the experiment. After spinal cord transection, two observers who were blind to groups of the experiment graded each animal according to Basso, Beattie, Bresnahan (BBB) open field locomotion test (Basso et al., 1995). Each session lasted 5 min. The BBB score was determined by observation of voluntary hindlimbs movement, body weight support, foot placement and coordination. Scores given by 2 observers were averaged for each rat.

**Statistical analysis**

All quantitative data (above-mentioned) was analyzed using one-way ANOVA or post hoc Student’s t test. A significance criterion of p < 0.05 was used for statistical analysis.

**Results**

**EA promoted NT-3 expression in the tissue of injured spinal cord**

Two weeks after spinal cord transection, 4 rats from each group were used for the detection of NT-3 expression in T6-L1 spinal cord segments by ELISA. It was found that NT-3 in the EA group and EA+MSCs group was more expressed than that in the control group and MSCs group (Table 1). The result indicates that NT-3 expression in the tissue of injured spinal cord may be increased by the use of EA treatment.

**Regeneration of nerve fibers**

The regeneration of nerve fibers was first detected by immunohistochemistry (IHC). Neurofilament (NF) positive nerve fibers could be observed at the injury site, rostral site and caudal site nearby injured spinal cord for all groups, except the injury site in the control group. In the EA or MSCs group, NF positive nerve fibers were short and tiny (Fig. 2B and C), while the nerve fibers in the EA+MSCs group were longer and branching (Fig. 2D). The number of NF positive fibers in the EA+MSCs group was more

**EA enhanced the differentiation of MSCs transplanted in injured spinal cord**

Thirty days after transplantation, many blue nuclear fluorescent labeled MSCs were found in the transplanted and nearby sites of spinal cord in the MSCs and EA+MSCs group. In the longitudinal sections of spinal cord containing injured site, nuclear fluorescent labeled MSCs were observed along the rostral–caudal axis of the spinal cord and these cells migrated in the spinal cord rostrally and caudally over 8 mm to the injury center 30 days after transplantation. Transplanted MSCs in both MSCs and EA+MSCs groups were able to differentiate into either neuron-like cells or oligodendrocyte-like cells. However, the number of neuron-like cells was significantly higher in the EA+MSCs group (Fig. 1B and C) than that in the MSCs group (Fig. 1A and C). Interestingly, the percentage of oligodendrocyte-like cells (MOSP positive) was also significantly higher in the EA+MSCs group (Fig. 1D and F) than that in the MSCs group (Fig. 1E and F).

**Table 1**

Comparisons of NT-3 content (ng/g, mean ± SD) in and around the injured site of spinal cord (T6-L1 segments).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Content of NT-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>1.52 ± 0.38</td>
</tr>
<tr>
<td>EA</td>
<td>4</td>
<td>2.93 ± 0.54</td>
</tr>
<tr>
<td>MSCs</td>
<td>4</td>
<td>1.72 ± 0.59*</td>
</tr>
<tr>
<td>EA+MSCs</td>
<td>4</td>
<td>3.83 ± 1.27*</td>
</tr>
</tbody>
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One-way ANOVA: *p < 0.05 vs. the control and EA groups.

**Fig. 1.** Distribution of microtubulin associated protein 2 (MAP2) positive cells (arrows) into the injured site of spinal cord in the MSCs group (A) and EA+MSCs group (B). (C) showed the comparison of percentages of MAP2 positive cells between MSCs group and EA+MSCs group, *p < 0.05 vs. the MSCs group. Distribution of mouse anti-oligodendrocyte special protein (MOSP) positive cells (arrows) into the injured site of spinal cord in the MSCs group (D) and EA+MSCs group (E). (F) showed the comparison of percentages of MOSP positive cells between MSCs group and EA+MSCs group, *p < 0.05 vs. the MSCs group. Immunohistochemistry staining; bar=20 μm.

than other groups (Fig. 3). The findings above prompted us to further define the nature of these regenerated nerve fibers. We did 5-HT and calcitonin gene-related peptide (CGRP) IHC staining. 5-HT is synthesized by a small group of neurons within the raphe nuclei of the brain stem and plays an important role in regulating voluntary movements, which attracts the most attention in this study. 5-HT positive nerve fibers could frequently be observed in the injury site and rostral site nearby injured spinal cord for all groups (Fig. 2E, F, G and H), but no 5-HT positive nerve fiber was observed in the caudal site nearby injured spinal cord in any group. These nerve fibers seemed especially fine and short in the control, EA and MSCs groups (Fig. 2E, F and G). However, these 5-HT nerve fibers showed obviously longer and string-of-beads in the EA+MSCs group (Fig. 2H). Nerve fiber counting showed that the number of 5-HT positive nerve fibers was the most in the EA+MSCs group (Fig. 4). These results suggested a group of nerve fibers could regenerate through the injury area by combination of EA and MSCs transplantation, an anatomic base for the functional recovery. CGRP is expressed in and released from a subset of sensory neurons. It can label local sensory axons. Our result showed there were more CGRP positive fibers in injury site in the EA+MSCs group (data not showed).

Functional recovery

Both hindlimbs of all rats in the experiment were paralyzed completely after spinal cord transection. Rats had to propel themselves with forelimbs, and their hindlimbs were dragged passively behind without joint extension. 14 days after operation the rats in the EA+MSCs group had an extensive movement of one joint and got a BBB score of $1.8 \pm 0.57$, which was significantly higher compared with other groups. This result was sustained until the accomplishment of experiment (Fig. 5). At 28th day, the
mean of BBB scores in the EA+MSCs group reached 6.3 ± 1.44 and especially one of the rats reached 8.5, which meant it could sweep with no weight support or occasionally with weight support, while rats in the control group only could have slight movement of one joint.

Discussion

Transplantation of cultured MSCs has been successfully used in repairing neural loss due to neurodegenerative diseases or traumatic damages. The actions of transplantation of cultured MSCs have also been intensively studied in spinal cord injury models. However, the application of MSC grafts for the treatment of damaged CNS is restricted by two major drawbacks: the poor survival rate and low differentiation rate into neurons. Although several neurotrophic factors such as NT-3 and brain-derived neurotrophic factor have been demonstrated to promote the survival of MSCs and induce MSCs to differentiate into neurons in vitro, the effect of these factors on MSCs in vivo is not clear. More importantly, these neurotrophic factors cannot maintain long-term effect on transplanted MSCs due to their relatively short half-life.

EA is a traditional method for the treatment of spinal cord injury. Treatment with EA has been shown to increase the expression of endogenous NGF on both the mRNA and protein level, and BDNF and NT-3 on the protein level in the lamina II of the spinal cord of cats subjected to dorsal rhizotomy (Wang et al., 2007). NT-3 is a member of neurotrophic factors, which play an important role in nervous system development, neuronal survival and differentiation, and neural repair (Yamamoto et al., 1996). Previously, we found that combination of EA on Governor Vessel and neural stem cells (NSCs) transplantation could significantly enhance NT-3 content (Chen et al., 2008). We have investigated whether EA treatment can enhance endogenous NT-3 expression and promote neuronal differentiation of MSCs. Similar to our previous findings on NSCs, we found that EA treatment increased neuronal differentiation of MSCs and expression of NT-3 in a closely parallel manner, suggesting that the effect of EA on neuronal differentiation of MSCs should be mediated by the elevation of endogenous NT-3. Elevated NT-3 expression can improve the microenvironment of injured spinal cord, thus enhance survival and differentiation of MSCs.

Combination of EA on Governor Vessel and NSCs transplantation has been shown to promote differentiation of NSCs into neuron-like cells in our previous study (Chen et al., 2008). Although NSCs are attractive candidates for cell therapy to treat nervous system diseases, its clinic application is limited due to the potential ethical problems. Unlike NSCs, MSCs do not have ethical issues and can also be induced into neuronal phenotypes in vitro (Zhang et al., 2006; Dezawa et al., 2004). Recently, a large number of studies have shown that MSCs transplantation is effective for treating neurological disorders and spinal cord injury (Phinney and Isakova, 2005). However, it is the bottleneck that the differentiation of MSCs into neurons is not satisfactory in vivo. Given that EA can enhance the neuronal differentiation of MSCs in vivo without any side effect, our findings have immediate clinical potential.

The differentiation of MSCs and regeneration of nerve fibers in injured spinal cord, and combination of EA and MSCs transplantation can improve partial functional recovery of rats with spinal cord transected completely.

In summary, the present study has shown that EA can promote the differentiation of MSCs and regeneration of nerve fibers in injured spinal cord, and combination of EA and MSCs transplantation can improve partial functional recovery of paralyzed hindlimbs. These effects might be mediated through induction of endogenous NT-3. The present study may offer a new avenue for the treatment of spinal cord injury as well as other neuronal degenerative diseases.

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