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Long-term cultured human umbilical cord neural-like cells transplanted into the striatum of NOD SCID mice

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Abstract

The use of stem cells and other cells as therapies is still in its infancy. One major setback is the limited survival of the grafts, possibly due to immune rejection. Studies were therefore performed with human umbilical cord blood cells (HUCB) to determine the ability of these cells to survive *in vivo* and the effect of the immune response on their survival by transplantation into the normal striatum of immunodeficient NOD SCID mice.

Long-term culture of HUCB cells resulted in several different populations of cells, including one that possessed fine processes and cell bodies that resembled neurons. Their neuronal phenotype was confirmed by immunohistochemical staining for the early neuronal marker TuJ1 and the potentially neural marker Nestin. Five days after cell transplantation of this neuronal phenotype, immunohistochemical staining for human mitochondria confirmed the presence of living HUCB cells in the mouse striatum, with cells localized at the site of injection, expressing early neural and neuronal markers (Nestin and TuJ1) as well as exhibiting neuronal morphology. However, no evidence of surviving cells was apparent 1 month postgrafting. The absence of signs of T cell-mediated rejection, such as CD4 and CD8 lymphocytes and minimal changes in microglia and astrocytes, suggest that cell loss was not due to a T cell-mediated immune response.

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Conflict of interest PRS is cofounder and owner of Saneron CCEL therapeutics Inc.; AEW, TZ, and JSR are consultants to Saneron CCEL Therapeutics Inc.; PRS, JSR, and AEW are coinventors on HUCB-related patents.

In conclusion HUCB cells can survive long-term *in vitro* and undergo neuron-like differentiation. In mice, these cells do not survive a month. This may relate to the differentiated state of the cells transplanted into the unlesioned striatum, rather than T cell-mediated immunological rejection.

Keywords

Human umbilical cord blood cells; Immunodeficient NOD SCID mice; Transplantation; Striatum; Long-term culture; Neural-like

1. Introduction

Nervous tissue diseases have relatively well-described etiology and pathology, but their therapy has always been a challenge and with few exceptions, effective treatment for many brain diseases and injuries does not exist. Advancements in stem cell research have raised hope that new therapeutic applications might emerge from this field. Transplantation of stem cells into diseased nervous tissue (or by peripheral administration) could provide beneficial effects by introducing a reservoir of progenitor cells with the potential to distribute widely in the brain and differentiate into missing neuronal phenotypes, or by trophic factor support rescuing degenerating endogenous cells [8,10,11,17,31,33,41]. The cell transplantation approach has already been implemented as clinical trials for the treatment of Parkinson's disease [15,35, 36] or stroke [1,27,34]. While the results of these studies were encouraging, it became clear that more basic research needs to be done before we can expect spectacular clinical results. Issues that must be addressed before further clinical trials proceed include those of increasing cell survival, ensuring cellular distribution in those diseased or injured areas, clearly defining the differentiation repertoire of the stem cell population and most importantly, resolving the issue of graft-host interactions and how to enhance axonal outgrowth and afferent/efferent wiring. The first step in this process must be the characterization of the cell candidates, but this can only partly be performed *in vitro* since the environment of the living organism is much more complex.

Cells which are to be considered for transplantation into humans are preferred to be of human origin. This presents a dilemma—is it reasonable to test human cells in animals? Xenografting of human cells into the rodent is a widely used research paradigm that often produces encouraging results. However, it is easy to show the presence of xenografted cells and even their phenotypic features, but assessment of their function is usually more complex. It has been shown that human umbilical cord blood (HUCB) derived cells can repopulate blood lineages in transgenic NOD SCID (non-obese diabetic severe combined immunodeficient) mice and produce immunoglobulins [19]. This suggests that at least some human cell types can execute their function in the mouse host. In the case of neural tissue xenografting, proof of a functioning xenograft *in vivo* is more challenging. Judgement has to be based on the cellular morphology, specific proteins expressed or behavioral testing.

The NOD SCID mouse has impaired T and B cell lymphocyte function, and lacks natural killer cells and the ability to stimulate complement activity. These mice are therefore severely immunodeficient, but they do still possess some ability to reject grafts, as shown by the increased graft acceptance of HUCB cells following anti-CD122 treatment [32]. CD122 is found on macrophages and monocytes as well as natural killer cells.

Our previous experiments showed that, under culture conditions, HUCB cells are able to generate cells with neural characteristics [4]. When transplanted into the neurogenic region of neonatal rat brain, a small fraction of these cells migrate and express neuronal and astroglial proteins [46]. Further, when intravenously transplanted into the rat middle cerebral artery

occlusion stroke model, behavioral deficits were reduced [43]. However, transplantation of HUCB cells into adult or aged rat brains was associated with vigorous rejection and strong immunosuppression was required to protect the graft [42].

The environment of the aging brain appears to be different from that of the neonate. Transplants in aged animals are considerably less successful than the same transplant in neonates. This may be a result of the immunological/inflammatory status of the aging brain. Microglia, the only immune competent cells in the brain, become more abundant with age and express proteins that are characteristic of an activated state [28]. The response to injury is also age-dependent. More activated effector cells are present in aged, compared to young, rats [29], as are greater quantities of pro-inflammatory molecules such as interleukin-1beta (IL-1beta), tumor necrosis factor-alpha (TNF-alpha) or interleukin-6 (IL-6) [39]. The combination of all these factors makes the aging brain a more 'hostile' environment than the developing one.

Due to the difficulties in maintaining graft survival in the aging rat brain we selected the NOD SCID mouse for our *in vivo* characterization of the HUCB cells; this mouse model is widely used for characterization of human hematopoietic [9] and non-hematopoietic [12] grafts and the engraftment rate is high. HUCB cells were stereotactically transplanted into the striatum, and the brains were examined for cell survival and neuronal differentiation at either 5 or 30 days after transplantation. At early time points, many differentiated HUCB cells expressing characteristic neuronal proteins were detected. However, at 1 month postgrafting, HUCB cells were no longer detected.

2. Materials and methods

2.1. Short-term culture of HUCB cells

Cryopreserved full population of HUCB mononuclear fraction (supplied by Cambrex, Poietics Inc., North Brunswick, NJ or Saneron CCEL Therapeutics Inc., Tampa, FL) were thawed in a 37 °C water bath, and suspended in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS, Gibco) and 0.1% Gentamicin (Sigma, St. Louis, MO). The cells were then plated on poly-L-lysine-coated 8-well dishes (Nunc, Rochester, NY) at a seeding density of 100,000 cells/cm², as described previously by Zigova et al. [46], and incubated in 5% CO₂ at 37 °C for 7 days with a change of media at day 4. The cells were washed with 0.1 M PBS, fixed with cold 4% paraformaldehyde (Sigma) in 0.1 M PBS for 10 min and washed in cold PBS. Soon after fixation, the slides were used for immunocytochemical detection of cell type-specific antigen expression.

2.2. Long-term culture of HUCB cells

Cryopreserved full population of HUCB mononuclear fraction (supplied by Cambrex, Poietics Inc. or Saneron CCEL Therapeutics Inc.) were thawed in a 37 °C water bath, suspended in DMEM with 10% FBS and 0.1% Gentamicin, then plated in a 25 ml flask (Nunc) at a density of 10⁷ cells and incubated in 5% CO₂ at 37 °C. After 1 day, the floating population was replated in a fresh 25 ml flask with identical medium for 60 days (p2). We then manually selected a cluster and replated it in the same medium for up to 160-224 days (p10-p16) with media exchange every 4-5 days. The cells were passaged every 10 days or so, i.e. once they had reached 80% confluence, the adhesive fraction was trypsinized and the floating cells collected, centrifuged and lightly triturated. The viability of initial full- and floating populations was determined by trypan blue (Sigma) to be 90-95%. The cells were then replated to the same density as before: 10⁷ cells per 25 ml flask. The cells to be injected were prepared the similarly, but not replated. For fixation, floating cells were trypsinized and then washed with 0.1 M PBS, fixed with cold 4% paraformaldehyde in 0.1 M PBS for 10 min and washed in cold PBS. The cells were then resuspended in a small volume of PBS and applied to either slides or chamber

slides treated with poly-L-lysine to enhance adherence at 4 °C overnight. Adhered cells were fixed in the 25 ml flasks as described above. Soon after fixation, the slides were used for immunocytochemical detection of cell type-specific antigen expression.

2.3. Animals

Transplantation experiments were performed on male 4-week-old NOD SCID mice (Jackson Laboratory, Bar Harbor, ME), housed two per cage ($n = 4$). Animals were maintained on 12 h light/dark cycles with a continuous supply of food and water. All animal procedures were performed in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and were approved by the University of South Florida Institutional Animal Care and Use Committee.

2.4. HUCB cells transplantation

Cells, grown as outlined in the long-term culture section above for 176 days, were loaded into the 10 μ l Flexifill microsyringe (WPI, Sarasota, FL), and placed into the motorized injector unit (Stoelting, Wood Dale, IL) attached to the stereotaxic device. Animals received an injection of cell suspension (total 100,000 cells in 2 μ l suspension) into the anterior part of the striatum [level skull, (AP) = 1.3, (ML) = 0.7, (DV) = -2.3, as measured from bregma and dura (Paxinos & Watson, 1996)]. Cells were slowly introduced into the brain at a rate of 0.5 μ l/min, using the motorized injector. The needle was left in place for 5 min and then slowly withdrawn. The incision was closed with surgical glue (Vetbond, Vet Products Labs, Phoenix, AZ). After surgery, animals were placed back into their cages and allowed to survive for either 5 days or 1 month.

2.5. Tissue processing

Five days, or 1 month, after transplantation, the animals were anesthetized with pentobarbital (150 mg/kg) and transcardially perfused with 200 ml of 0.1 M PBS, followed by 250 ml of 4% paraformaldehyde in the same buffer. The brains were removed and postfixed for 24 h in 4% paraformaldehyde and cryopreserved in 30% sucrose. Twenty micrometer thick cryostat-cut sagittal sections (from both hemispheres) were thaw-mounted on charged slides and completely dried before storing at -20 °C. When ready to use, the slides were equilibrated to room temperature and every sixth section was stained with thionin, dehydrated and sealed with permount.

2.6. Immunohistochemistry

Graft localization was first estimated with thionin (Sigma) staining. Sections from the brain area proximate to the injection site were processed for human-specific antigens (Human Nuclei or Mitochondria [HuMi], 1:50, Chemicon, Temecula, CA) to determine the presence of human cells. A human-specific Nestin antibody (1:200, Chemicon) that does not cross react with rodents, was also used for detection of HUCB neural progenitors. Double staining with human and cell type-specific markers (Doublecortin, 1:2000, Chemicon; TuJ1, 1:1000, Covance, Berkeley, CA; GFAP, 1:400, Dako, Carpinteria, CA; CD4, CD8, CD45 1:50, BD; CD11b, 1:50, Serotec, Raleigh, NC) were employed to identify the phenotype of the transplanted cells or reaction of the host. Sections were incubated overnight at 4 °C with the aforementioned primary antibodies diluted in 0.1 M PBS containing 10% normal goat serum. The following day, they were treated with the appropriate secondary antibodies. Negative controls involved the same procedures with the omission of the primary antibody. DAPI (Sigma) and vectashield mounting medium (Vectorlabs, Burlingame, CA) were used for counterstaining nuclei. The immunostained sections were analysed using the Olympus BX60 (Olympus, Center Valley, PA) and IX71 epifluorescence microscopes with Olympus DP-70 digital acquisition system.

Images were obtained using 20× and ×40 objectives and processed with Photoshop software (Adobe, San Jose, CA).

3. Results

3.1. Mononuclear HUCB fraction initially consists mostly of hematopoietic cells, but long-term culture under differentiating conditions induce neuronal features

Initial cultured HUCB mononuclear fraction presented heterogeneous morphologies when cultured with DMEM with 10% FBS. Initially, these cells formed clusters and subsequently differentiated (Fig. 1). Bigger flat round cells tightly attached to the flasks, acting as a feeder layer for these smaller cells. Small and round cells either 'piggy backing' the large cells or floating in the media, differentiated into a variety of cell types that were highly enriched with cells expressing the hematopoietic marker CD45 and to a lesser extent early neural markers such as Nestin (~26%) and the neuronal marker TuJ1 (3%) (Fig. 2).

The long-term culture of these cells (60-211 days) revealed the presence of mostly multipolar, elongated cells, often with an extended cytoplasmic rim resembling fibroblasts, as well as cells with fine processes and cell bodies appropriate for neurons (Fig. 1). Immunohistochemistry detected few CD 45 (pan leukocyte antigen) positive cells (less than 3% of total cell number) compared with that seen in the short-term cultures (greater than 80% of total cell number—Fig. 2; compare A and D) and the absence of mature lymphocyte or macrophage markers (*not shown*). Lymphocytes and macrophages are frequent in the mononuclear HUCB fraction but their content was reduced if not eliminated from the cell population over time. In the long-term culture (60-211 days) cells expressed the early neural marker Nestin (50%) and the early neuronal marker TuJ1 (90%) which were detected immunohistochemically (Fig. 2) and confirmed by Western Blot (see [4]). However the cells expressing TuJ1 do not appear to clearly possess all of the characteristic morphology of neurons mature enough to express this marker such as the fine, long or branched processes (see Figs. 1, 2C and F).

3.2. HUCB derived cells were detected 5 days postgrafting in the NOD SCID mouse striatum

Transplanted HUCB cells were detected at 5 days, but not 1 month postgrafting. Cells were detected within the striatum, using a human-specific antigen against mitochondria (HuMi). No major migration was observed along the needle track and in the surrounding tissue up to 2 mm from the deposit center (Fig. 3A). Of 100,000 transplanted cells, around 5% could be detected 5 days later. One month after transplantation, only autofluorescing round cells - possibly degenerating phagocytes - were visible in the injection area, meaning that it was devoid of surviving HUCB cells. The spleen was examined for HUCB cells, as the most probable organ for hematopoietic cells to migrate to. No cells were detected in the spleen allowing us to rule out the possibility that the cells had completely left the injection site and migrated through the blood stream.

3.3. Transplanted cells express neuronal markers and develop neuronal morphology

Five days after transplantation HUCB cells detected with antibody against HuMi appeared as round and polar cells often with long processes (Fig. 3A). Many of the grafted cells expressed Nestin - a protein found in neural progenitors (Fig. 3C). The antibody exclusively detects human cells and does not cross react with rodent tissue.

In line with results from culture studies, most of the human cells were positive for TuJ1 - an early neuronal marker (Fig. 3B) - and 50% of these cells also express the neural marker Nestin. None of them expressed the astroglial marker GFAP or pan leukocyte CD45.

3.4. NOD SCID mice did not develop detectable immune response against the graft but we were unable to detect the cells 1 month after transplantation

NOD SCID mice are completely depleted of competent lymphocytes and therefore also immunoglobulins, meaning these animals lack the adaptive immune response. Additionally, their innate immunity (natural killers, macrophages) is also highly reduced [13]. This suggests that rejection will not occur with either allo- or xeno-grafting.

The fact that transplanted cells were able to survive in the brain for less than 1 month encouraged us to check for a possible “leak” in immunodeficiency and rejection signs. Immunostaining against CD4 and CD8 T lymphocytes was negative in the injection area (not shown). To detect microglia/macrophage activation, immunostaining for CD11b antigen was performed (Fig. 4). A typical sign of activated microglia/macrophage around the graft in immunocompetent animals is the presence of a large number of unbranched cells and high intensity of staining. These signs were not observed in our grafts into NOD SCID animals. The staining intensity was slightly increased around the needle track but cells always had branched morphology and the number of positive cells did not suggest infiltration (Fig. 4).

3.5. Reaction of the host tissue in the area of the graft

At the time when human cells were present in the striatum (5 days postgrafting) they were found scattered among endogenous mouse cells without astrocytic or microglial limiting borders. Contrary to our previous experiments in immunocompetent animals, the number of glial cells as well as their appearance was not different from other sites in the brain. Endogenous neurons were seen in close apposition to the human cells allowing for their interactions (Fig. 5). Transplanted cells in the center of the graft deposit were surrounded by fibronectin (Fig. 5); a substance produced by HUCB cells which is important in axon sprouting and guidance.

4. Discussion

Transplantation of HUCB cells into the striatum of NOD SCID mice led to the integration and differentiation of these cells at 5 days postgrafting, but their absence by 30 days. By contrast, transplantation of these cells by intracerebroventricular injection into the mouse model of Sanfilippo Syndrome Type B led to both long-term survival (7 months) as well as neural morphology for these cells [16] and a 20% survival was seen following transplantation into rat neonates after 1 month [46]. In addition to the mode of delivery and the different mouse models, the Sanfilippo study differs from both this study and Zigova's [46], since it involved immunosuppressive treatments. The fact that the cells transplanted into the striatum did not survive to 30 days may be due to the absence of this immunosuppressive therapy, suggesting that inhibition of the aspects of immunity remaining in the SCID mouse that can be blocked by immunosuppressive drugs, are crucial in the survival of these transplants. Of interest with respect to this, is the successful treatment of a SCID child with her normal sister's cord blood cells; she still has excellent health 18 months later [40]. Surprisingly, no evidence of an immune response was apparent at either time point in our study. Repetition of our study using immunosuppressive drugs may allow us to determine if there is an immune response that the antibodies used and observations made cannot pick up, causing the lack of survival of these cells by 30 days.

Immunosuppressive drugs such as cyclosporine A, work by blocking calcineurin, a major component of intracellular calcium signaling, which in turn blocks activation of T cells and the production of pro-inflammatory cytokines. These drugs therefore inhibit cytokine production, and curtail T cell proliferation as well as reducing MHC II immunoreactivity and shifting the T cell helper response from Th2 (acute vascular rejection) to Th1 (cell-mediated rejection), promoting acceptance of the graft.

Alternatively, the striatum may be a more 'hostile' environment in which HUCB cells do not readily survive due to an absence of necessary survival signals or the presence of 'death' signals. These cells have been transplanted into NOD/SCID mice before, but only peripherally and they survive for at least 2 months, based on human-specific antibody detection [12]. Prior grafting studies in rats (with immunosuppression) also demonstrate migration of HUCB cells from the injection site, when the SVZ was used [42]. In contrast, our study showed no cell migration in the NOD SCID mouse following striatal grafting. This is in contrast to the study by Zietlow et al. [45] wherein they observed a greater migration of long-term cultured neural precursor cells following transplantation into the ibotenic acidlesioned striatum of rats. It is important to note that first these cells did not survive long-term (absent by 20 weeks) and that they were transplanted into lesioned rather than intact brain. This latter effect may be an explanation for discrepancies with our findings. Transplantation of human expanded neural precursors into the striatum of 6-OHDA lesioned rats demonstrated widespread migration of the cells throughout the brain, but no specific targeting of the lesioned areas [22]. This suggests that, in the parkinsonian rat at least, additional cues are required for the specific targeting of transplanted cells, though the study by Jain et al. [22] does confirm that migration of transplanted cells does occur. Previous studies in rats rather than mice, have shown significant migration of grafted human tissue from the striatum at 6 months, but interestingly very little migration was observed by 1 month, at which point in our study no transplanted cells survived [20]. It is therefore unclear whether migration would have occurred if the cells had survived for longer. One further notable difference is the presence of TuJ1 positive and therefore potentially neuronal cells in this study compared with their absence in the previous immunosuppressed rat study, though this may be explained by the long-term culturing of the transplanted cells here compared with the 1 week culture in the prior study [42]. Additionally there are likely to be different differentiating factors in the striatum, compared with the SVZ, though it should also be pointed out that a more dilute (onefold) and potentially less specific antibody solution was used in the SVZ study to detect the presence of TuJ1. The striatum is also rich in retinoic acid compared with the SVZ, which promotes neuronal differentiation.

A further consideration is that the cells were grown for 176 days before transplantation and during this time developed neural characteristics as shown by enhanced Nestin and TuJ1 staining. These cells appear to have begun to differentiate into a neural phenotype and are therefore not as pluripotent as in short-term studies. However, it should be remembered that Nestin is not solely expressed by neural precursors, but may also be present in non-neural cells including endothelial cells, which considering the hematopoietic origin of these cells is significant. Fortunately the presence of TuJ1 staining does help support the fact that some of the Nestin-staining cells could be neuronal precursors. Of interest is a recent study that suggests that all neural precursor cells express Nestin [44], but it does not follow that all Nestin expressing cells are neural. Unfortunately, the morphology of these cells does not fully support their suggested neuronal phenotype. A recent study from our lab has shown that the floating cells possess greater proliferation capacity and more neural characteristics than the adherent fraction of cells [5]. Such a distinction was not made in any major capacity in this study and may contribute to this.

Transplantation of fetal tissue has been shown to be time dependent, with embryonic neural precursor cells grown in long-term culture not surviving for as long as short-term cultured cells following transplantation [45], as well as there being an apparent optimal window of successful transplantation of tissue less than 65 days old [14]. It is therefore possible that the cells maybe too old or differentiated to survive long-term *in vivo*, since Zietlow et al. [45] demonstrated profound changes in the expression of a number of different classes of genes following long-term growth. Repetition of this study using short-term cultures would help to determine if this is a limiting factor in these mice. A short-term study in which the HUCB cells were treated with a number of growth and neurotrophic factors demonstrated enhanced survival, though

this was not found to result in long-term survival [5]. Interestingly, both Bouhan et al. [2] and Kelly et al. [25] have demonstrated that culture of embryonic neural precursors with either a chemically defined or EGF and FGF-2 containing media, results in growth of neurons. The latter study showed that this was embryonic age-dependent and also species-specific, since mice precursors proliferate indefinitely whereas the rat cells did not, with the longest period of proliferation occurring in the cells isolated earliest. Christophersen et al. [6] have also shown that growth factor-dependent expansion of human fetal forebrain cells promotes the induction of dopaminergic neurons. This all provides evidence of the importance of the age of the cells at harvesting and their ability to proliferate, as well as the conditions under which the cells are expanded and implanted being important with respect to their potential survival and use as a treatment for neurodegenerative disorders.

A final important point is that the cells were transplanted into 'normal' tissue rather than lesioned tissue. This means that there may not be adequate survival signals to keep the graft alive as well as the surrounding 'normal' tissue. Previous studies of fetal grafts, or marrow stromal cells into normal brain tissue do show some evidence of detrimental effects [7,18,30] and therefore it may be interesting to see whether HUCB cell survival is enhanced following transplantation into lesioned NOD SCID mice. Coyne et al. [7] found that marrow stromal cell transplants induced an inflammatory response which led to loss of the transplanted cells by 14 days, and Hagenmeyer-Houser and Sanberg [18] showed a time-dependent decrease in striatal transplant size of embryonic striatal tissue at 10 weeks compared to 3 weeks, though they still had a surviving graft at the later time point. In our case, there was little evidence of an inflammatory response, but the transplant died off anyway. Some hope is provided by the transplantation studies of Sun et al. [38], who demonstrated enhanced survival of neural stem cells into the 6-OHDA lesioned striatum of rats and observed greater survival and more differentiation into TH-positive cells in these mice than in controls. Further evidence that the adult intact striatum is less conducive for transplantation compared to denervated striatum was demonstrated by Sable et al. [37], whereas Joannides et al. [23] and, as mentioned above, Sun et al. [38] demonstrated that pathological conditions enhance neural formation. This may explain why the HUCB cells did not survive to 1 month in our study. Several studies have shown that cell survival is dependent on a number of factors. Kelly et al. [26] determined that striatal graft survival is influenced more by the origin of the cells than the immunogenic background. Our study would appear to support this, since the absence of an immunogenic background in the NOD/SCID mice did not aid survival. Chen et al. [5] also demonstrated enhanced proliferation and survival following growth factor treatment.

There is a growing body of studies using non-neural stem cells to generate neural cells. Recent studies demonstrate that long-term culture of HUCB enhanced neural content of cell cultures (Jurga et al. [24]; an observation which we also see), however, Jain et al. [21] saw an overall decrease in neuronal number with long-term culture, but an increased migration of cells with a neuronal phenotype (but not of total cells) in the hippocampus in intact rat brain. Buzanska et al. [3] have also generated a neural stem-like line from the non-hematopoietic fraction of HUCB. These cells could potentially be included in our cell culture since we do not remove the non-hematopoietic cells. Since the number of CD45+ cells was reduced in our long-term culture, this suggests that our long-term culture could be transforming into the neural stem-like line detected by Buzanska et al. [3].

The results of our long-term culture of HUCB cells, in conjunction with the studies by others already mentioned, show that these cells can survive for long periods of time *in vitro* and that they can also exhibit neural (or other) characteristics dependent on their environmental conditions. It is unclear why the same is not true *in vivo*. In fact, during long-term culture, the cells are more prone to differentiate since more neural-like (Nestin and/or TuJ1 staining) and fewer hematopoietic cells (CD45+ staining) were apparent compared with short-term cultures.

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This paper is dedicated to the memory of Dr. Zigova, an erudite scholar, a visionary scientist, and a gentlewoman. She was a beloved mentor, an inspiration and a friend.

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Fig. 1.

The morphological comparison of HUCB cells between short- and long-term culture. The bright field micrographs showed that (A) HUCB mononuclear cells formed clusters 1 DIV after culture. (B) HUCB cells morphologically presented high heterogeneity on day 20 in culture, bigger flat round cells adhered to the surface of vessels (asterisks) with small round and differentiated cells sitting on top of the adherent cells (arrows). (C) and (D) show the morphology of long-term cultured HUCB cells. (C) These cells grew well and were able to form clusters. (D) Differentiating cells had long single or multiple processes (arrows). Scale bar: 20 μm (A and C) and 40 μm (B and D).

Fig. 2.

Immunofluorescence microphotographs showing different incidences of the expression of hematopoietic and early neuronal antigens in short- and longer-term cultured HUCB cells. (A) More than 90% of cells expressed CD45 on 5-day culture (green) and a low number of CD45 positive cells were present in 192-day culture (D, green). (B) A small amount of cells that scattered in the short-term culture (5 DIV) presented Nestin staining (green). However, numerous cells in longer-term culture (143 DIV) expressed Nestin antigen (E, green). Most Nestin positive cells were clustered and resembled a neurosphere. (C) A few cells at day 15 were TuJ1 positive (red) and 60-80% of cells at day 143 exhibited TuJ1 positivity (red). Blue DAPI counterstaining was used for clear identification of all cultured cells in (D-F). Scale bar: 20 μm (A), 40 μm (B and C) and 50 μm (D-F).

Fig. 3.

Immunofluorescence microphotographs showing the pattern of human cell markers at the injection site for HUCB cells in mouse striatum, 5 days after grafting. (A) Human mitochondria (HuMi; green) reveals the presence of transplanted cells amongst a background of non-staining mouse cells. Note the variety of cell shapes, with some possessing long processes. (B) HuMi staining (green) is shown against a background of TuJ1 (red) endogenous neuronal staining. (C) The human neural-specific Nestin stain (green) demonstrates the presence of HUCB cells at the injection site in the mouse striatum. In B and C nuclei are visualized with DAPI staining (blue). A subpopulation of the grafted HUCB are also TuJ1 positive (white/yellow in

appearance), suggesting differentiation into neuron-like cells. Notice the lack of migration of these cells away from the injection site. Scale bar = 100 μ m in all figures.

Fig. 4.

Immunofluorescence microphotograph depicting the presence of microglia at the injection site for HUCB cells in mouse striatum, 5 days after grafting. CD11b staining (red) was used to demonstrate the increased presence of microglia and macrophages at the injection site of the HUCB cells. The morphology of the stained cells - typically branched - reveals a general lack of activated cells (shown by unbranched, rounded cells) of these cells at 5 days postgrafting, demonstrating the absence of a fully developed immune response. Scale bar = 200 μm .

Fig. 5. Immunofluorescence microphotograph demonstrating the incorporation of the transplanted HUCB cells into the striatum at the injection site. The HUCB cells, stained by Nestin (green) are surrounded by fibronectin (red). Fibronectin is an important substrate for axonal sprouting and guidance and its colocalisation (yellow) with the HUCB cells suggests their attempted integration into the striatum. DAPI staining (blue) reveals the presence of all endogenous (and non-neuronal HUCB) cells within the injection site.