Intraspinal Transplantation of hNT Neurons in the Lesioned Adult Rat Spinal Cord

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ABSTRACT: ***Background:*** The role of neural transplantation as a restorative strategy for spinal cord injury continues to be intensely investigated. Ideally, the tissue source for transplantation must be readily available, free of disease and able to survive and mature following implantation into the adverse environment created by the injury. We have studied the use of a commercially available cell line of cultured human neurons (hNT neurons) as a tissue source for neural transplantation in spinal cord injury. ***Methods:*** Following a left lateral thoracic hemisection, 54 immunosuppressed, female Wistar rats were randomly allocated into different treatment groups; hemisection only or hemisection and hNT cell transplantation (via a bridge, double or triple graft). Grafting occurred three days after spinal cord injury. After thirteen weeks the animals were sacrificed and tissue sections were stained with human neuron specific enolase and human specific neural cell adhesion molecule. ***Results:*** Immunohistochemical evidence of graft survival was displayed in 66.7% of the surviving, grafted animals. Fibre outgrowth, greatest in the bridge and triple grafts, was observed in both rostral and caudal directions essentially bridging the lesion. Double grafts were smaller, displaying less fibre outgrowth, which did not cross the lesion. Long fibre outgrowth was evident up to 2 cm from the graft as assessed by tracing and immunohistochemical studies. ***Conclusions:*** Bridge and triple grafts displayed greater growth and enabled the hNT graft to essentially bridge the lesion. This suggests that hNT neurons have the potential to structurally reconnect the proximal and distal spinal cord across the region of injury.

RÉSUMÉ: Transplantation intraspinale de neurones hNT dans la moelle épinière de rats adultes ayant subi une lésion. ***Introduction:*** Le rôle de la transplantation neurale comme stratégie de restauration des lésions de la moelle épinière continue de faire l'objet de recherches intensives. Idéalement, la source de tissus pour la transplantation doit être facilement disponible, indemne de toute maladie, et capable de survivre et de se développer après implantation dans un environnement hostile à l'endroit de la lésion. Nous avons étudié l'utilisation d'une lignée cellulaire de neurones humains en culture (neurones hNT), disponible commercialement, comme source de tissus pour la transplantation neurale dans les traumatismes de la moelle épinière. ***Méthodes:*** Cinquante-quatre rats Wistar femelles ayant subi une hémisection latérale gauche de la moelle thoracique ont été répartis au hasard en deux groupes de traitement: l'hémisection seule ou l'hémisection avec transplantation de cellules hNT par greffe en pont, greffe double ou triple. La greffe a été effectuée trois jours après la lésion de la moelle. Après treize semaines, les animaux ont été sacrifiés et des coupes de tissus ont été colorées au moyen de l'énolase neurospécifique humaine et de la molécule d'adhésion neurospécifique humaine. ***Résultats:*** On a observé des manifestations immunohistochimiques de survie du greffon chez 66.7% des animaux greffés survivants. Des excroissances, plus importantes dans les greffons en pont et dans les greffons triples, ont été observées en direction rostrale et caudale unissant les deux côtés de la lésion. Les greffons doubles étaient plus petits, avaient moins d'excroissances et ces excroissances ne franchissaient pas la lésion. Des études de traçage et d'immunohistochimie ont montré la présence de longues excroissances se projetant jusqu'à deux cm du greffon. ***Conclusions:*** Les greffons en pont et les greffons triples avaient une meilleure croissance et permettaient au greffon hNT de d'enjamber la lésion. Cette observation suggère que les neurones hNT ont le potentiel de rétablir la connexion entre la moelle proximale et distale au niveau de la région lésée.

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Neural transplantation as a restorative strategy for spinal cord injury (SCI) has been aggressively investigated for the past two decades.[1] Tissue sources for transplantation have included peripheral nerve,[2-4] dorsal root ganglia,[5] Schwann cells,[6-8] adrenal tissue[9] and fetal spinal cord tissue, derived from rat[10-13] and human.[14,15] The studies utilizing fetal tissue have shown regeneration and fibre outgrowth across the lesion and into the distal cord.[16-18] Fetal spinal cord grafts have been shown to replace lesioned neurons[19,20] and to rescue damaged neurons through the provision of trophic factors.[21-23] There is also evidence that fetal transplants serve as inhibitors of gliosis, which is a major impediment for axonal regeneration.[12,24,25] However, the use of fetal tissue has serious disadvantages; which include limited availability, potential for transmission of infectious agents and the ethical issues related to abortion. A

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potential alternative for neural transplantation in SCI are immortal cell lines,[26-28] which could be used as a readily available tissue source in transplantation procedures[29] and therefore circumvent the disadvantages of fetal tissue.

One such cell line is the NT2N line (commercially referred to as hNT cells), initially derived from a human teratocarcinoma.[30] Following *in vitro* treatment with retinoic acid, the progeny of this cell line is restricted to a neuronal phenotype yielding postmitotic neuronal cells. These cells have been shown to retain their neuro-chemical, -physiological and -morphological properties.[31-38] They have also been successfully transplanted into the brains of immunodeficient mice and been shown to survive for up to one year with no evidence of tumour formation, graft rejection, significant apoptosis or necrosis.[39-41] Furthermore, the NT2N transplanted cells demonstrate the capability to integrate into the surrounding host neural tissue by extending dendritic and axonal processes.[42] hNT cells have also been implanted into experimental models of neurological conditions, including: ischemia,[43-45] Huntington's disease,[46] Parkinson's disease[47] and traumatic brain injury.[48] These studies have shown survival and integration of the hNT grafts and, in the case of the ischemic models, promoted functional recovery.[43-45] As a result of these studies, a phase 1 human trial of transplantation of hNT cells in stroke patients has been reported. In this study there were apparently no deleterious effects of transplantation and there was some suggestion of functional effects in six of 12 patients.[49]

Recently, it has been reported that following transplantation into the spinal cord of intact mice, hNT cells can survive up to 15 months and integrate into the surrounding tissue, projecting axons to distances greater than 2 cm.[50] Furthermore, a recent study has suggested that transplantation of hNT cells leads to enhanced electrophysiological recovery following a contusion model of SCI.[51] These results suggest that hNT cells may be a potential source for neural transplantation in SCI. The objective of the present study was to investigate the ability of hNT neurons to survive in an acute SCI model. In this investigation, hNT cells were grafted in the thoracic hemisection rat model of SCI. A multitarget strategy was used to determine the ability of hNT cells to survive within the lesion as well as the proximal and distal spinal cord segments surrounding the lesioned area.

Materials and Methods

Spinal cord lesions and immunosuppression

The following protocol was reviewed and approved by the local University Animal Care Committee and all procedures performed throughout this investigation were conducted in accordance with the guidelines of the Canadian Council of Animal Care and the University Council on Laboratory Animals. Female Wistar rats (200-225 g; Charles River, St Constant, PQ, Canada) were anaesthetized using a mixture (2.0 mL/kg) of 25% ketamine hydrochloride (Ketalean, MTC Pharmaceuticals, Cambridge, Ontario), 6% xylazine (Rompun, Miles Canada, Etobicoke, Ontario), 2.5% acepromazine maleate (Wyeth-Ayerst Canada, Montreal, Quebec) in 0.9% saline. Betadine surgical scrub (Purdue Frederick Inc., 7.5% povidone-iodine) was used for sterile preparation of the surgical field after shaving. A dissecting microscope was used to facilitate surgical exposure and laminectomies of T8-T10 were performed. Hemostasis and obliteration of epidural veins in the region of durotomy was achieved with low temperature cautery (Solan, Jacksonville, Florida). Following a midline durotomy, microscissors were used to perform a left-sided hemisection of the exposed spinal cord from the posterior median sulcus laterally. A sharp probe was then used to ensure that a complete lateral hemisection was achieved. Hemostasis was obtained, the dura closed with a 10-0 monofilament suture, the wound irrigated and the muscle and skin closed in layers with 000 polyglactin sutures (Ethicon, Sommerville, New Jersey). Following lesioning the animals were randomly placed into one of the treatment groups; control, bridge graft, double graft, triple graft or killed cell triple graft. To decrease the possibility of graft rejection Cyclosporin A (Novartis, Dorval, Quebec) was employed to immunosuppress the animals. All animals, regardless of treatment group, began receiving daily intraperitoneal injections of Cyclosporin A (10 mg/kg) at the time of their hemisection. The immunosuppression was then continued for the duration of the experiment. While recovering from anaesthesia, the animals were kept warm by placing them on a heating pad turned to a low setting. All animals received 5 mL 0.9% saline subcutaneously. Analgesia was provided with 0.6% bupremorphine (1.25 mL/kg s.c.; Reckitt and Colman Products, Hull, England) in 0.9% saline. Animals were housed singly and provided with softened food and fresh hay. Tubes and wooden blocks were made available in each cage to serve as a distraction to reduce autotomy. All animals had twice daily bladder palpations and expression of urine until their normal bladder reflexes returned (10-14 days). When signs of urinary tract infections were identified, the animals were treated with 10% enrofloxacin (1.0 mL/kg s.c. twice daily for seven days; Baytril, Bayer Inc., Etobicoke, Ontario). Daily weights were obtained for the first two weeks postoperatively and then twice weekly. Any animal that experienced a weight loss greater than 20% of its' preoperative weight or exhibiting evidence of significant autotomy, greater than one digit, or experiencing uncontrolled infection was sacrificed.

hNT Cell Suspensions

Frozen hNT cells, provided by Layton Bioscience (Sunnyvale, California), were stored at -80ºC until required. Preparation of hNT neurons prior to freezing has been previously described.[32,36,37,39] At the time of transplantation, the cells were thawed rapidly in a water bath at 37ºC with no mixing during the thaw. The cells were transferred gently into a 10 mL solution of DMEM/0.05% DNase (Sigma, Oakville, Ontario), and then centrifuged for five minutes. The supernatant was aspirated and the pellet resuspended in 1 mL of DMEM/0.05% DNase. This suspension was centrifuged in a fixed angle tabletop centrifuge (Microfuge 12, Fisher Scientific) at room temperature, 1000 rpm (= 100 g) x 5 min, no brake applied. The supernatant was aspirated, and resuspended in 1 mL DMEM/0.05% DNase. For the Trypan Blue exclusion viability assay, 2 μL of the suspension was added to 49 μL DMEM/0.05% DNase + 49 μL 0.4% Trypan Blue (Sigma, Oakville, Ontario). Finally, the concentration was calculated and volume adjusted to yield a final concentration of 160 x 10^3 hNT cells per μL. To produce killed hNT cells, the

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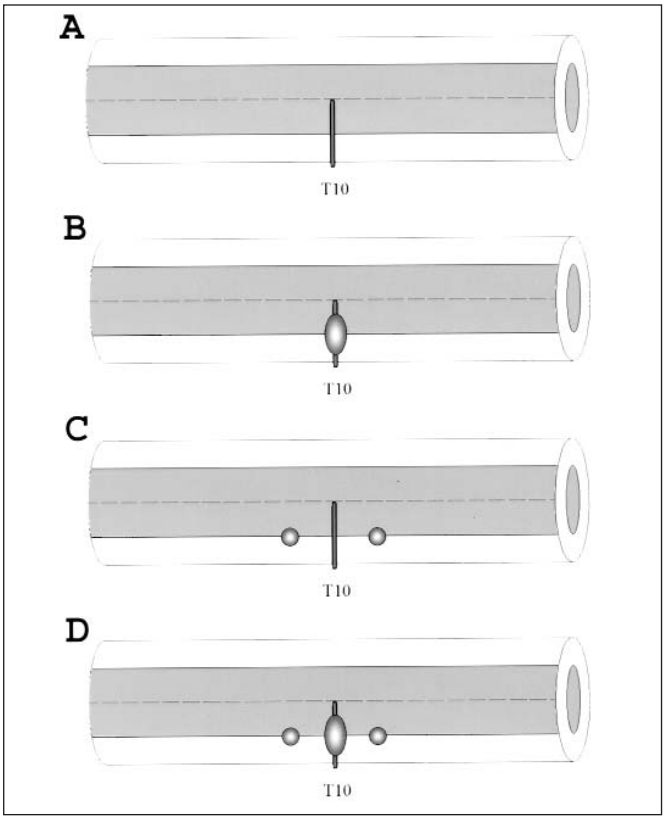

Figure 1: *Diagrammatic representation of multiple grafting strategies.* ***A:*** *left thoracic hemisection only.* ***B:*** *left thoracic hemisection and bridge graft.* ***C:*** *left thoracic hemisection and double graft.* ***D:*** *left thoracic hemisection and triple graft.*

vials were exposed to three freeze (-80ºC) and thaw (37ºC) cycles prior to transfer into the DMEM/0.05% DNase solution. The remainder of the preparation was as described above.

Transplantation procedure

On the third day after lesioning, the animals to be grafted were re-anaesthetized and the area of the lesion exposed. The transplantation sites are illustrated in Figure 1. After identifying the desired locations for transplantation, a small dural opening was made. A glass microcapillary connected to a Hamilton syringe was used to inject the cell suspensions into the left-hand side of the spinal cord. Each injection was made at a rate of 1 μL/minute and the needle was left in place for five minutes to aid in diffusion. Animals receiving bridge grafts had 160 x 10^3 hNT cells injected into the region of the hemisection. Animals receiving double grafts had 80 x 10^3 hNT cells injected 1 mm rostral as well as caudal to the region of the hemisection. The dosage of hNT cells for implantation was based on previous studies of hNT transplantation in the brain and spinal cord.[45,50] Injections were made at a depth of 0.75 mm and 0.05 mm lateral to the midline. The animals which received a combination of bridge and double grafts (triple graft group) had both of the above described injections performed. Animals receiving killed hNT grafts underwent the triple graft procedure.

Retrograde labelling and tissue preparation

A double retrograde labelling technique was used to help differentiate between unlesioned pathways and those re-established by the grafts. At the time of the initial surgery, following closure of the dura, 0.6-0.8 μL of a 2% solution of Fast Blue (Sigma, Oakville, Ontario) was injected bilaterally into the spinal cord 1 cm caudal to the lesion, utilizing the initial laminectomies. Twelve weeks post-transplantation the animals were anaesthetized as previously described above. A laminectomy was performed caudal to the site of the initial surgery and 1 μL of a 2% solution of Fluoro-Ruby (Molecular Probes, Eugene, Oregon) was injected bilaterally 2 cm caudal to the lesion site. One week later the animals were sacrificed with an overdose of anaesthetic and perfused transcardially with 150 mL of 0.1 M phosphate buffer, 4ºC, pH 7.4, followed by 300 mL of 4% paraformaldehyde, 4ºC. The brain and spinal cord were dissected and post-fixed overnight in 4% paraformaldehyde at 4ºC followed by cryoprotection overnight in 30% sucrose in 0.1 M phosphate buffer at 4ºC. The brains were cut into 30 μm coronal sections on a freezing microtome at -37ºC and stored in Millonig's solution (16.88 mg/mL $NaH_2PO_4H_2O$, 3.86 mg/mL NaOH, 0.006% NaAzide in distilled water). The spinal cords were embedded in hardened gelatin and then placed in 4% paraformaldehyde, followed by 30% sucrose at 4ºC prior to sectioning. Spinal cord specimens were cut longitudinally into 30 μm sections on a freezing microtome at -37ºC and stored in Millonig's solution at 4ºC.

Lesion assessment

Cresyl violet staining was used to identify the lesion sites and the extent of the hemisection. Sections to be stained were mounted on gelatin-coated slides and air dried overnight. The slides were then placed in 0.1 M phosphate buffered saline for five minutes, dipped in distilled water and then 50% followed by 70% ethanol, four times each. The slides were then placed for five minutes in 95% ethanol containing 10 drops of glacial acetic acid/200 mL of ethanol. Again the slides were dipped four times each in 70% and 50% ethanol then distilled water before placement in 0.1% cresyl violet acetate in distilled water. After eight minutes the slides were dipped twice in distilled water and then four times in each 50% and 70% ethanol and then placed in 95% ethanol with acetic acid for one to two minutes. Further dehydration was achieved by passage through ethanol solutions, including twice in both 95% and 100% ethanol for five minutes. Prior to cover-slipping the slides were immersed in xylene.

Immunohistochemistry

Standard immunohistochemical techniques for gelatin embedded samples were used to assess graft survival and integration. Sections were immunostained for human neuron specific enolase (NSE) and human neural cell adhesion molecule (N-CAM). These antibodies are specific for human cells and do not react with the analogous rat antigens. Both NSE and N-CAM have been previously shown to identify transplanted hNT cells *in vivo*.[47] Briefly, sections were washed in 0.1 M phosphate buffered saline, followed by a 30 minute period of quenching in a solution of 10% methanol and 3% hydrogen peroxide in 0.1 M PBS. After washing, the sections were then blocked with 5% normal horse serum (Sigma, Oakville, Ontario), 0.3% Triton X-

100 (Sigma, Oakville, Ontario) in 0.1 M PBS. The sections were then placed in block with primary antibody and left overnight at 4ºC. The primary antibody was visualized using either a peroxidase-antiperoxidase reaction, 1:250 biotinylated horse anti-mouse IgG Ab (Vector Laboratories Canada Inc., Burlington, Ontario) and the ABC kit (Vector, Burlington, Ontario) with diaminobenzidine (Sigma, Oakville, Ontario) as the chromagen or by using a fluorescent secondary antibody (Alexa 488, 1:300; Molecular Probes, Eugene, Oregon). Staining for the presence of NSE was performed using the primary mouse anti-NSE2 monoclonal antibody (1:100; Vector Laboratories Canada Inc., Burlington, Ontario). The presence of N-CAM utilized the primary mouse anti-human N-CAM monoclonal antibody (MOC-1, 1:1000; DAKO Diagnostics Canada, Inc., Mississauga, Ontario). The following are exceptions of the preparation described above; when using the MOC-1 antibody we used 0.05 M TRIS-HCl as our buffer solution instead of 0.1 M PBS. When the Alexa 488 secondary was used the Triton X-100 was omitted from the reaction solutions.

Confocal, routine fluorescent and light microscopic digital images were enhanced for clarity by adjusting the brightness and contrast options in Adobe Photoshop 6.0 (Adobe Systems, Mountain View, California).

Results

hNT cell suspensions

The cell preparation protocol resulted in excellent viability of hNT neurons for transplantation. The Trypan-Blue exclusion technique demonstrated a mean pre-operative viability of 80.4 ± 7.6%. The control group of animals receiving killed hNT neurons had a pre-operative viability of 0%, which indicated that all the cells were dead prior to transplantation. The cell suspensions used for transplantation had a mean concentration of 163.5 ± 8.6 x 10^3 cells per μL.

Animal survival

A total of 54 animals were lesioned ± grafted. Thirty-six of those animals survived the duration of the experiment. Of the 18 (33.3%) animals that did not survive the entire experiment; 44.4% (8/18) were sacrificed at the request of our animal care facility due to weight loss (four animals), autotomy (three animals) and urine scalding (one animal). Of the remaining animals which died, five succumbed to gastrointestinal complications, nonresolving ileus, perforation or impaction; two had uncontrollable infections, one suffered a spontaneous intracerebral hemorrhage and no cause for death was found in two animals. The grafted animals exhibited a trend towards greater survival rates but this did not reach statistical significance ($p>0.07$).

Cell survival and growth

Overall, 66.7% of grafted animals surviving the duration of the experiment showed evidence of graft survival as assessed by immunohistochemical staining with NSE2 or MOC-1. There was no evidence of tumour formation in any of the animals.

Control groups

There was no positive staining for NSE2 or MOC-1 in any of the animals that received grafts of killed hNT cells (Figure 2A), nor was there any staining seen in the animals which received a lesion only and no cell transplantation. The control animals exhibited a greater degree of cavitation than did the grafted animals (Figure 2B,C).

Bridge grafts

Transplant survival in the bridge graft group was 87.5% (seven out of eight) surviving animals staining positive for hNT cells. Bridge grafts ranged in size from 500 μm to 1300 μm (mean 920 ± 299 μm) in diameter. Immunohistochemical staining revealed a dense pattern of staining in both NSE2 and MOC-1 preparations, which precluded accurate cell counts in any of the grafts. In the grafts, neurons could be identified and their fibres seen coursing both within the graft and extending from the graft into the host white matter. There was evidence of growth in both a rostral and caudal direction from the location of the bridge graft (Figure 2D-F). This was only evident if the graft was placed exactly within the lesion site. If the grafts were placed even a few hundred microns distal to the lesion they grew only in a caudal direction, with no projections rostral to the lesion. A fine network of fibres containing numerous varicosities could be seen projecting caudally and coursing primarily through the white matter.

Double grafts

Graft survival was the least in the double grafted group with only three of nine (33.3%) grafted animals displaying immunohistochemical evidence of hNT cells. Surviving grafts in the vicinity of the lesion appeared small, ranging from 200 μm to 500 μm (mean 360 ± 151 μm) in diameter, and contained within the area of implant. There was no evidence of growth between the two grafts and no processes were seen to cross the site of the lesion (Figure 2G-I). No significant outgrowth was identified from the proximal grafts.

Triple grafts

There was excellent graft survival in animals receiving triple grafts, with six of seven (85.7%) animals displaying positive immunoreactivity for hNT neurons. The triple grafts ranged in size from 600 μm to 1400 μm (mean 1055 ± 332 μm). There was less cavitation in the spinal cords containing viable triple grafts compared to the other two grafting strategies, which suggests that the grafts may have grown to fill the necrotic cavities produced by the lesions. It was noted in a number of the triple grafts that considerable outgrowth of fibres projected both rostrally and caudally from the graft into the host spinal cord, essentially spanning the region of the lesion (Figure 2J-M). These projections were measured up to 600 μm from the graft deposit, in both the rostral and caudal directions. However, the majority of outgrowth from the grafts was directed caudal from the area of the lesion. It was in these caudal projections that the most significant growth occurred (Figures 3 and 4). Fibre outgrowth was primarily in the white matter and the fibres exhibited numerous varicosities (Figure 3) along their course. Once the fibres entered the grey matter they appeared to terminate within a short distance. No long distance growth was noted within the spinal grey matter.

Retrograde labelling

There was no evidence of regeneration of rubrospinal axons through the areas grafted. Due to the crossed nature of the

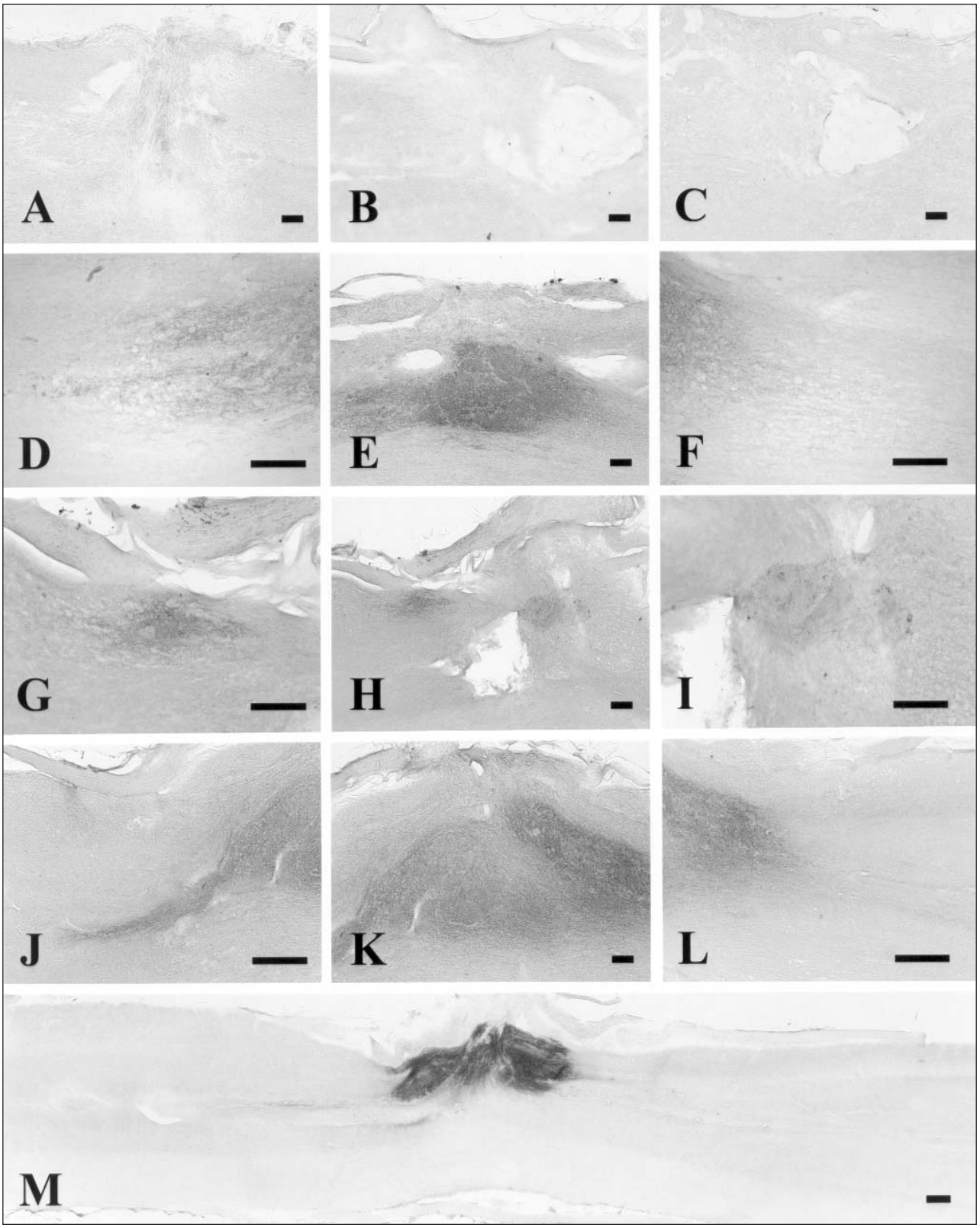

Figure 2: *Representative longitudinal rat spinal cord sections depicting outgrowth rostral (A,D,G,J and M left side) and caudal (C,F,I,L and M right side) from hNT grafts.* ***A:*** *demonstrates negative staining for NSE2 in a graft of killed hNT neurons.* ***B,C:*** *negative staining for MOC-1 and NSE2, respectively, in lesioned, ungrafted rats.* ***D-F:*** *bridge graft animal stained for NSE2.* ***G-I:*** *double grafted animal stained for NSE2 illustrating no fibres crossing the site of the lesion.* ***J-L:*** *triple grafted animal stained for NSE2.* ***M:*** *triple grafted animal stained for MOC-1. Scale bar = 200μm.*

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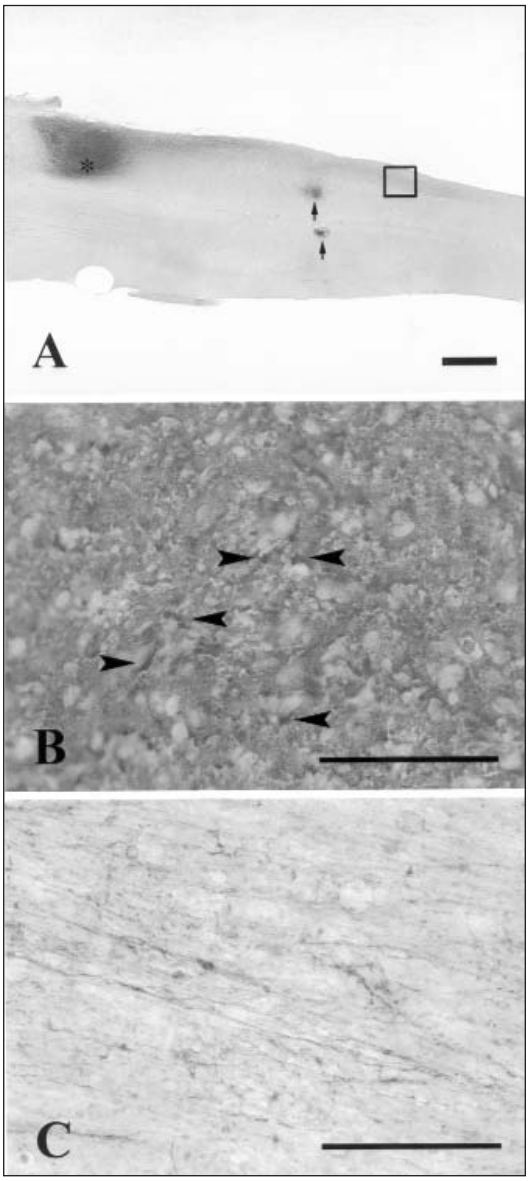

Left: Figure 3: *Longitudinal section of rat spinal cord following transplantation of hNT cells, immunostained for human NSE2.* ***A:*** *low power view depicting fibre outgrowth. Arrows indicate Fast Blue injection sites. Scale bar = 1mm.* ***B:*** *high power view of area indicated by the asterix in (A). Arrow heads indicate hNT neurons. Scale bar = 100μm.* ***C:*** *high power view of area indicated by the box in (A). Numerous varicosities are seen along the fibres. Scale bar = 100μm.*

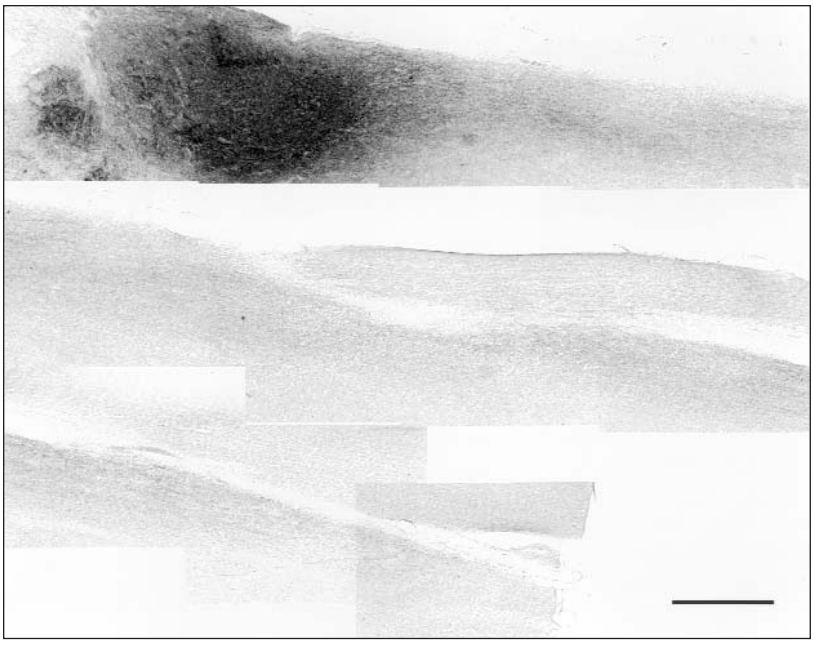

Figure 4: *Longitudinal spinal cord sections from a rat receiving a triple graft. Continuous series of eleven photomicrographs sequentially arranged in a montage to demonstrate fibre outgrowth of hNT neuronal processes caudal to the lesion, stained for NSE2. Scale bar = 500μm.*

rubrospinal tracts, we would have expected Fast Blue (first tracer) to be visible ipsilateral to the lesion, with no uptake in the contralateral red nucleus if a complete rubrospinal lesion was made. Conversely, our second tracer would have been visible in both red nuclei had regeneration of the rubrospinal tract through the grafted area occurred. All brains showed good uptake of the first tracer, Fast Blue, ipsilateral to the lesion and no uptake contralateral to the lesion side. Uptake of the second tracer, Fluoro-Ruby was only seen on the side ipsilateral to the lesion. This strongly suggests that there was no regeneration of rubrospinal fibres distal to the lesion. These results were consistent in all the treatment groups as well as in the control animals. However, when the grafted sites were examined, further evidence of long distance fibre growth was displayed. Confocal microscopic images (Figure 5) were obtained following immunohistochemical staining of hNT transplants with NSE2. Co-labelling of hNT cells within a graft by both NSE2 and through the uptake of the tracer Fluoro-Ruby strongly suggests that these cells have projected processes at least to the point of injection, 2 cm caudal to the lesion. There was no evidence of double labelling of cells with NSE2 and the tracer Fast Blue.

Discussion

This study has demonstrated the ability of hNT cells to survive after transplantation and extend fibres for considerable distances in the lesioned rat spinal cord.

Fibre outgrowth up to 2 cm from the site of the graft deposit, at 13 weeks post-transplantation, was observed in the hemisection model of SCI. This would yield a growth rate of 0.2 mm/day or 1.5 mm/week which is similar to that reported in the intact mouse spinal cord.[50] The potential of a neuronal graft to serve as a synaptic relay[1] may be enhanced if the grafted cells exhibit the ability to extend fibres for considerable distances,

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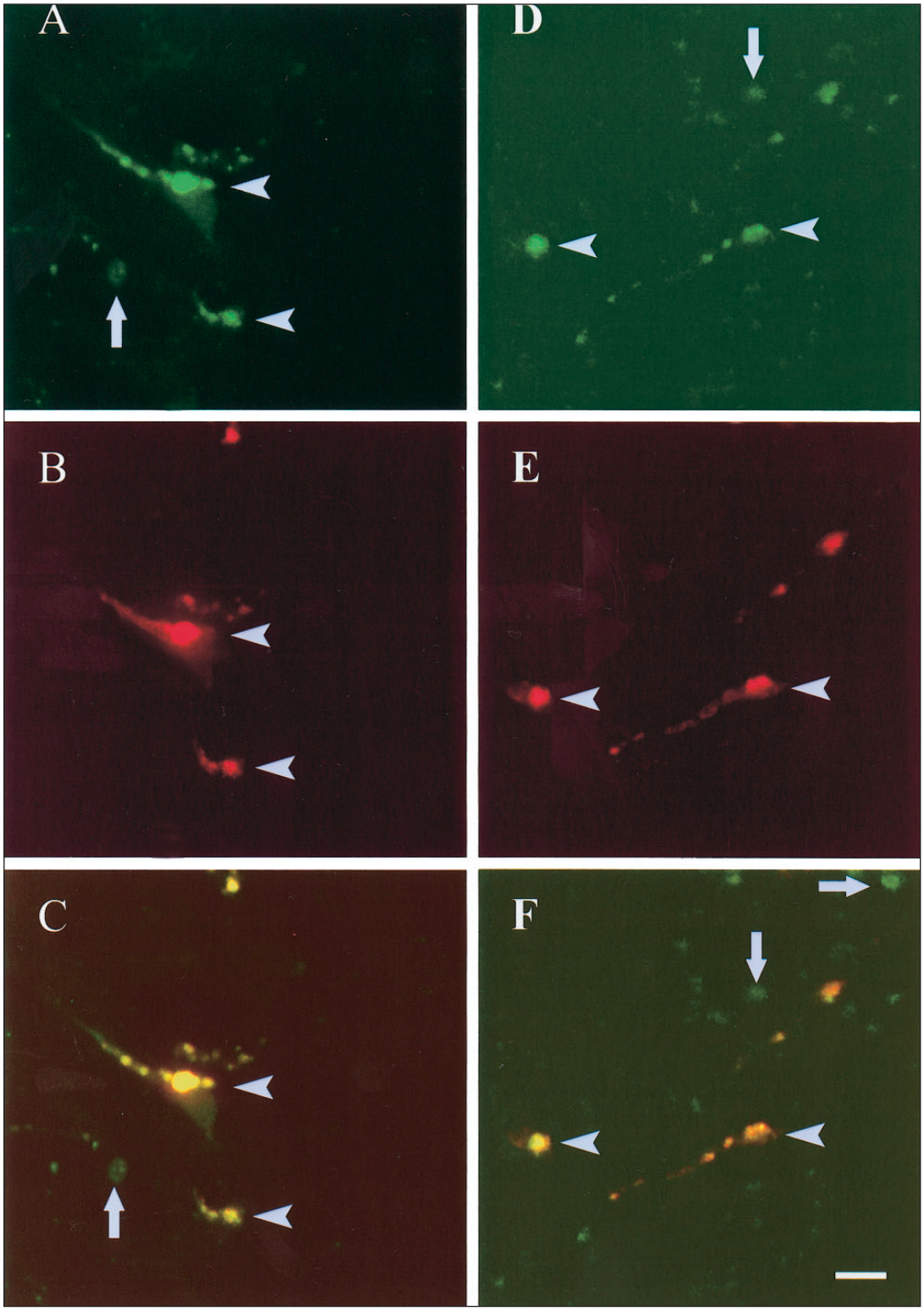

Figure 5: *Longitudinal rat spinal cord section viewed as confocal microscopic images depicting grafted hNT neurons on the caudal side of a lesion.* ***A-C:*** *taken from a rat receiving a bridge graft, the labelled cells are from the mesiocaudal aspect of the graft.* ***D-F:*** *taken from a rat receiving a double graft, the labelled cells are from the laterocaudal aspect of the graft.* ***A*** *and* ***D*** *are stained for NSE2 with Alexa 488 as a secondary, viewed through an FITC filter.* ***B*** *and* ***E*** *display uptake of the tracer Fluoro-Ruby, injected 2 cm caudal to graft, viewed through a rhodamine filter.* ***C*** *and* ***F*** *are the overlay composite images illustrating the double labelling. Arrow heads indicate double labelled neurons. Arrows indicate neurons only labelled with NSE2. Scale bar = 10 μm.*

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such as the hNT cell line appears to demonstrate in these experiments.

One of the more difficult problems in neural transplantation for SCI is enticing axons to regrow through the graft and penetrate extensively into the distal host spinal cord.[52,53] Previous work in a Parkinson's model has demonstrated that a double grafting strategy leads to enhanced outgrowth of axons between the two grafts.[47] Our experiments attempted to take advantage of this observation to promote growth across the area of injury. We have shown that our grafting strategy, particularly the triple grafts (Figure 1D), supports growth in both rostral and caudal directions enabling the graft to essentially bridge the lesion (Figure 2). This observation supports the idea that when grafted in this fashion, hNT neurons may have the potential to functionally reconnect the proximal and distal spinal cord across the region of injury by becoming a synaptic relay station.

The overall survival of the hNT grafts was 67%. Survival appeared to be related to the site and number of cells transplanted, as the bridge and triple grafts showed greater survival than the double grafts (88% and 86%, respectively versus 33% for the double grafts). Previous work with hNT neurons in an ischemic rodent model demonstrated that there was a dose-response effect with regard to cell survival and functional effect.[45] The decrease in graft survival in the double graft group may be related to the smaller number of cells deposited within each graft site. Smaller grafts may be more susceptible to damage from the deleterious secondary biochemical effects which are known to follow acute SCI.[54] Therefore, it is possible that we may require large numbers of cells to be transplanted to produce viable grafts in the adverse environment of acute SCI.

The site of implantation also appears to be important. The presence of a graft in the lesion area (bridge and triple grafts) appears to improve graft survival and promote fibre outgrowth across the lesion. The triple grafts had the best survival and fibre outgrowth suggesting that grafting in the lesion and adjacent proximal and distal spinal cord segments promotes further bridging of the lesioned area. Conversely, poor graft survival and no fibre growth across the lesion were observed in the double graft group, which lacked a bridge graft in the lesioned area. These factors may play a role in any functional benefit that these cells may provide. Saporta et al[51] reported that hNT cells transplanted into a contusion model of SCI showed functional effects as demonstrated by motor evoked potential recordings. The modest gains reported were achieved via a grafting technique similar to our double grafts reported in this paper. It is possible that if the graft size and fibre outgrowth is enhanced by utilizing a triple graft strategy, meaningful functional improvements may result from hNT transplantation.

The mechanisms by which transplants may improve function are still being investigated.[1] The ability of a transplant to enhance the regeneration of damaged host axons has been well-described.[55-57] In order to determine if our grafting strategy using hNT neurons would enable regeneration of rubrospinal axons, after a thoracic hemisection, examination of the uptake of two tracers in the red nucleus was performed. Due to the crossed nature of this pathway we would expect to find only the second tracer in the red nucleus contralateral to the grafted side, if regeneration of this pathway into the distal spinal cord had occurred following complete axotomy. However, no labelling of either tracer was seen in the red nucleus contralateral to the lesion. These results strongly suggest that no axons from the red nucleus regenerated through the lesion and into the distal spinal cord as a result of the hNT grafts. The lack of regrowth seen in this experiment is consistent with previous studies looking at rubrospinal regeneration, in which minimal or no regeneration through a transplant is seen in adult rodents compared to neonatal pups.[10,58] However, descending axons may not need to grow through the lesion into the distal cord if they have a synaptic relay with a graft that has bridged the lesion and extended fibres into the distal cord. The present study suggests that hNT grafts may be able to be such a synaptic relay.

It is likely that a successful treatment paradigm for the management of SCI will be multifaceted, including minimizing secondary injury, enhancing regeneration and facilitating repair of the lesion. Having a graft that can act as a synaptic relay to establish functional connections with the distal cord may be of crucial importance for a repair strategy. Of considerable relevance is the question of what type of tissue is best to be transplanted. The hostile environment of oxidative cascades and lipid peroxidation involved in the secondary injury following trauma is well-recognized.[54] Following acute SCI the biological response to injury initiates a cascade of effects, which includes oxidative stress and lipid peroxidation.[54] This severe stress does not appear as significant in other models of neurological conditions in which transplantation occurs more than a month after the insult.[45,47] It is clear from the results of this study that hNT neurons can survive and mature despite such adverse conditions. A successful neuronal source not only needs to be able to survive and grow in this environment but must also be readily available, produced under controlled circumstances and have no risk of infectious transmission. We have now shown that a commercially, readily available source of human neurons (hNT cells) can survive and mature after transplantation in the environment following acute SCI.

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