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hNT neurons delay onset of motor deficits in a model of amyotrophic lateral sclerosis

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ABSTRACT: Amyotrophic lateral sclerosis (ALS) is a degenerative motor neuron disease that manifests as a progressive muscular weakness leading to paralysis and death. Because of the diffuse nature of the motor neuron death, this disease is not considered a good candidate for treatment through neural transplantation. The purpose of this study was to show that transplantation of human neuron-like cells (hNT neurons) into the spinal cord of a transgenic ALS mouse model would improve motor deficits. The hNT neurons were transplanted bilaterally into L4-L5 spinal cord of the transgenic mice (~8 weeks of age), and the animals were evaluated on health and behavioral measures. The animals were perfused, and immunohistochemistry was performed to identify the transplanted cells. Transplantation of the hNT neurons into the spinal cord delayed the onset of motor behavioral symptoms. This was the first demonstration that even localized transplantation of neural cells directly into the parenchyma could improve motor function in an ALS model. Further study is needed to delineate the mechanism underlying these effects. This therapeutic approach has the potential to restore neural transmission, thereby improving quality of life for the ALS patient and possibly extend life expectancy. © 2002 Elsevier Science Inc.

KEY WORDS: Motor neuron, Degeneration, Transplantation, Human, Amyotrophic lateral sclerosis.

INTRODUCTION

The human neuron-like cells (hNT neurons) have been used as a substitute for fetal tissue in neural transplantation studies for the treatment of such neurodegenerative diseases and brain injury as Parkinson's disease [2], Huntington's disease [12], spinal cord injury [16,25], and stroke [4,22]. These cells are produced when the NTera2/D1 human teratocarcinoma cell-line is exposed to retinoic acid and induced to differentiate into postmitotic neuron-like cells [1]. Upon transplantation of the differentiated hNT cells into the striatum, the cells survive for at least a year *in situ* without reverting to a tumorigenic phenotype, and they develop the morphological characteristics of neurons [13]. They express a variety of neurotransmitters, including dopamine [17,27,28], acetylcholine [26], serotonin [6], and GABA [9] and have been shown to form synaptic contacts *in vitro* [9]. When the hNT cells were transplanted into the rat middle cerebral artery occlusion model of cerebral ischemia, the animals with this lesion recovered both motor and cognitive function in a dose-dependent fashion [4,22]. In the first clinical safety trial of hNT neurons in patients with basal ganglia stroke and fixed motor deficits, significant improvements occurred in the neurological scores of six patients that were also associated with increased fluorodeoxyglucose uptake as demonstrated by positron emission tomographic (PET) scan, indicating increased cellular metabolism on the side of implantation [14].

Because this is the first human neuronal cell-line to go through U.S. Food and Drug Admnistration (FDA)-regulated preclinical and clinical safety, toxicity, and tumorigenicity testing as well as appearing to be progenitor-like neurons that may undergo site-specific differentiation [23,29], it is of great interest to determine whether these cells are efficacious for cell transplantation into the spinal cord or brain of ALS patients as well as for other neurodegenerative diseases. Makoui et al. [16] recently showed that when these cells were implanted into the contused spinal cord partial recovery of motor evoked potentials and neurological scores was observed within 8 weeks of transplantation; neuritic outgrowth was observed throughout the contused region of the spine and extended 2 mm from the contused region. When these cells were examined over one year after transplantation in the rat spinal cord, the processes extended for even greater distances—in some cases, more than 2 cm within the white matter [10]. The neuronal features of these cells, their putative ability to reestablish fiber pathways, and their demonstrated efficacy for the treatment of other neural degenerative states suggest that the hNT neurons may be an excellent alternative source of neural tissue for transplantation to repair injured/dying motor neurons. The purpose of this study was to examine whether bilateral transplantation of the hNT cells into the ventral spinal cord at L4-L5 would delay the progression of hind limb weakness and paralysis that develops in the Cu/Zn SOD1 G93A transgenic mouse model of ALS.

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MATERIALS AND METHODS

Animals

Fourteen transgenic mice overexpressing human SOD1 carrying the $Gly_{93}\rightarrow Ala$ mutation [8] (G93A; obtained from Jackson Laboratories, Bar Harbor, MA, USA) were used in this study. These mice have been shown to develop progressive motor neuron disease similar in character to familial ALS. A second group of transgenic mice carrying the normal allele for the human SOD1 gene (hTg) were used as controls ($n = 4$). All mice were maintained on a 12:12 h dark:light cycle (lights on at 0600 h). Room temperature was 23°C. The food and water were available *ad libitum*. This study was reviewed by the University of South Florida before commencement and was conducted in accordance with the National Institutes of Health guidelines for animal treatment.

Cell Transplantation

At approximately 8 weeks of age, the transplant surgery was performed in two steps. First, burr holes were made bilaterally (0.5–0.7 mm from midline) through the lamina of the L1 vertebra, which corresponds to the L4-L5 segment of the spinal cord. We chose to place the transplants here because motor neurons in this region innervate the hindlimbs, which are affected first in this ALS model. The incision was sutured, and movement was monitored for a few days to ensure the spine had not been damaged.

On the day of transplant, the hNT neurons (Layton BioScience, Inc., Atherton, CA, USA) were thawed rapidly at 37°C and transferred to a 15-ml centrifuge tube containing Isolyte S, pH 7.4 (B. Braun, Bethlehem, PA, USA). The cells were centrifuged (1000 rpm/7 min), the supernatant was discarded, and the process was repeated. After the final wash, the viability of the cells was assessed using 0.4% trypan blue dye exclusion method, and cell concentration for each transplant was adjusted to 75,000 cells/μl. After this, mice from each of the groups were transplanted bilaterally with either hNT cells (75,000/μl/site) or media (Isolyte S, pH 7.4) through the previously prepared holes in the vertebra. Group assignment (hNT ($n = 8$) or media ($n = 6$)) was done randomly. Briefly, the cells were drawn into a 10-μl Hamilton syringe with a 31-gauge needle held stable in a stereotaxic frame. The needle was lowered through the prepared holes in the lamina into the ventral horn (1–1.3 mm below dura). The needle was allowed to remain in place for 2 min; after which, 1 μl of cells or media was injected into the transplant site over a 5-min period. After waiting an additional 5 min, the needle was slowly withdrawn and the incision was closed. All animals were immunosuppressed with cyclosporine (25 mg/kg per day orally) during the post-transplantation period.

Evaluation of Animal Status

Body weight was measured weekly. Behavioral tests were performed both pretransplant and then at 10, 13, and 16 weeks of age and then at weekly intervals until the animals either could not perform the tests or were euthanized. The first test was the extension reflex test. Mice were suspended by the tail above the cage bottom, and a score from 0 to 2 was assigned depending on the whether both hind limbs were fully extended (normal, 2), only one hind limb was extended or limbs were partially flexed (1), or fully flexed (0). The mice were also tested for motor coordination on the Rotarod apparatus (AccuScan Instruments, Columbus, OH, USA.) The animals were placed on a rotating axle (16 rpm), and the latency to the first fall and the number of falls in a 3-min test were measured.

Histology and Immunohistochemistry

After the last behavioral test, or when the disease progression reached the point of paralysis, the mice were sacrificed under deep chloral hydrate (10%) anesthesia and perfused transcardially with 4% paraformaldehyde in 0.1-M phosphate buffer (pH 7.2). The L4-L5 segments of the spinal cord were removed, postfixed, cryoprotected in 20% sucrose in 0.1-M phosphate buffer (pH 7.2) overnight, and 30-μm frozen sections cut. Selected sections from each animal were stained with cresyl violet for a histological examination of the transplant site. In addition, the transplanted neurons were immunolabeled with an antibody complex of human nuclear matrix antigen antibody (Calbiochem, San Diego, CA, USA, 1:200) and a monovalent goat anti-mouse Fab' fragment antibody conjugated to rhodamine (Jackson Immunoresearch, Westgrove, PA, USA, 1:200). Sections were mounted, coverslipped with Vectashield with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Burlingame, CA, USA), and examined under epifluorescence using an Olympus BX60 microscope (Melville, NY, USA).

Statistical Analysis

The behavioral data were analyzed using repeated measures analysis of variance with data being reported as means ± standard error of the mean (SEM). If tests of homogeneity of variance suggested that the populations were not normally distributed, the post hoc tests were performed using the Mann–Whitney U-test; otherwise, the Newman–Keuls test was performed.

A survival analysis using the Kaplan–Meier method was performed, comparing the G93A animals with and without hNT neuron transplants to determine if a difference in survival rate existed between the two groups. Further analysis was applied to the last datapoint at which animals in the G93A+ media group were still alive (16 weeks of age) to determine if the difference between groups was significant at this time and reported as a chi-square value (χ^2).

RESULTS

Beginning at 6 weeks of age, the G93A and transgenic control mice expressing the human normal Cu/Zn SOD1 gene were weighed and baseline behavioral measures of the extension reflex and motor coordination were obtained. At approximately 8 weeks of age, the hNT neurons and sham transplants were performed and behavior was examined for an additional 11 weeks.

Body weight was a good indicator of animal health, with weight gain slowly continuing until the development of behavioral symptoms. By 12 weeks of age, the G93A mice that received a sham, media transplant peaked at 119 ± 3.8% of their initial body weight and thereafter consistently lost weight. G93A mice with hNT transplants increased and maintained their body weight to a maximum of 123 ± 5.0% of initial body weight until 18 weeks of age when a dramatic reduction in body weight to just below baseline weight was observed (98 ± 8%). The transgenic controls (both hNT and media transplants), which did not differ from each other and were therefore considered as one group for statistical purposes, continued to gain weight throughout the study.

The extension reflex has previously been validated as a sensitive test for measuring the progression of motor neuron death in the transgenic ALS mouse model [3]. With repeated measures analysis of variance, significant differences were found between the groups ($F_{1,12} = 35.7$, $p < .0001$), and over time ($F_{9,108} = 25.9$, $p < .0001$) with a group by time interaction also noted ($F_{9,108} = 2.17$, $p < .02$). Only those animals in the G93A + hNT or media group were included in the analysis because of surgical losses in

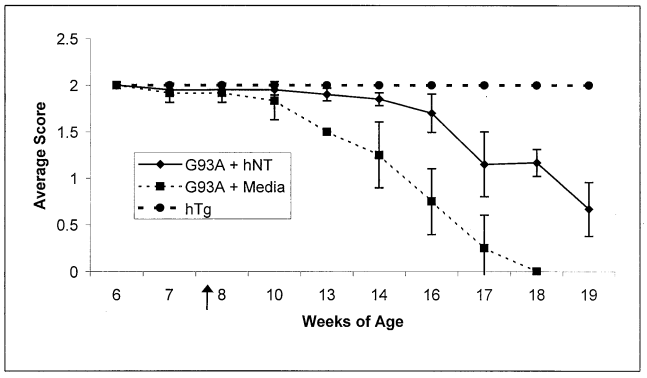

FIG. 1. The extension reflex test. By 13 weeks of age, media-injected G93A mice performed significantly worse than did both G93A mice with human neuron-like cells (hNT) transplants and human SOD1 gene (hTg) controls ($p < .02$). The performance of G93A mice with transplants was not significantly different from that of controls until 17 weeks of age ($p < .02$). Arrow indicates time of transplant.

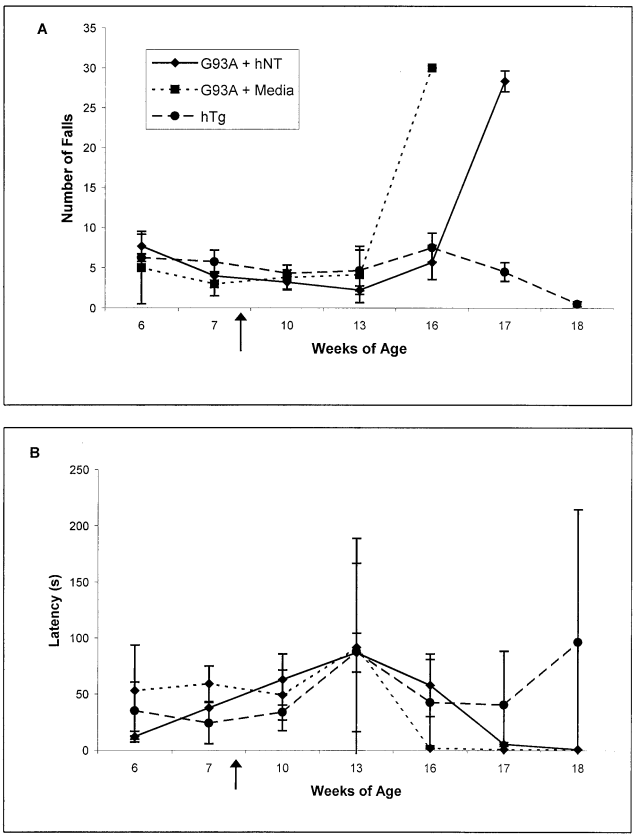

FIG. 2. Motor coordination. (A) The number of falls in a 3-min test were measured. A significant decrease occurred in performance over time for both media and hNT-transplanted animals. The deterioration occurred between 13 and 16 weeks of age for the media-injected animals, who fell 30 times during the test ($p < .03$). Further testing of these animals was discontinued at this time. The deterioration in performance occurred between 16 and 17 weeks in the hNT transplant G93A mice ($p < .04$). (B) Latency to first fall failed to distinguish motor performance of the experimental groups. Arrow indicates time of transplant.

the hTg groups; the behavioral data for these animals are included in the figures for comparison only. The media-injected G93A mice exhibited a significant deterioration of this reflex by 13 weeks of age compared with the G93A + hNT animals (see Fig. 1; $U = 2.5$, $p = .04$). It was not until 16 weeks of age that the hNT transplanted animals exhibited a similar decline in function, and even in the final test, these animals scored relatively higher than did media-injected controls during their final test.

Performance of the G93A mice was also impaired on the Rotorod test of motor coordination. Animals were placed on a 3-cm diameter axle that rotated at 16 rpm. Significant differences were found between the groups ($F_{1,12} = 15.6$, $p < .002$), over time ($F_{6,72} = 233.4$, $p < .0001$) with a group by time interaction ($F_{6,72} = 34.6$, $p < .001$) in the number of times that an animal fell in a 3-min test (see Fig. 2A). By 16 weeks of age, the media-injected G93A mice could no longer remain on the Rotorod, whereas animals transplanted with hNT neurons still performed at baseline levels ($p = .04$). For these latter mice, the deterioration in performance occurred between 16 and 17 weeks of age. Although latency to the first fall was also measured, this variable was not a sensitive indicator of performance in this model ($p < .32$; Fig. 2B).

Transplantation of hNT neurons only enhanced the survival of the recipient mice as determined in a survival analysis marginally ($p = 0.12$). At 16 weeks of age, significantly more G93A mice with hNT transplants were alive compared with the media-injected mice ($\chi_1 = 9.2$, $p < .002$, see Fig. 3). The last G93 + hNT neuron-transplanted mouse lived almost 2 weeks longer than did the media-injected controls and was euthanized before the final stages of the degeneration.

In addition, we could identify the transplant sites and surviving hNT neurons using an epifluorescence immunoassay (Figs. 4 and 5).

DISCUSSION

The origin of ALS is not yet understood, but hypotheses include excitotoxicity, mutation of the superoxide dismutase gene, production of auto-antibodies to calcium channels, and neurofilament accumulation [11]. Treatments are directed at either easing symptoms or addressing these hypothesized causes, as in the pharmacological treatments of administering glutamatergic antagonists [7] or trophic factors [18,21,29]. In mice models of motor neuron disease, these latter have been shown to slow the development of motor symptoms and extend survival, but only the glutamate antagonist riluzole has shown any clinical efficacy to date [5], and that has been modest. As yet, no treatments are aimed at

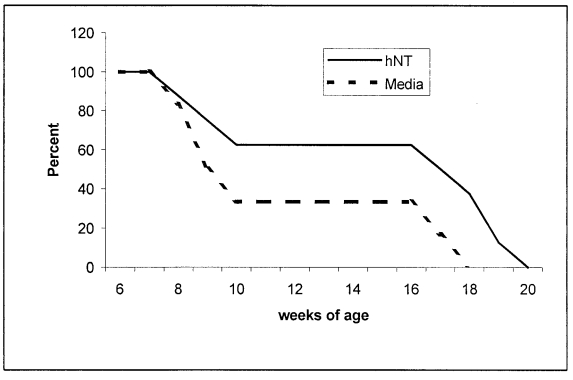

FIG. 3. Survival of G93A transgenic mice with transplanted hNT neurons or media injection alone. Transplants were performed at approximately 8 weeks of age.

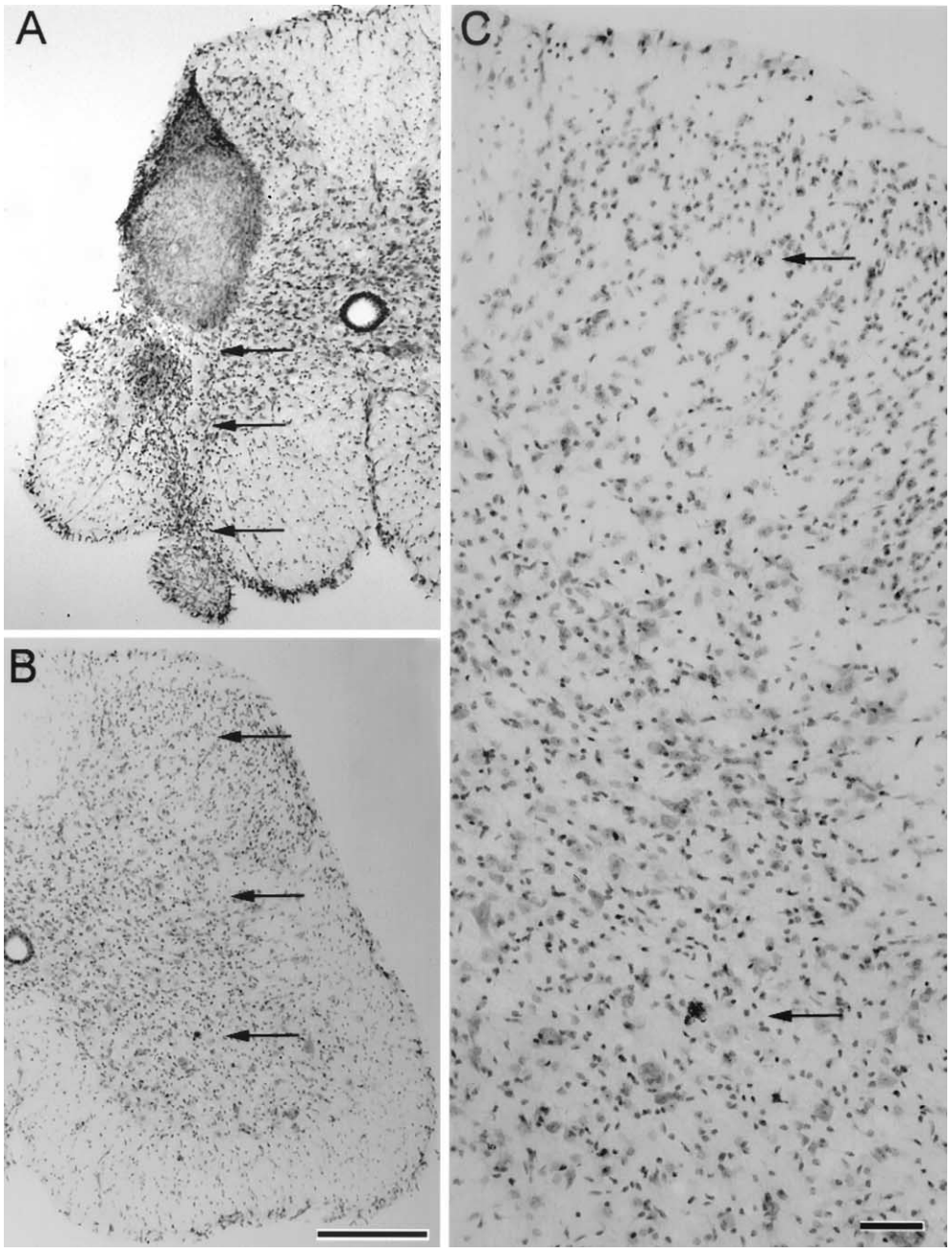

FIG. 4. Representative photomicrographs of Nissl staining of the lumbar spinal cord. (A) A graft of hNT neurons in the ventral horn in lumbar spinal cord. This graft extended past the boundaries of the ventral horn into the adjacent lateral white matter regions as well as more dorsally. (B) A Nissl section from a G93A animal that was injected with media alone. (C) A higher magnification view of the injection site shown in (B). Arrows indicate the transplant in (A) and the injection tract in (B) and (C). Scale bar = 200 μm in (A) and (B) and 25 μm in (C).

blocking the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, which has been specifically implicated in motor neuron death [24]. Although the other treatments also show efficacy, cellular therapy may represent a more effective form of treatment.

One mechanism by which the hNT neurons may have enhanced survival and motor performance could be through replacement of the dying motor neurons and reestablishing the neuronal circuitry in the spinal cord. Even though these cells have been shown to develop extensive neuritic outgrowth in both normal [10] and contusion rodent models [16], the quick progression of the neurodegeneration coupled with the relatively slow development of these [13] and other transplanted human cells [19,20] may have prevented the hNT cells from demonstrating their full capabilities. This study was designed to establish the efficacy of neural transplantation for the treatment of ALS and therefore did not address

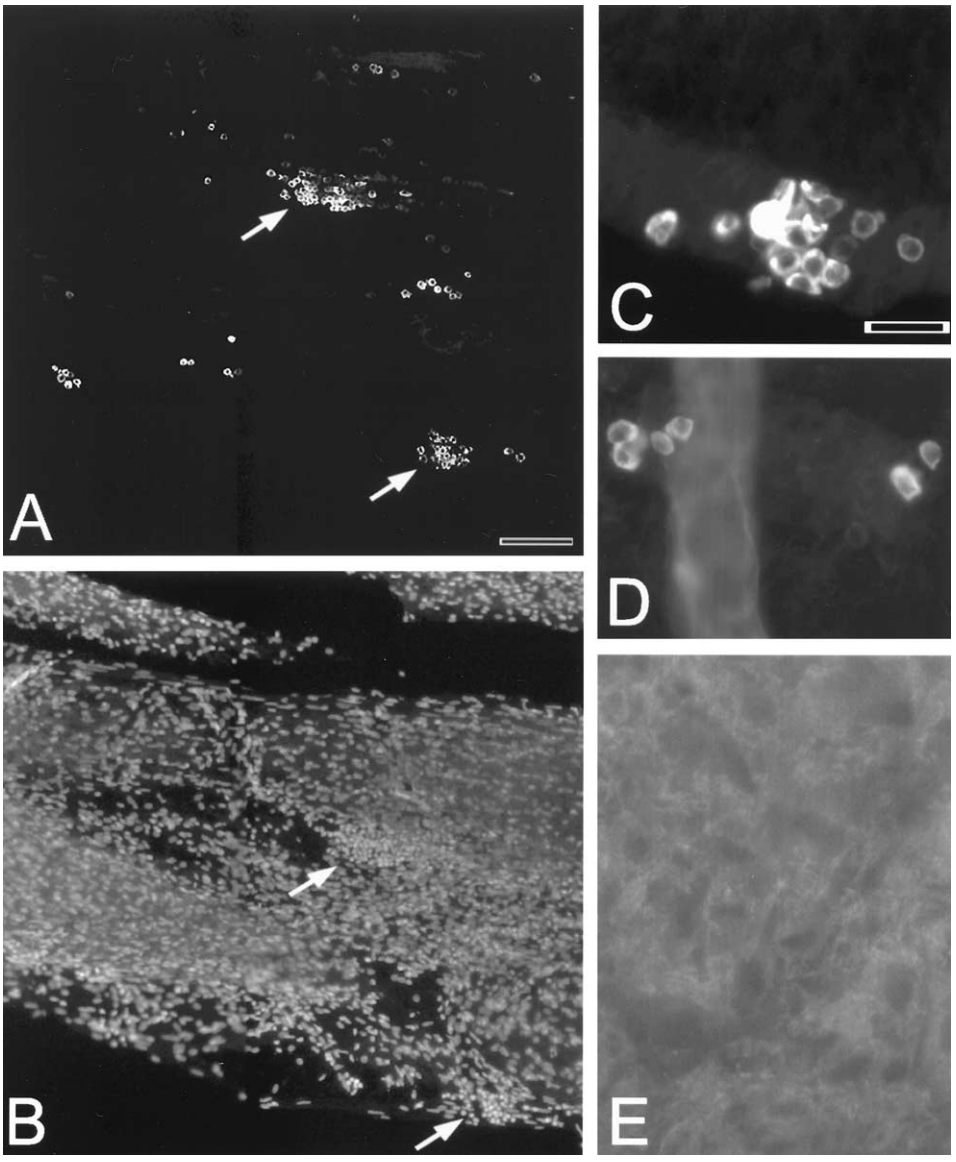

FIG. 5. Graft survival in lumbar spinal cord. (A) The spinal cord was sectioned in the dorsal to ventral direction, and cells were labeled with an antibody complex of the human nuclear matrix antigen antibody and a monovalent goat anti-mouse Fab' fragment antibody conjugated to rhodamine. Surviving hNT neurons were observed in the ventral horn. (B) An alternative view of the same section shows DAPI-labeled cells in the spinal cord. Arrows in (A) and (B) show the same group of transplanted cells. (C) A photomicrograph of NuMA-labeled cells in a cross-section of the lumbar spinal cord from another G93A + hNT animal. The hNT cells in this image are located in lateral, ventral spinal cord. (D) Labeled NuMA-positive cells located more medially in ventral spinal cord. (E) No NuMA-positive cells were ever found in sections from media-injected animals. Scale bar = 100 μm in (A) and (B). Scale bar = 25 μm in (C)–(E).

whether the number of cells necessary to produce an optimal effect were transplanted, whether specific regions existed in which the transplant would be more effective than in others, or whether transplants at multiple levels produce better results. These issues are currently under investigation, and with optimization of the transplant, increased efficacy may be demonstrated.

Another means by which the hNT neurons may have improved motor function is through a trophic mechanism providing support to the host motor neurons, prolonging the life of the afflicted neurons or stimulating the function of those neurons that are still surviving. These cells have been shown to express glial-derived neurotrophic factor [15]. Further study will delineate the mechanism by which these cells have produced their effects and define the optimal transplantation strategy.

In conclusion, even though the diffuse motor neuron degeneration of ALS provides a daunting task for repairing the neural circuitry through neural transplantation, we have shown that grafting hNT neurons directly into the ventral spinal cord in a transgenic mouse model of ALS can reduce motor symptoms and while not statistically significant, may extend the survival of these mice.

These results are as good as those demonstrated for pharmacological treatments that have been tested in this animal model and suggest that repairing the neural circuitry at a rudimentary level or providing direct trophic support could be an effective therapeutic approach. These cell-line–derived cells that are manufactured under Good Manufacturing Practices and are the only FDA-approved cells undergoing clinical testing may prove to be beneficial for improving the quality of life for ALS patients and possibly extending their life expectancy.

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