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Full Length Research Paper

Ultramicrostructure of tissue repair in the rat with spinal cord injury following transplantation of neural stem cells

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We observed ultramicrostructure of the contact or the connection between neural stem cells (NSCs) and host cells following transplantation of NSCs to treat the spinal cord injury (SCI). NSCs were labeled with Feride prior to transplantation. pEGFP-C2-Trk was transfected to transplanted NSCs under liposome medication. The EGFP-TrkB fusion protein was located with the colloidal gold technique. The contact or connection between transplanted NSCs and host cells were observed through distinguishing the cell body and the process according to whether Feridex or Colloidal gold particles existed under the transmission electron microscope. Feridex could label the cell body instead of the cell process, while the colloidal gold technique to locate the EGFP-TrkB fusion protein could label both the cell body and the cell process. There was body-body and body-process contact between transplanted NSCs and host cells, but no typical synaptic structures were observed. The cell body and the cell process of transplanted NSCs can be located by the colloidal gold technique, though technical improvement is required. There are no synaptic structures despite contact or connection between transplanted NSCs and host cells.

Key words: Neural stem cells, spinal cord injury, enhanced green fluorescent protein (EGFP), colloidal gold, Feridex.

INTRODUCTION

It has been shown that neural stem cells (NSCs) of different origins after transplantation promote neural recovery and motion of hind limbs in animals with the spinal cord injury (SCI) (Teng et al., 2006; Wang et al., 2008; Tang et al., 2007; Tarasenko et al., 2007). However, few report on the contact or connection between transplanted NSCs and host cells. The reason underlying this is that the current labeling techniques can only label the cell body but not the axon or the dendrite, being unable to identify the origin of the axon or the dendrite under the electron microscope. Thus the contact or connection among transplanted NSCs and host cells cannot be defined.

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Feridex (FE) is a specific super-paramagnetic iron oxide sanctioned by US Food and Drug Administration as a contrast agent in MRI. It can effectively label cells where poly-lysin accumulates (Cai et al., 2007; Nishida et al., 2006; Dai et al., 2007), and be observed under electron microscope. Protein-tyrosine kinase receptor B (TrkB) is a specific receptor for the brain-derived neurontrophic factor (BDNF) distributed in the axon, the dendrite, and the presynaptic membrane (Ogawa et al., 2002; Lykissas et al., 2007; Pereira and Chao, 2007; Huang et al., 2005; Dai et al., 2004). If exogenous TrkB gene with a specific marker is transfected into the transplanted cells and the marker is detectable under the electron microscope, the origin of the axons and the dendrite can be identified according to whether the marker is observed.

In the current research, the established pEGFP-C2-Trk plasmid (Dai et al., 2004) was transfected into transplanted NSCs under liposome medication. The transplanted NSCs

were labeled with FE prior to transplantation. The EGFP-TrkB fusion protein was located with the colloidal gold (CG) technique. The contact or connection between transplanted NSCs and host cells was observed through distinguishing the cell body and the process according to whether FE or CG particles existed under transmission electron microscope.

MATERIALS AND METHODS

Materials

The experimental animals were male, clean-grade syngeneic 9L/Fisher344 rats weighing 200-250 g. They were purchased from the Center of Animals, Sun Yat-Sen University (Guangzhou, China). They met the requirements for experiment use according to inspection and quarantine. Instruments included the CO_2 thermostatic culture box, the Eppendorf® Model 5810R Refrigerated Centrifuge (Eppendorf International, Hamburg, Germany), the Leica VT1000 S vibrating blade microtome (Leica Microsystems, Wetzlar, Germany), Leica DMIRE2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany), the Leica DMIRE2 inverted microscope (Leica Microsystems, Wetzlar, Germany), and the JEOM2100SX transmission electron microscope (JEOL, Tokyo, Japan). Main reagents included leukemia inhibitory factor, the basic fibroblast growth factor, the GFP immunohistochemistry kit, CG (Sigma-Aldrich, Saint Louis, Missouri, US), and FE (Advanced Magnetics, Pittsburgh, Philadelphia, USA). The CYTOKINE neural stem cells culturing solution was prepared by our laboratory (Patent No. ZL02134314.4).

Cell preparation

Isolation, culturing and detection of bone marrow-derived stromal cells (BMSCs) from femur of rat, neural stem cells (NSCs) induced from BMSCs applying NSCs medium (Patent number: ZL02134314.4) were referred to routine procedures (Ke et al., 2005; Alberti-Amador and Garcia-Miniet, 2003). Transfection of pEGFP-C2-TrkB plasmid and detection of target genes were performed by Huang et al., 2005. At 1 w before NSCs were transplanted, 25 μg/ml FE (final concentration) and 0.75 μg/ml PLL (final concentration) were mixed and shaken for 30 min to form the FE-PLL complex. After NSCs were labeled by FE-PLL, they were stained by Prussian blue and observed for FE under the transmission electron microscope.

Pretreated NSCs were cultured for 1 w and observed for the cell process under the microscope. Cells were digested by 0.125% pancreatin and 0.02% EDTA at pH 7.4 at room temperature for 10-15 min, and the digestion was terminated using media. The cell suspension was removed to the centrifuge tube by a burette for centrifugation at 1000 rmp for 5 min. The supernatants were discarded and the cells on the tube ground were collected to a mini-centrifuge tube. Culturing medium was added until 0.01 mL to prepare 107/ml solution to be stored in the ice box for future use.

Model establishment

After the rat was successfully anesthetized by peritoneal injection of chloral hydrate 4 ml/kg, it was fixed on the prone position on the operating table. A midline incision was conducted on the lumbar back aseptically. The skin and subcutaneous tissues were cut layer by layer until the spine. The vertebral lamina was severed until the bilateral articular processes to expose the T7-9 spinal segments. The spinal cord was severed ventrally using the microsurgical scissors at this site. After complete hemostasia, the muscle and the skin were sutured layer by layer. Complete hinder limb paralysis was achieved in the rats, suggesting successful model establishment (Schallert et al., 2000).

Postoperative observation and care

The cage was cleaned and each rat was raised individually. Penicillin 0.8 million units, twice/day, was administered intramuscularly to prevent infection. Urine and stool were induced through abdominal pressure 2 twice a day until the bladder function recovered. The change of the vital signs was observed.

Transplantation of NSCs

At 2 w after the SCI model was successfully established, well living rats were selected. The surgical route was the same as that in the SCI establishment surgery. After the endorachis was exposed, the cell suspension was injected into the injury site using the microinjector.

Tissue treatment

The rat was deeply anesthetized by peritoneal injection of 10% chloral hydrate at 4 w after NSCs transplantation. The heart was exposed, and the left ventricle-aorta was intubated to perfuse 200 ml normal saline. After the blood was drained, precooled 4% paraformldehyde 200 mL was perfused. The rat was fixed on the ice for 30 min, and the spinal cord at the injury site was then separated. The separated spinal cord was fixed in 4% paraformldehyde at 4℃ for 24 - 48 h. The rat brain was fixed in 0.01 mol/L PBS containing 20% sucrose. After the fixation solution was completely replaced by the sucrose, the tissue was sectioned at 50 μm.

Ultramicroscopic detection of the connection or contact among transplanted NSCs and host cells

The EGFP-TrkB fusion protein was located with the CG labeled GFP technique specified as follows. Gradient dehydration was performed after 4% paraformldehyde fixation. After the spinal cord tissue was saturated in the LR white resin at room temperature for 1 h, the tissue was irradiated by the ultraviolet lamp for 36 h. Then it was sectioned, blocked by 20% goat serum and 1% BSA for 20 min, and cultured with GFP monoclonal antibody (primary antibody, 1:200) for 2 h. The sections were recultured with CG labeled goat anti-rat IgG secondary antibody (1:200) for 1 h. They were re-stained using 1% uranyl acetate and lead citrate, respectively for 20min and 3min, and observed under the transmission electron microscope.

RESULTS

FE observations

NSCs were positively stained by Prussian blue contained small blue FE particles. The cells were mainly blue in the cell body (Figure 1A) with a positive staining rate of 90% (Figure 1B). Under transmission electron microscope,

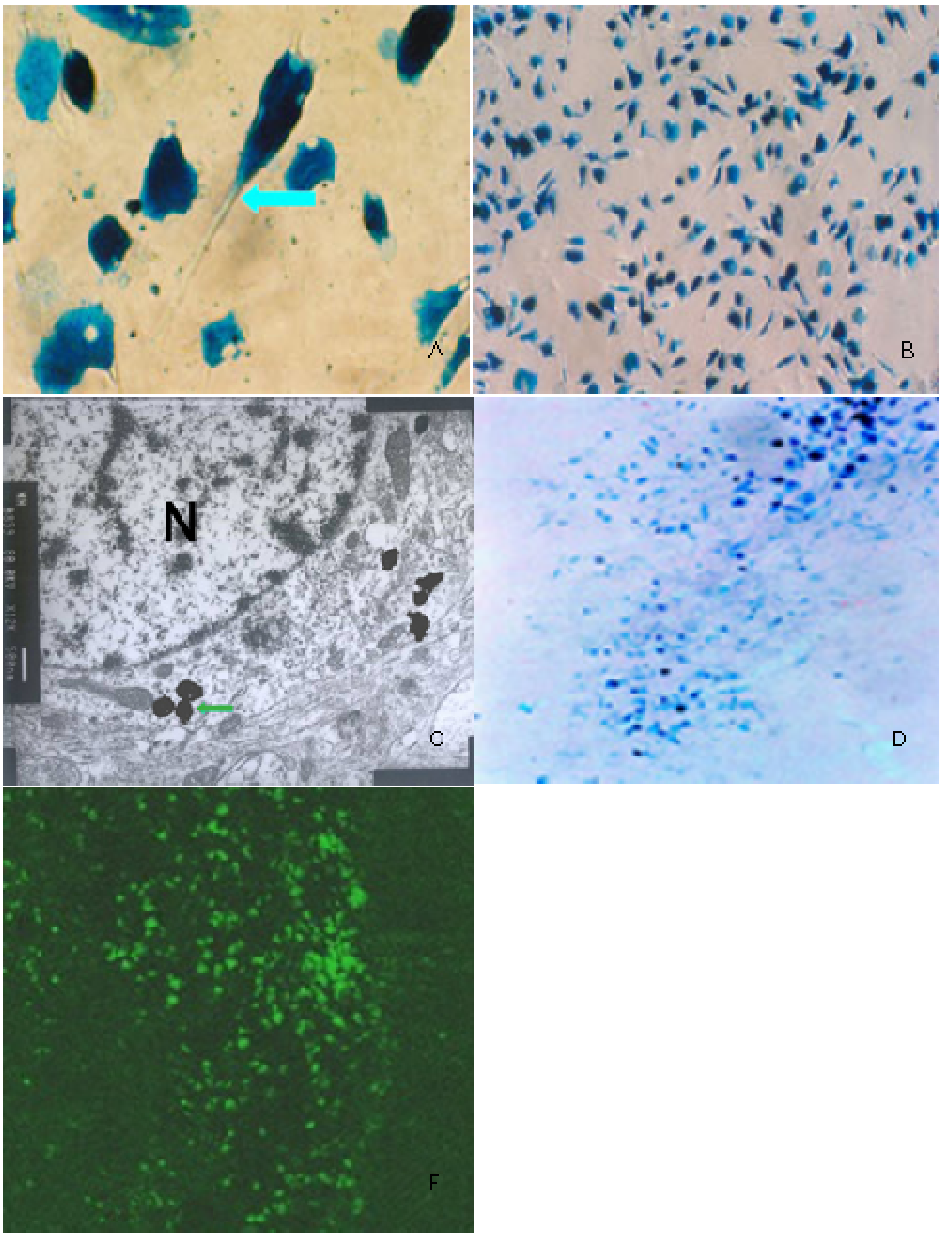

Figure 1. Observation under electron microscopy. A. FE particles mainly in the cell body and the axon hillock (green arrow) (inverted microscope: 3 d; 200 x); B Cells labeled with FE-PLL were blue (inverted microscope: 3d; 200x); C, Ultramicroscopic structure of NSCs labeled with FE-PLL (transmission electron microscope: N: nucleus; FE: containing vesicle (green arrow); scale: 0.5 μm; 3 d); D, Site of NSCs transplantation (inverted microscope: Prussian Blue staining; 4 d; 100 x); E, Transplanted NSCs were bright-green (fluorescence microscope: 4 w; FE containing vesicle (green arrow); scale: 0.5 μm; 3 d).

NSCs labeled with FE-PLL had many vesicles with a diameter of 0.8 -1.5 μm that contained FE particles. There were small FE particles in the vesicles, mainly in the cell body (Figure 1C).

Tissue sections

There were blue stained areas at the cell transplantation site following Prussian blue staining (Figure 1D). Near the injection route, transplanted cells were observed, and fewer were distributed far from the route.

Histological observations

Macroscopically, there were apparent tissue defects at the NSCs transplantation site. The spinal cord was thin,

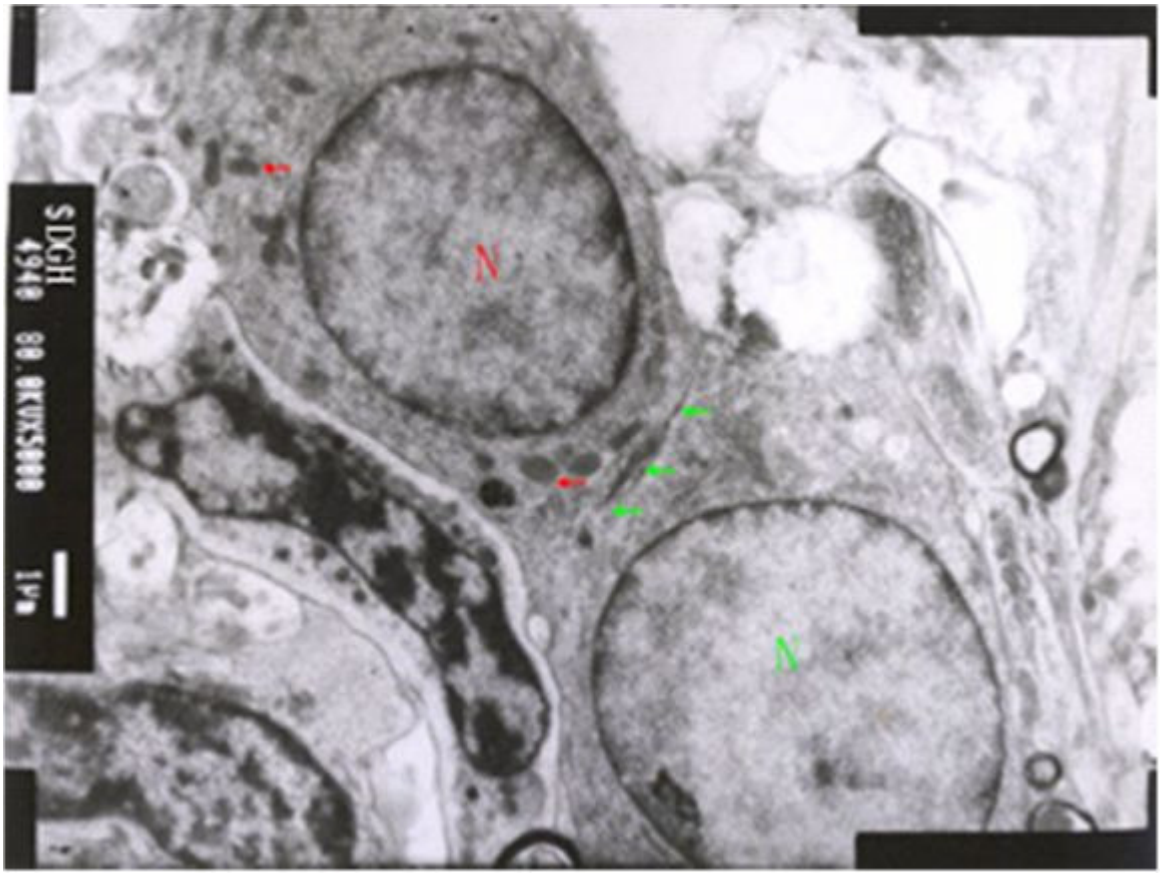

Figure 2. Body-body contact between transplanted NSCs (positively labeled with FE-PLL) and host cells (positively labeled with FE-PLL) (transmission electron microscope: N: nucleus labeled with FE-PLL; N: unlabeled nucleus; FE containing vesicle (red arrow); body-body contact (green arrow) between transplanted NSCs and host cells; 4 w; 200 x; scale: 1 μm).

and there incomplete pseudo envelope formed adhesion with adjacent tissues at different degrees. Under fluorescence microscope, the labeled cells at the transplantation site were bright-green (Figure 1E).

Ultramicroscopic observations of transplanted NSCs and host cells

Under the transmission electron microscope, the cytoplasm of transplanted NSCs had vesicle-like inclusion bodies that contained high-density nanometer FE particles. The inclusion bodies were mainly in the cell body, and some in the axon hillock. No inclusion bodies spread to the cell processes. There were no observations of FE particles in the axon or the dendrite in pathology examination. The contact formed between cells positively labeled by FE-PLL and non-labeled cells, including the body-body contact, while no typical synaptic structures existed (Figure 2). Under electron microscope, CG particles were distributed randomly in the membrane, and some cell processes possessed similarly high-density particles. The myelin sheaths from labeled cells were fewer than from normal axons or dendrites. The contact formed between the body of transplanted NSCs (positively labeled by FE-PLL) and host cell process (negatively labeled by CG) or the process of transplanted cells (positively labeled by CG) (Figures 3 and 4), as well as between the process of transplanted cells (positively labeled by CG) and the body of transplanted NSCs (positively labeled by FE-PLL) (Figure 5). However, no typical synaptic structures formed.

DISCUSSION

In recent years, as research on neuropathology and neural development advances, the traditional idea on non-regeneration of neural tissues is challenged. The neural tissues or cell transplantation are gradually successfully applied in SCI experiments, and especially NSCs are the focal point (Woodbury et al., 2000; Lee et al., 2007). Currently, application of NSCs of different origins to treat SCI is a concern in neural regeneration and recovery, and is demonstrated as effective in animal models. Currently, the efficacy of the treatment can only

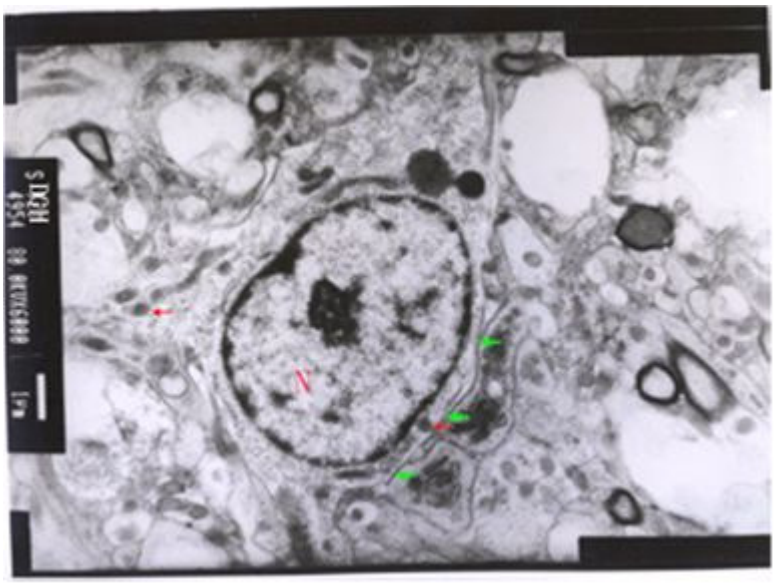

Figure 3. Contact between the body of the transplanted NSC (positively labeled with FE-PLL) and the process of the host cell (positively labeled with CG) (transmission electron microscope: N: nucleus labeled with FE-PLL; FE containing vesicle (red arrow); Body-process contact (green arrow); 4 w; 200 x; scale: 1 µm).

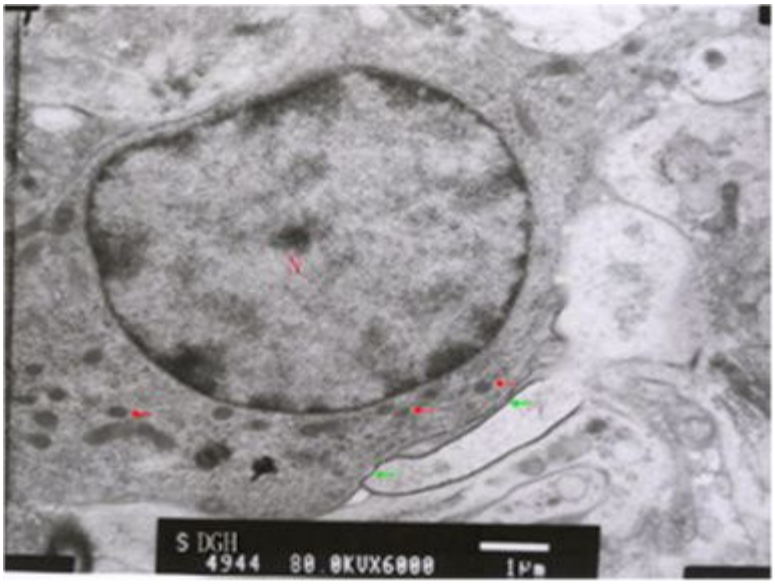

Figure 4. Contact between the body of the transplanted NSC (positively labeled with FE-PLL) and the process of the host cel (positively labeled with CG) (transmission electron microscope: N: nucleus labeled with FE-PLL; FE containing vesicle (red arrow); body-process contact (green arrow) between transplanted NSCs and host cells; 4 w; 200 x; scale: 1 µm).

be evaluated in respect to the survival of transplanted NSCs, the reconnection of neural fibers, and the functional improvement of hinder limbs. As current cell labeling techniques cannot label cell processes under the electron microscope, the efficacy of the treatment of SCI by NSCs is not evidenced by ultramicroscopic structures.

FE is widely recognized in labeling transplanted cells. Its average diameter is 80 nm, and the core ferric oxide is 20 nm in diameter. It forms the FE-PLL complex with PLL. As its particle is small, it may spread to the axon and the

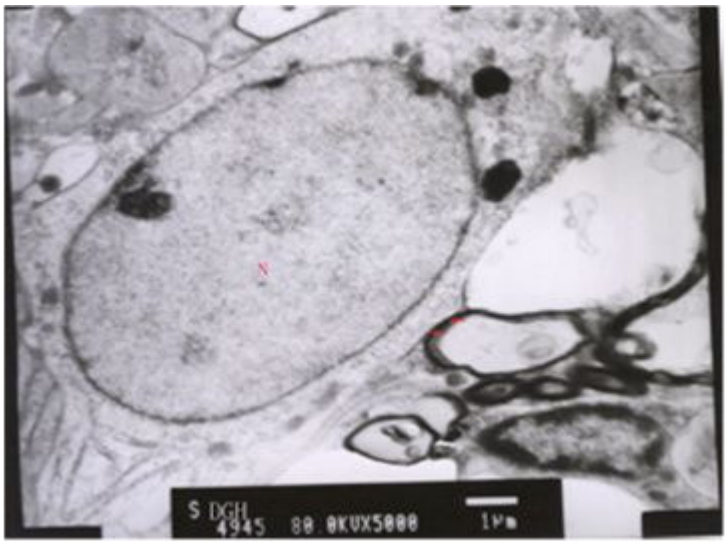

Figure 5. Contact between the process of the transplanted NSC (positively labeled with FE-PLL) and the body of the host cell (positively labeled with CG) (transmission electron microscope: N: host cell nucleus; FE containing vesicle (red arrow); body-process contact (green arrow); 4 w; 200 x; scale: 1 μm).

dendrite during transportation by the cytoplasm. Under the electron microscope, the origin of the axon or the dendrite may be identified through detection of FE particles. With the nanometer technology emerging, nanometer particles with unique physical or chemical features, quantum size effect, and surface effect are widely used in immune labeling, biological marker identification, and protein location (Lai et al., 2007; Kerman et al., 2007; Salih et al., 2007; Peng et al., 2007; Noh et al., 2007; Lu et al., 2006). The gold nanoparticles at the stable state in the solution are named GC. GC produces strong plasmon resonance, and appears claret-red at small diameters, having a bright prospect in immune labeling (Bokor et al., 2002; Kar et al., 2003; Barth et al., 2002). In the EGFP immunohistochemistry, CG can be applied to label the secondary antibody, and then the EGFP is labeled by CG sequentially. Similarly, if CG-labeled EGFP is to location the EGFP-TrkB protein, exogenous TrkB can be labeled by exogenous CG particles, and can be reflected under the transmission electron microscope. Using this technique, the cell body with EGFP-TrkB infusion protein as well as even the cell process can be distinguished.

The current research aimed to distinguish transplanted NSCs and host cells, as well as their axon and dendrite using the CG in location of EGFP-TrkB infusion protein and regarding whether FE particles existed. The ultramicroscopic structures between transplanted cells and host cells were also observed. Results showed that the electron microscope could only identify the cell body but not the cell process using the FE-PLL labeling technique. Through this technique, the contact between cell bodies can be detected, while the contact or connection between cell processes cannot. Using the CG protein location technique, the EGFP-TrkB infusion protein can be specifically located. Observations showed that the contact formed between the body of transplanted NSCs and host cells. However, no typical synaptic structures formed. Other experimental results indicated the SCI rat model nervous system function recovered at a degree after NSCs transplantation, which was different from our result in theory. We thought it was possibly because NSCs could secrete multi-neurotrophic factors, which nourish and conserve the transplanted cells and host rudimentary nervous system cells at injured region, and partial function of the rudimentary cells might recover. In addition, endogenous NSCs could play an important role in process of function recover. In the current research, we find ultramicroscopic structures of the contact between transplanted NSCs and host cells, but not synapse. Possible reasons may be that the animal model live short after NSCs transplantation, so CG cannot spread to the axon or the dendrite or that the CG location technique is not mature in use. A further study is required for improvement.

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