Original Article

Comparison of P19-Derived Neuroprogenitor and Naive Cell Survival after Intracerebellar Application into B6CBA Mice

(P19-derived neuroprogenitors / naive P19 cells / intracerebellar application / graft survival)

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Abstract. Mouse embryonic carcinoma cells (P19 line) were studied for both their survival and developmental potential in the intact cerebellum of B6CBA mice. The P19 cells were cultured and labelled with green fluorescent protein using transfection. Cells were used for transplantation either in the undifferentiated stage or after 3 days of neurodifferentiation induced by retinoic acid. The intracerebellar application was performed in 43 mice: group A (N = 21) received neuroprogenitors and group B (N = 22) received undifferentiated cells. The morphology of transplanted cells within the context of the surrounding cerebellar tissue was evaluated after 3 weeks. Naive P19 cells engrafted and survived in the cerebellum of 7 of the 22 adult mice (survival rate 31.8 %). Neuroprogenitors survived in 13 of the 21 mice (survival rate was 61.9 %). Since the cut-off is $P < 0.05$, the difference is not statistically significant ($P = 0.069$). An expansive appearance of the graft was significantly more frequent ($P = 0.0047$) in naive P19 cells than in neuroprogenitors. In mice in which the grafts did not survive, no marks of grafted cells or only fluorescing detritus were found. In conclusion, this is the first study to track the fate and morphology of embryonic carcinoma cells transplanted into the cerebellum, confirming that neuroprogenitors derived from embryonic carcinoma cells can settle in the host tissue and differentiate according to the surrounding conditions. With further validation, the embryonic carcinoma cells could become a valuable model with which to study the impact of cell therapy on neurodegenerative diseases.

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Abbrevations: BDNF – brain-derived neurotrophic factor, bw – body weight, CNS – central nervous system, DMEM – Dulbecco's modified Eagle's medium, EC cells – embryonic carcinoma cells, FBS – foetal bovine serum, GFP – green fluorescent protein, IIF – indirect immunofluorescence, ITS – insulin, transferrin, selenium, MAP-2 – microtubule-associated protein 2, N-CAM – neural-cell adhesion molecule, NGF – nerve growth factor, RA – retinoic acid, RT – reverse transcription, SC – stem cells, SDS – sodium dodecyl sulphate, WB – Western blot.

Introduction

Stem cell-based therapies have great potential for future treatments of various diseases. However, the limited knowledge concerning basic stem cell biology (i.e. survival, migration, differentiation and integration in real time) when transplanted into the damaged central nervous system (CNS) remains a bottleneck in attempts to design stem cell therapies for CNS diseases.

Degenerative diseases (including those of cerebellum (Manto, 2005)) and their detrimental influence on the tissue could potentially be treated using stem cells (SC), which would disrupt this vicious cycle. However, we

first have to understand how various cell types influence their surroundings.

In clinical practice, transplantation of foetal ventral mesencephalic tissue has been used in the treatment of Parkinson's disease since the late 1980s (Lindvall et al., 1990; Björklund and Lindvall, 2000). The treatment of other CNS diseases with neurotransplantation is still at the research stage. However, the treatment of other neurological diseases with neurotransplantation has already been tested in human patients with, e.g., spinal cord injuries (Sykova et al., 2006), Huntington's disease (Bachoud-Lévi et al., 2000), strokes (Kondziolka et al., 2004; Hicks and Jolkkonen, 2009) and ataxia teleangiectasia (Amariglio et al., 2009). For a review, see Kim and de Vellis (2009); Ali and Bahbahani (2010).

As part of the research on both the ideal source for cellular therapy and the regulatory pathways of regeneration, it is important to establish the role of cell differentiation on graft survival. So far, multiple papers have hypothesized that differentiated elements have a lower survival rate than naive SC (Hildebrand et al., 2005; Gulino et al., 2010).

It is still uncertain which kind of cells would be an ideal source for cellular grafts and what are the mechanisms by which stem cell transplantation leads to functional recovery and structural reorganization. In this regard, little is known, especially about the cerebellum.

The aim of our study was to compare the short-term survival of mouse embryonic carcinoma (EC) SC (line P19)-derived neuroprogenitors and naive P19 cells grafted into the cerebellum of adult B6CBA mice. SC derived from cancers were recently used for the treatment of various neurodegenerative diseases in the mouse model (Baker et al., 2000; Garbuzova-Davis et al., 2002; Bliss et al., 2006; Hara et al., 2008).

Material and Methods

Cell culture and permanent plasmid transfection

The P19 line of EC cells was isolated from a teratocarcinoma induced in the C3H/He strain of mice (McBurney and Rogers, 1982). The EC cells were purchased from the European Collection of Cell Culture, Wiltshire, UK.

Undifferentiated EC cells were cultured on tissue culture dishes pre-treated for 10 min with 0.1% aqueous solution of gelatin in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) containing 10% foetal bovine serum, 0.05 mM β-mercaptoethanol, 100 i.u. /ml penicillin, and 0.1 mg/ml streptomycin (all GIBCO BRL, Chemos CZ, Prague, Czech Republic), here referred to as standard culture medium (Pachernik et al., 2005a). These cells were genetically modified to express the green fluorescent protein (GFP) so that they could subsequently be detected after transplantation in histological sections using direct fluorescent microscopy. The permanent transfection of P19 cells was performed using GFP plasmid (Clontech Laboratories, Takara Bio Inc., Mountain View, CA) according to the CalPhos™ Mammalian Transfection Kit (Clontech).

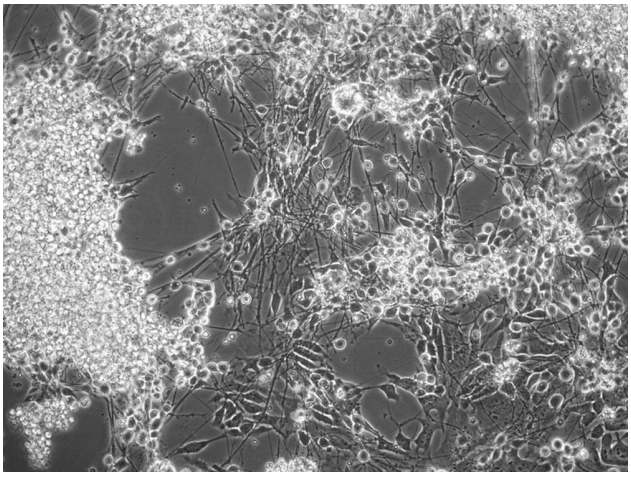

Fig. 1. Neural morphology of EC P19 cells after RA induction and 10-day culture

Differentiation of EC cells to neuronal lineages

The EC cells were cultured under serum-free conditions as described above for three days (neural progenitors) or 10 days (matured neuronal cells) in DMEM/F12 (1 : 1) media supplemented with a mixture of insulin, transferrin, selenium (ITS) and antibiotics. To induce neurogenesis, they were initially treated with retinoic acid (RA, $c = 5 \times 10^{-7}$M) for the first two days, followed by an additional day of culture without RA (Pachernik et al., 2005a, 2007). At that point, the neural progenitors were used for transplantation.

Neuroprogenitors (after three days of neurodifferentiation) and matured neuronal cells (after 10 days of neurodifferentiation, Fig. 1) were used to characterize the cell population using the Western blot, indirect immunofluorescence and quantitative reverse transcriptase real-time PCR samples of undifferentiated (naive) cells.

Western blot (WB) analysis

The cells were washed with PBS and lysed in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCl, pH 7.5; 1% SDS; 10% glycerol). Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Czech Republic, Prague, Czech Republic). Lysates were supplemented with bromophenol blue (0.01%) and β-mercaptoethanol (1%), and equal amounts of total protein (10 μg per line) were subjected to 10% SDS-PAGE. After being electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, Sigma-Aldrich), the proteins were immunodetected using the appropriate primary and secondary antibodies and visualized by ECL+Plus reagent (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. The following primary antibodies were employed: mouse monoclonal antibody against the neural-cell adhesion molecule (N-CAM; C0678, Sigma-Aldrich), mouse monoclonal antibody

against neuron-specific class III β-tubulin isotype (11-264-C100, ExBio, Vestec, Czech Republic), rabbit polyclonal antibody against p27 (sc-528, Santa Cruz Biotech., Santa Cruz, CA), rabbit polyclonal antibody against Oct-3/4 (sc-9081, Santa Cruz Biotech.), and rabbit polyclonal antibody against Cdk4 (sc-260, Santa Cruz Biotech.).

Indirect immunofluorescence (IIF)

The cells were fixed in 4% formaldehyde for 30 min on ice and permeabilized in 0.2% Triton X 100 with 0.5% Nonidet NP 40 for 10 min. These conditioned cells were then quenched with 1% FBS in PBS for 10 min and incubated with the appropriate primary (overnight at 4 min) and FITC-conjugated secondary antibodies (10 min at room temperature) (FITC: anti-mouse IgG+IgM-Invitrogen /Carlsbad, CA/ and anti-mouse IgG-Sigma-Aldrich; Alexa Fluor 568: anti-mouse IgG-Invitrogen; Table 1). The specimens were mounted in DAPI/Antifade solution and viewed under an epifluorescent microscope (Olympus BX 41, Olympus Czech Group, Prague, Czech Republic). The following primary antibodies were employed: mouse monoclonal antibody against microtubule-associated protein 2 (MAP-2; M4403, Sigma-Aldrich), rabbit polyclonal antibody against Oct-3/4 (sc-9081, Santa Cruz Biotech.), and mouse monoclonal antibody against Lewis X antigen (FORSE-1, developed by Dr. Patterson); the latter was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, http://www.uiowa.edu/~dshbwww). The dilutions used for the detection are shown in Table 1.

Quantitative reverse transcriptase real-time PCR

The entire RNA was isolated from each cell preparation using the Fast RNA Pro Green Kit (Q-BIOgene, Irvine, CA). The reverse transcription (RT) was performed with 3 μg of the entire RNA using Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA) and oligo $d(T)_{21}$ as a primer.

The *HPRT* was detected as a housekeeping gene, *Pax6* and *Mash1* as markers of the neuronal phenotype. The sequences of the primers used for mRNA quantification are shown in Table 2 (Esner et al., 2002; Pachernik et al., 2005a). These primers were synthesized by GeneriBiotech (Hradec Králové, Czech Republic). A quantitative estimation was performed using an iCycler apparatus (Bio-Rad). The melt curve analysis was used to verify specificity. PCR products used for the quantification were separated on 1.5 % agarose gels and visualized by ethidium bromide staining.

Graft preparation

For the transplantation, naive P19 cells or neuroprogenitors (after three days of neurodifferentiation) were isolated by trypsin, which was neutralized by adding DMEM with serum and then centrifuged and resuspended in DMEM to give a final cell concentration of 50 000 viable cells/μl. The cells were then grafted within one hour.

Animals

Normal adult mice of the B6CBA strain were used (N = 43, 17 males, 26 females). Their mean age at the time of transplantation was 284.6 days (SD = 45.6 days, minimum = 209 days, maximum = 385 days). The mice were reared in standard conventional conditions with 12 : 12 hours light : dark cycle (6 am – 6 pm), temperature 22–24 °C. Water and food were available *ad libitum*. The mice were housed in plastic cages with metal mesh covers (11 × 25 × 14 cm for 1–2 mice or 18 × 25 cm × 14 cm for 2–4 mice). Twenty-two mice (9 males and 13 females) were treated with naive P19 cells. Twenty-one animals (8 males and 13 females) received neuroprogenitors.

Table 1. Antibodies used for immunocytochemistry

EC cells	Primary antibodies	Dilution	Secondary antibodies	Dilution
undifferentiated	Hybridoma FORSE-1 (Developmental Studies Hybridoma Bank, University of Iowa)	1 : 1	anti-mouse IgG+IgM (Invitrogen)	1 : 750
	Oct-3/4 (Santa Cruz Biotechnology, Inc.)	1 : 200	anti-mouse IgG Alexa Fluor 568 (Invitrogen)	1 : 200
differentiated	MAP-2 (Sigma-Aldrich)	1 : 500	anti-mouse IgG (Sigma-Aldrich)	1 : 150

Table 2. Primers used for gene expression analysis, HPRT (a housekeeping gene), Pax6 and Mash1 (neuronal phenotype)

Genes	Forward primer	Reverse primer	Length (bp)
Pax6	5‘-TGCCCTTCCATCTTTGCTTG-3‘	5‘-TCTGCCCGTTCAACATCCTTAG-3‘	178
Mash1	5‘-CTCGTCCTCTCCGGAACTGATG-3‘	5‘-CGACAGGACGCCCGCCTGAAAG-3‘	303
HPRT	5‘-CTTGCTGGTGAAAAGGACCTCTC-3‘	5‘-CAAATCAAAAGTCTGGGGACGC-3‘	350

Transplantation

The host mice were anaesthetized with intraperitoneal administration of a combination of Ketamine (100 mg/kg bw) and Xylazine (16 mg/kg bw). The parietal and occipital area of the head was shaved and disinfected. Then the mice were fixed on a stereotaxic holder. The soft tissues of the occipital and dorsal parietal area of the head were cut at the midline. A hole (2 mm in diameter) was drilled in the occipital bone (Bregma – 7.0 mm, midline), and 1 μl of cell suspension (a total amount of 50 000 cells) was injected with a Hamilton syringe at a constant speed for 120 s. The tip of the needle was inserted 1.6 mm under the surface of the cerebellum and was elevated to a depth of 1.5 mm during the administration of the cell suspension. Upon completion of the administration, the needle remained *in situ* for 5 min to prevent the return of the cells out of the host head. Finally, the wound was sutured in one layer with Chirlac rapid (Chirmax GmbH, Ratingen, Germany) and disinfected. Immunosupression was not used in spite of using allografts because the CNS is an immuno-priviledged organ (Rosenfeld et al., 2003; Li et al., 2006; Kwiatkowska-Patzer et al., 2009).

Histopathological analysis

The mice were sacrificed 21 days after the transplantation by overdosing them with Thiopental (0.2 ml of a 10% solution intraperitoneally) and transcardially perfused with phosphate-buffered solution (pH 7.4) and 4% phosphate-buffered paraformaldehyde. Their brainstems and cerebella were stored for several days in 4% paraformaldehyde for post-fixation and then overnight in 20% sucrose for cryoprotection. The specimens were then sectioned with a cryostat (40 μm frontal sections). The sections were pooled in a phosphate-buffered solution (pH 7.4) and examined under a fluorescent microscope. The grafted and graft-derived GFP-positive cells were detected according to their green fluorescence in the native sections. The sections were mounted on gelatinized glass slides, and each second section was stained with haematoxylin-eosin, while the remaining sections were stained according to the Nissl technique with 1% Thionin Blue to visualize the histological structure of the graft and surrounding host tissue.

Statistical evaluation

The number and percentage of mice in which the graft survived (the presence of at least some GFP cells) and the frequency of the occurrence of various features of the grafts (the destruction of the tissue and characteristic growth of the graft) were assessed. Differences between the groups of mice treated with naive P19 cells and mice that received neuroprogenitors were evaluated using Fisher's test. In all cases, the differences were considered significant if $P < 0.05$.

All experiments reported here were performed in full compliance with the EU guidelines for scientific experimentation on animals and with the permission of the Ethical Commission of the Charles University in Prague – Faculty of Medicine in Pilsen.

Results

Neural differentiation of P19 cells in vitro

Prior to the transplantation experiments, we verified the *in vitro* neural commitment of P19 cells according to the protocol described above. The undifferentiated cells had a uniform morphology and were positive for the Oct-3/4 (Fig. 2) and LewisX antigen (IIF). However, the neural/glial markers were not detected (not shown).

After three days of differentiation the cells were positive for MAP-2 and the RT-PCR analysis confirmed an elevated expression of Pax-6 and Mash-1 (Babuska et al., 2010).

After 10 days of differentiation, cells formed colonies with expanding neuron-like phenotypes (Fig. 1). The elements adopted neural morphology by first rounding up, creating protrusions, and finally organizing into neurosphere-like colonies with expanding neurites positive for MAP-2 (IIF). According to their morphology and differentiation status, the cells that differentiated into neural lineages tested positive for both neural markers (N-CAM and β III-tubulin) according to the WB (Fig. 2), and the maternal, non-differentiated cells tested positive for Oct-3/4. The results of the RT-PCR analysis also confirmed the expected cell phenotype (Babuska et al., 2010).

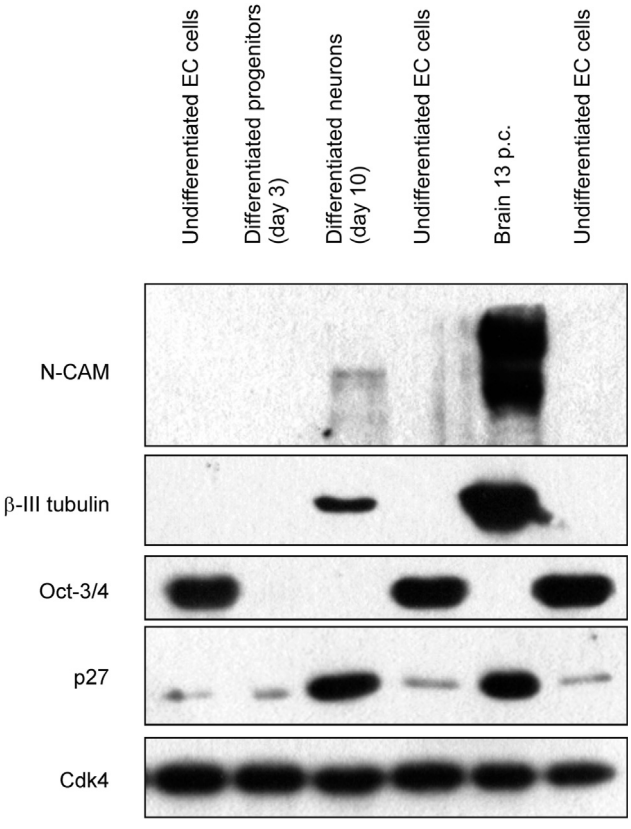

Fig. 2. Characterization of undifferentiated EC cells, neuroprogenitors and mature neural cells using WB

Survival of naive cells and neuroprogenitors in the cerebellum

Naive P19 cells grafted into the cerebellum of adult mice survived in seven animals and were not detectable in 15 mice (no marks of grafted cells or only fluorescing detritus).

Grafted neuroprogenitors survived in 13 of the 21 mice. Accordingly, the naive P19 cells survived in 31.8 % of the mice, while the survival rate of neuroprogenitor grafts was 61.9 %. This difference is statistically insignificant ($P = 0.069$).

In cases of survival, both P19 cells and neuroprogenitors formed a separated mass of GFP-positive cells (either grafted or graft-derived) and of non-fluorescent tissue, which means that the GFP cells were dispersed in non-fluorescent tissue (Fig. 3A, B, C, D). The mass was usually situated in the vicinity of the injection site in the middle of the cerebellum. In one case (neuroprogenitors), the mass extended to the mesencephalon, in another case (neuroprogenitors), it was localized between the cerebellum and the mesencephalon, and in one mouse (naive P19) GFP cell-containing tissue was found on the surface of the brainstem, inside the aqueduct and in the mesencephalon (Fig. 3E). In all cases, the mass was delimited from the host cerebellum, and no signs of dispersion outside of the mass were observed (Fig. 3A, B, C, D). Some of the cells clearly differentiated further in the cerebellum into a neuron-like phenotype and showed multiple processes (Fig. 4). Besides the cells, a fluorescing detritus was found in the masses.

In six of the 13 animals with surviving neuroprogenitor grafts, a cavity in the middle of the accumulation of the GFP-positive cells was observed (Fig. 3F). We assume that the cavity is a sign of tissue destruction. In the mice that received naive P19 cells, no cavity or other signs of tissue destruction were observed. A comparison of the presence of tissue destruction in the neuroprogenitors and P19-derived tissue masses had not shown any statistical significance yet ($P = 0.0515$).

In four mice that received neuroprogenitor grafts and in seven mice that were transplanted with naive P19 cells, the grafts formed singular large spherical masses, which disrupted the surface of the host cerebellum and/or affected its shape (Fig. 3A; compare with non-expansive appearance shown in Fig. 3B). This expansive ap-

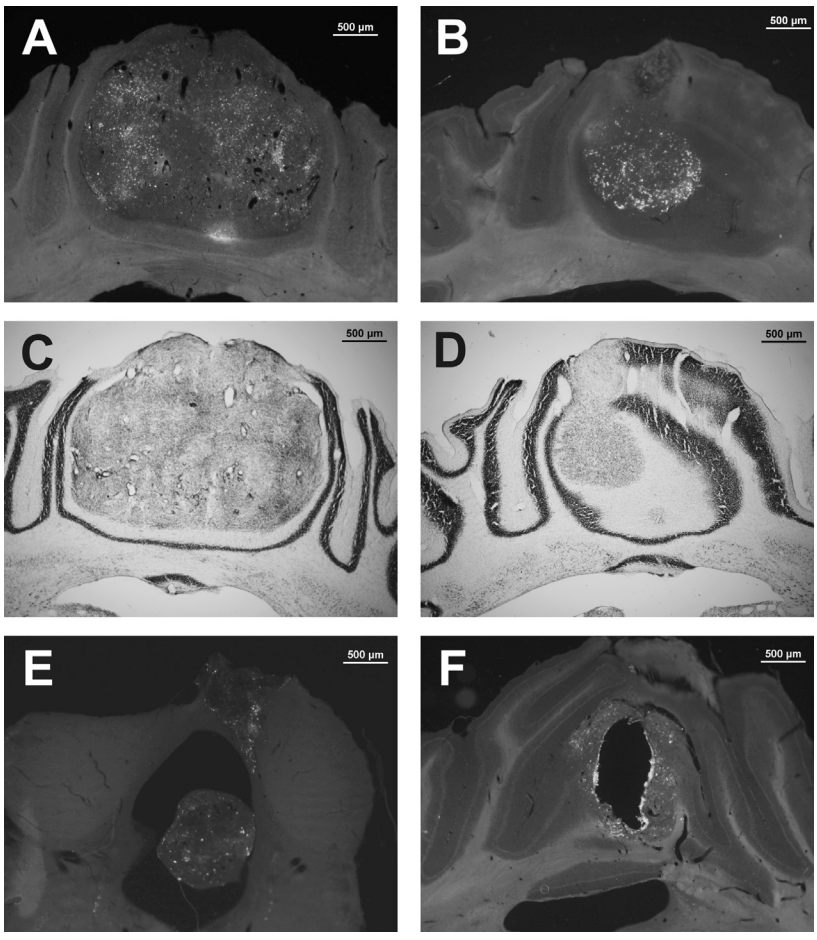

Fig. 3. Examples of graft morphology. Expansive growth showing a graft in a mouse treated with naive P19 cells, native GFP fluorescence (A), Nissl staining (C). Non-expansive growth showing a graft in a mouse treated with neuroprogenitor cells, native GFP fluorescence (B), Nissl staining (D). GFP cells containing tissue in the aqueduct in a mouse treated with naive P19 cells, native GFP fluorescence (E). A cavity in the middle of the accumulation of the GFP-positive cells in a mouse treated with neuroprogenitor cells, native GFP fluorescence (F).

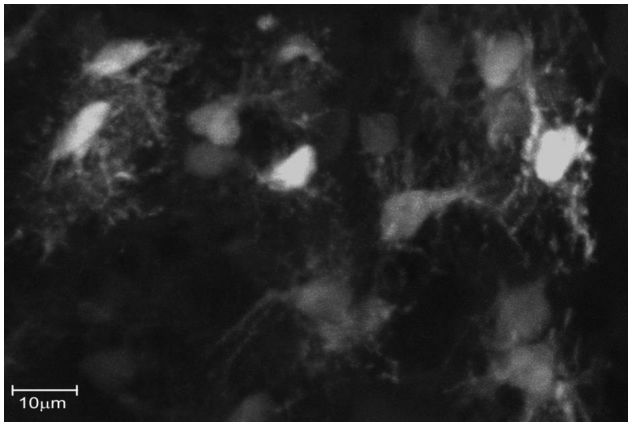

Fig. 4. Neuron-like phenotype of GFP-positive cells grafted into the cerebellum

pearance of the graft was significantly more frequent ($P = 0.0047$) in naive P19 cells than in neuroprogenitors.

These 11 cases of the expansive appearance were examined for the presence of tissue destruction. The signs of destruction could only be found in one case compared with nine cases showing no signs of expansive growth of the grafted cells in which five cases of tissue destruction were detected. This proves that tissue destruction was significantly more frequent in tissue masses without any signs of expansive growth ($P = 0.0498$).

Discussion

Most current studies agree that neurodegeneration may be influenced by SC therapies and neurotransplantation. Many alternatives have been examined, including xenografts, genetically engineered cells, immortalized cell lines, or paraneural cells that secrete specific neurotrophic or growth factors. Accumulating evidence suggests that exogenous treatment with neurotrophic or growth factors, immunosuppressants, free radical scavengers or anti-apoptotic agents can enhance the survival and functional effects of grafts (Ali et al., 2009; Buzanska et al., 2009; Karimi-Abdolrezaee et al., 2010).

The P19 embryonic carcinoma-derived cell line consists of undifferentiated pluripotent cells, which irreversibly differentiate into neuroprogenitors and then into mature neurons after exposure to RA (Pachernik et al., 2005b). Additionally, the cancer cell lines can be beneficial in cases of certain neurodegenerations (Baker et al., 2000; Nakao et al., 2000; Garbuzova-Davis et al., 2002). It has also been suggested that P19 might be considered for cell replacement strategies in neurodegenerative disorders of the CNS (Morassutti et al., 1994; Watanabe et al. 1996).

Our study is the first to follow the fate of transplanted EC cells into the cerebellum. The survival of cells was examined in animals that were not affected by any neurodegenerative process. Our results confirm that EC cells can survive in the host tissue. The survival rate of the neuroprogenitor grafts was comparable to that of solid embryonic cerebellar grafts (Cendelin et al., 2009a, b). The naive P19 cells survived less than neuroprogenitor grafts (31.8 % versus 61.9 %). The fact that there is no statistically significant difference in the survival of naive cells and neuroprogenitors is in contrast with the results published in some previous studies (Hildebrand et al., 2005; Gulino et al., 2010).

The destruction of the graft-derived tissue mass appeared exclusively in neuroprogenitor grafts and never in the case of naive P19 cells. Although the difference was not statistically significant, it could be hypothesized that the degree of differentiation might have an impact on the presence of cavities and tissue destruction. A larger study is needed to evaluate this further, probably with a longer survival of the experimental mice.

Our results prove that the expansive appearance of the tissue mass is significantly more frequent after transplantation of naive P19 cells, which is probably in accordance with the pluripotency of these naive cells.

The presence of destruction is significantly more frequent in the masses without any signs of expansive growth. We hypothesize that the destruction of the graft-derived tissue mass is a P19-specific effect. In P19 cells, neurogenin, a proneural basic helix-loop-helix transcription factor, plays a central role in promoting neuronal specification and differentiation in many regions of the CNS and is highly unstable compared to other stem cell lines (Vosper et al., 2007). That is why we plan to compare our results with other stem cell lines in both conditions – with and without the presence of neurodegenerative processes in the experimental mice.

Grafted cells did not show any tendency to migrate and spread through the host cerebellum, contrary to some previous experiments with grafted granule and Purkinje cells (Kawamura et al., 1988). The detected morphology does not suggest any strong and specifically functional effect which requires the involvement of grafted cells in the neural circuitries of the host (Kawamura et al., 1988; Rossi and Cattaneo, 2002; Zawadska et al., 2009; Rolando et al., 2010). It might be useful to enhance nerve fibre growth, cell migration and synaptic plasticity with the addition of some of the neurotrophic factors (NGF, BDNF) (Vaynman et al., 2003, 2004, 2006; Molteni et al., 2004) which also have anti-apoptotic effects on neural cells and can therefore improve the survival of grafted neuroprogenitors and prevent tissue destruction (Perrelet et al., 2000).

In this pilot study, we focused on the basic examination of graft survival and structure, which provided information for the design of future experiments. We are aware of the fact that it will be necessary to determine the cell types in which grafted cells differentiated in the host cerebellum. The functional impact of the transplantation into the cerebellum will also have to be assessed with a series of behavioural tests in a cerebellar disorder model.

Transplantation of EC cells is considered to be dangerous due to the tendency of these cells to create donor-derived carcinoma. Nevertheless, it has been shown that

once they have differentiated, they lose their tumorigenic capacity (Garbuzova-Davis et al., 2002). We have shown that the *in vitro* differentiation of EC cells into neuroprogenitors does not lead to a decrease of the survival rate of the graft and that it decreases the tendency of the graft towards expansive growth. With further validation, EC cells might well become a valuable model for the cell therapy of neurodegenerative diseases.

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