ELSEVIER

Developmental Brain Research 89 (1995) 56–66

DEVELOPMENTAL BRAIN RESEARCH

Research report

Ciliary neurotrophic factor promotes the terminal differentiation of v-*myc* immortalized sympathoadrenal progenitor cells in vivo

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Accepted 23 May 1995

Abstract

Survival and differentiation of a sympathoadrenal progenitor cell line (termed MAH), transduced with a v-*myc* oncogene, was studied subsequent to transplantation in the peripheral and central nervous system of adult rats. In the brain, MAH cell survival depended on the secretion of ciliary neurotrophic factor (CNTF) by co-grafts of genetically modified glioma cells. No trophic factor supplement was required for development of the MAH cells in the peripheral nerve environment. Transplanted progenitor cells withdrew from the cell cycle within 48 h and differentiated into a prominent population of large sympathetic-like neurons. The neurons expressed the α subunit of the CNTF receptor and appropriate spatial distributions of cytoskeletal proteins and catecholamine related enzymes. The results identify a role for CNTF in the development of the sympathoadrenal cell lineage and support the concept of immortalized progenitor cells as alternatives to primary cells for cell replacement strategies in the nervous system.

Keywords: Progenitor cell; Transplant; Ciliary neurotrophic factor; Oncogene; Cytoskeleton; Peripheral nerve

1. Introduction

Sympathoadrenal progenitor cells, derived from the neural crest tissue during development, migrate to the sympathetic ganglia and differentiate into sympathetic neurons or alternatively, enter the primordium of the adrenal gland to become chromaffin cells [44]. This bipotential progenitor cell has been isolated from the embryonic rat adrenal medulla and infected with a recombinant retrovirus vector containing the v-*myc* oncogene. This cell line, abbreviated as MAH (v-*myc* infected, adrenal derived, HNK-1 positive) has retained many cellular characteristics of the counterpart cells in situ and has been used to study cellular and molecular aspects of sympathetic neuron and chromaffin cell development [6,61]. In culture the MAH cell can be directed, according to the presence of glucocorticoids or trophic factors, to develop into chromaffin-like cells or sympathetic-like neurons respectively. Fibroblast growth factor (FGF) can induce a small proportion of MAH cells to acquire neuron-like characteristics in vitro and these cells become dependent on nerve growth factor (NGF) for subsequent survival [6].

Considerable attention has recently centered on ciliary neurotrophic factor (CNTF), a molecule that is related to the cytokine subfamily and shown to influence the survival and differentiation of various classes of neurons and glial cells throughout the nervous system [3,32,37,42,51]. In culture, CNTF can also act in synergy with FGF to induce neuronal differentiation in MAH cells [30]. The developmental potential of the MAH cell has not been assessed in vivo. With interest in the potential use of genetically modified cells for cell replacement strategies in the brain, we transplanted MAH cell suspensions to the peripheral and central nervous system of adult rats. In this study, we identify the expression of a functional CNTF receptor on the MAH cell and describe the morphology of differentiated MAH cells in vivo. Furthermore, the cells show no continued proliferation after transplantation in the nervous system.

2. Materials and methods

All experiments with the rodents adhered to the guidelines set out by the Canadian Council on Animal Care and

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SSDI 0165-3806(95)00095-X

were approved by the Animal Research Ethics Board of McMaster University.

2.1. Cell cultures

The MAH-B2 cell line was derived by Birren and Anderson [6] by preparative flow cytometry of HNK-1 positive precursors from the adrenal medullas of Sprague–Dawley E14.5 rat embryos. These precursor cells were immortalized with a recombinant retrovirus vector containing the v-*myc* oncogene. The v-*myc* gene is transcribed from the viral long terminal repeat (LTR) under control of the SV40 early promoter [50]. MAH cells were plated at high density on poly-D-lysine (0.5 mg/ml) and laminin (2.0 μg/ml) coated dishes and grown in a modified L-15 CO_2 media supplemented with 10% fetal bovine serum. After 3–5 days in vitro the cells were removed with trypsin and washed with lactated Ringer's solution in preparation for transplantation.

Cultures of C6 glioma cells transfected with secretory and non-secretory CNTF expression constructs were grown on plastic tissue culture dishes without attachment factors in RPMI medium 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine. The full length rat CNTF cDNA was driven by the glial fibrillary acidic promoter and the secretory construct contained an in frame human growth hormone signal sequence at the start of the CNTF cDNA [28]. Three types of glioma cells designated GS6-1 (CNTF secretory), GNS1-1 (CNTF non-secretory) and parent C6 cells (ATCC CCL #107) were used. All glioma cell types were grown to near confluency, trypsinized and washed before grafting.

2.2. Transplantation

The cell concentrations were adjusted to deliver approximately 2.0×10^4 viable MAH cells into the peripheral nerves and equal numbers (5.0×10^3) of viable MAH and glioma cells into the striatum. Viable cells (Trypan blue exclusion or Cell Tracker™ orange [Molecular Probes] uptake) were transplanted in 5.0 μl of lactated Ringer's. Viability of the cell suspensions prior to grafting ranged between 95 and 97%.

Adult female Wistar 200–220 g rats ($n = 27$) were deeply anaesthetized with an intraperitoneal injection of Somnotol. Animals were placed in a rodent stereotaxic frame (Kopf). The skin over the skull was incised and the muscles retracted from the skull surface over Bregma. A 1.0 mm^2 area of bone immediately anterior to Bregma was removed to allow entry of a 25 gauge needle fitted to a 10-μl Hamilton syringe. The syringe was filled with one of the following cell suspensions (MAH, MAH/GS6-1, MAH/GNS1-1 or MAH/C6) and the needle tip lowered into the striatum at the following coordinates relative to Bregma and the dural surface (+1.0 AP, +2.5 ML, +5.0 DV). The needle was left in place for an additional 5 min after the injection and then slowly withdrawn. Autoclips (9 mm) were used to close the skin on the top of the head.

For injection of the cells into peripheral nerves, the skin over the left thigh of adult female Wistar rats ($n = 25$) was incised and the muscle mass cut just enough to expose the sciatic nerve trunk. The nerve trunk was completely transected and the nerve ends tied with 4-0 silk suture. This step was performed to initiate clearing of the host axons from the distal nerve segment and provide an internal milieux that offers less resistance to the needle and requires reduced pressure to inject the cells, thereby reducing physical trauma to both the grafted cells and the endogenous cells of the nerves. One week after the transection step, the animals were anaesthetized and the distal nerve re-exposed. With the aid of a dissecting microscope, a small incision was made in the epineurium just distal to the 4-0 suture. For these injections, the syringe was hand held and the needle tip was pushed into the core of the nerve and 5.0 μl of the cell suspension was pressure injected into the nerves over 1 min. After the injection, the needle was held in place for an additional minute and then slowly withdrawn. Finally, the cut edges of the epineurium were tied with 10-0 suture, and the muscle incision and the skin sutured with 4-0 silk.

2.3. Cell tracing and bromodeoxyuridine (BrdU) incorporation

To trace the implanted MAH cells and determine if the MAH cells underwent DNA replication subsequent to transplantation, the cells were initially labelled with Cell Tracker™ orange (CMTMR). This dye contains a chloromethyl reactive group and this dye passes freely through cell membranes. Inside the cell, the dye is thought to undergo a glutathione *S*-transferase mediated reaction which conjugates the chloromethyl moiety to intracellular thiols producing a cell-impermeant fluorescent dye-thioether adduct. Twenty-four h prior to transplantation, the MAH cells (100% labelled) were trypsinized from the flasks and suspended in L15 media (200 μl) containing 2.5 μl of the Cell Tracker™ for 20 min at 37°C. Cells were washed in L15-CO_2 and replated. The cells (100% labelled) were harvested for transplantation as described above. Grafted animals ($n = 18$) received an intraperitoneal injection of the thymidine analog bromodeoxyuridine (50 mg/kg) 6 h prior to grafting ($n = 5$), and after 1 ($n = 5$), 2 ($n = 4$) and 4 ($n = 4$) days post transplantation, the animals were given an overdose of anesthetic and perfused with saline. A second labelling paradigm involved a series of three multiple BrdU injections (50 mg/kg) given 30 min apart at 1, 2 or 4 days post implantation. These animals were perfused and processed 30 min after the final injection. The nerves or brains were dissected and cryostat sections (10 μm) of the transplants collected on microscope slides. Tissue sections were fixed with absolute methanol at 4°C for 10 min, air-dried, trans-

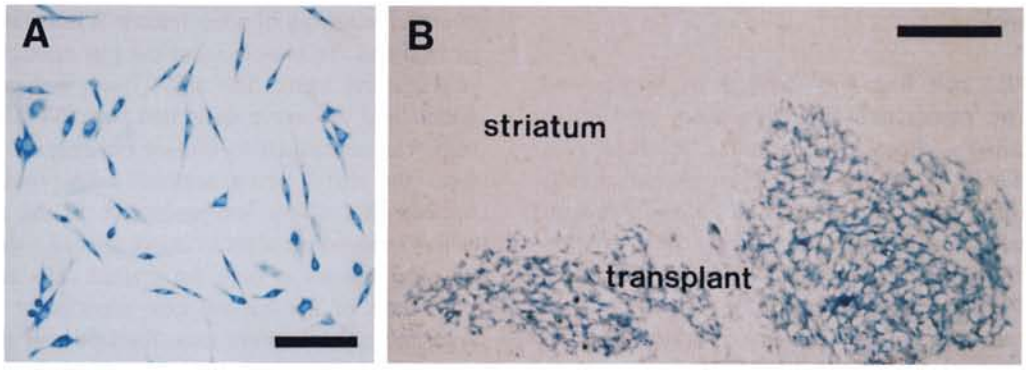

Figure1

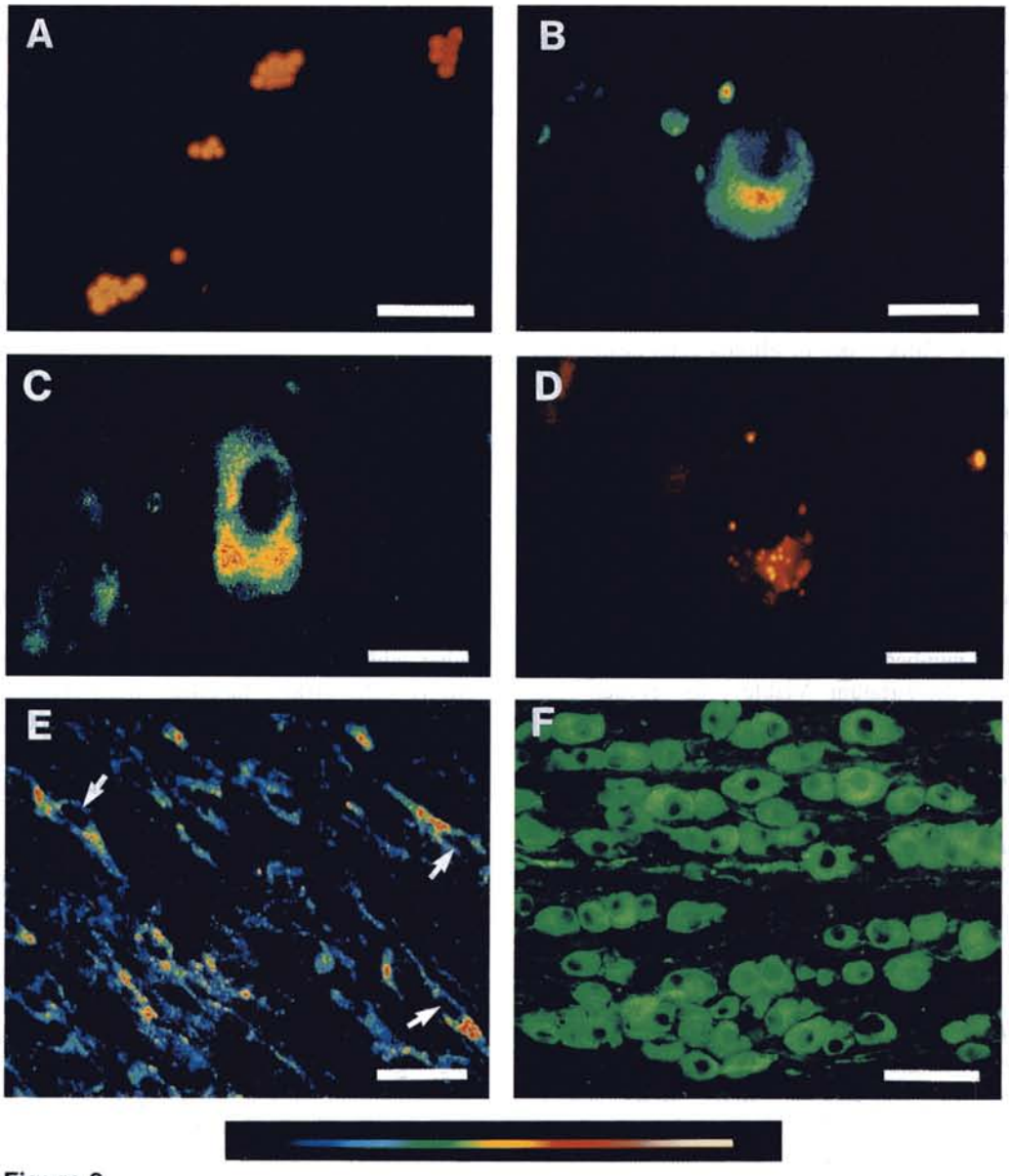

Figure 2

ferred to 0.1 M glycine for 10 min at room temperature and then washed with 0.01 M phosphate-buffered saline (PBS). The DNA was denatured in 2 N HCl for 10 min at 37°C and then neutralized with 0.1 M sodium borate for 10 min. Anti-bromodeoxyuridine (Boehringer Mannheim) was used at 6 μg/ml and applied for 1 h. The primary antibody was omitted for the control sections. After a brief wash in PBS, a secondary antibody linked to FITC was applied for 30 min. The slides were rinsed and then coverslipped. MAH cells identified with the Cell Tracker™ and cells that underwent DNA replication were observed with FITC and rhodamine filter packs respectively. Control sections processed without the antibody showed no nuclear staining.

2.4. Stereology

To estimate the number of differentiated MAH cells in the grafts, the animals were perfused with 4.0% buffered paraformaldehyde. After an overnight fixation, the brain or nerve/transplant complex was immersed in 15% sucrose for at least 3 hours in preparation for cryostat sectioning. Every 3rd pair of sections (10 μm) through the entire graft complex in the peripheral nerves was transferred to gelatin-coated microscope slides and stained with Cresyl violet to identify the neurons. For the transplants in the striatum, the MAH cells were identified by the presence of the Cell Tracker™ orange. Reference volumes were calculated by the Cavalieri technique [10] and quantitative estimates of the neuron populations (labelled with Cell Tracker™) were determined by the dissector method [54] as previously described [15].

2.5. β-Galactosidase histochemistry

The GS6-1 cells, grown on glass coverslips, were rinsed with PBS and fixed for 5 min at 4°C in 2.0% formaldehyde and 0.2% glutaraldehyde in PBS. Animals were initially perfused with saline followed by 4.0% paraformaldehyde. Cryostat sections (10 μm) of the striatum/MAH cell implants were collected in PBS. The histochemical reaction mix contained 1.0 mg/ml of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, Boehringer Mannheim), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in PBS. The X-gal was initially diluted in dimethylsulfoxide (DMSO) at 40 mg/ml. Incubation times for visualization of the β-galactosidase ranged from 15 to 30 min for cultures and 1–3 h for tissue sections depending on the desired intensity of the blue reaction product. The brain sections were floated on microscope slides and dried. Finally, the slides and coverslips were dehydrated, cleared and mounted with Permount.

2.6. Immunocytochemistry

After 2 weeks, 1 and 2 months post transplantation, the animals were perfused with 4.0% paraformaldehyde in 0.1 M phosphate buffer (PB) and the host tissue/graft complex dissected and post-fixed in the same type of fixative at 4°C overnight. The brains and nerves were cryoprotected in 15% sucrose (v/v in PB) for 2 h and then frozen for cryostat sectioning. Longitudinal sections of the nerves and coronal brain sections were cut at 10 μm and collected in PBS. All immunocytochemical dilutions were made with PBS. Free-floating sections were incubated with one of the following established antibodies overnight at 4°C: tyrosine hydroxylase (TH) [[48]-Boehringer Mannheim], dopamine β-hydroxylase (DBH) (Eugene Tech Inc.), PNMT [7], phosphorylated M_r 150 and 200 kDa neurofilament subunits (RT97 and SMI-31) [40], non-phosphorylated M_r 168 and 200 kDa neurofilament subunits (SMI-32) [[55]-Sternberger Monoclonals Inc.], microtubule associated protein (MAP2) [[60]-Amersham Inc.], ciliary neurotrophic factor (CNTF) [19], CNTF receptor-α subunit (CNTFRα; antibody designated RG30) (Regeneron Pharmaceuticals Inc.). The sections were rinsed for 10 min and then immersed in goat anti-mouse (monoclonal) or goat anti-rabbit (polyclonal) IgG secondary antibodies conjugated to FITC, DTAF or TRITC (Jackson Immunoresearch Inc.). Control sections were not incubated in the primary antibodies. Sections were positioned on glass mi-

Fig. 1. Expression of β-galactosidase in C6 cells modified to secrete CNTF. A: appearance of GS6-1 cells in vitro visualized by β-galactosidase histochemistry. Many cells have a long thin bipolar morphology. An increased cytoplasmic content is apparent in other cells. Scale bar = 100 μm. B: co-graft of MAH/GS6-1 cells in the adult striatum. Section of a one month old transplant processed for β-galactosidase and counterstained with eosin. Scale bar = 150 μm.

Fig. 2. Confocal laser scanning microscopy of sympathetic-like neurons derived from MAH cells. Increasing immunoreactivity is represented from blue to white on the colour bar. A: undifferentiated MAH cells in suspension labelled with Cell Tracker ™ orange (CMTMR) prior to transplantation. Scale bar = 100 μm. B: differentiated sympathetic-like neuron in a 1-month-old mixed MAH/GS6-1 graft identified with the SM1-32 antibody. Intense non-phosphorylated neurofilament immunoreactivity adjacent to the nucleus is characteristic of these cells. Scale bar = 25 μm. C: expression of the CNTFRα (RG30) in the cytoplasm of a sympathetic-like neuron within a MAH/GS6-1 co-graft. Scale bar = 25 μm. D: same neuron as (B) double-labelled with the fluorescent probe Cell Tracker™ (CMTMR). This tracer appears concentrated in vesicles within the cytoplasm. Scale bar = 25 μm. E: confocal image of CNTF immunoreactivity in the GS6-1 cells of a MAH/GS6-1 co-graft. Arrows indicate the nuclei of these bipolar cells. Scale bar = 30 μm. F: localization of MAP2 in sympathetic-like neurons in peripheral nerve. Dendrites from the neurons are also evident in this 2-month-old transplant. FITC epifluorescence microscopy. Scale bar = 75 μm.

croscope slides, rinsed with distilled water, dried and then covered with a 1:1 ratio of glycerol and sodium carbonate buffer containing 0.04% *p*-phenylenediamine. Immunoreactivity was visualized by epifluorescence microscopy or by confocal microscopy with a Zeiss (LSM) laser scanning microscope. An argon laser was used to observe FITC and TRITC immunoreactivity at excitation wavelengths of 488 and 514 nm respectively.

3. Results

3.1. Characteristics of MAH cells and GS6-1 cells in culture

In vitro characteristics of MAH-B2 cells have been previously described by Birren and Anderson [6]. Briefly, these cells continually divide and grow as tight clusters of

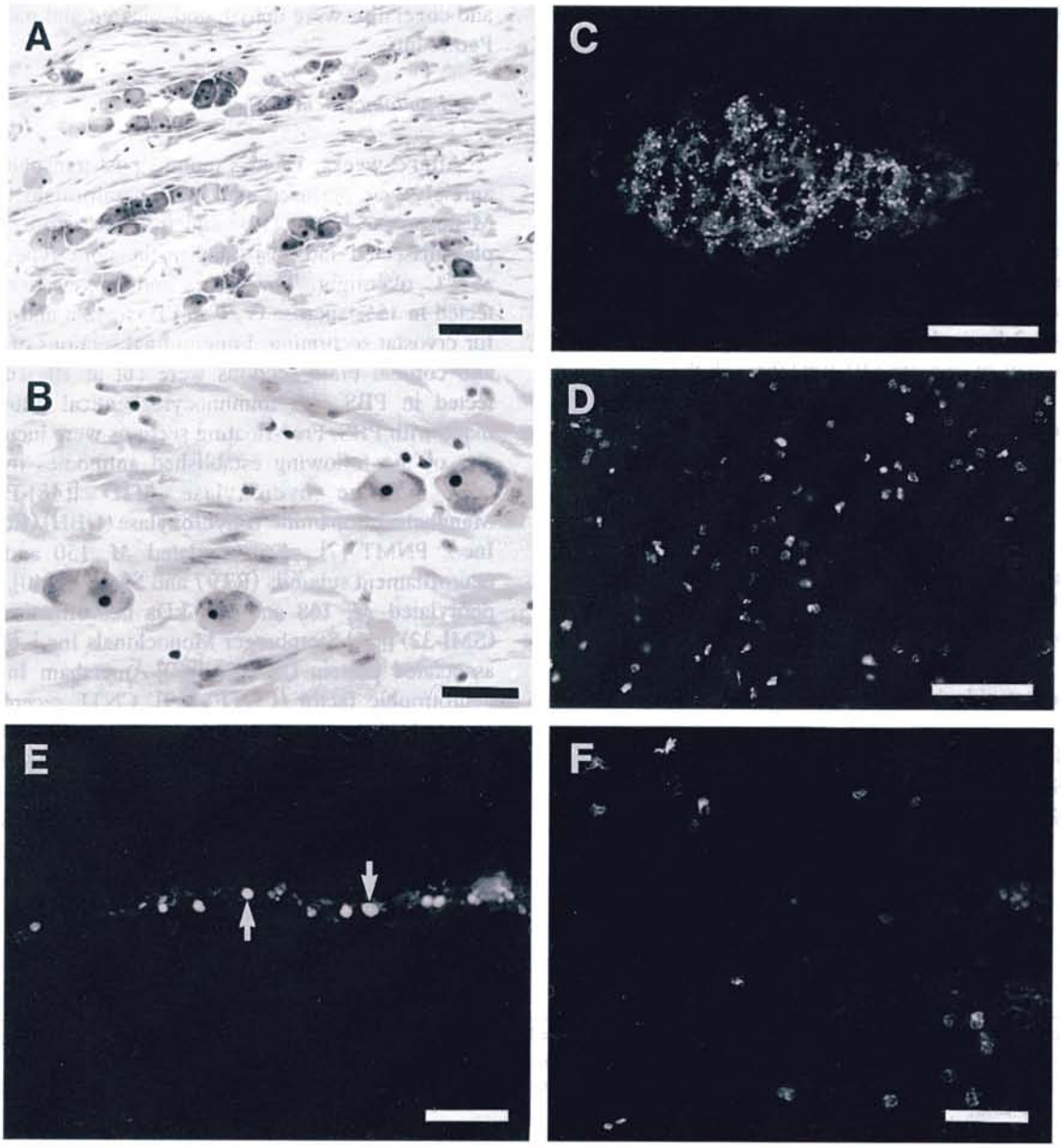

Fig. 3. Light microscopy of differentiated MAH cells and absence of DNA replication in MAH cells. A: rows of large sympathetic-like neurons stained with Cresyl violet in a 1-month-old transplant are aligned parallel with the Schwann cell columns. Nissl substance and nuclei with prominent nucleoli are evident. Scale bar = 150 μm. B: large neurons with prominent nucleoli are visible in the peripheral nerve matrix at high magnification. Scale bar = 30 μm. C: a graft of MAH cells labelled with the Cell Tracker™ orange probe. Fluorescent spots represent the undifferentiated MAH cells in this 24-h graft. Scale bar = 200 μm. D: incorporation of bromodeoxyuridine (BrdU) in mitotic cells of the peripheral nerve immediately distal to the transplant site, 24 h after grafting. Many cells including fibroblasts and Schwann cells undergo DNA replication subsequent to peripheral nerve transection or physical disruption of the nerve matrix from the needle insertion. Scale bar = 100 μm. E: a row of sympathoadrenal progenitors (arrows) labelled with the Cell Tracker™ fluorescent dye after 48 h post-grafting. Scale bar = 50 μm. F: same field as (E) doubled labelled with anti-BrdU to indicate peripheral nerve cells with DNA replication. No MAH cells show the synthesis of DNA. Scale bar = 50 μm.

undifferentiated cells. The cells are small and round with a high nuclear to cytoplasmic ratio. After 3–5 days in vitro, large aggregates develop that sometimes separate from the substrate and float in the culture media. There was no MAH cell differentiation (increase in somata size or development of processes) in culture prior to transplantation. The MAH cells in culture expressed the CNTFR α as visualized by RG30 immunoreactivity (not shown).

The genetically modified C6 cells expand rapidly in culture. The GS6–1 line is characterized by many bipolar cells with long thin processes. These cells were identified by the β-galactosidase reaction product (Fig. 1A) and CNTF immunoreactivity.

3.2. CNTF induces MAH cell differentiation in the brain

Single injections of MAH cells into the adult rat striatum showed poor survival. In animals examined at 2 weeks, occasional small cells were located along the path of the needle tract. These cells also identified with the Cell Tracker™ showed a thin cytoplasmic rim of weak immunoreactivity to the TH antibody, lacked sharp morphological definition and were apparently in a process of cell death. By 4 weeks, no transplanted cells were observed in the striatum.

In striking contrast, MAH cells survived and developed when co-grafted with the GS6-1 cells modified to secrete CNTF. In the striatum, transplants were observed in 15/16 (94%) of the animals that received the mixed MAH/GS6-1 cell combination (Fig. 1B). By 2 weeks post transplantation, the MAH cells (labelled with the Cell Tracker™ orange) had differentiated into a population of cells that expressed a variety of neuronal markers. These transplants consisted of two distinct neuronal populations (based on the cell body diameter) that expressed strong immunoreactivity to the non-phosphorylated M_r 168 and 200 neurofilament (NF) subunits (SMI-32) and to the microtubule associated protein MAP2. One group revealed the characteristics of large sympathetic-like neurons ($5.4 \pm 0.65 \times 10^2$ per transplant) (Fig. 2B,D). The second group consisted of smaller cells with a neuronal phenotype ($1.2 \pm 0.21 \times 10^3$ per transplant). Both groups of neurons expressed immunoreactivity for tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). The differentiated MAH cells were observed as isolated neurons, rather than groups or clusters of neurons in close association within the glioma cell matrix. All the grafted neurons were surrounded by the glioma cells that expressed intense CNTF immunoreactivity (Fig. 2E). Co-grafts of C6 glioma cells carrying CNTF without the signal sequence or the parent C6 line failed to support the survival of the MAH cells.

3.3. MAH cells express the CNTF receptor

Undifferentiated MAH cells in vitro and cells that developed into sympathetic neurons within the peripheral

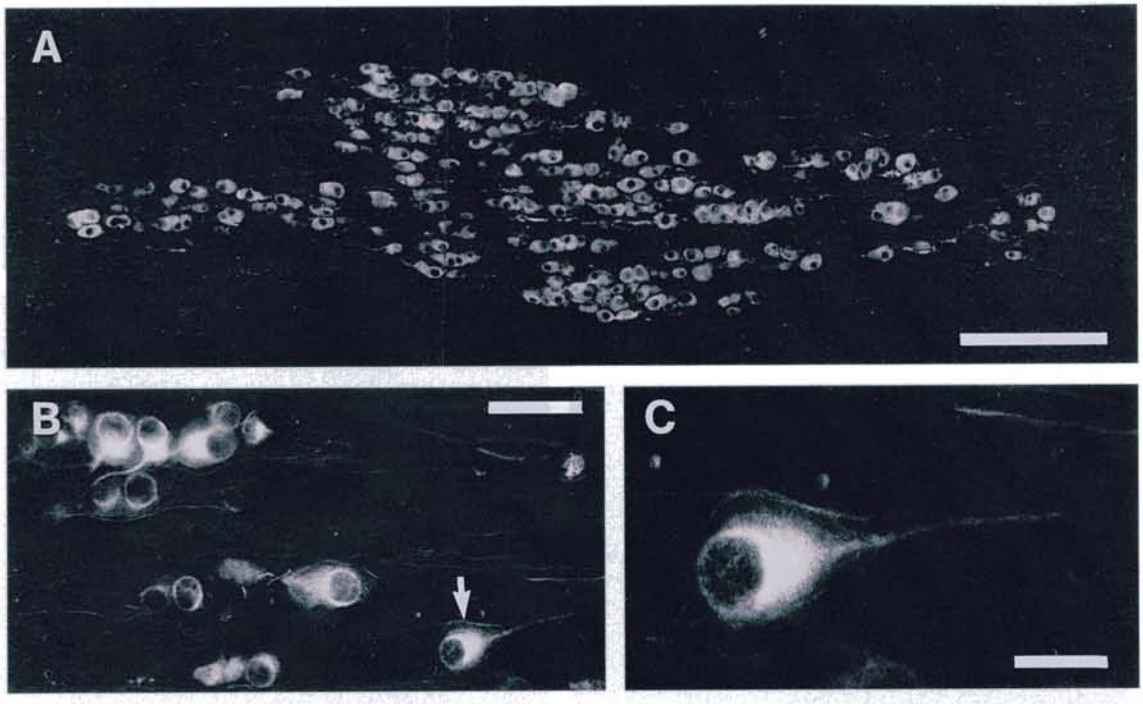

Fig. 4. Spatial distribution of MAP2 and non-phosphorylated neurofilaments in MAH cell derived sympathetic-like neurons within the PNS. A: longitudinal section of peripheral nerve containing a 2-month-old transplant with numerous sympathetic-like neurons. The cells show strong immunoreactivity in the somata and the dendrites. Scale bar = 250 μm. B: morphology of grafted neurons defined by SM1-32 neurofilament staining in a 1-month-old preparation. Scale bar = 50 μm. C: neuron indicated by the arrow in (B) magnified to show the spatial distribution of SM1-32 immunoreactivity. The antibody defines a strong central region that diminishes towards the periphery. Epitope expression in the primary dendrite shaft and cell borders is seen. Scale bar = 20 μm.

nerves and the striatum were labelled with the RG30 (CNTFR α) polyclonal antibody. Confocal microscopy revealed a distribution of the receptor throughout the cytoplasm of the sympathetic-like neurons (Fig. 2C). An additional control to ensure specificity of the RG30 was demonstrated by complete elimination of RG30 immunoreactivity in adsorption experiments with a 1/100 dilution of the NT2 peptide (0.7 mg/ml) used to generate the RG30 antibody. In addition, differentiated sympathetic-like neurons expressed CNTF immunoreactivity. The CNTF antibody was localized to nuclear profiles and to the cytoplasm of these neurons (Fig. 5D).

3.4. Transplants of MAH cells withdraw from the cell cycle

The cells labelled with the Cell Tracker™ orange prior to implantation and assessed for the incorporation of bromodeoxyuridine (BrdU) did not show any DNA replication after 48 h post transplantation. In the peripheral nerves, numerous cells within the nerve matrix showed immunoreactivity to the BrdU antibody (Fig. 3D). However, no MAH cells (clearly identified with the Cell Tracker™) displayed BrdU immunoreactivity. Likewise, in the brain, the C6 glioma cells revealed DNA replication while the MAH cells were unlabelled with the BrdU antibody. Even after only 24 h, less than 5% of the Cell Tracker™ labelled MAH cells in the implants showed DNA immunoreactivity (Fig. 3E,F). For the animals that received the multiple BrdU injections, the nuclear staining appeared stronger, but only 5% of the CMTMR cells showed DNA replication.

3.5. MAH cells are viable in peripheral nerve

Viable transplants in the PNS were found in 33/37 (89%) of the animals. These peripheral grafts contained $5.3 \pm 0.25 \times 10^3$ MAH cell derived large neurons as determined by the disector analysis. In sections stained with cresyl violet, clusters of the grafted MAH cells were easily identified (Fig. 3A,B). These large cells (31.6 ± 0.65 μm in diameter) were often aligned in rows reflecting the parallel arrangement of Schwann cells within the peripheral nerves. The neurons in these preparations were definitively derived from the injected progenitor cells since peripheral nerves contain no endogenous neurons.

The sympathetic-like neurons within the transplants were recognized by SMI-32 immunoreactivity as a strong network of neurofilaments throughout the cytoplasm. Particularly intense SMI-32 immunoreactivity was observed adjacent to the nucleus giving these cells the aspect of polarity (Fig. 4B,C). Stellate patterns of NF staining were observed by confocal microscopy. Phosphorylated epitopes to the M_r 200K NF (RT97 and SMI-31) were also detected in the perikarya of these cells (not shown). The sympa-

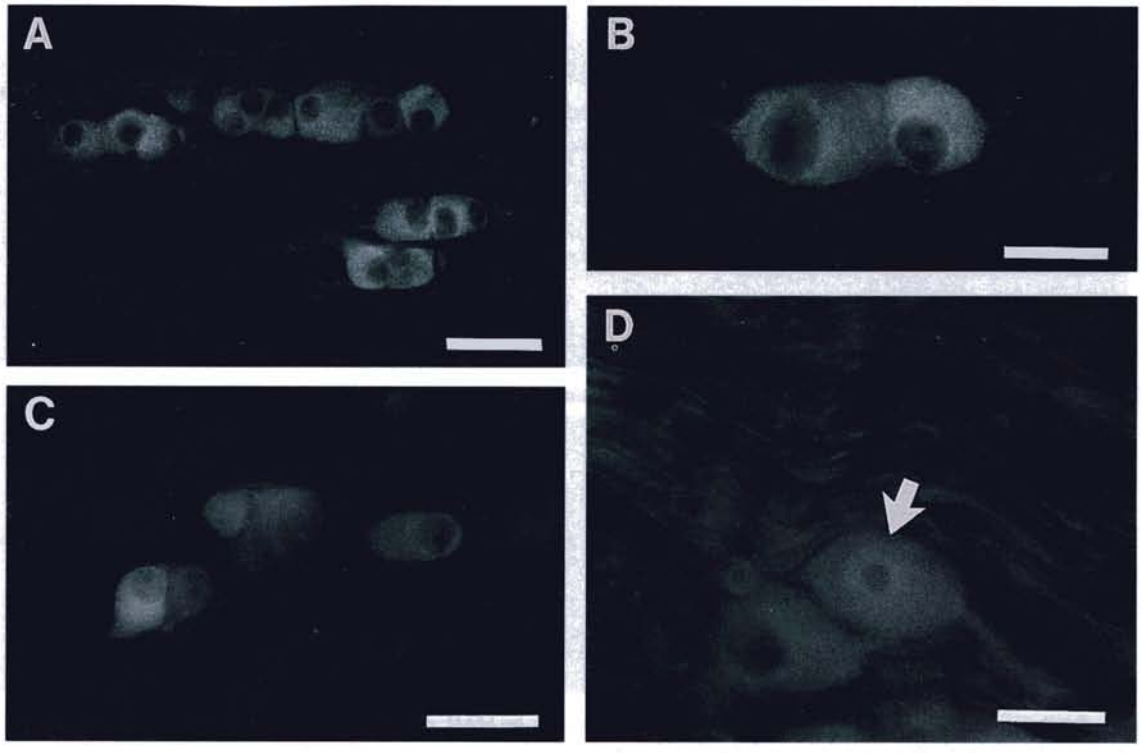

Fig. 5. Localization of catecholamine synthetic enzymes and CNTF in differentiated MAH cells within a peripheral nerve. A,B: TH expression in a 1-month-old MAH cell graft. Note the row of neurons orientated parallel to long axis of the nerve. Scale bar = 50 μm for (A) and 25 μm for (B). C: differentiated neurons display various levels of DBH expression. Scale bar = 40 μm. D: CNTF immunoreactivity is observed in the cytoplasm and nucleus (arrow) of the sympathetic-like neurons. Schwann cell profiles (shown above the neurons) also express CNTF. Scale bar = 25 μm.

thetic-like neurons expressed strong immunoreactivity to MAP2 (Fig. 4A) and there was prominent labelling of the dendrites. In the peripheral nerves, dendrites could be followed for several hundred microns along the Schwann cell columns.

Although all the differentiated sympathetic-like neurons expressed the epitope for the SMI-32 antibody, adjacent sections reacted with either TH or DBH, indicated that only 30% of the SMI-32 positive cells expressed TH and DBH (Fig. 5A,B,C). This same population of neurons exhibited robust immunoreactivity to the MAP2. In addition, there were numerous chromaffin-like cells within the grafts, arranged in rows, that expressed a punctate type of TH and DBH immunoreactivity. It was rare that these groups of chromaffin-like cells expressed immunoreactivity to phenylethanolamine *N*-methyltransferase (PNMT).

4. Discussion

The criteria of morphology, phenotype and developmental potential in vitro indicate that MAH cells retain many of the properties of their normal progenitor cell counterparts [2]. We have now extended these observations by showing MAH cell differentiation in vivo. The present experiments demonstrate that endogenous PNS trophic factors and elevated levels of exogenous CNTF in the CNS environment can induce the differentiation of this immortalized progenitor cell line. Co-grafting MAH cells with cell lines that did not secrete CNTF failed to drive neuronal differentiation. Moreover, the transplanted MAH cells withdraw from the cell cycle in vivo and show no sign of continued proliferation.

4.1. MAH cells withdraw from the cell cycle after transplantation

It is significant that MAH cells became post-mitotic almost immediately upon grafting. Our BrdU data indicate that MAH cells rapidly withdraw from the cell cycle subsequent to transplantation. No BrdU incorporation in the MAH cell nuclei was evident after 2 days. Snyder et al. [52] have shown that cerebellar progenitor cell lines generated via the v-*myc* oncogene are also non-tumorigenic when transplanted back into the cerebellums of newborn mice. It is currently unknown if v-*myc* continues to be expressed after the cells are grafted. Under experimental conditions, down regulation of *myc* expression is correlated with the differentiation of murine F9 teratocarcinoma cells [25]. When cells enter into the S phase of the cell cycle, decreased *myc* levels affect DNA replication in a manner that initiates a commitment to differentiation [25]. On the other hand, proliferation of v-*myc* transfected cells can apparently be suppressed without down regulation of the v-*myc* product [34,43].

4.2. Multiple trophic factors support MAH cell survival

Sympathoadrenal precursor cells and MAH cells have been extensively studied in culture to elucidate some of the molecular events that underlie the progressive differentiation of the sympathoadrenal progenitors into end cells that closely resemble chromaffin cells and sympathetic neurons [2,6,39,53]. Most recently, the low affinity p75 form of the NGF receptor has been shown to regulate *trk* signal transduction and the differentiation of MAH cells when exposed to NGF [61].

In culture, CNTF and leukemia inhibitory factor (LIF) dramatically inhibit MAH cell proliferation [30]. A similar CNTF dose also terminates the proliferation of normal progenitors from embryonic chick sympathetic ganglia [17]. In contrast, basic FGF triggers neuronal differentiation of MAH cells as well as being mitogenic. The addition of basic FGF to the tissue culture medium can lead to p75 NGF receptor expression and a small percentage (less than 1%) of the cells differentiate into sympathetic-like neurons that become dependent on NGF for their survival [6,41]. Our experiments suggest that CNTF is a factor that can also modulate the survival of MAH cells. We have showed that elevated levels of CNTF supplied by genetically modified glioma cells can drive the development of this sympathoadrenal precursor into a prominent population of sympathetic-like neurons. In addition, CNTF immunoreactivity was localized to the nuclear and cytoplasmic compartments of the sympathetic neurons in the grafts. Various subgroups of neurons in the CNS (e.g. hippocampal neurons, spinal motoneurons, Purkinje cells) have been identified by CNTF immunoreactivity in the cytoplasm and the nucleus [27]. The functional significance of CNTF in the nucleus is presently unknown.

We have identified the CNTF receptor on the MAH cells by immunocytochemical methods. The CNTF receptor consists of an α chain component anchored to the cell membrane by a glycosyl-phosphatidylinositol linkage [13], a signal transducing component referred to as gp130 [30,58] and a leukemia inhibitory factor binding protein termed LIFβ [21]. The CNTF α chain (identified with the RG30 antibody in our experiments) is a required component of the functional CNTF receptor complex and can function in a soluble form as part of a heterodimeric ligand [12].

It is not surprising that trophic factor supplement was not required to obtain post-mitotic MAH cell survival in the peripheral nerves based on the rich source of trophic factors produced by the constituent cells of peripheral nerve. Trophic factors endogenous to Schwann cells and fibroblasts include CNTF [19,38,46,56], FGF [16,24] and NGF [4]. The combination of these trophic factors may have led to the enhanced efficiency of sympathetic-like neuron development in the nerves. Indeed, synergy between CNTF, FGF and NGF drives MAH cells into post-mitotic neurons in vitro [31]. It is also interesting to note that in comparison to in vitro conditions, we showed that a

significantly greater proportion of cells (up to 25%) can form neurons in situ. This finding demonstrates the limitations of manipulating these cells in vitro.

4.3. Sympathetic neurons express phosphorylated neurofilaments in the perikarya

The antibody RT97 recognizes the medium to high molecular weight NF subunit and is specific for the phosphorylated form [40]. In the normal adult CNS this antibody identifies only axons and predominantly the distal axon segments. In contrast to central neurons, the perikarya of certain peripheral neurons are immunostained with RT97. For example, neurons in the dorsal root ganglion are recognized as sub-populations of neurons known as the light and small dark cell types [35,45]. Other antibodies that identify epitopes on the high molecular weight subunit have also been shown to recognize the neuronal perikarya in peripheral (sympathetic) ganglia [59].

In the present experiments, phosphorylated NF were detected in the somata of the large neurons. The RT97 immunoreactivity in the cytoplasm of the sympathetic neurons was not as uniform when compared to the results of Perry et al. [45] and Trojanowski et al. [59]. The differentiated sympathetic-like neurons revealed a particular strong band of RT97 antigenicity around the nucleus and this band usually contained exceptionally intense spots of staining. Clumping of NF in the sensory neurons was also indicated by Perry et al. [45].

4.4. Genetically modified cells as alternatives for cell replacement strategies

In the field of neural transplantation, genetically modified cells have the potential to increase the utility of CNS transplantation [20]. The introduction of desired genes into somatic cells offers the potential to generate cells which synthesize trophic factors [49,57] or catecholamines for neurotransmitter production [29,33,62]. In addition to the physical and chemical procedures used to transfect cells, replication-defective viruses represent a current focus for gene delivery in the nervous system. Viral mediated gene transfer into neural cells has been accomplished with defective HSV-1 vectors [14,22,23], retrovirus vectors [5,11,18] and adenovirus vectors [1,8,36].

It is now clear that clones of progenitor cells can be selectively isolated and propagated with specific growth factors from the fetal and the adult nervous system [9,26,47]. Long term primary cultures or immortalized cell lines can be fully characterized in vitro, are unlimited in supply, and can also be genetically altered before transplantation.

In conclusion, these experiments illustrate that v-*myc* oncogene transduction is a very valuable method to immortalize pluri-potential cells without unwanted proliferation subsequent to transplantation in vivo. In the present study, given the correct environmental cues and trophic factors (eg. CNTF), MAH cells rapidly become post-mitotic and differentiate into neurons with appropriate expression of neuronal specific markers. Based on the in situ survival and the absence of continued proliferation, cells modified with v-*myc* potentially represent excellent candidates in consideration of neuronal replacement approaches.

Acknowledgements

This research was supported by grants from The Parkinson Foundation of Canada and the Natural Sciences and Engineering Research Council of Canada (NSERC). Susan Birren and David Anderson kindly supplied the MAH cells. Gifts of antibodies were made available by John Wood (RT97) and Martha Bohn (PNMT). Beth Friedman and Neil Stahl of Regeneron provided helpful discussions during the course of this work. The antibodies to CNTF (#0007), CNTFRα (RG30) and the peptide NT2 were generously supplied by Regeneron Pharmaceuticals Inc. Excellent technical assistance was made by Kristen Young. We thank Tracy Roketta for outstanding secretarial help.

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