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RAPID REPORT

HUMAN NEURAL STEM CELL-DERIVED CHOLINERGIC NEURONS INNERVATE MUSCLE IN MOTONEURON DEFICIENT ADULT RATS

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Abstract—Motoneuron damage occurs in spinal cord injury and amyotrophic lateral sclerosis. Current advances offer hope that human embryonic stem cells [Science 282 (1998) 1145] or neural stem cells (NSC) [Exp Neurol 161 (2000) 67; Exp Neurol 158 (1999) 265; J Neurosci Methods 85 (1998) 141; Proc Natl Acad Sci USA 97 (2000) 14720; Exp Neurol 156 (1999) 156] may be donors to replace lost motoneurons. Previously, we developed a priming procedure that produced cholinergic cells that resemble motoneurons from human NSCs grafted into adult rat spinal cord [Nat Neurosci 5 (2002a) 1271]. However, effective replacement therapy will ultimately rely on successful connection of new motoneurons with their muscle targets. In this study, we examined the potential of human fetal NSC transplantation to replace lost motoneurons in an animal model of chronic motoneuron deficiency (newborn sciatic axotomy) [J Comp Neurol 224 (1984) 252; J Neurobiol 23 (1992) 1231]. We found, for the first time, that human neural stem cell-derived motoneurons send axons that pass through ventral root and sciatic nerve to form neuromuscular junctions with their peripheral muscle targets. Furthermore, this new cholinergic innervation correlates with partial improvement of motor function.

Key words: neuromuscular junction, axotomy, motoneuron disease, cell therapy.

To replace lost motoneurons, important issues are 1) to direct stem cells to differentiate into cholinergic motoneurons in the ventral horn of the spinal cord, 2) to have enough cues to allow new motoneurons to send axons through appropriate nerves to form synapses on muscle cells, and 3) to show concomitant improved motor function. Several groups generated cholinergic neurons from mouse embryonic stem (ES) cells *in vitro* by treating them with inductive factors such as retinoic acid and sonic hedgehog (Renoncourt et al., 1998; Wichterle et al., 2002; Barberi et al., 2003). However, these ES or neural stem cells (NSCs) do not form typical motoneurons in adult spinal cord presumably because of lack of sufficient environmental cues (Svendsen et al., 1997; Sheen et al., 1999; Shihabuddin et al., 2000; Cao et al., 2001; Fricker et al., 1999; Vroemen et al., 2003). For example, most human embryonic germ cells, when grafted into rats with diffuse motor neuron injury, remain undifferentiated and only a few become cholinergic, and these do not connect to muscle (Kerr et al., 2003). To obtain a higher proportion of stem cell-derived cholinergic neurons, we developed an *in vitro* priming technology that resulted in human fetal NSCs successfully differentiating into cholinergic neurons *in vitro*, and many acquire a cholinergic phenotype when grafted into intact adult spinal cord (Wu et al., 2002a). In this study, we show that these primed NSCs, when grafted into adult rat spinal cord with motoneuron degeneration, develop into cholinergic neurons that innervate peripheral muscle with concomitant improvement of motor function.

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Abbreviations: bFGF, basic fibroblast growth factor; ChAT, choline acetyltransferase; CTB, cholera toxin subunit B; ES, embryonic stem; LAMP2, lysosomal-associated membrane protein 2; NSC, neural stem cells; SFI, sciatic function index; WGA, wheat germ agglutinin.

EXPERIMENTAL PROCEDURES

Human NSCs and AAVegfp vectors

The K048 line of hNSCs (Svendsen et al., 1998) were propagated as neurospheres in growth medium supplemented with epidermal growth factor, basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (Wu et al., 2002a). For priming, 2-day neurospheres (passages 25–31) were treated with 20 ng/ml bFGF, 5 μg/ml heparin, 1 μg/ml laminin and 50 ng/ml mouse sonic hedgehog amino-terminal peptide (R&D Systems, Minneapolis, MN, USA) for 5 days and then cultured with 1× B27 (Invitrogen, Carlsbad, CA, USA) diluted in DMEM/F12 (Invitrogen) for 2 days *in vitro*. Four days before transplantation, primed cells were labeled by transduction with a recombinant adeno-associated viral AAVegfp (Wu et al., 2002b). For unprimed cell transplants, proliferating neurospheres were treated with AAVegfp directly and maintained in medium containing growth factors for 4 days.

Neonatal sciatic axotomy and transplantation

Left sciatic nerves of newborn Sprague–Dawley rats were crushed mid- thigh for 30 s using smooth-tipped forceps. Two months later, axotomized rats were divided into three groups to receive 1) primed cell grafts, 2) unprimed cell grafts or 3) vehicle. The spinal cord transplantation protocol was established according to the NIH guidelines for the care and use of laboratory animals, and approved by The University of Texas Medical Branch IACUC. Approximately 10^5 primed or unprimed hNSCs/2 μl or 2 μl vehicle were implanted into L_4 motoneuron deficient spinal ventral horns (ML: +0.7 mm; DV: −1.5 mm from dura). All animals were treated with the immunosuppressor NEORAL cyclosporine (Novartis Pharmaceuticals Co., East Hanover, NJ, USA), 100 μg/ml in drinking water 3 days before surgery and thereafter.

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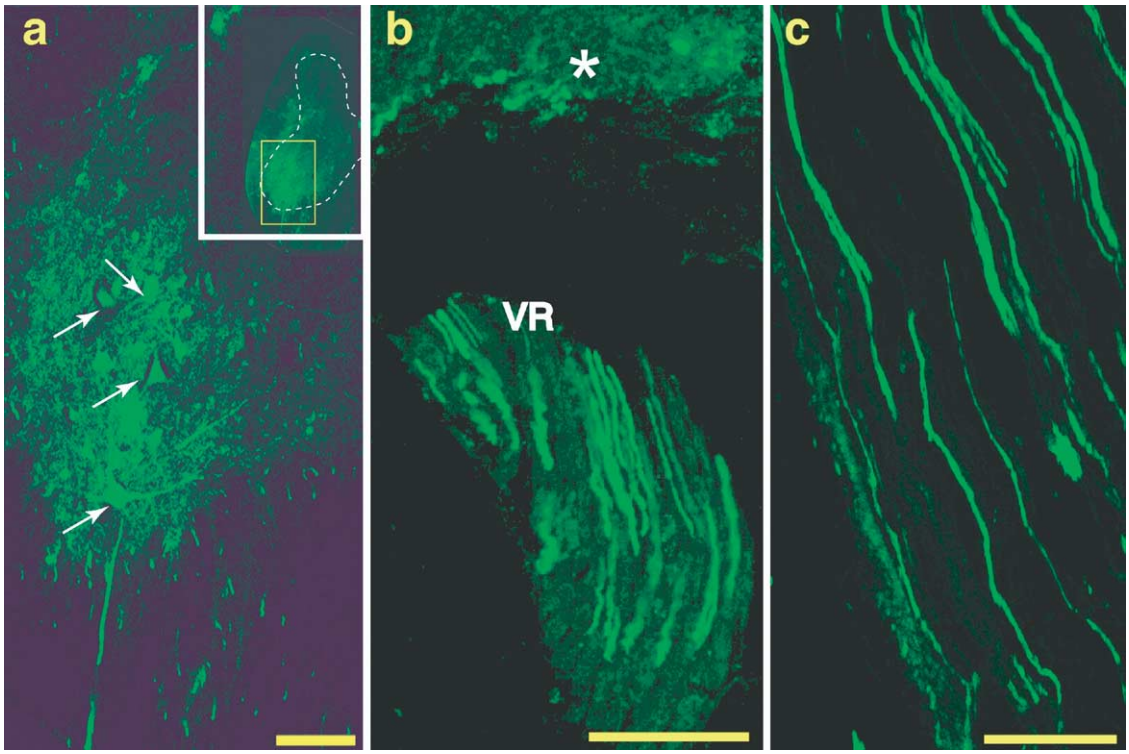

Fig. 1. Confocal images of primed human NSCs grafted in adult rats with motoneuron deficiency. (a) A view of the ventral horn with many GFP-labeled cells that resemble motoneurons; inset, a low power view showing the whole hemi-cord at the graft site. Grafted hNSCs send numerous large GFP fibers into the ventral root (VR) (b) and sciatic nerve at mid-thigh (c). * Ventral white matter of the spinal cord. Scale bars=200 μm.

Gait analysis

Motor function of rats with neonatal sciatic axotomy was evaluated before transplantation and then 1 or 3 months after lesion by a sciatic function index (SFI; de Medinaceli et al., 1982). SFI of −100% represents a complete lost of gait, SFI of 0±11% is a normal gait.

Retrograde tracing

Animals received injections of 7.5 μl of Alexa 594-wheat germ agglutinin (WGA; 10 μg/μl; Molecular Probes, Eugene, OR, USA) into the sciatic nerve, or 10 μl of Alexa 594-cholera toxin subunit B (CTB; 1 μg/μl; Molecular Probes) into the left medial and lateral gastrocnemius muscles. Three days post-WGA injection or 10 days after CTB administration, animals were perfused with 4% paraformaldehyde.

Immunohistochemistry

Five animals in each group were perfused with 4% paraformaldehyde 3 months after grafting. L4–L6 segments of spinal cords and ventral roots plus the sciatic nerves were cryo-sectioned at 45 μm. Primary antibodies included choline acetyltransferase (ChAT; Chemicon) at 1:100, Hb9 (Chemicon) at 1:100, human specific lysosomal-associated membrane protein 2 (LAMP2) at 1:1600 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA; Chen et al., 1985), human specific neurofilament 70 (Chemicon) at 1:200 and rat specific neurofilament 200 (Chemicon) at 1:200.

Neuromuscular junction labeling

Gastrocnemius muscles from two animals in each group were sectioned at approximately 500 μm with a razor blade. The muscle slices were incubated with 1 μg/ml of rhodamine-conjugated α-bungarotoxin (Molecular Probes) for 1 h at room temperature. Some of the slices were double-labeled with either GFP or human specific neurofilament 70.

Quantitative analyses

Sections were imaged confocally. Numbers of GFP-labeled hNSCs in the ventral horn were determined stereologically using a fractionator analysis (Coggeshall, 1992). Percentages of double- or triple-labeled cells or neuromuscular junctions with and without an attached GFP or human neurofilament axon were also determined. Motor function (SFI) was analyzed using repeated measures ANOVA (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Grafted human NSCs survived and differentiated in motoneuron deficient spinal cords

Neonatal sciatic axotomy resulted in an approximately 40% loss of myelinated axons in the L4 ventral root, which is equated to a 40% motoneuron loss, and an average SFI of −50%. Axotomized animals were screened by gait analysis just before implantation, and those with SFI values of −40 to −70% were randomly divided into three groups to receive primed or unprimed hNSC grafts or vehicle. Three months after grafting with primed cells, approximately 500 GFP-labeled cells (514±64) appeared in each transplanted ventral horn (Fig. 1a). Many of these cells had radiating dendrites and sent axons into ventral roots (Fig. 1b) and sciatic nerves (Fig. 1c). In contrast, most trans-