ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research Paper Acta Neurobiol Exp 2013, 73: 88–101

Different methods of immunosuppresion do not prolong the survival of human cord blood-derived neural stem cells transplanted into focal brain-injured immunocompetent rats

Anna Jablonska[1], Mirosław Janowski[1,2], and Barbara Lukomska[1]*

[1]NeuroRepair Department, Mossakowski Medical Research Centre, PAS, Warsaw, Poland *Email: barbara.lukomska@imdik.pan.pl; [2]Russell H. Morgan Department of Radiology and Radiological Science, Division of MR Research, the Johns Hopkins University School of Medicine, Baltimore, USA

Cerebrovascular diseases are the leading cause of severe disability worldwide, with an enormous financial burden for society. There is growing evidence that stem cell-based therapy may positively influence recovery from stroke. Cord blood is an attractive source of ontogenetically young, yet safe, stem cells. Conceptually, preclinical studies in which donor cells were of human origin have been the most valuable, and thus, it is likely that these cells will be used in clinical trials. Unfortunately, immunological barriers impede discordant xenotransplantations. We have previously observed acute rejection of cord blood-derived neural stem cells (HUCB-NSC) after transplantation to the brains of intact animals. Since it was reported recently that a brain lesion may actually improve the chances of graft survival, in this study, we used infarcted animals as graft recipients. In ongoing studies, we tested three immunosuppressive regimes: group I received cyclosporine A (CsA: 10 mg/kg i.p.); group II received a triple-drug therapy (CsA: 10 mg/kg i.p., azathioprine: 5 mg/kg i.p., and methylprednisolone: 1.5 mg/kg i.m.); group III included rats that were formerly desensitized with HUCB, group IV had not undergone immunosuppression.. Animals were sacrificed at five time-points: 1, 3, 7, 14, and 21 days post-transplantation to evaluate graft survival and the time-course of immunological response. We observed a gradual decrease in the number of transplanted cells, with complete disappearance by day 14, surprisingly, with no difference among the experimental groups. The involvement of the innate immune system in the process of graft rejection dominated over an adaptive immunoresponse, with the highest activity on day 3, and subsequent fading of immune cell infiltration. In this work, we have shown that none of our immunosuppressive strategies proved adequate to prevent rejection of human stem cell grafts after transplantation into immunocompetent animals.

Key words: cord blood cells, neural progenitors, focal brain injury, cell transplantation, desensitization, immunosuppression

INTRODUCTION

Cerebrovascular diseases are the leading cause of severe disability worldwide, with an enormous financial burden for society. Brain injuries, and, in particular, ischemic stroke, are one of the most difficult therapeutic challenges for physicians. Brain ischemia is caused by cerebral artery occlusion, inducing the infarction of brain tissue with acute loss of neurons, astroglia, and oligodendroglia and disruption of the synaptic architecture in the brain (Anderson et al. 2003, Wechsler and Kondziolka 2003, Haas et al. 2005).

Recently, it has been shown that the adult brain has the capacity to repair itself. Spontaneous neural tissue recovery occurs in neurological disorders by endogenous neural progenitor cell activation in response to ischemia. However, this process is not robust enough to promote a functional and stable recovery of the central nervous system (CNS) architecture (Pluchino et al. 2005, Munoz et al. 2005, Lindvall and Kokaia 2005, Jin et al. 2006). For this reason, there is a great interest in the possibility of repair of the nervous system through the transplantation of new cells that can replace those lost through damage.

Correspondence should be addressed to B. Lukomska, Email: barbara.lukomska@imdik.pan.pl

Received 11 February 2013, accepted 19 March 2013

Potential sources of stem cells are bone marrow, peripheral blood, and cord blood. Stem cells from these sources were shown to be capable of transdifferentiating *in vitro* into neural lineages (Buzanska et al. 2002, Zhao et al. 2003, Hermann et al. 20 04, McGuckin et al. 2004, Habich et al. 2006). During the past two decades, the human umbilical cord blood has garnered great interest as a novel, valuable source for stem cells that possess several unique characteristics (Zigova et al. 2002, Sun et al. 2005, Buzanska et al. 2006, Jurga et al. 2009, Ali et al. 2012). In contrast to adult bone marrow, stem cells in cord blood are at the intermediate point between embryonic/fetal and adult life, with high proliferative potential associated with an extended life span and longer telomeres (Kogler et al. 2004). Apart from this biological superiority, cord blood is also a valuable source of stem cells in terms of access and supply, and the noninvasive and ethically accepted method of collection.

The clinical use of exogenous stem cells for neuroregeneration is critically dependent on preclinical studies in animal models that are relevant to human disease. Over the last several years, several models of brain injury, mainly in rodents, have been established. Most experimental studies have been performed using a middle cerebral artery occlusion (MCAO), either permanently (Hicks et al. 2009, Dubois-Dauphin and Julien 2010) or transiently (Rosen et al. 2005, Takahashi et al. 2008, Steiner et al. 2012). However, while this model is widely used by researchers, it causes extensive damage to the brain, with symptoms related to brain edema (Hofmeijer et al. 2004). To limit the ischemic injury related to the small deep brain structures, Veldhuis and coauthors (2003a) invented a chemical insult using the Na/K ATP-ase pump inhibitor, ouabain. The lesion induced by the intraparenchymal injection of ouabain brings metabolic and structural brain changes that mimic focal ischemic injury. This model of lacunar deep-brain ischemic lesions observed in clinical practice has been successfully used in morphologic and neuroprotection studies (van der Stelt et al. 2001, Veldhuis et al. 2003b, Janowski et al. 2008).

Conceptually, studies in which the donor cells were of human origin are the most valuable because it is highly likely that only human-derived cells will be approved for use in clinical trials. However, xenotransplantation of human cells raises the high risk of rejection in discordant recipients. Donor cells evoke an immunological response triggering host-*versus*-graft reaction, which abrogates the beneficial effect of the graft due to its limited survival.

Our previous data demonstrated that intracerebral transplantation of human umbilical cord blood-derived neural stem cells (HUCB-NSC) into the intact brain of healthy rats resulted in their acute rejection by the host. Surprisingly, it was reported that CNS injury, when compared with intact tissue, apparently improved the viability of stem cell grafts (Olstorn et al 2007).

The goal of the present study was to analyze the survival, proliferation rate, and differentiation patterns of HUCB-NSC transplanted into ouabain-injured rat brains. In addition, several strategies, including different immunosuppression protocols, or desensitization, were investigated to avoid eventual rejection problems with HUCB-NSC grafted into xenogeneic recipients.

METHODS

Cell culture

A human umbilical cord blood-derived neural stem cell (HUCB-NSC) line was derived from $CD34^-$ and $CD45^-$ cord blood mononuclear cells, as described previously by Buzanska and colleagues (Buzanska et al. 2006), and cultured in DMEM/F12 supplemented with 2% fetal bovine serum, 1% insulin-transferrin-selenium, and 1% antibiotic/antimycotic solution (Invitrogen). At 24 h before transplantation, HUCB-NSC were moved into serum-free NeuroBasal medium supplemented with B27 to avoid serum content and the risk of immunological reaction of the host. Immediately before grafting, HUCB-NSC were pre-labeled with CellTracker™ Green (Molecular Probes) to facilitate their identification in the recipients. For transplantation, HUCB-NSC were suspended in PBS at a concentration of 1×10^5 cells/µl.

Animals

The experiments were performed in adult male Wistar rats weighing 250 g. The animals were kept in standard conditions with a 12:12 photoperiod and unlimited access to water and food. All procedures were approved by the First Warsaw Local Ethics Committee, and were consistent with EU guidelines.

Neonatal desensitization

On the day of birth (P0), the rat pups were anesthetized by hypothermia on ice for three-to-four minutes and injected i.p. with 1 µl of HUCB-NSC (1×10^5), suspended in PBS, using a Hamilton syringe equipped with a 33G micro-needle. Animals were allowed to recover from cryoanesthesia, and, after reaching normothermia, and a normal respiratory rate, they were returned to their mothers. Six weeks after desensitization, these rats received HUCB-NSC grafts.

Ouabain model of brain injury

Adult rats were anesthetized with ketamine (10%; 90 mg/kg b.w., given i.p.) and xylazine (2%; 10 mg/kg b.w., given i.p.), and immobilized in a stereotactic apparatus (Stoelting). A small burr hole was drilled in the cranium over the right hemisphere. The needle (length 15 mm, gauge 33), connected to a 10-µl syringe (Hamilton), was lowered into the right striatum (coordinates A 0.0, L 3.0, D 5.0 mm). To minimize brain shift, there was a delay of five min between needle insertion and the injection of the active substance. A 1-µl of 5 mmol ouabain (Sigma) was then injected into the brain at a rate of 0.1 µl/min *via* a microinfusion pump (Stoelting) mounted on the stereotaxic apparatus. After injection, the needle was left *in situ* for five min to avoid leakage of injected fluid through the needle tract. The needle was then withdrawn and the skin closed with a suture.

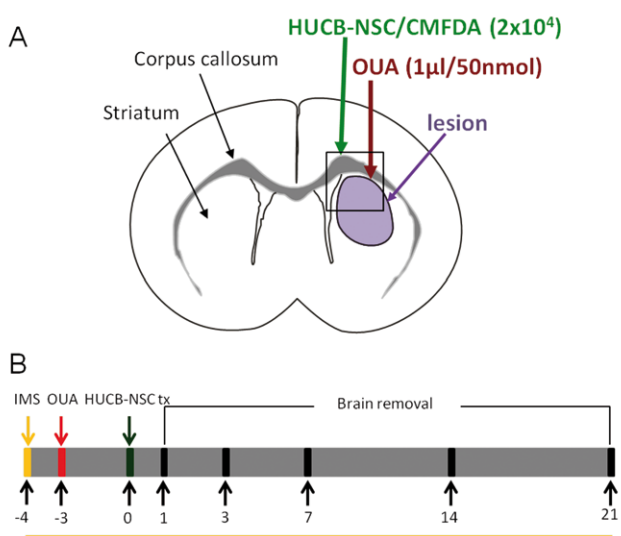

Fig. 1. Scheme of surgical procedures; the area bounded by the box depicts the brain region analyzed histochemically for the presence of transplanted human cells and immune response activation of the host effectors (A), and the timetable of experimental procedures (B).

Transplantation procedure

Three days after the ischemic insult, the rats were anesthetized with ketamine/xylazine 90/10 mg/kg i.p., and immobilized in a stereotactic apparatus. After a median skin incision on the skull, a burr hole was drilled in the cranium over the right hemisphere, and a 33G micro-needle attached to a 5-µl Hamilton syringe was lowered into the corpus callosum using the following coordinates: A 0.0, L 4.0, D 3.0, in reference to the bregma. Animals were injected with 2 µl of CMFDA-labeled HUCB-NSC (2×10^5), at a rate of 0.5 µl/min. Then, the needle was withdrawn, the surgical wound was suture-closed, and animals were allowed to recover from anesthesia. Four experimental groups of ouabain brain-injured rats that received HUCB-NSC transplantation were divided according to the type of immunosuppression: group I, which received cyclosporine A (CsA, 10 mg/kg i.p.); group II, which received a three-drug therapy regimen (CsA: 10 mg/kg i.p., azathioprine: 5 mg/kg i.p., and methylprednisolone: 1.5 mg/kg i.m.); group III, which included rats that were formerly desensitized with HUCB-NSC; group IV, which had not undergone immunosuppression. The immunosuppression treatment was started two days prior to transplantation, with daily injections of CsA or three-drug therapy, which continued for the entire survival period of the animals (Fig. 1). The control group had undergone ouabain brain injury without HUCB-NSC transplantation.

Immunohistochemistry and image acquisition

Rats were sacrificed sequentially at post transplantation day 1, 3, 7, 14, or 21, and then, the brains were removed and snap frozen. Twenty micron-thick coronal tissue sections were cut using a cryostat and immunoassayed with the primary antibodies: anti-Ki67 (Leica, 1:100), recognized marker of proliferating cells; anti-NF200 (Sigma, 1:400); anti-βTubulin-III (TUJ1) (Covance, 1:750); and anti-GFAP (Cappel, 1:100) to determine the neural fate of implanted HUCB-NSC. For characterization of the host immune response, anti-CD5 (Serotec, 1:25) as T cell, anti-

CD45RA (Serotec, 1:100) as B cell, anti-CD68 (ED1) (Serotec, 1:100), as macrophages/microglia, anti-CD15 (Serotec, 1:50) as neutrophils, and anti-IgM (Southern Biotech, 1:500) specific antibodies for the following molecules were applied. Positive cells were visualized by secondary staining with goat anti–rabbit (Alexa Fluor-488, green) and goat anti–mouse IgG (Alexa Fluor-594, red) (1:200, Molecular Probes) antibodies. Cell nuclei were stained with 5 μM Hoechst 33258. Images were acquired using a Zeiss confocal LSM. Following acquisition, images were processed using the software Zeiss LSM 510.

Cell counting

To calculate the neural phenotypes of transplanted HUCB-NSC or immunological induction of the host response in the brain, the positively stained cells present in the implantation site and at the border of infarction, including the lesioned and perilesioned areas, were randomly analyzed *via* confocal laser scanning microscopy (Fig. 1 box). To determine the number of desired cells, 12 slide-mounted coronal sections were sampled from the brain of each of the five rats used in one experimental group. In each brain section stained with antibodies, the specific marker of double-positively labeled cells with CMFDA for HUCB-NSC or Hoechst for rat cells were counted (at least 2 500 cells). Quantitative analysis was presented as the percentage of immunopositive cells and the data are expressed as mean ± SE.

Statistical analysis

All values are given as mean ± SE. All statistical calculations were performed using GraphPad Prism 5.0. One-way ANOVA with the Bonferroni Multiple Comparison test was used for comparison between mean values. Statistical significance was deemed to be present if $P<0.05$.

RESULTS

Characterization of HUCB-NSC after their transplantation into the ouabain-injured brain of rats without immunosupression

To investigate the proliferation, capacity, and phenotype distribution of HUCB-*NSC in vivo* after their transplantation into ouabain-injured rats, immunohistochemistry was performed on brain tissue samples at 24 hours as well as at 3, 7, and 14 days post-grafting. One day after transplantation into the ouabain-injured rat brain, all HUCB-NSC were detected in the injection bolus, most of them as undifferentiated precursors. Identification of the proliferation marker Ki67 revealed 4.1 ± 0.3% positive cells. Immunohistochemical staining of donor cells located in the graft area showed the expression of neuronal NF-200 (22.8 ± 0.6%) and astrocyte GFAP (22.8 ± 0.6%) proteins. After three days, a smaller proportion of human cells expressed NF-200

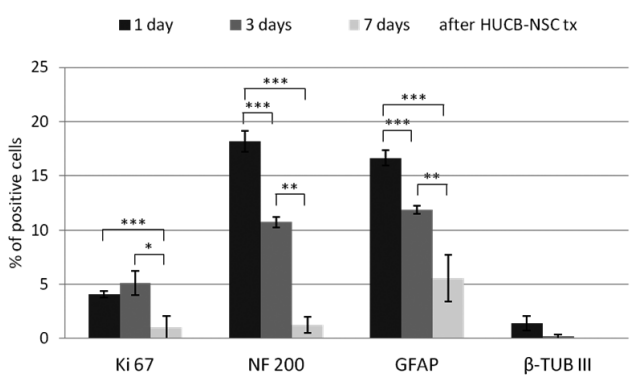

Fig. 2. Analysis of cell-counts of the different phenotypes of HUCB-NSC, expressed as the percent of positive cells in the ipsilateral hemisphere of the ouabain-injured rat brain after human cell transplantation revealed significant differences at various time-points of observation. The results are expressed as the mean ± SE, n=6. *P<0.05; **P<0.01; ***P<0.001.

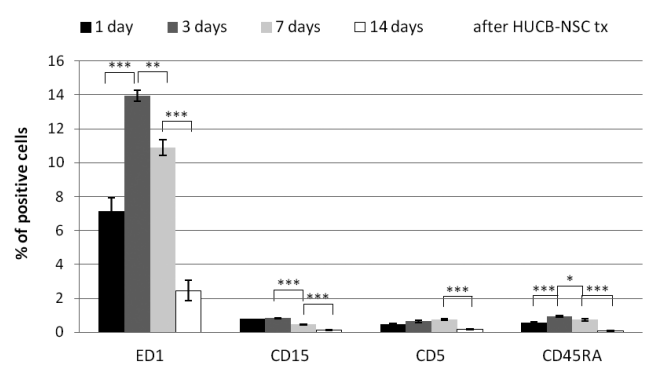

Fig. 3. Analysis of cell counts of immune cells expressed as the percent of positive cells in the ipsilateral hemisphere of the ouabain-injured rat brain after HUCB-NSC transplantation revealed significant differences at various time-points of observation. The results are expressed as the mean ± SE, n=6. *P<0.05; **P<0.01; ***P<0.001.

(10.73 ± 1.5%) and GFAP (11.8 ± 0.6%) markers. Some occasional HUCB-NSC migrated from the graft core to the surrounding parenchyma. The percentage of Ki67-positively labeled HUCB-NSC was 5.1 ± 1.1%. Seven days after transplantation, significantly more donor cells migrated along white matter fibers toward the lesion. Cells situated between the graft and the lesion border rarely expressed NF-200 (1.3 ± 0.7%) or GFAP (5.5 ± 2.1%) and Ki67 (1.0 ± 1.0%) markers (Fig. 2). At 14 days, there was no evidence of viable human cells remaining either at the transplant site or in the injured area.

The effectors of innate and adaptive immune response in the brain of untreated rats after HUCB-NSC transplantation

To demonstrate the reaction of the host to human cells (HUCB-NSC) being transplanted in the setting of our model, immunostaining of rat brain tissue was performed using phenotypic markers of cell characteristics for innate and adaptive immunity effectors. At 24 hours, intensive staining for ED1, a marker for microglia/macrophages, was evident in the ischemic region and close to the cell graft. A similar activation of astrocytes was also observed, with the appearance of a cell population that demonstrated the expression of CD15, a cell-surface marker for neutrophils, CD5, a marker of T lymphocytes, and CD45RA, a phenotypic marker of B lymphocytes. The analyses of brain tissue three days after HUCB-NSC transplantation revealed a significant increase in $ED1^+$ and $GFAP^+$ cells relative to the first day, whereas no differences were observed between the numbers of $CD15^+$, $CD5^+$, and $CD45^+$ cells. At seven days post-graft, a significant decrease of microglia/macrophages, neutrophils, and T and B lymphocytes occurred, representing a shift in the population of inflammatory cells observed during the follow-up after HUCB-NSC administration. Two weeks after human cell grafting, a few cells expressing ED1, CD15, CD5 and CD45RA markers were found in the rat brain, located mainly at the border of the scar formation area. The activity of immune cells positively correlated with the presence of transplanted HUCB-NSC, and no viable human cells were observed at this time-point (Figs 3, 4).

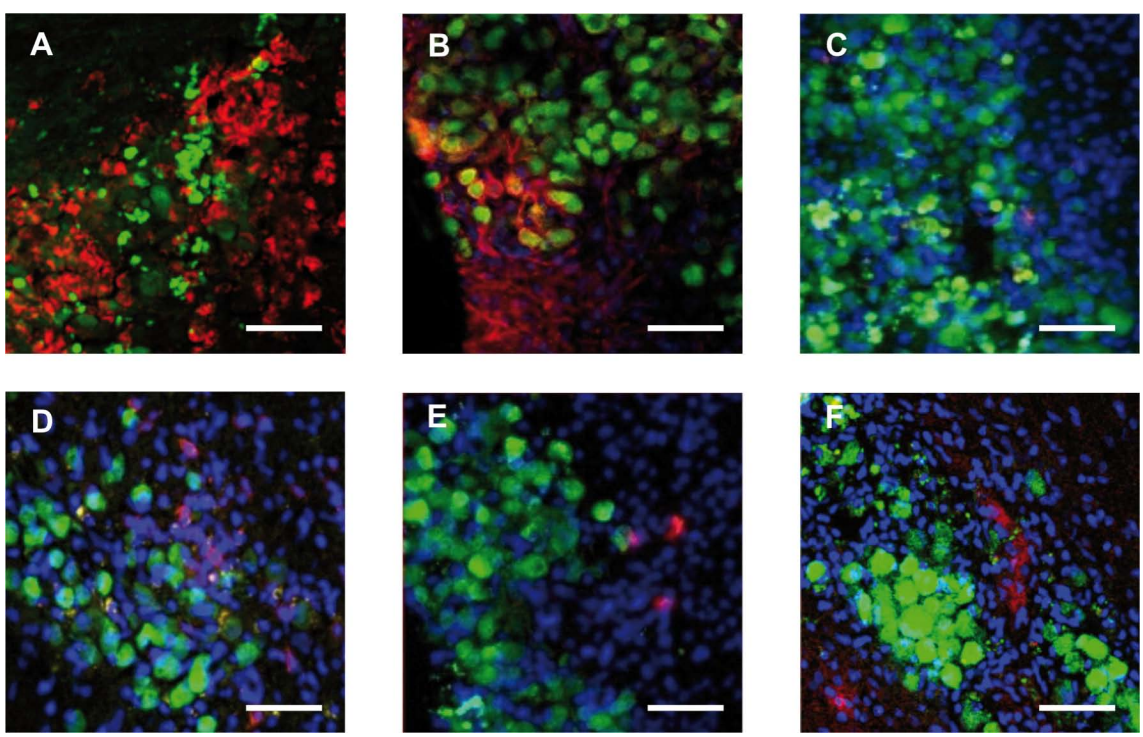

Fig. 4. Detection of host response in the ischemic brain three days after HUCB-NSC (green by CMFDA) transplantation. Cells stained with Texas Red (red) after specific immunoreactions for ED1 (A); GFAP (B); CD15 (C); CD5 (D); CD45RA (E); IgM (F); and Hoechst 33258 staining (blue) showed their nuclei. Scale bar is 50 μm.

Characterization of donor cells and the immune cells of the host after HUCB-NSC grafting into the brain of CsA-treated rats

The effectiveness of CsA immunosuppression of the human graft recipients was studied in our experimental model by comparing the survival rate of HUCB-NSC and their functional potential to proliferate and mature in the brain tissue to that of untreated and CsA-treated rats. The results of our study showed no difference in the survival time of human cells transplanted into ouabain-injured rat brain between both groups of animals at any of the time-points compared (24 hours, 3, 7, 14, 21 days). Despite the increasing total cell number of HUCB-NSC ($<10^5$), the viable donor cells in the brains of the CsA-immunosuppressed group could be detected only up to two weeks post-grafting. Moreover, human cells transplanted into CsA-treated rats displayed the same properties as HUCB-NSC in the control untreated recipients. Most of the grafted HUCB-NSC were found at the injection site, showing no or only a little migration capacity. Immunohistochemical staining for neuronal and astrocyte markers performed on brain sections to analyze the state of differentiation of the grafted cells revealed no significant differences between cohorts in the number of certain phenotypes observed in rats with and without CsA immunosuppression.

It is worth noting that CsA treatment did not affect the inflammatory response in the brain of lesioned animals after HUCB-NSC transplantation. Interestingly, CsA, which exerts its immunosuppressive action to depress the cellular immune response of T cells, did not reduce the number of T lymphocytes appearing at 24 hours in human cell grafts, and persisted during two weeks of observation. Similarly, B lymphocytes were noticed in the transplant area. The pattern of adaptive immune response seen in CsA-immunosuppressed rats was comparable to that observed in the non-suppressed graft recipients. The analysis of the innate immune reaction revealed quantitative and morphological changes in reactive microglial ED1$^+$ cells and GFAP$^+$ astrocytes, and infiltration of CD15$^+$ neutrophils in post-ischemic tissue and at the HUCB-NSC injection site of CsA-immunosuppressed rats. This inflammatory reaction reached its peak on the 3rd day after cell transplantation; then, the number of cells was gradually reduced with much less detected at a later time, i.e., two-to-three weeks, when surviving human cells were not observed. In our experimental model, no significant differences in the activation of the immunological response were detected between the two groups of human graft recipients (with and without CsA immunosuppression (Fig. 5).

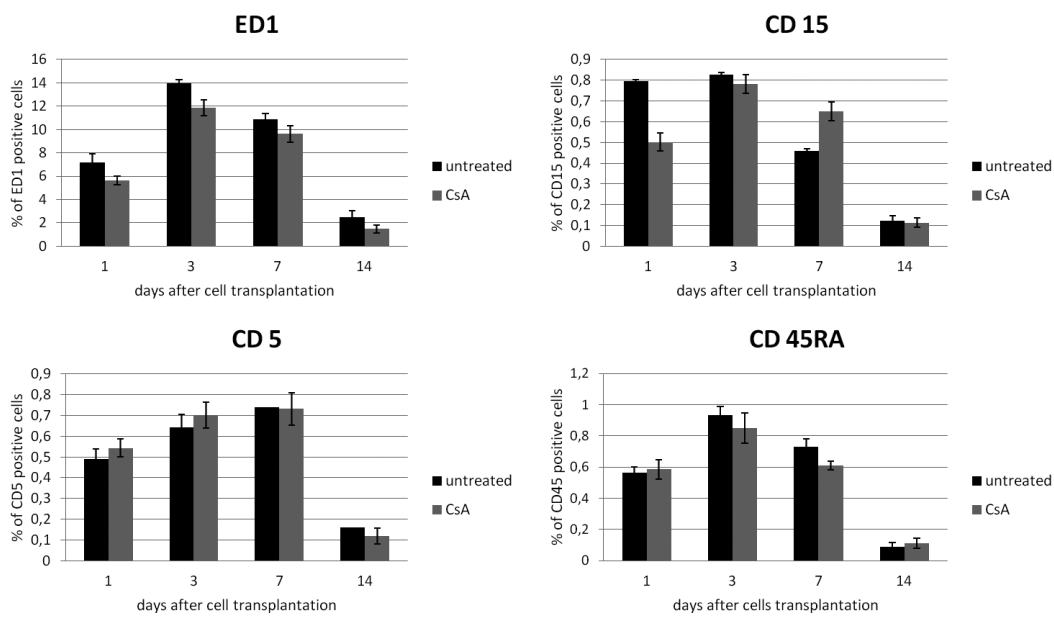

Fig. 5. Analysis of cell counts of immune cells expressed as the percent of positive cells in the ipsilateral hemisphere of the ouabain-injured rat brain after HUCB-NSC transplantation. No significant differences in the cell counts were observed, either without immunosuppression or with CsA treatment of graft recipients, at various time-points of observation. The results are expressed as the mean ± SE, n=8.

Characterization of donor cells and the immune cells of the host after HUCB-NSC grafting into the brain of triple-immunosuppression-treated rats

Since CsA treatment appeared to be insufficient to protect HUCB-NSC grafts, we utilized a three-drug pretreatment regimen to interfere with a much wider range of immunological response parameters. The combination of CsA, azathioprine, and methylprednisolone was chosen to suppress both adaptive and innate immunity. Unfortunately, we did not prove a positive effect for the triple-immunosuppression protocol. After HUCB-NSC transplantation into the brain of ouabain-lesioned rats, most of the cells remained at the site of deposit with only minimal ability to disperse and migrate into surrounding tissue. Graft cells expressed the proliferation marker Ki67, as well as neuronal NF-200 and astrocyte GFAP markers similar in expression to those observed in rats without immunosuppression. There was gradual depletion of donor cells, indicating the inability of the immunosuppressant drugs used in our experimental settings to prolong graft survival. There was a strong negative correlation between the number of inflammatory cells that represented adaptive and innate host immunity in the rat brain and the survival rate of transplanted HUCB-NSC cells. Again, between triple-immunosuppression-treated and non-treated rats, there were no statistically significant differences in the number of activated host immune cells at various time-points. A strong GFAP and ED1 expression was observed in brain tissue located close to the injection site of HUCB-NSC at 24 hours post-graft. Simultaneously, CD15, CD5, and CD45RA immunoreactive cells were found. The increase in innate and adaptive immunity effectors could be clearly discerned in confocal laser scanning microscopy over the next seven days despite continuous immunosuppression. Then, the number of immune cells was reduced after 14 days in the almost absolute absence of previously transplanted human cells (Fig. 6). Heavy immune suppression using CsA, azathioprine, and methylprednisolone was unable to protect HUCB-NSC from acute rejection; however this treatment regimen was associated with adverse effects. Moreover, triple-immunosuppression was highly detrimental to the animals. Several rats developed superficial infections, as indicated by symptoms of respiratory and gastrointestinal tract failure. All animals lost weight. Three rats died, presumably due to side effects of the three-component immunosuppression treatment. A few animals had to be euthanized before the end of the experiments in accordance with the rules of the animal license.

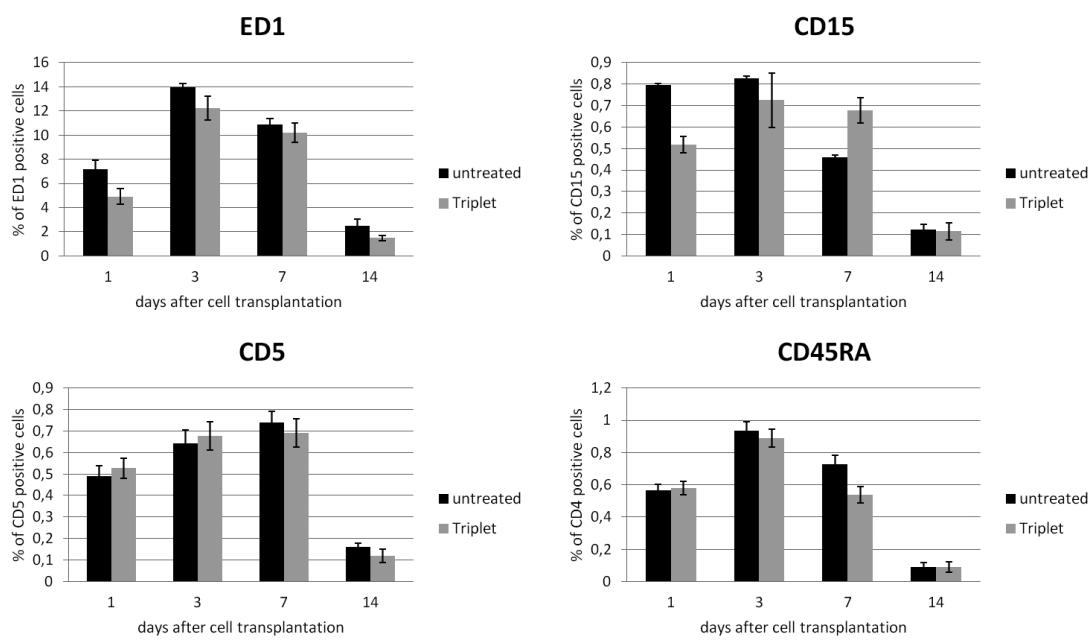

Fig. 6. Analysis of cell counts of immune cells expressed as the percent of positive cells in the ipsilateral hemisphere of the ouabain-injured rat brain after HUCB-NSC transplantation. No significant differences in the cell counts were observed, either without immunosuppression or with CsA + azathioprine + methylprednisolone treatment of graft recipients, at various time-points of observation. The results are expressed as the mean ± SE, n=8.

Characterization of donor cells and the immune cells of the host after HUCB-NSC grafting into the brain of desensitized rats

To improve graft survival, selective tolerance induction was substituted for the immunosuppression paradigm. Encouraged by the study of Kelly and coauthors (2009), which proposed neonatal desensitization that induced selective tolerance of the host to transplanted cells, we followed the same method in our experiments. Immunohistochemistry used for visualization of HUCB-NSC transplanted into the brain of ischemic rats, six weeks after their desensitization, revealed an abundance of donor cells at one and three days after grafting. However, the number of HUCB-NSC decreased substantially at later time points, with hardly any cells detectable in rat brain tissue after 21 days. The immunological/inflammatory rejection of human cells was associated with activated astrocytes and microglial cells, and infiltration of neutrophils and lymphocytes. The number of ED1, CD15, CD5, and CD45RA cells detected in the proximity of the graft increased on day 3 and 7 after HUCB-NSC transplantation. The loss of human cells in the host brain at day 14 coincided with a gradual decrease of immune cell activation and infiltration (Fig. 7). Overall, in our experiments, the neonatal desensitization method failed to induce specific tolerance of the recipients to transplanted HUCB-NSC.

DISCUSSION

Cord blood is widely used for the purpose of hematopoietic stem cell replacement therapies. Recently, it has been demonstrated that human umbilical cord blood cell transplantation is not limited to the treatment of hematopoietic diseases, and the cells have the ability to enhance regeneration in numerous tissue types, including the nervous system. This has, in part, been attributed to the heterogeneous population of cord blood stem cells.

Several experimental studies employing cord blood transplantation in animal models of CNS disease raised hope that the application might be expanded to the treatment of neurological disorders. To assess and quantify the resulting functional improvements after cord blood cell transplantation various behavioral tests were performed. The first study exploring the therapeutic effects of HUCB cells in the treatment of stroke was performed by (and colleagues (Chen 2001). Other studies have also demonstrated the beneficial influence of cord blood cell transplantation on lesion-in-

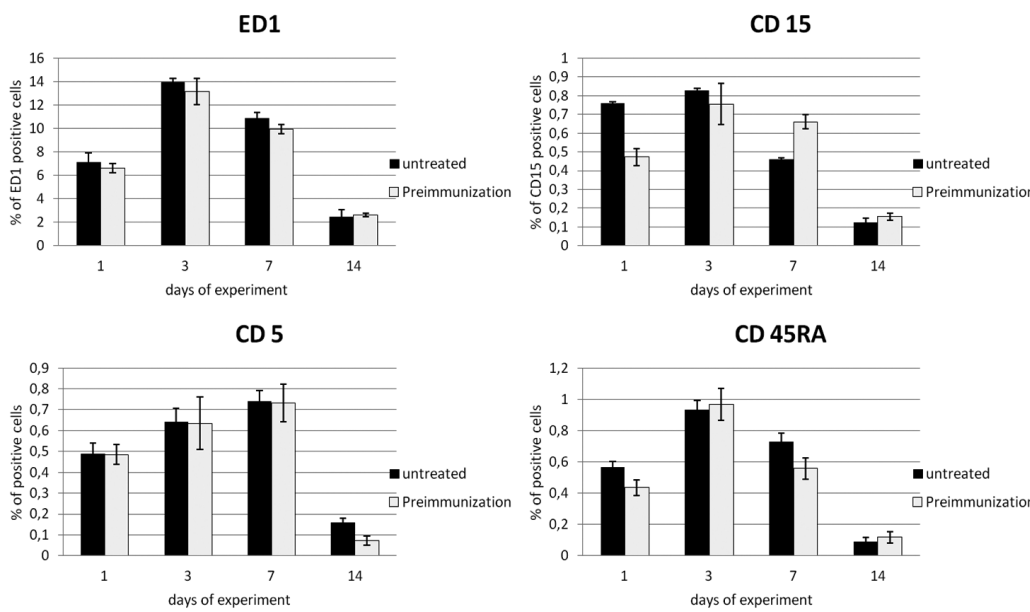

Fig. 7. Analysis of cell counts of immune cells expressed as the percent of positive cells in the ipsilateral hemisphere of the ouabain-injured rat brain after HUCB-NSC transplantation. No significant differences in the cell counts were observed, either without any treatment or with desensitization of graft recipients, at various time-points of observation. The results are expressed as the mean ± SE, *n*=8.

duced paresis (Willing et al. 2003, Vendrame et al. 2004, Borlongan et al. 2004, Meier et al. 2006). Also, in our previous studies, we have shown that systemic treatment of ouabain brain-injured rats with freshly isolated HUCB cells ameliorated the impaired limb movements of graft recipients (Gornicka-Pawlak et al. 2011). However, despite the positive effect of HUCB cell grafting on brain lesion-induced functional impairments, the mechanism of how transplanted cord blood cells exert their beneficial effects is unknown. Previous studies described the expression of neural marker proteins by mononuclear cord blood cells under specific culture conditions (Sanchez-Ramos et al. 2001, Buzanska et al. 2002, Neuhoff et al. 2007). Based on this evidence from *in vitro* work, results from the behavioral tests in animal models suggest that a cell replacement mechanism is likely responsible for the observed beneficial effects. In order to address these issues, we transplanted a human umbilical cord blood-derived neural stem cell (HUCB-NSC) line, established previously in our lab (Buzanska et al. 2006), into the ischemically injured rat brain to follow the fate of the donor cells in the host microenvironment. The phenotypic and functional identity of the HUCB-NSC line, their stability, ability to form neurospheres, and differentiation potential into all neural cell types were confirmed in several studies (Buzanska et al. 2006, Jurga et al. 2006, Ruiz et al. 2008, Jurga et al. 2009, 2011, Zychowicz et al. 2012). 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.

The grafted HUCB-NSC ceased to survive up to two weeks after their implantation in the ouabain-injured rat brain. Most of the donor cells were observed to persist in the injection bolus. Approximately 5% were immunoreactive for Ki-67, indicating that some of the transplanted cells continued ongoing proliferation in the host brain. One day after transplantation, HUCB-NSC expressed astroglial (GFAP) and neuronal (NF-200, β-TubIII) proteins; however, these markers were present in low numbers at seven days. In our experiments, HUCB-NSC never fully acquired the terminal phenotype of mature neurons *in vivo*. Scant migration of donor cells was observed in the corpus callosum, radiating toward the peri-infarct region of the striatum. These results are in accordance with other observations, where few HUCB cells transplanted into the brain of rats with cerebral ischemia were observed in the lesion site displaying various neural markers (Kuh et al. 2005, Liu et al. 2006, Kozlowska et al. 2007). In some studies, neural differentiation of human cord blood cells was not observed *in vivo* at all (Taguchi et al. 2004, Meier et al. 2006); however, the presence of transplanted HUCB cells has been demonstrated in the vicinity of brain lesions. Despite the indication of targeted migration of transplanted cells as a response to injury, the survival of human cells in the rat brain is equally poor. We did not find a significant difference in the total survival of grafted HUCB-NSC between healthy and ischemic animals.

The local inflammatory response associated with a brain lesion seems to work against transplanted cells. In addition, xenogeneic human cells grafted into the brain of adult rats are the subject of considerable immune surveillance. The transplantation procedure is also another critical issue and invariably leads to inflammation. All these factors initiate the effector mechanisms that coordinate the innate and adaptive immune responses. The dynamic interactions between immune cells, the injured neural tissue, and transplanted cells enable the CNS to respond to the graft. As a consequence, the majority of HUCB-NSC transplanted to the adult brain of rats exhibit poor survival and meager differentiation due to the short time of observation. From a clinical perspective, it must be noted that human neurons require several months to fully acquire their terminal functional phenotype after transplantation (Kelly et al. 2004). The previously view that the brain was an absolute immunologically privileged site, allowing indefinite survival without rejection of cell grafts, has proven to be in error. Thus, the brain should be regarded as a place where immune responses can occur, albeit in a modified form, and, under certain circumstances, these responses are as vigorous as those seen in other peripheral sites.

The elimination of human cord blood cells after their transplantation might be the result of graft rejection, since a remarkable immunological response was observed in adult rat ischemic brain around the HUCB-NSC injection site. In our studies, brain injury obtained after ouabain injection into the striatum of adult rats revealed activation of macrophage/microglia and astrocytes in the host brain. Human cord blood cell transplantation potentiates the acute inflammatory response initiated by the ischemic insult. The accumulation of ED1- and GFAP-positive cells adjacent to the implanted HUCB-NSC clusters was detected at 24 hours after

transplantation. There was a two-fold increase in the density of microglia at three days. Reactive astrogliosis was observed in locations similar to those of activated microglia. Further analysis of the reactive response to brain injury and cell transplantation revealed a less pronounced activation of microglia and astrocytes that indicated the disappearance of donor cells. These results are in accordance with previously published data describing a time-dependent increase in innate immune effector activation of human neural stem cells implanted near the brain injury site of immunocompetent animals (Walczak et al. 2004, Olstorn et al. 2007, Molcanyi et al. 2007, Chen and Palmer 2008). Microglia and astrocytes are the major players involved in the neuro-inflammatory response, and their activation results in a change in the balance between different mediators of the brain environment. The local innate mechanisms of immune response play the main role in xenogeneic transplantation, but they also function in allogeneic, syngeneic, or even autologous cell transplants into the CNS (Coyne et al. 2006, Tambuyzer et al. 2009, Bergwerf et al. 2009, Glover et al. 2010).

In our studies, low levels of infiltrating neutrophils and T and B lymphocytes were consistently found from the earliest time-point examined after HUCB-NSC transplantation into the ischemic rat brain. The number of immune cells was constant beyond two weeks of observation. First, both neutrophils and T and B lymphocytes localized to the graft area; then, they were seen close to the scar formation area. A similar pattern of lymphocyte infiltration in rejecting xenografts has been reported previously (Armstrong et al. 2001, Larsson et al. 2001). Wood and colleagues (1996) showed that T-cytotoxic/suppressor lymphocytes ($CD8^+$) were unable to mediate xenogeneic graft rejection in the absence of T-helper cells ($CD4^+$). Indeed, depletion of $CD4^+$ lymphocytes reduced the incidence of human cell rejection in the mouse brain. The adaptive immune response plays an important role in the mechanism underpinning graft rejection in the brain, as investigators have observed the prolonged survival of allogeneic or xenogeneic grafts among nude rats or NOD/SCID mice deprived of T lymphocytes (Walczak et al. 2007).

Previous studies have demonstrated that graft rejection was never a problem in studies utilizing neonatal hosts (Zigova et al. 2002, Jablonska et al. 2010). However, with adult animals, a remarkable immunological reaction was accompanied by donor cell elimination. Several strategies have been proposed to avoid these problems, including immunosuppression of the graft recipients, the use of genetically immunodeficient animals, or desensitization of the host immune system to xenogeneic transplanted cells (Pedersen et al. 1997, Walczak et al. 2004, Kelly et al. 2009, Gorelik et al. 2012). The immunosuppressive drug most commonly used in neuronal transplantation has been cyclosporin A (CsA). It produces specific, reversible inhibition of pro-inflammatory cytokine transcription; thus, the effect of CsA relies on T-lymphocyte suppression. To date, the effectiveness of CsA treatment in xenotransplantation is debatable. A few groups have shown that immunosuppression with CsA prolonged the graft survival of human cells transplanted into the ischemic rat brain (Olstorn et al. 2007, Rota-Nodari et al. 2010, Hovakimyan et al. 2012); however, immunosuppression did not affect the inflammatory response in ischemic brain-lesioned animals. This was in contrast to our results. CsA treatment of ouabain-injured rats (recipients of HUCB-NSC) had no favorable effect on graft survival, and did not even ameliorate T cell response at any of the time-points studied. Similarly, the studies that directly compared the percentage of surviving donor grafts between untreated and CsA-treated rats showed no difference in the survival rate between groups (Larsson and Widner 2000, Kogler et al. 2004, Walczak et al. 2004, Pan et al. 2005, Niranjan et al. 2007, Hicks et al. 2009). Although the lymphocytic infiltrate was scant or absent, the immunological/inflammatory rejection was associated with activated microglial cells.

To cope with the adaptive as well as the native immunity of the host, we used immunosuppression with combined CsA, azathioprine, and methylprednisolone. This triple-drug immunosuppression interferes with a much wider range of immunological response parameters than CsA monotherapy, and it seems valuable for experimental xenotransplantation studies. The protection of intracerebral mouse-to-rat hippocampal grafts upon treatment with three drugs has been reported previously (Pedersen et al. 1997). Nevertheless, our studies applying HUCB-NSC in an ouabain model of rat brain injury did not confirm these findings. The combination immunosuppression therapy was ineffective for the sustained survival of human cells. Host microglia activation and leukocyte infiltration, together with the astrogliosis observed in triple-medicated

recipients, was comparable with that seen in rats that received CsA alone. Walczak and coworkers (2004) found no prolongation of human cord blood cell survival in the brain of rat recipients pretreated with a combination of CsA, azathioprine, and methylprednisolone. Despite the decreased microglial activity, donor cell survival was poor. The authors suggested that the observed differences were related to concordant and discordant xenografts because of phylogenetically closely related (e.g., mouse-rat) or distant pairings (e.g., human-rat) of donor and recipients. In concordant systems, the graft rejection seems to follow the allograft-like mechanism, with relatively longer cell survival, whereas, in discordant systems, graft rejection is an acute or hyperacute mechanism.

The limited survival of human cells in the rat brain necessitated the search for some other methods to ensure acceptance of the graft by the recipients. The strategy of desensitization of the rodent to human cells in the neonatal period proposed by Kelly and colleagues (2009) was very attractive. We followed the same procedure to induce tolerance in the host to the cells transplanted in the same animals in their adulthood. However, in our experiments, the neonatal desensitization method failed to prolong HUCB-NSC survival and induced a native inflammatory reaction (e.g., microglial activation and astrogliosis) and an adaptive immunological response (T and B lymphocyte infiltration) in the rat brain. No difference was observed in the dynamics of donor cell elimination between desensitized and control animals. Recently, a group from the Johns Hopkins University confirmed our findings using human glial-restricted precursors derived from fetal brains. Transplantation of these cells into desensitized mice revealed immune cell infiltration in the area of the graft, followed by elimination of donor cells at two weeks (Janowski et al. 2012).

CONCLUSIONS

Over the past few years, extensive data have suggested that human cord blood cell grafts have a tremendous potential to improve functional recovery from motor deficits due to ischemic brain damage. However, clinical cell transplantation for the treatment of neurological disorders is still in a nascent stage due to the preliminary and insufficient experimental data. The elimination of human cells transplanted into discordant xenogeneic animals is a major setback. Establishing the experimental models for long-term survival of human cells in immunoprivileged, e.g., immune-deficient or humanized (human/animal chimeras**)** graft recipients, is one of the greatest challenges in stem cell transplantation research.

ACKNOWLEDGMENTS

The study was supported by Polish Ministry of Scientific Research and Higher Education, grant No. 401014235, and the Mossakowski Medical Research Centre, statutory fund No 23.

REFERENCES

Ali H, Forraz N, McGuckin CP, Jurga M, Lindsay S, Ip BK, Trevelyan A, Basford C, Habibollah S, Ahmad S, Clowry GJ, Bayatti N (2012) In vitro modelling of cortical neurogenesis by sequential induction of human umbilical cord blood stem cells. Stem Cell Rev 8: 210–223.

Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS, Nilsson M (2003) Astrocytes and stroke: networking for survival? Neurochem Res 28: 293–305.

Armstrong RJ, Harrower TP, Hurelbrink CB, McLaughin M, Ratcliffe EL, Tyers P, Richards A, Dunnett SB, Rosser AE, Barker RA (2001) Porcine neural xenografts in the immunocompetent rat: immune response following grafting of expanded neural precursor cells. Neuroscience 106: 201–216.

Bergwerf I, De Vocht N, Tambuyzer B, Verschueren J, Reekmans K, Daans J, Ibrahimi A, Van Tendeloo V, Chatterjee S, Goossens H, Jorens PG, Baekelandt V, Ysebaert D, Van Marck E, Berneman ZN, Linden AV, Ponsaerts P (2009) Reporter gene-expressing bone marrow-derived stromal cells are immune-tolerated following implantation in the central nervous system of syngeneic immunocompetent mice. BMC Biotechnol 7: 9–11.

Borlongan CV, Hadman M, Sanberg CD, Sanberg PR (2004) Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. Stroke 35: 2385–2389.

Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K (2002) Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 115: 2131–2138.

Buzanska L, Jurga M, Stachowiak EK, Stachowiak MK, Domańska-Janik K (2006) Neural stem-like cell line derived from a nonhematopoietic population of human umbilical cord blood. Stem Cells Dev 15: 391–406.

Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, Sanchez-Ramos J, Chopp M (2001) Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke 32: 2682–2688.

Chen Z, Palmer TD (2008) Cellular repair of CNS disorders: an immunological perspective. Hum Mol Genet 17(R1): R84–92.

Coyne TM, Marcus AJ, Woodbury D, Black IB (2006) Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 24: 2483–2492.

Dubois-Dauphin M, Julien S (2010) Stem cell-derived neurons grafted in the striatum are expelled out of the brain after chronic cortical stroke. Stroke 41: 1807–1814.

Glover JC, Boulland JL, Halasi G, Kasumacic N (2010) Chimeric animal models in human stem cell biology. ILAR Journal 51: 62–73.

Gorelik M, Janowski M, Galpoththawela C, Rifkin R, Levy M, Lukomska B, Kerr DA, Bulte JW, Walczak P (2012) Non-invasive monitoring of immunosuppressive drug efficacy to prevent rejection of intracerebral glial precursor allografts. Cell Transplant 21: 2149–2157.

Gornicka-Pawlak E, Janowski M, Habich A, Jablonska A, Drela K, Kozlowska H, Lukomska B, Domanska-Janik K (2011) Systemic treatment of focal brain injury in the rat by human umbilical cord blood cells being at different level of neural commitment. Acta Neurobiol Exp (Wars) 71: 46–64.

Haas S, Weidner N, Winkler J (2005) Adult stem cell therapy in stroke. Curr Opin Neurol 18: 59–64.

Habich A, Jurga M, Markiewicz I, Lukomska B, Bany-Laszewicz U, Domanska-Janik K (2006) Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured in vitro. Exp Hematol 34: 914–925.

Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J, Brenner R, Storch A (2004) Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci 117: 4411–4422.

Hicks AU, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J (2009) Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. Eur J Neurosci 29: 562–574.

Hofmeijer J, Veldhuis WB, Schepers J, Nicolay K, Kappelle LJ, Bär PR, van der Worp HB (2004) The time course of ischemic damage and cerebral perfusion in a rat model of space-occupying cerebral infarction. Brain Res 1013: 74–82.

Hovakimyan M, Müller J, Wree A, Ortinau S, Rolfs A, Schmitt O (2012) Survival of transplanted human neural stem cell line (ReNcell VM) into the rat brain with and without immunosuppression. Ann Anat 194: 429–435.

Jablonska A, Kozlowska H, Markiewicz I, Domanska-Janik K, Lukomska B (2010) Transplantation of neural stem cells derived from human cord blood to the brain of adult and neonatal rats. Acta Neurobiol Exp (Wars) 70: 337–350.

Janowski M, Gornicka-Pawlak E, Kozlowska H, Domanska-Janik K, Gielecki J, Lukomska B (2008) Structural and functional characteristic of a model for deep-seated lacunar infarct in rats. J Neurol Sci 273: 40–48.

Janowski M, Jablonska A, Kozlowska H, Orukari I, Bernard S, Bulte JW, Lukomska B, Walczak P (2012) Neonatal desensitization does not universally prevent xenograft rejection. Nat Methods 9: 856–858.

Jin K, Wang X, Xie L, Mao XO, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg DA (2006) Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A 103: 13198–13202.

Jurga M, Markiewicz I, Sarnowska A, Habich A, Kozłowska H, Lukomska B, Bużańska L, Domańska-Janik K (2006) Neurogenic potential of human umbilical cord blood: neural-like stem cells depend on previous long-term culture conditions. J Neurosci Res 83: 627–637.

Jurga M, Lipkowski AW, Lukomska B, Buzanska L, Kurzepa K, Sobanski T, Habich A, Coecke S, Gajkowska B, Domanska-Janik K (2009) Generation of functional neural artificial tissue from human umbilical cord blood stem cells. Tissue Eng Part C Methods 15: 365–372.

Jurga M, Dainiak MB, Sarnowska A, Jablonska A, Tripathi A, Plieva FM, Savina IN, Strojek L, Jungvid H, Kumar A, Lukomska B, Domanska-Janik K, Forraz N, McGuckin CP (2011) The performance of laminin-containing cryogel scaffolds in neural tissue regeneration. Biomaterials 32: 3423–3434.

Kelly CM, Precious SV, Scherf C, Penketh R, Amso NN, Battersby A, Allen ND, Dunnett SB, Rosser AE (2009) Neonatal desensitization allows long-term survival of neural xenotransplants without immunosuppression. Nat Methods 6: 271–273.

Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK (2004) Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci U S A 101: 11839–11844.

Kögler G, Sensken S, Airey JA, Trapp T, Müschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Müller HW, Zanjani E, Wernet P (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200: 123–135.

Kozłowska H, Jabłonka J, Janowski M, Jurga M, Kossut M, Domańska-Janik K (2007) Transplantation of a novel human cord blood-derived neural-like stem cell line in a rat model of cortical infarct. Stem Cells Dev 16: 481–488.

Kuh SU, Cho YE, Yoon DH, Kim KN, Ha Y (2005) Functional recovery after human umbilical cord blood cells transplantation with brain-derived neutrophic factor into the spinal cord injured rat. Acta Neurochir (Wien) 147: 985–992.

Larsson LC, Widner H (2000) Neural tissue xenografting. Scand J Immunol 52: 249–256.

Larsson LC, Frielingsdorf H, Mirza B, Hansson SJ, Anderson P, Czech KA, Strandberg M, Widner H (2001) Porcine neural xenografts in rats and mice: donor tissue development and characteristics of rejection. Exp Neurol 172: 100–114.

Lindvall O, Kokaia Z (2005) Stem cell therapy for human brain disorders. Kidney Int 68: 1937–1939.

Liu HY, Zhang QJ, Li HJ, Han ZC (2006) Effect of intracranial transplantation of CD34+ cells derived from human umbilical cord blood in rats with cerebral ischemia. Chin Med J (Engl) 119: 1744–1748.

McGuckin CP, Forraz N, Allouard Q, Pettengell R (2004) Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp Cell Res 295: 350–359.

Meier C, Middelanis J, Wasielewski B, Neuhoff S, Roth-Haerer A, Gantert M, Dinse HR, Dermietzel R, Jensen A (2006) Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. Pediatr Res 59: 244–249.

Molcanyi M, Riess P, Bentz K, Maegele M, Hescheler J, Schäfke B, Trapp T, Neugebauer E, Klug N, Schäfer U (2007) Trauma-associated inflammatory response impairs embryonic stem cell survival and integration after implantation into injured rat brain. J Neurotrauma 24: 625–637.

Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ (2005) Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. Proc Natl Acad Sci U S A 102: 18171–18176.

Neuhoff S, Moers J, Rieks M, Grunwald T, Jensen A, Dermietzel R, Meier C (2007) Proliferation, differentiation, and cytokine secretion of human umbilical cord blood-derived mononuclear cells in vitro. Exp Hematol 35: 1119–1131.

Niranjan A, Fellows W, Stauffer W, Burton EA, Hong CS, Lunsford LD, Kondziolka D, Glorioso JC, Gobbel GT (2007) Survival of transplanted neural progenitor cells enhanced by brain irradiation. J Neurosurg 107: 383–391.

Olstorn H, Moe MC, Røste GK, Bueters T, Langmoen IA (2007) Transplantation of stem cells from the adult human brain to the adult rat brain. Neurosurgery 60: 1089–1098.

Pan Y, Nastav JB, Zhang H, Bretton RH, Panneton WM, Bicknese AR (2005) Engraftment of freshly isolated or cultured human umbilical cord blood cells and the effect of cyclosporin A on the outcome. Exp Neurol 192: 365–372.

Pedersen EB, Zimmer J, Finsen B (1997) Triple immunosuppression protects murine intracerebral, hippocampal xenografts in adult rat hosts: effects on cellular infiltration, major histocompatibility complex antigen induction and blood-brain barrier leakage. Neuroscience 78: 685–701.

Pluchino S, Zanotti L, Deleidi M, Martino G (2005) Neural stem cells and their use as therapeutic tool in neurological disorders. Brain Res Brain Res Rev 48: 211–219.

Rosen CL, Dinapoli VA, Nagamine T, Crocco T (2005) Influence of age on stroke outcome following transient focal ischemia. J Neurosurg 103: 687–694.

Rota Nodari L, Ferrari D, Giani F, Bossi M, Rodriguez-Menendez V, Tredici G, Delia D, Vescovi AL, De Filippis L (2010) Long-term survival of human neural stem cells in the ischemic rat brain upon transient immunosuppression. PLoS One 5: e14035.

Ruiz A, Buzanska L, Gilliland D, Rauscher H, Sirghi L, Sobanski T, Zychowicz M, Ceriotti L, Bretagnol F, Coecke S, Colpo P, Rossi F (2008) Micro-stamped surfaces for the patterned growth of neural stem cells. Biomaterials 29: 4766–4774.

Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR (2001) Expression of neural markers in human umbilical cord blood. Exp Neurol 171: 109–115.

Steiner B, Roch M, Holtkamp N, Kurtz A (2012) Systemically administered human bone marrow-derived mesenchymal stem home into peripheral organs but do not induce neuroprotective effects in the MCAo-mouse model for cerebral ischemia. Neurosci Lett 513: 25–30.

Sun W, Buzanska L, Domanska-Janik K, Salvi RJ, Stachowiak MK (2005) Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells derived from the nonhematopoietic fraction of human umbilical cord blood. Stem Cells 23: 931–945.

Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, Tsukamoto Y, Iso H, Fujimori Y, Stern DM, Naritomi H, Matsuyama T (2004) Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest 114: 330–338.

Takahashi K, Yasuhara T, Shingo T, Muraoka K, Kameda M, Takeuchi A, Yano A, Kurozumi K, Agari T, Miyoshi Y, Kinugasa K, Date I (2008) Embryonic neural stem cells transplanted in middle cerebral artery occlusion model of rats demonstrated potent therapeutic effects, compared to adult neural stem cells. Brain Res 1234: 172–182.

Tambuyzer BR, Bergwerf I, De Vocht N, Reekmans K, Daans J, Jorens PG, Goossens H, Ysebaert DK, Chatterjee S, Van Marck E, Berneman ZN, Ponsaerts P. (2009) Allogeneic stromal cell implantation in brain tissue leads to robust microglial activation. Immunol Cell Biol 87: 267–273.

van der Stelt M, Veldhuis WB, van Haaften GW, Fezza F, Bisogno T, Bar PR, Veldink GA, Vliegenthart JF, Di Marzo V, Nicolay K (2001) Exogenous anandamide protects rat brain against acute neuronal injury in vivo. J Neurosci 21: 8765–8771.

Veldhuis WB, Floris S, van der Meide PH, Vos IM, de Vries HE, Dijkstra CD, Bär PR, Nicolay K (2003a) Interferon-beta prevents cytokine-induced neutrophil infiltration and attenuates blood-brain barrier disruption. J Cereb Blood Flow Metab 23: 1029–1039.

Veldhuis WB, van der Stelt M, Wadman MW, van Zadelhoff G, Maccarrone M, Fezza F, Veldink GA, Vliegenthart JF, Bär PR, Nicolay K, Di Marzo V (2003b) Neuroprotection by the endogenous cannabinoid anandamide and arvanil against in vivo excitotoxicity in the rat: role of vanilloid receptors and lipoxygenases. J Neurosci 23: 4127–4133.

Vendrame M, Cassady J, Newcomb J, Butler T, Pennypacker KR, Zigova T, Sanberg CD, Sanberg PR, Willing AE (2004) Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. Stroke 35: 2390–2395.

Walczak P, Chen N, Hudson JE, Willing AE, Garbuzova-Davis SN, Song S, Sanberg PR, Sanchez-Ramos J, Bickford PC, Zigova T. (2004) Do hematopoietic cells exposed to a neurogenic environment mimic properties of endogenous neural precursors? J Neurosci Res 76: 244–254.

Walczak P, Chen N, Eve D, Hudson J, Zigova T, Sanchez-Ramos J, Sanberg PR, Sanberg CD, Willing AE (2007) Long-term cultured human umbilical cord neural-like cells transplanted into the striatum of NOD SCID mice. Brain Res Bull 74: 155–163.

Wechsler LR, Kondziolka D (2003) Cell therapy: replacement. Stroke 34: 2081–2082.

Willing AE, Lixian J, Milliken M, Poulos S, Zigova T, Song S, Hart C, Sanchez-Ramos J, Sanberg PR (2003) Intravenous versus intrastriatal cord blood administration in a rodent model of stroke. J Neurosci Res 73: 296–307.

Wood MJ, Sloan DJ, Wood KJ, Charlton HM (1996) Indefinite survival of neural xenografts induced with anti-CD4 monoclonal antibodies. Neuroscience 70: 775–789.

Zhao Y, Marín O, Hermesz E, Powell A, Flames N, Palkovits M, Rubenstein JL, Westphal H (2003) The LIM-homeobox gene Lhx8 is required for the development of many cholinergic neurons in the mouse forebrain. Proc Natl Acad Sci U S A 100: 9005–9010.

Zigova T, Song S, Willing AE, Hudson JE, Newman MB, Saporta S, Sanchez-Ramos J, Sanberg PR (2002) Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. Cell Transplant 11: 265–274.

Zychowicz M, Mehn D, Ruiz A, Frontczak-Baniewicz M, Rossi F, Buzanska L (2012) Patterning of human cord blood-derived stem cells on single cell posts and lines: Implications for neural commitment. Acta Neurobiol Exp (Wars) 72: 325–336.