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ORIGINAL RESEARCH REPORT

Bilateral Interaction Between Cord Blood–Derived Human Neural Stem Cells and Organotypic Rat Hippocampal Culture

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The umbilical cord blood–derived neural stem/progenitor cells (HUCB-NSCs) potentially represent a rich source of transplantable material for treatment of a wide range of neurological diseases. Although, recently reported effects of their implementation in animal models of brain pathology are still controversial. As a simplified alternative to in vivo transplantation in this work we have applied a long-term organotypic rat hippocampal slice culture (OHC) as a recipient tissue to study bilateral graft/host cells interactions ex vivo. This type of culture can be considered as a kind of reductionistic model of brain transplantation where direct influence of systemic immunological responses to transplanted human cells would be excluded. The transplantation material derived from a HUCB-NSC line developed and characterized in our laboratory and delivered to the slices either as a single-cell suspension or after formation of typical neurospheres in serum-free medium in vitro (N-HUCBs). Experiments were focused on space-temporal context of cell transplantation in relation to their ability to ingrown, migrate, and differentiate within the slice cytoarchitecture. We gain evidences that these responses are strictly dependent on the engraftment site and that cell movement reflects typical routes used for migratory neuroblasts in vivo. The cells implanted at the second week of slice cultivation ingrown readily and deeply into host cytoarchitecture then matured to the level never observed in our transplantation animal models in vivo. Importantly, transplanted neurospheres, in addition to yield exogenous migratory cells to the host tissue can locally inhibit astrocytosis and promote outgrow of DCX-reactive neuroblasts in the surrounding OHC tissue.

Introduction

Since the physiological capacity to regeneration is not sufficient to restore brain structure and function when compromised by variety of acute or progressively chronic diseases, the novel supportive strategies for this naturally occurring process has to be considered. One of them is the stem cell–based therapy that might either support endogenous process of neurogenesis or directly replace the cell losses in different damaged brain areas [1]. However, there are still no convincing experimental data for long-term survival and stable engraftment of the alien human donor cells into damaged animal tissue. Even, when these cells were shown to migrate to the site of injury shortly after transplantation, they survived transiently and were too few in number to account for suggested reparative ability [2]. Thus, it seems that most of transplanted human cells are acutely immunorejected shortly after xenografting due to existence of an innate (immunosuppression-resistant) immunological barrier. While the above observations concern NSC-derived form cord blood, similar data have been already published also in the case of other xenotransplantations concerning human mesenchymal [3,4] or even embryonic stem cells [5]. Looking in clinical perspectives, there is still an urgent need for hasten efforts to develop animal models with higher tolerance to transplanted human cells for relevant verification of their therapeutic potential before setting of any clinical trials.

Meantime, as compromised solution of this methodological problem, a simplified model of ex vivo human cell transplantation could be proposed. Rat hippocampal organotypic culture, the model presumptively free of systemic immunological influence, permits stable and controlled conditions for studying cell transplantation and interactions between recipient brain tissue and transplanted human

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neural stem cells. The experiments described in this work were performed in the attempt to investigate whether human neural stem cells transplanted to central nervous system (CNS) tissue may undergo maturation and incorporation into the host neuronal network. The other important issues addressed here were to investigate how histological region of transplantation can modify transplant behavior and a vice versa, how grafted cells influence recipient neural tissue. As a source of neural stem cells we have used well characterized line of a non-transformed, clonal, and karyotypically stable neural stem cells derived from human umbilical cord blood (HUCB-NSCs) [6–8]. This source of human stem cells is free from ethical controversies, easy to obtain, and significantly less expensive in relation to other embryonic or adult sources [9,10]. In further perspective of clinical translation of the experimental results, important is that HUCB has been used successfully for over 20 years in treatment of almost 80 different human diseases. In our experiments we have confirmed that HUCB-NSCs can be differentiated in vitro into functional neurons [11] which are able to generate spontaneous, TTX-sensitive electric potentials (Jurga et al., in progress), suggesting their usefulness as an ideal material for neurotransplantation.

In this study, we have used the heterogeneous mixture of neural stem cells with their more mature progenitors (HUCB-NSCs) growing in monolayer culture [8] and transplanted as the cell suspension or after generation of neurospheres (N-HUCBs) [12], the multicellular aggregates of nondifferentiated NSCs, previously described as the typical entities for brain-derived neural stem cell cultured in vitro. Single-cell suspensions of HUCB-NSCs have been transplanted equally all over the surface of hippocampal slices and these experimental designs may represent systemic cell delivery in vivo. The other approach comprise delivery of a single 3-D N-HUCB at distinguished region of OHC (gyrus dentatus—DG, cornu ammonis—CA, enthorinal cortex—EC), mimics behavior of the solid graft after inraparenchymal transplantation. Transplantations were performed at various time points after OHC establishment. Considering slice preparation as a moment of a severe brain tissue injury such timing may reflects different periods of post-traumatic cell transplantation and help to find the best therapeutic window for cell administration in vivo. In our experiments we gain evidences that effect of transplantation is strictly dependent on the time and side of cells engraftment and that cell movement reflects typical routes used by migratory neuroblasts in vivo (DG toward CA). The cell migration, clearly evidenced when transplant was delivered shortly after OHC establishment, decreased subsequently in the older cultures in favor of increased cell differentiation. Moreover, the hippocampal slices seem to prefer neuronal differentiation of transplanted cells with concomitant suppression of glial commitment either in grafted HUCB-NSCs or in the host tissue.

Materials and Methods

Neurospheres and neural stem cells derived from hematopoietic progenitor-depleted (CD34 negative) cord blood isolates

Neural progenitors have been obtained from nonhematopoietic, CD34-negative fraction of HUCB mononuclears as described in Buzanska et al. [7,12].

HUCB-NSC was developed by repetitive passages of nonadherent, clonogenic neural progenitors in the presence of EGF (10 ng/mL; Sigma, Manchester, UK) [6,12,13].

HUCB-NSCs were cultured in low-serum (LS) medium, Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 2% fetal bovine serum, insulin-transferrin-selenium (1:100), amphotericin A and streptomycin (1:100) (all from Gibco). In these conditions cells grow as heterogeneous population of the nondifferentiated floating and attached cells. Then, a substantial part of adherent cells differentiated spontaneously into committed neural progenitors and differentiating neuronal, astroglial, and oligodendroglial cells [8].

Growing of neurospheres: after moving HUCB-NSCs to serum-free (SF) medium containing DMEM/F12 supplemented with B27 (1:50; Gibco) and EGF (20 ng/mL; Sigma), the small regular aggregates of nondifferentiated cells start to be formed during the 1st week. Selection of aggregates with ~200 μm in diameter leads to prolonged culture of regular, free-floating, ball-like neurospheres (N-HUCBs) [13]. Neurospheres growing ~1 month in SF conditions became nondistinctive from that derived from human brain tissue [14]. HUCB-NSCs and N-HUCBs grown in continuous, long-lasting (over 2 years) cultures at 37°C, 5% CO_2 and in a fully humidified atmosphere, were fed twice a week then dissected/passaged when neurospheres grow >200 μm in diameter or 2–3 $\times$ 10^6 cells appeared in a culture (25 cm^2) bottle.

Organotypic hippocampal culture

Hippocampal slices were prepared from 7 to 10 days old Wistar rats according to the method of Stoppini [15] slightly modified in our laboratory. After brief anesthesia with Vetbutal (pentobarbital; Sigma), ice-cooled pups were plunged into 70% alcohol solution, decapitated with scissors, and then brains were quickly removed to ice-cold HBSS (Gibco). The desired brain areas were separated and cut into 400-μm slices using McIlwain tissue chopper. Slices were transposed to Millicell-CM (Millipore) membranes for further growth, four slices on each. Millicell-CM membranes in six-well plates were pre-equilibrated with 1 mL of culture medium (pH 7.2, 50% DMEM, 10 mM HEPES, 25% HBSS, 25% horse serum (Gibco), 2 mmol/L l-glutamine, 5 mg/mL glucose, 1% amphotericine B and 0.4% penicillin–streptomycin prepared according to Aitken and modified by Gahwiler [16,17]. Cultures were started in a regular, 25% horse serum–containing medium which was gradually replaced (from DIV 4th until 7th) by SF, defined-solution-based medium. This SF medium contained DMEM/F12, 10 mM HEPES, 25% HBSS, 2 mmol/L l-glutamine, 5 mg/mL glucose, 1% amphotericine B and 0.4% penicillin–streptomycin, N2A (1:10; Gibco) and B27 (1:100; Gibco) supplements. Cultures were performed in a moist atmosphere of air and 5% CO_2, at 36°C for 14–16 days.

Cell labeling

Dispersed HUCB-NSCs were labeled with fluorescent cell tracker (CMFDA). After short trypsinization, HUCB-NSCs were moved to 0.4 mL of DMEM/F12. Then 1 μL of 10 mM 5-cloromethyl-fluorescein-diacetate (CMFDA) cell tracker (Molecular Probes) diluted in DMSO (Sigma) was added. Final concentration of DMSO in culture medium was not higher than 0.5%. Cells were incubated at 37°C for 30 min, washed twice with DMEM/F12 medium to remove not incorporated tracker and transplanted on OHC.

Free floating N-HUCBs cultured in SF medium were moved to 0.4 mL of DMEM/F12. Then 1 µL of 10 mM 5-cloromethyl-fluorescein-diacetate (CMFDA) cell tracker (Molecular Probes) diluted in DMSO (Sigma) was added. Final concentration of DMSO in culture medium was not higher than 0.5%. The neurospheres were incubated at 37°C for 30 min, washed twice with DMEM/F12 medium to remove not-incorporated tracker and transplanted on OHC. It should be noted that penetration of CMFDA into a core of N-HUCBs was substantially limited by the compact neurosphere structure. This labeling was used preferentially for visualization of N-HUCBs position after transplantation and was followed by immunocytochemical staining of particular cells with human-specific antigens.

Alternatively, for better visualization of all neurosphere-forming cells we have transfected HUCB-NSCs with enhanced green fluorescent protein (eGFP) gene.

Generation of GFP+ line

eGFP gene was cloned into pTRIP-WPRE backbone plasmid (with 5′ and D3′ LTR—long terminal repeats flanking the expression cassette, TRIP—central polipurine tract [18], and WPRE—woodchuck posttranscriptional regulatory element [19] under control of −115/+54 fragment of calretinin promoter [20,21]. Transient co-transfection of 293T cells with pVSV (pseudotyping plasmid encoding the vesicular stomatitis virus envelope), pΔ8.7 encapsidation plasmid, and TRIP-CR-eGFP-WPRE plasmid, resulted in generation of replication-defective lentiviral vectors in cell culture medium [22] that were incubated with DNaseI (Sigma; 1 µg/mL) before ultracentrifugation (22,000 rpm, 2 h), re-suspended in phosphate-buffered saline (PBS), and used to infect HUCB cells: 10^5 cells/well were incubated with 10^6–10^7 of the lentivector particles for 3 h, which then served as a source of fluorescence-emitting green cells and neurospheres.

Transplantation of HUCB-NSCs and N-HUCBs on hippocampal organotypic slices

HUCB-NSCs and N-HUCBs were transplanted on OHC at two different time points and then cultivated for following 7 days. First round of transplantation was performed immediately after OHC preparation (DIV0). Approximately 10^5 CMFDA-traced HUCB-NSCs were transplanted all over the whole surface of already grounded, freshly isolated OHC. One day after transplantation, the cells that did not stick to slices, were washed out. Then the slices were cultivated up to 1 week at 36°C in air + 5% CO_2 atmosphere of 100% humidity with medium changed every 2 days. To observe the difference in migrating and differentiating potential of cells transplanted into acutely prepared tissue (OHC-DIV0) and more stable tissue (OHC-DIV7), the other group of traced cells was transplanted consecutively into 7-days old slices. The procedure of transplantation was identical as described before. N-HUCBs either labeled with eGFP or with fluorescent cell-tracker and selected in respect of their size (200–300 µm in diameter), were transplanted using pipette into defined regions of the hippocampus: CA, DG, and EC, then cultivated as described before. The second round of transplantation (adequately to transplantation with dispersed cells) took place 1 week after OHC preparation into the 7-days old culture.

Immunocytochemistry

Hippocampal slices were fixed in 4% paraformaldehyde for 24 h. After washing in PBS, slices were cut out from the membrane and moved to PBS in 24-well culture plates. Free-floating slices fixed on the pieces of membranes were permeabilized with 1% Triton X-100 (Sigma) for 15 min and blocked with 5% NGS or 5% bovine serum albumin (for antinestin staining) diluted in PBS. All blocking media were applied for 1 h at room temperature (RT). Primary antibodies were diluted as follows: monoclonal antibodies anti-mouse NeuN (IgG1, 1:100; Chemicon), monoclonal antimouse MAP2 (IgG1, 1:1,000; Sigma), monoclonal anti-mouse nestin (IgG1, 1:50; Chemicon), monoclonal anti-mouse TUJ1 (IgG2a, 1:1,000; Covance), monoclonal anti-mouse NF200 (IgG1, 1:400; Sigma), monoclonal antimouse GalC (IgG3, 1:10; Chemicon), monoclonal antimouse O4 (IgM, 1:10; Chemicon) and then incubated overnight in 4°C. Polyclonal antibodies antirabbit glial fibrillary acidic protein (GFAP) (1:200; DAKO), antirabbit S100β (1:1,000; Swant), antigoat doublecortin (H+L1:100, Santa Cruz), anti-Human Nuclei (1:100, Chemicon) were also incubated overnight in 4°C. After washing in PBS, following secondary antibodies were applied: goat antimouse IgG1 for MAP2, nestin, NF200, NeuN (Pharmingen), goat antirabbit IgG (H+L) for GFAP, S100β (Chemicon), donkey antigoat for doublecortin (Dianova), goat antimouse IgG3 for GalC (Southern Biotech.), goat antimouse IgGm for GAlC and goat antimouse IgG2a for TUJ1. All secondary antibodies were conjugated with fluorescein isothiocyanate, or Texas Red and were applied for 60 min in RT. Cell nuclei were stained with 5 mM Hoechst 33258 (Sigma) for 30 min. After final wash, the slides were mounted in Fluoromount-G (Southern Biotechnology Association). As a control, the first antibodies were omitted during immunocytochemical staining.

Image analysis and statistics

The specimens were analyzed by inverted microscope Axiovert 25 or Axiovert 200M and captured by AxioCam MRm (Carl Zeiss), AxioCam MRc5 (Carl Zeiss), or CCD Videotronic CCD-4230 (SONY) cameras. To obtain detailed images of OHC with transplanted HUCB-NSCs and N-HUCBS, a confocal laser scanning microscope Zeiss LSM 510 (Carl Zeiss) was used. Following acquisition, the images were processed using Zeiss LSM 510 software package v. 2.8 and Corel Draw v. 11.0.

Statistical analysis of the raw data was conducted by one-way analysis of variance followed by Bonferroni's Multiple Comparison Test. The values were considered as significant when $P < 0.05$. Data were presented as mean ± SE. The number of different experiment (n) and the number of slices used per group and per experiment were indicated for each experiment.

Results

HUCB-derived NSCs differentiate and incorporate into slices depending on place and time of their delivery

As described in the Methods, we transplanted suspension of single cells (HUCB-NSCs) directly into rat hippocampal organotypic slice culture (OHC). The cells were seeded at two time points: immediately (OHC-DIV0) or consecutively

7 days (OHC-DIV7) after OHC preparation. Time of observation was established 1 week after cell transplantation in both experimental settings.

After cell transplantation into OHC-DIV0 (Fig. 1A), numerous of HUCB-NSCs were found to integrate stably with hippocampal tissue. However, in spite of firm incorporation into cultured tissue, majority of the transplanted cells stayed undifferentiated, round-shaped with tiny cytoplasm and without distinguishable processes (Fig. 1A, insert). The cells migrated well deeply inside of the OHC structure whereas only limited number of them acquired bipolar morphology (<13%). Confocal scanning revealed round, undifferentiated CMFDA-labeled HUCB-NSCs migrating even 100–200 μm in depth below surface of slices. Small part of differentiated cells displayed immunoreactivity for neural progenitor or more advanced neuronal cell markers (nestin: 1B, type III β-tubulin: 1C; MAP-2: 1D; NF200: 1E; GluR1: 1F).

In contrast, the HUCB-NSCs transplanted into OHC-DIV7 acquired neuron-like, highly ramified cell morphology after 7 days of coculturing (Fig. 1I). The numerous cells showed a single, neurite-like processes (Fig. 1I, upper insert) or multipolar, long and branched extensions (Fig. 1I, lower insert). Confocal scanning revealed HUCB-NSCs neuron-like cells that constituted a three-dimensional network inside of the OHC slice (0–200 μm). Most of them revealed neural progenitor marker (nestin: Fig. 1J) or neuronal markers (type III β-tubulin: Fig. 1K; MAP-2: Fig. 1L; NF200: Fig. 1M; GluR1: Fig. 1N).

Expression of oliogodendrocyte surface markers: O4 and GalC (indicated by arrows) was also clearly visible in both experimental settings (Fig. 1G, O and Fig. 1H, P, respectively).

Despite that HUCB-NSCs were transplanted equally all over the whole OHC surface (Fig. 1A), their maturation

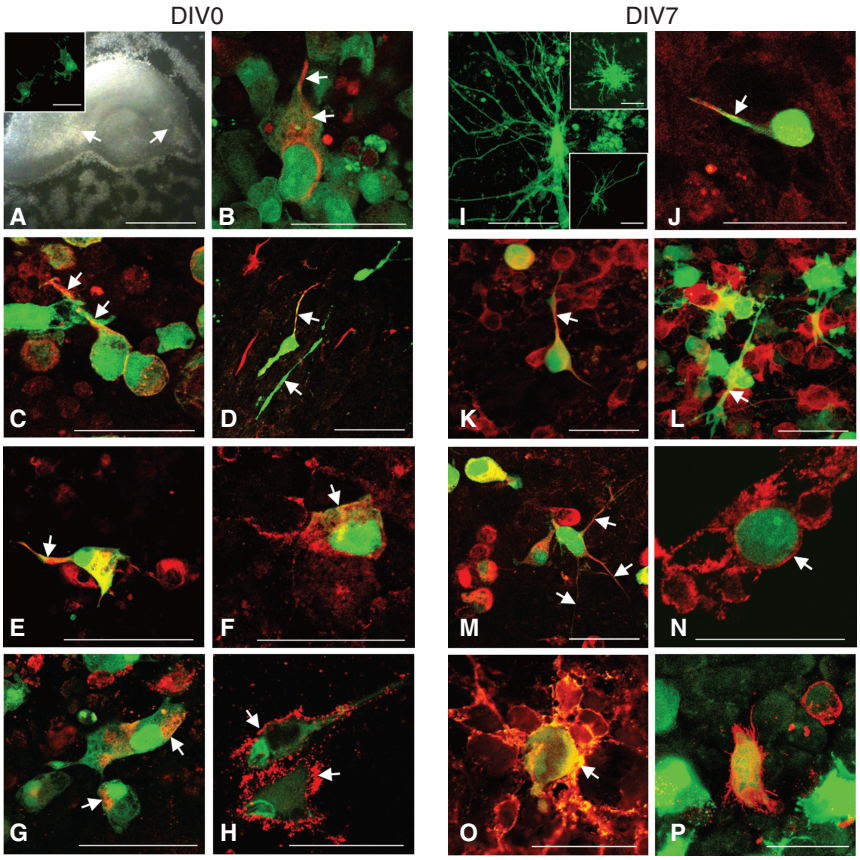

FIG. 1. HUCB-NSCs (green after CMFDA labeling) transplanted into hippocampal organotypic slice culture. CMFDA-traced HUCB-NSCs were transplanted all over the whole surface of already grounded OHC (DIV0, *left panel*) (**A**, arrows). During 7 days of co-culture majority of HUCB-NSCs remained undifferentiated, round-shaped, or extended one or two relatively short processes (**A**, insert). The part of those differentiated cells displayed immunoreactivity for neural progenitor and neuronal markers (nestin: **B**; type III β-tubulin: **C**; MAP-2: **D**; NF200: **E**; GluR1: **F**, arrows). On the contrary HUCB-NSCs transplanted into OHC DIV7 (DIV7, *right panel*) often revealed multipolar, neuron-like morphology with numerous branched processes, some of them extending over 200–400 μm (**I**) or with only one neurite as a typical feature of migrating neuroblasts (**J**). Immunocytochemical analysis revealed strong expression of neural progenitor or neuronal markers (nestin: **J**; type III β-tubulin: **K**; MAP-2: **L**; NF200: **M**; GluR1: **N**, arrows). Expression of oliogodendrocyte surface markers: O4 and GalC (indicated by arrows) was also clearly visible in both experimental settings (**G**, **O** and **H**, **P**, respectively). Surprisingly, neither GFAP- nor S100β-positive cells were detected among transplanted HUCB-NSCs. Co-localization of red (specific for neuronal and oligodendrocyte-specific antigens) and green (for CMFDA) labels in HUCB-NSCs appeared yellow after overlaying (arrows indicated co-localization in cell processes). Scale bars: **A**: 2 mm, **C**: 100 μm, **B**, **D**–**P**, and inserts: 50 μm.

strongly depended on the region of incorporation, independently from the age of brain tissue (DIV-0 or DIV-7). Bipolar, neuroblast-like HUCB-NSCs were observed mainly in *radiatum* layer of CA region, in parallel to the host's neuronal processes (Fig. 1D). Within that region numerous connections between CMFDA-labeled cells and the host cells could be noticed under a confocal microscope. In contrast, HUCB-NSCs transplanted into DG remained round shaped and did not elaborated ramified, branched processes.

HUCB-derived cells differentiate preferentially toward neurons under co-culture with the brain tissue

The exact immunofluorescence analyses performed after transplantation of the dispersed HUCB-NSCs revealed that many of exogenous, CMFDA-labeled cells start to co-express neuronal markers: NF200, TUJ1, MAP2 under influence of OHC environment (Fig. 1). However, the extent of acquisition of neuronal phenotype by transplanted cells obviously depended on the particular experimental setting. The part of HUCB-NSCs seeded on the freshly isolated OHC-DIV0 acquired expression of committed neural progenitors and neuronal markers: TUJ1 in 35 ± 7%; MAP2 in 18 ± 9% and NF200 in 36 ± 3% of all counted green cells. The nestin, an undifferentiated neural cell marker, was immunodetected in 9 ± 0.6% of these cells (Fig. 2).

When HUCB-NSCs were transplanted into OHC-DIV7, a significant increase in number of cells differentiated toward neuronal, morphologically advanced phenotypes was noticed in comparison with the first group. These cells were positive for TUJ1, MAP2, and NF200 in 49 ± 4%, 43 ± 13%, and 74 ± 2%, respectively, whereas the nestin was still immunodetected in 18 ± 9% of morphologically less differentiated green cells (Fig. 2).

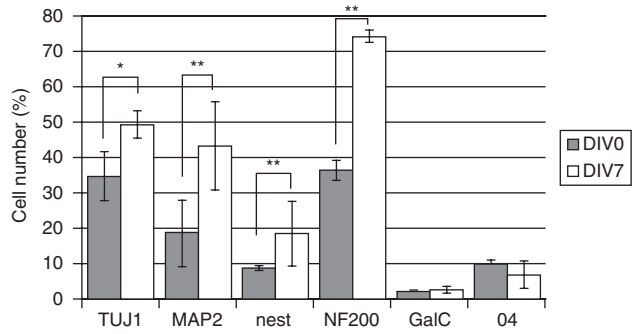

FIG. 2. Quantitative analyses of neural differentiation markers expression in OHC-engrafted HUCB-NSCs. The slices at the 7th day after HUCB-NSCs transplantation were immunostained and the cell phenotypes quantified using confocal laser scanning microscope Zeiss LSM 510 (Carl Zeiss). Grey bars: HUCB-NSCs transplanted into OHC DIV0; crossed bars: HUCB-NSCs transplanted into OHC DIV7. Immunofluorescence analyses revealed that at both time points a number of CMFDA-labeled cells expressed neuronal markers: TUJ1, MAP2, nestin, NF200 but at significantly higher level in OHC DIV7 group. In both groups, oliogodendrocyte markers, O4 and GalC, were expressed in similar, relatively low percentage of the total survived cells. Surprisingly, neither GFAP- nor S100β-specific markers were expressed in HUCB-NSCs. Data are presented as a mean ± SE from 3 to 5 independent cultures (each culture is an equivalent of four slices per staining, from each slice we counted 200 cells). The values were considered as significant when $P < 0.05$(*) and $P < 0.01$(**).

In both experimental settings the oliogodendrocyte markers, O4 and GalC, were expressed in similar percentages of total CMFDA-positive cells: 9 ± 2% and 2 ± 0.2% for OHC-DIV0, then equally 7 ± 3% and 2.5 ± 0.9% for OHC-DIV7 (Fig. 2). The most of O4-expressing cells morphologically displayed round cell bodies with fine branched processes (Fig. 1O). It is worth to note that neither GFAP nor S100β immunostaining were detected among transplanted, green-labeled HUCB-NSCs.

Migration and differentiation of transplanted N-HUCB into OHC is region-dependent

Similarly, to previous experiments also the neurospheres transplantation was performed in two different time points of the slice culture: on freshly isolated OHC-DIV0 and at OHC-DIV7. Since migration of neural stem cells outside the transplants occurred exclusively in the case of acutely prepared slices, further description would refer only to this experimental group.

Neurospheres were placed into three defined regions of OHC, namely at a CA, DG, and EC (Fig. 3A). Strong dependency between N-HUCBs activation/migration and the engraftment place was observed. Confocal scanning revealed that initially N-HUCBs plunged in OHC at 100–200 μm below its surface and then numerous cells started to migrate out of their ball-like, firm structure and further spread within the host tissue (Fig. 3B) creating a halo around the aggregates. Migration of this stream of single NSCs (Fig. 3E) occurred preferentially along endogenous fiber tract leading from the enthorinal cortex and DG to CA region (Fig. 3C). Cells derived from the N-HUCBs placed in close vicinity of DG region migrated either through a *hilus* or disseminate in the direction of the pyramidal cell layer (Fig. 3C). In contrast, the NSCs coming from the neurospheres placed into the CA region spread throughout the *oriens* and pyramidal cell layers but never in opposite, at *radiatum* layer and further toward DG region (Fig. 3D). The majority of N-HUCBs deposited into the EC remained quiescent during the whole observation period with all the cells situated inside of aggregate. Rarely, the NSCs migrated here out of the spheres but without specific direction and were confined to a relatively narrow halo around N-HUCBs engraftment site (Fig. 3B).

Similarly, to HUCB-NSCs, NSCs derived from N-HUCBs transposed to pyramidal layer of CA region differentiated predominantly into neurons (Fig. 3F and G) whereas those transplanted into neurogenic subgranular zone of DG remained mostly undifferentiated during the period of our observation (Fig. 3H). Immunochemistry of N-HUCBs after transplantation revealed that the cells within neurospheres as well as those migrating out of them, differentiated mostly to MAP2, TUJ1, NeuN, and doublecortin-positive neurons. Still, the cells differentiated only marginally toward astrocytes and expressed S100β exclusively as long as they remained within aggregates.

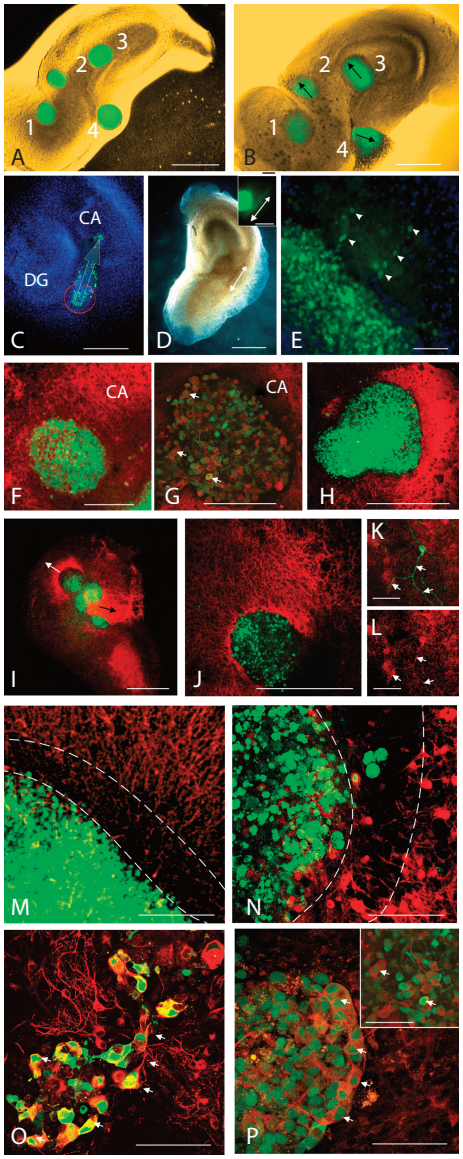

FIG. 3. Neurospheres transplanted into organotypic slice culture (OHC). N-HUCBs obtained from GFP+ stable line were transplanted into four different regions of hippocampus: EC (**A**1), CA (**A**2), DG (**A**3), or hippocampal fissure (**A**4). After 4 days N-HUCBs-derived cells plated in CA regions migrated outside the hippocampus (**B**2, arrow; cells visible here as regular or irregular halo around the aggregate) or in direction of hippocampal fissure (**B**4, arrow). N-HUCBs when deposited onto EC spread out in all directions and migrating cells are visible here as regular halo all around the neurosphere. (**B**1). N-HUCBs placed into DG or in the neighbourhood spread cells throughout hillary region that disseminate further alongside pyramidal cell layer (**B**3, arrow; **C**, arrow). Migrating HUCB cells formed migratory chains characteristic for neuroblasts derived from neurogenic niches (**D**, phase-contrast image; insert, magnification of green N-HUCBs; arrow, migrating NSCs; **E**, magnification of green N-HUCBs with stream of migrating NSCs, arrow). NSCs derived from N-HUCBs transposed to CA region differentiated predominantly into neurons (**F** and **G**; MAP2, red; N-HUCBs, green) whereas those transplanted into neurogenic subgranular zone of DG remained mostly undifferentiated (**H**, MAP2, red; N-HUCBs, green). Immunostaining for doublecortin (red: **I** and **J**) revealed activation of the host neuroblasts in the neurosphere-surrounding rat tissue. Some of the cells derived from transplanted neurospheres (green, **K**, arrows) also expressed doublecortin immunostaining (red, **L**, arrows) and neuroblast phenotype whereas others differentiated further into more mature neurons. In part of neurosphere-remaining cells an expression of S100β can be detected, (**M**) whereas immunopositive reaction to GFAP has never been achieved in HUCB cells in these co-culture model (**N**). Moreover, a significant reduction of astrocytes (expressing S100β or GFAP) occurred in the host tissue directly contacting neurospheres (**M** and **N**; area defined by dotted lines). On the contrary neurosphere-derived neurons not only created connections between different neurospheres (**O**, arrows) but also host cells infiltrated each other on the border of neurosphere (**P**, type III β-tubulin, red; N-HUCBs, green). This can facilitate incorporation of the transplanted cells within host neuronal network as can be judged from the contacts between red (species nonspecific type III β-tubulin staining) and green (N-HUCB-specific) or yellow (co-localized) cell markers. Cell nuclei stained with Hoechst (blue, **C**). Scale bars: **A**–**D**, **I**: 500 μm; **E**, **G**, **K**–**P**: 100 μm; and: **F**, **H**, **J**: 300 μm.

Neurospheres transplanted into OHC activate host neuroblasts

In this study, we used a doublecortin (a protein expressed transiently in migrating and differentiating neuroblasts) to examine host (GFP-negative) cell reaction induced by transplanted neurospheres. The evident activation of rat's neuroblasts was found around N-HUCBs transplanted at vicinity of DG region (Fig. 3I and J). Migration routes of these host neuroblasts overlaid with that of exogenous (green) NSCs coming out from the transplanted neurospheres (Fig. 3K and L). Interestingly, activation of doublecortin-positive host neuroblasts was evident even after transplantations performed on OHC cultured for 7 days in vitro (OHC-DIV7) when no signs of migration of seeded N-HUCBs could be observed.

Neurospheres transplanted on OHC inhibit astrocytosis

As it has been noted previously, N-HUCBs transplanted either on freshly isolated OHC-DIV0 or on those cultured already for 7 days (OHC-DIV7), differentiate poorly into GFAP and S100β-positive astrocytes. Unexpectedly, immunostaining for GFAP and S100β revealed significant decrease of the rat tissue immunoreactivity in hippocampal regions surrounding transplanted neurosphere (Fig. 3M and N) indicating marked depletion of host astrocytosis. This, evident and highly specific inhibitory influence of N-HUCBs was independent from the time and site of transplantation. That effect we could not observe with cells migrated out of a neurosphere expressing neuronal markers. Those neurons were able to make very close contacts with each other (Fig. 3O) and with surrounding host neurons (Fig. 3P).

Discussion

Based on the above-described results we can postulate that hippocampal organotypic tissue culture may provide a valuable, informative model for basic research on the mechanisms that govern processes of human neural stem cell engraftment and incorporation into brain structures. In this model, we can easily modulate grafting environment

and culture conditions in the absence of vascular component that provides an uncontrolled influx of peripheral macrophages, immunocytes, and circulating trophic factors [23]. Under these circumstances, we have observed a potent ability of human cord blood–derived neural stem cells to undergo maturation and differentiation to the level never observed after transplantation of these cells into rat brain in vivo [2]. Moreover, behavior of the cells after transplantation was strongly dependent on histological region of cell delivery. HUCB-derived neural cells, either these transplanted as a single cell suspension [8] or those which spread out from neurospheres, migrated from DG toward CA (neuron-rich) region utilizing the natural fiber pathways typical for endogenous neuroblasts movement [24–26]. Reaching CA region they differentiated and incorporated into existing host cytoarchitecture in parallel to endogenous pyramidal neuron processes in *striatum radiatum*. In contrary, the cells delivered as neurospheres to EC region and these which remained in DG, stayed undifferentiated (quiescent) for the whole period of our observation. It has been previously shown in vivo that CNS-derived neural progenitors [27,28] as well as HUCB-derived cells [29,30], when transplanted into subventricular zone region, can obey the instructive signals existing in developing rat brain for directed migration along the rostral migratory stream into olfactory bulb [29]. Similar directed migration of the endogenously born neuronal progenitors to replace died pyramidal neurons in CA1 were reported in post-ischemic rat hippocampus by ref. 25. In our experiments, the DG-transplanted HUCB-derived stem/progenitor cells utilized the same route of directed migration in hippocampal slices.

Apart of regional dependency, the other crucial factor for efficient migration and differentiation of grafted cells seems to be the moment of cell delivery. Our experiments utilizing OHC at DIV0 may represent an in vitro model of direct cell transplantation into acutely injured brain, whereas at DIV7 into metabolically more stabilized tissue. As we have already noticed, efficient migration was characteristic for cells seeded at OHC-DIV0. In contrast, the morphological and immunocytochemical signs of advanced neuronal differentiation were observed exclusively in the experiments settled at OHC-DIV7. Comparing with our previous data [8], these cells differentiated during the 7 days of co-culture ~2 times faster than those seeded at OHC-DIV0. In addition to expression of these typical neuronal markers, also clear immunostaining for GluR1–3 has been appeared on OHC-DIV7-seeded cells. This finding may indicate functional maturity acquired by the transplanted cells which make them ready to display excitatory signals. Of note, our previous patch-clamp experiments demonstrated such ability, but only after long-term treatment of HUCB-NSCs with potent neuromorphogen like dBcAMP [31].

The HUCB-NSCs, either transplanted as cell suspension or spread out from neurospheres, have acquired morphological and immunocytochemical characteristics exclusively of neuronal or oligodendroglial cells. Substantially lower number of O4- and GalC-reactive cells among all cells transplanted on OHC-DIV7 (9 and 2%, respectively) is in accord with our earlier report on HUCB-NSCs differentiation under defined neuromorphogenic signals introduced into monolayer cell culture in vitro [8]. Most probably a relatively weak commitment of the transplanted HUCB-NSCs into oligodendrocyte lineage, similar to that observed in other types of NSCs [32–34], could be explained by the need of still poorly defined specific signal(s) not readily supplied by the adult neural tissue itself.

One of the most interesting findings in our study was an obvious suppression of GFAP immunoreactivity after HUCB cell transplantation observed both in the host tissue as well as in the grafted cells. Here the host tissue reaction was most spectacular after neurosphere transplantation in CA region. However, similar observations were described previously by Jurga et al. [8] in monolayer HUCB-NSCs culture. These cells, when co-cultured with primary astrocyte cultures by 7 days revealed, mostly, type III β-tubulin and MAP-2 positive phenotypes with marked suppression of GFAP immunoreactivity. Recently, Shen [35] described in vivo reduction of scar wall thickness around the cystic ischemic core after bone marrow-derived SCs treatment in comparison with that of the control MCAO rats. Abovementioned results may suggest that NSCs after transplantation, especially in the form of aggregated entities, could inhibit glial scar formation. That observation seems to be particularly important since an excessive astrogliosis occurs in a variety of neuropathological disorders and is believed to be devastating for the restoration of neuronal circuits. Therefore, elucidation of the possible mechanisms of astrogliosis suppression by grafted cells should be of practical interest. The HUCB-derived mononuclear cells can secrete a high number of factors with anti-inflammatory and neurotrophic activity. That cells when stimulated by EGF/FGF-2- or NGF/RA-secreted high amount of anti-inflammatory interleukins: IL-1b, IL-6, IL-8, IL-10 whereas the pro-inflammatory cytokine IL-16 was barely detectable [36–38]. The profile of HUCB-NSCs cytokine secretion, additionally modulated by the local tissue signaling, could explain observed suppression of astrocytosis in graft-surrounding tissue and, in effect, diminution of glial scar formation.

In parallel with the described N-HUCBs ability to suppress local astrocytosis, the neighboring, doublecortin-positive host neuroblasts seem to be activated by grafted cells. This reaction corresponds with previous observations in vivo [37,39] showing that human umbilical blood–derived cell infusion can stimulate endogenous neurogenesis by altering numerous metabolic and growth-receptor-specific pathways.

Concluding, in this first systematic study performed on organotypic hippocampal slices in vitro we have proved capability of transplanted human cord blood–derived neural stem cells to integrate and respond directly to brain tissue–provided signals by differentiation to neuron-like cells expressing typical morphology and specific protein markers. At lower extend, these cells convert also into oligodendrocyte-like phenotypes after transplantation. The particular cells fate after transplantation depends strongly on the time and place of their delivery. Observed migration of transplanted HUCB-derived cells was directed most probably by specific, however, still non-defined signals provided by the host tissue and similar to these operating in vivo. Moreover, HUCB-NSCs transplanted into OHC seem to stimulate host tissue to neurogenic response with concomitant suppression of local astrocytosis. Despite of all these promising observations gained from the organotypic culture model, these findings have to be further confirmed in vivo to ensure the common biological mechanisms existing in the both experimental situations.

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