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Transplantation of neural stem cells derived from human cord blood to the brain of adult and neonatal rats

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Searching for a reliable source of alternative neural stem cells for experimental treatment of neurological disorders we have established neural stem cell line derived from human umbilical cord blood (HUCB-NSC) (Buzanska et al. 2006). These cells have been shown to differentiate along neuronal and glial lineages in the promoting *in vitro* conditions. In the current study we transplanted HUCB-NSC into rat brain to determine whether the neural progenitors would be able to survive, migrate and eventually adopt neural phenotypes after exposure to central nervous system (CNS) microenvironment. Our experiments revealed that HUCB-NSC grafting into the brain of adult rats limited their survival up-to two weeks probably due to their elimination by severe immunological host reaction evoked by xenotransplantation. HUCB-NSC graft in neonates survived longer time in rat brain, migrated, proliferated and differentiated into neuronal cells however their presence in the host tissue did not exceed more than five weeks after transplantation.

Key words: cord blood, cell transplantation, immune response, neural stem cells, rat brain

INTRODUCTION

Cell replacement therapy is a widely discussed novel concept of medical treatment in various diseases characterized by the loss of functional cells. The traumatic and ischemic injuries to the central nervous system are attributed to the acute as well as progressive loss of neurons and glial cells. Some interventions during the acute phase of the insult such as thrombolytic agents have been recognized to improve the outcome of patients however, since there is no treatment to halt or reverse neurodegeneration, stem cell-based approaches could be used therapeutically to restore function of CNS. This might be achieved by the replacement of lost cells or by inducing endogenous neurogenesis in the adult brain. Besides these mechanisms, grafted stem cells could encourage functional improvement by releasing different molecules that are neuroprotective or modulate inflammation.

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Due to the different studies, neural stem cells (NSC) are regarded as the ideal cell type for reparation of the damaged neural tissue because they most closely resemble the cell type lost in pathological processes and may respond better for differentiating guidance cues from the local microenvironment. Although brain-derived NSC have received the most attention as a source of cells for neural cell replacement the other sources of non-CNS-derived somatic stem cells that are capable of neural differentiation are also subjects of active investigation. The alternative sources of NSC might be that of bone marrow (Jiang et al. 2003, Hermann et al. 2004), peripheral blood (Zhao et al. 2003), umbilical cord blood (Sanchez-Ramos et al. 2001, Buzanska at al. 2002, Kögler et al. 2004, McGuckin et al. 2004, Ali et al. 2009), amniotic fluid (Tsai et al. 2006, De Coppi et al. 2007), Wharton jelly (Mitchell et al. 2003, Wang et al. 2004), skin (Joannides et al. 2004, Metcalfe and Ferguson 2008) or adipose tissue (Safford et al. 2002, Kim et al. 2007). For tissue regeneration, these stem cells are either pre-differentiated *in vitro* to the required tissue type before application or they are applied directly without prior neural

commitment following the concept that signals in the injured tissue will recruit the stem cells and induce their differentiation *in situ*. Besides proliferative and differentiation potential of the stem/progenitor cells, there are other parameters, which also have an impact on their suitability for therapy. These include sufficient cell numbers, biological and epidemiological safety as well as low immunogenicity after transplantation.

In contrast to other adult cell sources, stem cells in cord blood are less mature and have a higher proliferative potential associated with their immaturity, extended life span and longer telomeres (Kögler et al. 2004, Lindvall et al. 2004). Besides this biological superiority, cord blood is abundantly available, routinely harvested without risk to the donor and viral infection is rare exception.

During the past two decades human umbilical cord has emerged a great interest as a novel valuable source for stem cells which possess several unique characteristics (Sanberg et al. 2005, Zandonella 2005, Habich et al. 2006, McGuckin et al. 2008, Copeland et al. 2009). Reports of cord blood stem cells turning into brain cells, both *in vitro* and *in vivo* have been consistent and incessant. In the past few years many papers show how subsets of cord blood stem cells differentiate under defined conditions into neurons, astrocytes and microglia. Equally impressive, and not less frequent, are the reports of the myriad trophic factors secreted by cord blood cells for initiating and maintaining the process of CNS repair (Borlongan et al. 2004, Fan et al. 2005).

Our laboratory has successfully derived from human umbilical cord blood a non-immortalized cell line with properties of NSC (Buzanska et al. 2006). This cell line serves as a useful prototype for the ideal transplantable material and based on our *in vitro* studies it has a strong potential for clinical use (Jurga et al. 2006, Domanska-Janik et al. 2008, Jurga et al. 2009, Sypecka et al. 2009).

However, for the successful development of stem cell-based therapies for neurological diseases different issues in basic and experimental research need to be reached before such treatment can be tested in patients. In particular, we need to better understand the mechanisms of action of stem cells after transplantation and learn how to control stem cell proliferation, survival, migration and differentiation in CNS environment.

Cells which are to be considered for clinical transplantation are preferred to be of human origin. This presents a predicament to test the human cells in animals. Xenotransplantation of human cells into the rodent is a widely used research paradigm that often produces encouraging results in terms of reduction of behavioral deficits however the proliferation, long term survival and maturation of xenografted cells in the host milieu is more challenging.

In the current study we injected HUCB-NSC into the rat brain to determine whether these cells would be able to survive, migrate and eventually adopt neural phenotypes after exposure to CNS microenvironment. Since the long time observation of human cells after transplantation into adult rat brain could be less successful, due to immunological and inflammatory reaction, the same cell graft was applied in neonatal recipients. Our experiments revealed that HUCB-NSC grafting into the brain of adult rats limited their survival up-to two weeks. Severe immunological response of the host against transplanted cells suggests that hostile rat microenvironment dictates the fate of neural progenitors derived from human cord blood. HUCB-NSC grafted into immunologically immature neonatal rat brain survives longer time. The donor cells migrated, retained their proliferation and differentiation potential along neuronal lineage however their presence in the rat brain did not exceed five weeks.

METHODS

Human umbilical cord blood-derived neural-like stem cell line (HUCB-NSC)

The cell line was derived from human umbilical cord blood and cultured in low serum medium (LS) containing Dulbecco's modified Eagle medium (DMEM) and F12 (Gibco) supplemented with 2% fetal bovine serum (FBS; Gibco), insulin, transferrin, and selenium (ITS, 1:100; Gibco), and antibiotic antimycotic solution (AAS, 1:100; Sigma). Under these conditions HUCB-NSC grew as two subpopulations, floating and adherent cells. At 24 h before transplantation, HUCB-NSC were moved into serum free Neurobasal (NB) medium with B27 to reduce serum level and the risk of recipient immunological reaction. Only floating HUCB-NSC were taken for transplantation.

Animals

The experiments were performed on adult male rats weighted 250 g and newborn (P0) pups of Wistar

strain. Throughout the experiments, animals were housed in plastic cages with a 12 h light-dark cycle and free access to food and water. All procedures complied with EU guidelines for the use of animals in research and were approved by the Fourth Warsaw Local Ethics Committee.

HUCB-NSC labeling with CMFDA cell tracker

HUCB-NSC preconditioned with NB medium and B27 for 24 h were collected by and suspended in NB medium and 10 mM 5-chloromethyl-fluorescein-diacetate (CMFDA; 1µl/500µl NB) cell tracker was added. The cells were incubated at 37ºC for 30 min, washed twice with serum free NB medium and counted using light microscope. Immediately before transplantation HUCB-NSC were re-suspended in saline and assessed with Trypan Blue exclusion test for viability, which was not lower than 95–98% in all experiments.

CMFDA-labeled HUCB-NSC transplantation in adult rats

Adult Wistar rats were anaesthetized with ketamin (90 mg/kg) and xylazine (10 mg/kg) given by i.p. injection and immobilized in a stereotactic apparatus (Stoelting). Then an incision was made through the skin overlying the sagital suture and a small burr hole was drilled in the cranium over the right hemisphere. The needle (length 15 mm, gauge 33) connected with a 5 µl syringe Hamilton was lowered into corpus callosum (coordinates: A 0.0, L 4.0, V 3.0 mm where the bregma was adjusted to the same horizontal plane, and the ventral coordinates were calculated from the dura) and 2 µl of HUCB-NSC labeled with CMFDA ($2x10^4$) were injected at a rate of 0.5 µl/min via microinfusion pump (Stoelting) mounted on stereotactic apparatus. After injection, the needle was left in situ for 5 min to avoid the leakage of injected cells through the needle tract. Then the needle was withdrawn and the skin closed with a suture.

CMFDA-labeled HUCB-NSC transplantation in newborn (P0) rats

Newborn (P0) Wistar rats from separate litters were anesthetized by hypothermia according to the procedure described previously (Zigova 2002). Briefly, the newborn rats were mounted in Cunningham Mouse and Neonatal Rat Adaptor of the stereotactic apparatus (Stoelting) and an incision was made through the skin overlying sagittal suture. A small hole was created in the skull on the right hemisphere (1 mm right from the midline, 2 mm anterior to Bregma), and a Hamilton syringe was lowered (2 mm deep to the pial surface) through the opening in the skull and 2 µl of HUCB-NSC labeled with CMFDA ($2x10^4$) slowly delivered into the subventricular zone (SVZ) at a rate of 0.4 µl/min. After transplantation, the skin was repositioned and sealed with surgical glue. Then the pups were placed under a heat lamp for recovery before returning them to their mother.

Brain tissue preparation and fixation

Rats were deeply anaesthetized with pentobarbital (65 mg/kg i.p.) 1, 3, 7, 14, 21 or 35 days after HUCB-NSC transplantation. The brains were removed, immediately frozen with dry ice, and stored at -70ºC. Before sectioning, the brains were placed at -20ºC overnight. Coronal tissue sections, 20 µm thick, were cut in a cryostat and mounted on superfrost microscope slides and then stored at -70ºC until immunohistochemistry was performed.

Immunohistochemistry and confocal microscopy analysis

For immunofluorescence analysis the sections were air dried at room temperature 30 minutes, and fixed with freshly prepared 4% PFA in PBS (pH 7.4) for 15 minutes. Before incubation with primary antibodies, non-specific binding was blocked with normal goat serum or bovine serum albumin (1:10, diluted with 0.1% Triton X-100) for 60 minutes. Then the primary antibodies were applied and the brain slices were incubated overnight at 4ºC. To determine the neural fate of implanted HUCB-NSC specific antibodies were used: anti-NF200 (Sigma, 1:400), anti-β tubulin-III (TUJ1) (Covance, 1:750) and anti-MAP-2 (Sigma, 1:1000) as neuronal markers, anti-GFAP (Cappel, 1:100) and anti-S100β (Swant, 1:1000) as astrocyte markers and anti-GalC (Invitrogen, 1:500) as an oligodendrocyte marker. For characterization of the host immune response the following antibodies were applied: anti-CD5 (Serotec, 1:25) as T cell, anti-CD45 (Serotec, 1:100) as B cell, anti-CD68 (ED1) (Serotec, 1:100), as macrophages/microglia, anti-CD15 (Serotec, 1:50) as neutrophil and

anti-IgM (Southern Biotech, 1:500) markers. Additionally to identify the presence of transplanted HUCB-NSC in the rat brain tissue, the primary antibodies were used: anti-NuMA (Calbiochem, 1:50) against human nuclear antigens, anti-HuNu (Millipore, 1:100) against human nuclei, anti-HuMi (Millipore, 1:50) against human mitochondria proteins and anti-Ki67 (Leica, 1:100) as a marker of proliferating cells. After rinsing in PBS, the rat brain sections were exposed to goat anti-mouse IgG1 Alexa Fluor 546, goat anti-mouse IgG2a Alexa Fluor 546, goat anti-mouse IgG2b Alexa Fluor 546 (Invitrogen, 1:500), goat anti-mouse IgG3 Texas Red-conjugated (Southern Biotechnology, 1:500), or goat anti-rabbit IgG (H+L) Alexa Fluor 546-conjugated (Invitrogen, 1:500) secondary antibody, for 60 minutes at RT in the dark. Additionally cell nuclei were stained with 5 μM Hoechst 33258. The adjacent sections were used as negative controls. All procedures for negative controls were processed in the same manner except the primary antibodies were omitted. A confocal laser scanning microscope (Zeiss LSM 510) was used to obtain detailed images of the positively stained cells. A helium–neon laser (543 nm) was used for excitation of Alexa Fluor 546. An argon laser (488 nm) was used for the excitation of CMFDA and diode 405 nm was used for excitation of Hoechst. Following acquisition, images were processed using the software package ZEN 2008.

RESULTS

Morphology, proliferation capacity and phenotype of HUCB-NSC

HUCB-NSC cultured in NB medium revealed round shape with non-differentiated morphology. The proliferation rate of HUCB-NSC visualized by using Ki67 marker - a nuclear protein expressed only in the active phase of the cell cycle, was 30%. Immunocytochemical staining for neural specific markers showed 96.5% of Nestin-positive cells and 39.9% of GFAP-positive cells. The GFAP staining in HUCB-NSC was localized in cytoplasm and lacked the typical filamentous appearance characteristic to GFAP polymers in mature astrocytes. The similar type of Nestn/GFAP co-expressing has been demonstrated as typical for neural progenitor B cells observed in SVZ of developing brain (Doetsch 2003).

We also determined whether the culture environment being used before transplantation could induce HUCB-NSC to express more mature neural phenotypes. However no staining for more advanced neural phenotypes i.e. NF-200, β-tubulin III, MAP-2, S-100β and GalC was found in HUCB-NSC cultured in NB medium.

Distribution and phenotype of HUCB-NSC after transplantation into the brain of adult rats

One day after HUCB-NSC transplantation into the brain of adult rats, most donor cells remained in the injection site and only few cells migrated in the brain tissue. Among the donor cells visible in the graft core proliferating (Ki-67$^+$) cells was observed (Fig. 1A). Most of the transplanted HUCB-NSC stayed undifferentiated with only a few cells expressing more mature neuronal (NF-200$^+$ and TUJ-1$^+$) or astrocytic (GFAP$^+$) phenotypes (Fig. 1B, C, D). At the 3rd day after HUCB-NSC transplantation the proliferation rate of donor cells increased (Fig. 1E) and the most of cells visible in the graft displayed neural markers (NF-200, TUJ-1, GFAP) (Fig. 1F, G, H). After 7 days of HUCB-NSC grafting the migration of the donor cells along white matter fibers was evident. These cells stayed viable however proliferating cells (Ki-67$^+$) were scarcely found among them (Fig. 1I). Also only a few donor cells distinguishable in the rat brain at that time presented the neural differentiation markers (NF-200$^+$, TUJ-1$^+$ or GFAP$^+$) (Fig. 1J, K, L). At 14th day most of HUCB-NSC underwent successive degeneration and subsequent depletion with only single human cells retained viable in the host tissue. These cells did not reveal immunoreactivity to proliferation marker Ki-67 (Fig.1M) or to any other neural (NF-200, TUJ-1, GFAP) antigens (Fig. 1N-P).

Immune response of the host after HUCB-NSC transplantation into adult rat brain

Twenty four hours after HUCB-NSC transplantation into the brain of adult rats the infiltration of macrophage/microglia (ED1$^+$cells) and neutrophils (CD15$^+$cells) with the rare occurrence of T lymphocytes (CD5$^+$) cells was observed around the graft (Fig. 2A-C). At the 3rd day after HUCB-NSC transplantation the infiltrate of ED1$^+$ cells increased but the number of CD15$^+$ and CD5$^+$ cells remained unchanged (Fig.

2E-G). Additionally IgM-class immunoglobulin immunoreactivity was seen around the edges of the graft (Fig. 2H). Seven days after HUCB-NSC grafting, the total number of cells expressing ED1 and CD15 markers was reduced dramatically compare to the 3rd day and exceptional $CD5^+$ cells were visible (Fig. 2I-K). No clear temporal evaluation in humoral response could be discerned: specific IgM immunoreactivity through-

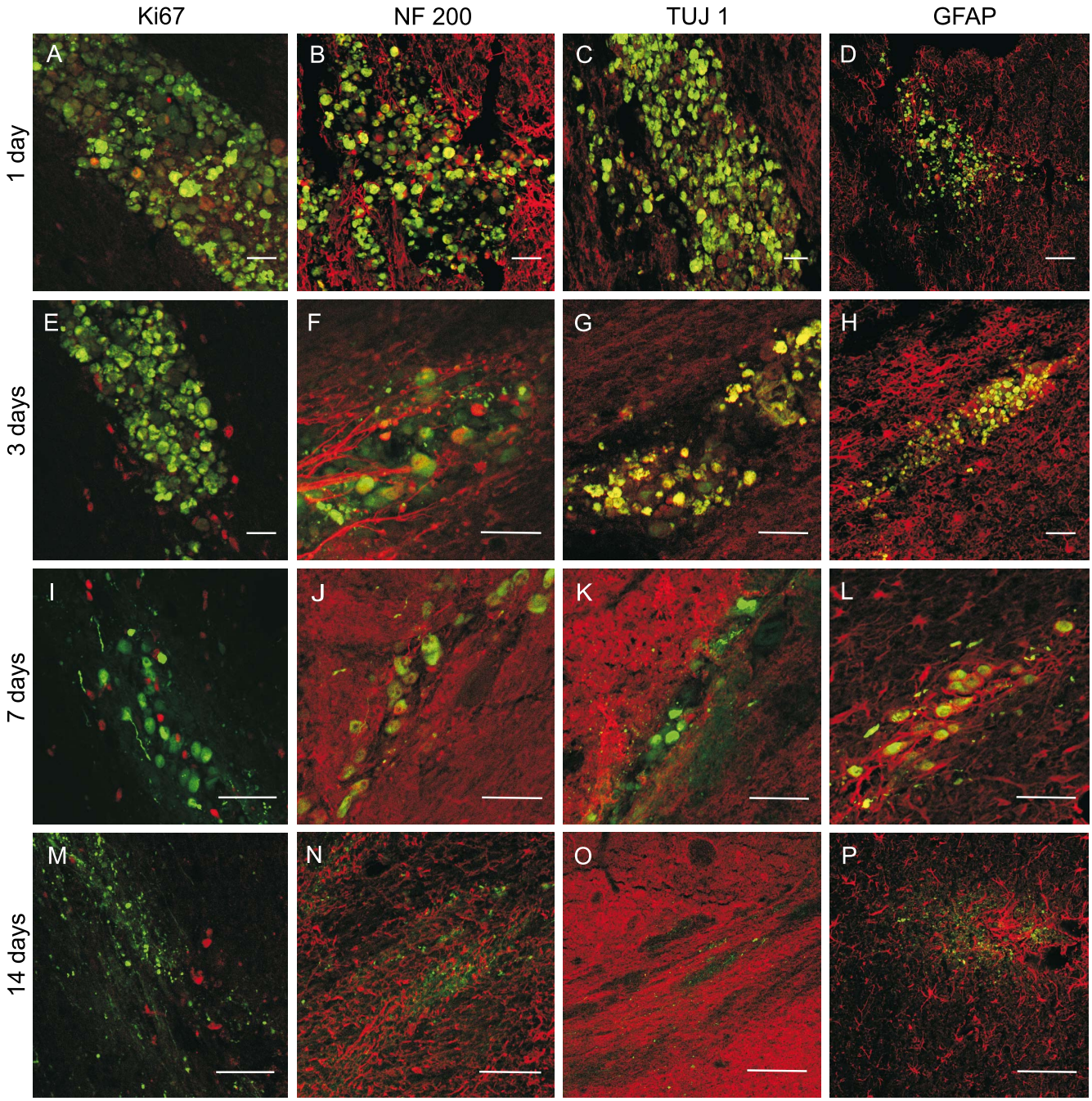

Fig.1. Immunocytochemical analysis of HUCB-NSC present in adult rat brain 1, 3, 7 and 14 days after transplantation. Double labeling studies followed by confocal imaging detected HUCB-NSC colored green by CMFDA and red by Alexa Fluor 546 after phenotype specific immunoreaction with antibodies against: Ki-67 – proliferation marker expressing cells (A,E,I,M), neuron specific immunostaining for NF-200 (B,F,J,N) and TUJ-1 (C,G,K,O) or GFAP- astrocytic marker (D,H,L,P). Co-localization of red and green labeling appears yellow and overlaying these images. Scale bar = 50 μm.

out the grafted corpus callosum and striatum was equally likely to be present at early time-points (Fig. 2L). By 14 days, only limited number of ED1$^+$ CD15$^+$ and CD5$^+$ cells were found most likely due to the scar formation and rejection of HUCB-NSC since no viable HUCB-NSC have been noticed remaining in the core of the graft (Fig. 2M-O). Concomitantly, low level of IgM was seen to bind to the grafted cells, the majority of which appeared dystrophic (Fig. 2P).

Transplantation of HUCB-NSC into SVZ of the newborn rats

One day after HUCB-NSC transplantation in close vicinity of SVZ in neonatal rats the donor cells appeared as round cells forming a dense deposit with minimal ability to disperse and migrate into the brain tissue. Few of the grafted cells present at the periphery of the transplant and among migratory cells expressed Ki67 (Fig. 3A). A small number of human cells adopted early neuronal marker NF-200 (Fig. 3B). At the 3rd day after HUCB-NSC transplantation in neonatal rats the donor cells were found in the anterior part of the SVZ and along the full extent of the rostral migratory stream (RMS). Occasionally they were also visible in the lumen of cerebral lateral ventricle and choroid plexus. Moderate proliferation of HUCB-NSC (Ki67$^+$cells) was observed mainly in the injection site. Most of HUCB-NSC remained undifferentiated but some of them resembled the morphology of migrating neuroblasts, usually with one or two relatively short processes. Phenotypically, these cells displayed presence of neuronal antigens NF-200 (Fig.3F and inset). At the same time, transplanted HUCB-NSC acquired more mature neuron-specific class III β-Tubulin (TUJ-1) immunoreactivity (Fig. 3G). Within one week, a lot of donor cells were observed within the vertical or horizontal limb of the RMS. The number of HUCB-NSC expressed neuronal markers (NF-200; TUJ-1) increased (Fig. 3J, K). Three weeks after grafting, HUCB-NSC expressing neuronal phenotypes (NF200$^+$ and TUJ-1$^+$cells) were found in the different locations of the brain (Fig. 3N, O). Transplanted HUCB-NSC survived in the rat brain up to 5 weeks. With increased survival time some human cells became MAP-2$^+$. These cells exhibited morphology of mature neurons with long processes (Fig. 3P) however, they were barely detectable. Interestingly, with line of our results none of transplanted HUCB-NSC into SVZ of neonatal rats expressed the astroglial marker GFAP.

Histochemical analysis of rat brain tissue showed a scarcely activation of microglia (ED1$^+$cells) appeared 24 hours after HUCB-NSC transplantation and persisted only few days. This host response might be initiated following surgical procedure related to cell transplantation. However, we did not observe the infiltration of neutrophils and T and B lymphocytes visualized by immunostaining for CD15, CD5 and CD45 over a time period of five weeks.

DISCUSSION

From the time of discovery that in cord blood there are cells capable of changing their fate towards the neural lineage our attempts have been focused on isolation and characterization of these cells. The first positive results confirming the assumptions that cord blood contains a subpopulation of stem cells with flexible phenotype which allow them to differentiate into neural cell types were published in 2002 (Buzanska et al. 2002). Thereafter, through repeated passages of floating round shaped, Nestin-positive and clonogenic cells we have established the first expanding indefinitely growing HUCB-NSC line (Buzanska et al. 2006). Neuronal character of this cell line was further evaluated by analysis of gene expression, protein design and electrophysiological properties of HUCB-NSC descendents (Sun et al. 2005, Buzanska et al. 2006, Jurga et al. 2006, Domanska-Janik et al. 2008, Jurga et al. 2009). But based on *in vitro* studies it is very difficult to predict what stem cells are capable of doing *in vivo*, where the cellular and molecular environments are more complex and dynamic. Moreover, an important stage in their preclinical evaluation of human cells for transplantation in neurological disorders has been to validate the survival, safety and functional efficacy of these cells by grafting into animal models of the disease.

The first experimental transplantation studies performed with human umbilical cord blood (HUCB) cells have reported promising results after ischemic insults (Chen et al. 2001, Willing et al. 2003) and amyotrophic lateral sclerosis (Garbuzova-Davis et al. 2003). However, the results of other studies are more controversial (Coenen et al. 2005, Nystedt et al. 2006, Kozlowska et al. 2007, De Paula et al. 2009). In our study HUCB-NSC transplanted into adult rat brain survived maximum 2 weeks after grafting. After 1-3 days of transplantation approximately 4% of the donor

cells co-stained with Ki67, indicating that some of transplanted cells continued to proliferate in the new host tissue. The rate of proliferation in the present study is comparable to what has been reported when xenografting embryonic or adult neural human neural precursors into the rodent brain (Le Belle et al. 2004, Olstorn et al. 2007).

Transplanted HUCB-NSC could be mainly encountered along the white matter tracts, whereas the density of donor cells in the rest of the brain was low and

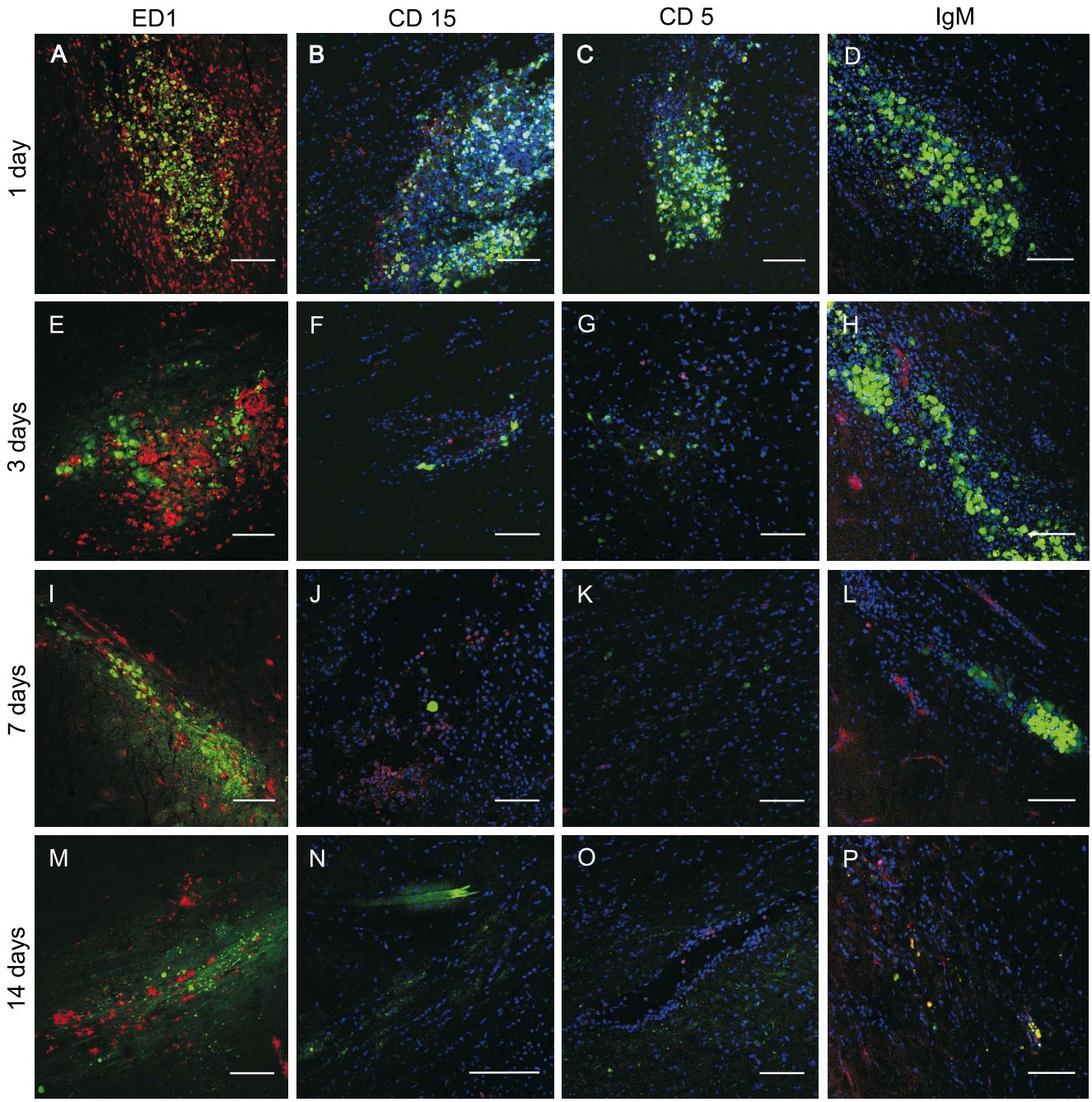

Fig.2. Immunohistochemical staining of adult brain tissue 1, 3, 7 and 14 days after HUCB-NSC transplantation. Double staining followed by confocal imaging revealed the presence of HUCB-NSC colored green by CMFDA and host immune response effectors stained red by specific antibodies coupled with Alexa Fluor 546 or Texas Red against: CD68 (ED-1) – macrophages/microglia (A,E,I,M), CD15 – neutrophils (B,F,J,N), CD5 – T lymphocytes (C,G,K,O) or IgM (D,H,L,P). Cell nuclei were counterstained with Hoechst 33252 (blue). Scale bar = 50 μm.

apparently random. A similar pattern of distribution has been observed after grafting of human neural stem cells (Burnstein et al. 2004, Wennersten et al. 2004, Olstorn et al. 2007). But in contrast to our own observation (Kozlowska et al. 2007) no significant difference was found in the total survival of the grafted human cells between healthy and ischemic animals (Olstorn et al. 2007).

In our hands, when kept *in vitro* conditions favouring differentiation, HUCB-NSC were shown to differentiate into cells expressing antigens of mature neurons, astrocytes and oligodendrocytes (Jurga et al. 2006). Compared with this, differentiation *in vivo* was much protracted because for example we never found MAP-2$^+$ cells among transplanted HUCB-NSC into the rat brain. The fact that we observed rather slow progression in neuronal differentiation 7 days after HUCB-NSC transplantation as visualized by NF-200 and TUJ-1 marker expression in only few donor cells indicates that the end-stage may not have been reached at the short time of possible observation due to elimination of human cells by the discordant recipient. The incomplete neuronal differentiation were observed in other studies where relatively low number of xenogeneic cells transplanted into the brain of adult rats expressed antigens typical for mature neurons (Kuh et al. 2005, Liu et al. 2006, Hicks et al. 2007, Kozlowska et al. 2007).

The limited survival of the human cells in rats possibly due to immune rejection is the major setback. While several reports ascribe low immunogenic profile of HUCB cells in *in vitro* experiments the immune-privileged status of these cells *in vivo* remains controversial (Walczak et al. 2004, Park et al. 2009). The local inflammatory response in the rat brain associated with surgical injury and/or xenogeneic cell transplantation seems to work against the donor cells as well as endogenous progenitors (Ekdahl et al. 2003, Kelly et al. 2004, Hicks et al. 2008). Indeed, majority of reported HUCB cell transplantation into adult or aged rat brains was associated with vigorous rejection (Walczak et al. 2004).

The involvement of immune cells in human transplant paradigms is well illustrated in evaluation of the graft fate in the rodent tissues. The innate immune response serves as the first line defence in any transplantation scenario, whether it involves xenogeneic but also allogeneic and even autologous transplantation (Bergwerf et al. 2009, Glover et al. 2010). Central nervous system strongly relies on microglia for innate protection. The results of our studies indicate the massive activation of ED1$^+$ cells in the adult rat brain 24 hours after implantation of HUCB-NSC. Furthermore, additional immuno-staining, including CD15 for neutrophils and CD5 for T-lymphocytes revealed the involvement of these inflammatory cell types in immune-mediated response in rat brain. The increased activation of macrophage/microglia was observed for few days after HUCB transplantation whereas, the number of lymphocytes diminished over time. Transplantation of human cells into the adult rat brain accompanied by the activation of microglia and lymphocyte markers immunoreactivity has been demonstrated previously (Walczak et al. 2004, Olstorn et al. 2007, Chen and Palmer 2008). However, our results suggest that microglia, rather than T-cells, are the main contributors to xenograft rejection in CNS. It was also shown by xenografting of human cells into NOD/SCID mouse being completely depleted of competent lymphocytes (Walczak et al. 2007). These severe immunodeficient mice possessed the ability to reject graft since at 4 weeks postgrafting HUCB cells were no longer detected in the brain of the recipients. It suggests that the disappearance of implanted cells was not due to a T and B-cell-mediated immune response. Although there is no evidence for involvement of T-cells in clearing human cells in the rat brain we cannot exclude their subsequent effectors functioning in CNS.

Humoral mechanisms may also be responsible for the rapid rejection of xenogeneic transplants in the CNS. Previous studies have shown the presence of IgM immunoglobulins in rat brain after the transplantation of porcine neural precursor cells, especially in the area displaying features of graft rejection (Armstrong et al. 2001). In our studies rat IgM response was examined to address whether humoral factors were recruited in HUCB-NSC recognition. The intense IgM immunoreactivity was seen around the graft what suggests that antibodies directed against specific epitopes on HUCB-NSC-derived cells constitute a component of the response. However, the possibility that some IgM immunoreactivity may result from plasma leakage of a range of non-specific IgM cannot be excluded on the basis of these data. IgM is produced in the first of phase of a primary immune response and also constitutes the principal class of the natural, preformed xenoreactive antibodies that exist in all species

(Larsson and Widner 2000). Thus the specific IgM binding may be generated *de novo* as part of a primary response against HUCB-NSC graft or may reflect binding of pre-existing antibodies. Additionally, IgM may be produced by locally infiltrating cells of the B-cell lineage. The identity of these cells has been noted previously in neural xenografts (Larsson et al. 1999). Although, our experiments so far have not been able to demonstrate any B cells in rat brain tissue within two weeks after HUCB-NSC transplantation.

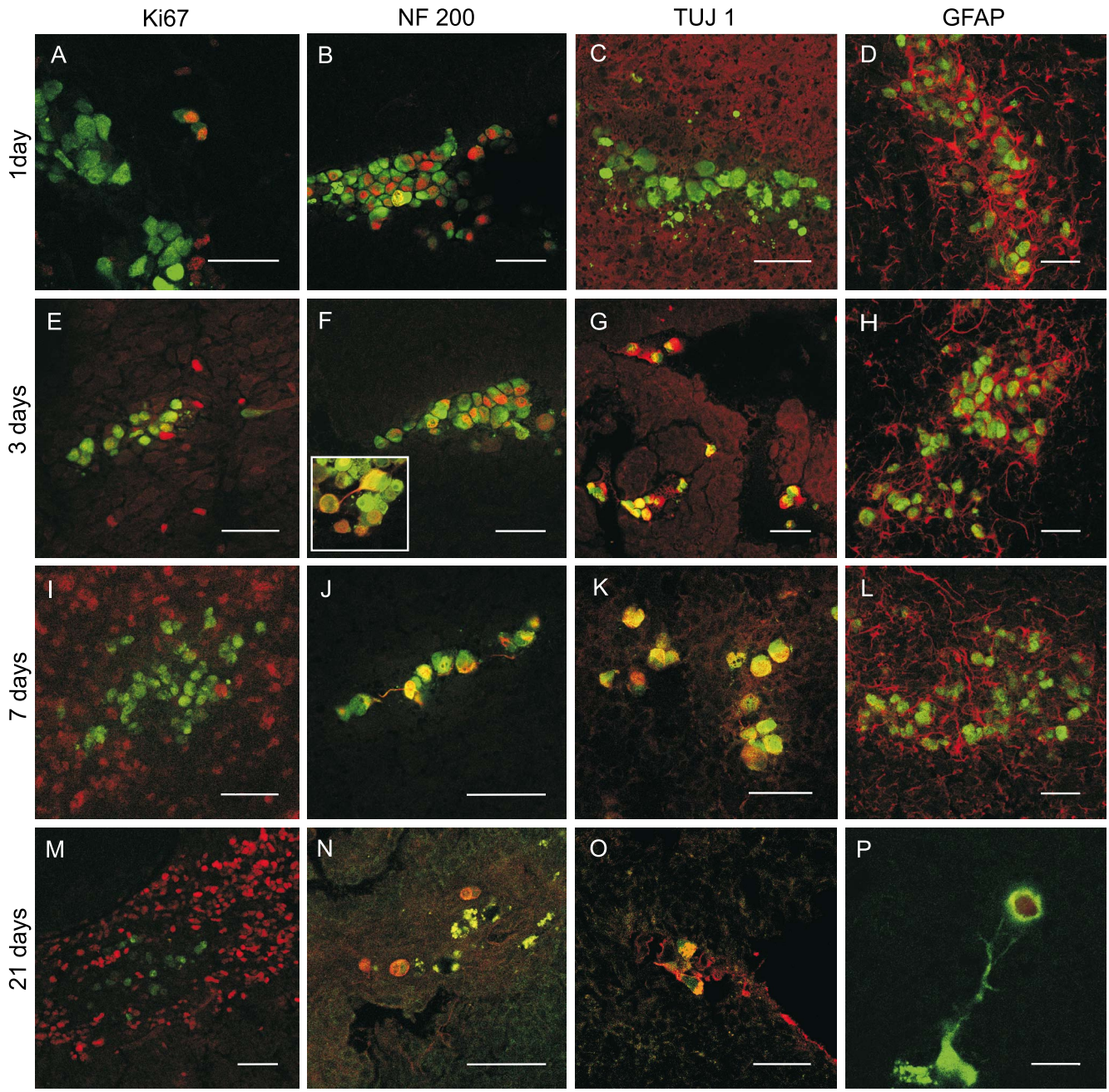

Fig.3. Immunocytochemical analysis of HUCB-NSC present in rat brain 1, 3, 7 and 21 days after their transplantation into SVZ of newborn recipients. Double labeling visualized HUCB-NSC colored green by CMFDA and red by Alexa Fluor 546 after phenotype specific immunoreaction with antibodies against: Ki-67 (A, E, I, M), NF-200 (B, F, J, N), TUJ-1 (C, G, H, O) or GFAP (D, H, L). Colocalization of red and green labeling appears yellow and overlaying these two markers. Scale bar = 50 μm. Five weeks after transplantation HUCB-NSC stained with NuMa – human specific nuclear antigens (red) showed neuronal morphology and expressed MAP-2 – mature neuronal marker (green) (P). Scale bar = 10 μm.

The results of our studies suggest that antibodies play a role in the initial phase of graft rejection. This is in concordance with Larsson report where porcine neural tissue was grafted to immunoglobulin deficient mice and the rejection was delayed for around 4 weeks compared to normal mice however, the transplanted cells were eventually rejected by 6 weeks (Larsson et al. 1999). The deteriorate effect of IgM in discordant xenografts seems to be multifunctional. Antibodies may not only attack the grafted cells by themselves but may also prime cells for lysis by host phagocytes. Significant correlation of IgM staining intensity with ED1 grading in rat brain tissue after HUCB-NSC transplantation could be related to indirect cytotoxic effect of antibodies.

Several reports describe the need for immune-suppressive therapy in order to avoid rejection of xenogeneic implants in CNS (Hayashi et al. 2005, Ronsyn et al. 2007). However, multiple studies including our own work described low human cell survival in rat brain despite of the usage of classical Cyclosporine A (CyA) (Kögler et al. 2004, Walczak et al. 2004, Pan et al. 2005, Janowski et al. 2006, Niranjan et al. 2007, Hicks et al. 2009) which suggests that CyA immunosuppression is not an effective strategy for enhancing survival. Other strategy to avoid this deleterious reaction of the host to the transplant included triple-immunosuppression with CyA, Azathioprin (Aza) and methylprednisolone (MP) (Finsen personal communication) but according to our experience CyA+Aza+MP-treated rats became unwell at 2 weeks and had to be killed in accordance with the rules of the animal license and graft survival could therefore not to be assessed beyond this time (unpublished). In context of cell therapies even if immune suppression would prevent rejection of non-autologous grafts, in parallel the restorative immune response is eliminated together with the immune mediated guidance cues that are required for donor cells to migrate and proliferate. The recent studies have shown that the infiltration of CNS by immune cells in following injury promotes neuroregeneration (Ziv et al. 2006, Glezer et al. 2007, Kulbatski 2010). The contradictory function of immune cells results in their detrimental or beneficial roles to the repair of CNS damage have been debated over the decade, arguing in favour or against the use of immunosuppressive drugs.

In trying to ameliorate the negative consequences of cell graft rejection by suppressing the host immune response we transplanted HUCB-NSC into the neonates with incompletely developed immunological system. Previous studies showed that xenogeneic transplants into the developing rat brain were more successful than the same transplant in aged animals and immunosuppression was not decisive for the survival and differentiation of human donor cells (Englund et al. 2002, Zigova et al. 2002, Walczak et al. 2004, Coenen et al. 2005). Our results are compatible with these studies showing that HUCB-NSC transplanted into newborn rats persisted in the host brain for longer time with no histopathologic signs of graft rejection.

In the current study, depending on the survival time, transplanted HUCB-NSC into the newborn rat brain were found around the injection site in the anterior part of the SVZ and along the initial part of RMS. These findings confirm the ability of HUCB-NSC to follow the migratory route of endogenous neural progenitors. Within one week $Ki67^{+}$cells were observed among the graft suggesting that the donor cells are still actively proliferating. At the same time many of HUCB-NSC acquired a neuron-specific NF-200 and TUJ-1 immunoreactivity. It suggests that transplantation of HUCB-NSC into neurogenically active region of the brain (SVZ) may support neural differentiation of these cells. Human cells transplanted into neonatal rats survived in the brain up to five weeks being visible in the most remote locations from the injection site. Morphologically a few single donor cells displayed long processes accompanied by the presence of neuronal MAP-2 protein. Our data are compatible with the previous studies showing that human cells transplanted into SVZ of neonatal rats become immunopositive for neuronal antigens (Englund et al. 2002, Zigova et al. 2002).

Overall, our data suggest that HUCB-NSC transplanted into SVZ of neonatal rats are capable of responding to cues present in the developing brain since introduced into it underwent advanced neuronal differentiation. However, despite of the little evidence of an inflammatory response in the rat brain the human cells died off during five weeks after transplantation. It seems that even when immunological barriers are overcome, survival of human cells implanted into animals is not guaranteed. It is highly likely that beyond immune destruction, mismatches in the requirements for and availability of trophic support lead to apoptosis of transplanted cells and their progeny. Such mismatches may arise because of species differences in the case of xenogeneic transplantation

(Glover et al. 2010). Overcoming this problem will require more knowledge to come to a greater end: the use of human stem cells to treat the patients.

CONCLUSIONS

The present study shows that HUCB-NSC transplanted into the brain of adult versus neonatal rats exhibit distinct engraftment kinetics with preference of the youngsters. Limited survival of the donor cells reveals that further studies are warranted to optimize the experimental conditions for preclinical testing of biological features of human cells *in vivo*. The strategy of desensitizing the host rat to human cells in the neonatal period proposed recently by Kelly (Kelly et al. 2009) is currently under investigation.

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