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Laminin promotes oligogliogenesis and increases MMPs activity in human neural stem cells of HUCB-NSC line

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Oligodendrocytes, the cells responsible for myelin formation and maintenance in CNS, are depleted in many acute and chronic conditions. The stem/progenitor cells stimulation or transplantation might be seriously considered as a long hoped-for therapeutic perspective. Better understanding of the mechanism(s) regulating the activation of the cell lineage from the endogenous progenitor reservoir might be helpful. Therefore an efficient source of donor cells for transplantation in humans is being craved for. In this study we show that the application of extracellular matrix component-laminin promotes oligogliogenesis from neural stem-like cells of human cord blood cells (HUCB-NSC). Although oligodendrocytes constitute a minor subpopulation of spontaneously differentiated HUCB-NSC, the manipulation of active compounds regulating the process of cell commitment results in a several fold increase in their number. Thus cells of the HUCB-NSC line could be considered as a potential source of glial cells, fulfilling the suitable candidate criteria for oligodendrocyte replacement therapy.

Key words: neural stem cells, HUCB-NSC line, laminin, metalloproteases, ooligodendrocytes, transplantations

INTRODUCTION

Oligodendrocytes (OLs) are glial cells responsible for myelin formation and maintenance in the central nervous system (CNS). There is also growing recognition, that glia-constituting approximately 90% of nervous cells in human brain-may exhibit communication skills that complement the neuronal network (Bauman and Pham-Dinh 2001). Inherited or acquired OLs deficiency or/and abnormalities lead to severe neurological disorders, common in humans and animals. The inherited leukodystrophies might be due to either mutations in myelin genes (for instance Pelizaeus-Merzbacher disease (PMD)-characterised by severe neurological impairment as nystagmus, paraplegia and mental retardation) (Hodes et al. 1994, Seitelberger 1995, Koeppen and Robitaille 2002) or to disorders of intermediary metabolism (for instance globoid cell leukodystrophy – Krabbe disease or organic aciduria – Canavan disease) (Höster et al. 2005). The central myelin loss (demyelination) is a critical element in numerous pathological clinical conditions, especially in multiple sclerosis (MS).

White matter is affected in many acute conditions, such as focal and global ischemia (cardiac arrest), stroke, brain and spinal cord trauma, heavy ions intoxication (Dewar et al. 2003). White matter injures are also common during the perinatal period, since 23–32 weeks of human gestation coincides with the immature, premyelinating stages of OL development and myelinogenesis is most active during the first year of human life (Back et al. 2001). Since developing OLs are particularly vulnerable to hypoxia and hypoglycemia, these insults usually result in periventricular leukomalacia (PVL), the underlying cause of cerebral palsy and cognitive impairment (Volpe 2003). Many of

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those pathological conditions are accompanied with changes in matrix metalloproteinases (MMPs) activity (Yong et al. 2001, Rosenberg 2002, Cunningham et al. 2005).

MMPs constitute a group of at least 25 Zn^{2+}-depending endopeptidases regulating the cellular activity in various ways (Seeds et al. 1997, Sternlicht and Werb 2001, Visse and Nagase 2003). These include extracellular matrix (ECM) degradation, alterations of the cell adhesion, proteolytic release of ECM sequestered molecules and shedding off cell surface proteins that transduce signals from extracellular environment. MMPs are recognized as proteolytic enzymes that are involved in the remodeling of extracellular environment during development and homeostasis. These proteins are synthesized in cells and secreted to the extracellular space as inactive enzymes where they are activated by the cleavage in the propeptide region. MMPs are classified on the basis of ECM substrate preference and similarities of structural domains. They contain the gelatinases, stromelysins, collagenases and membrane – type (MT-MMPs). The gelatinasaes (MMP-2 and MMP-9) possess a fibronectin domain within the catalytic region. The MMPs are tightly regulated at the transcriptional and post-transcriptional levels *via* their proenzyme activation or release of endogenous inhibitors (TIMPs) to avoid unwanted proteolysis (Baker et al. 2002, Dzwonek et al. 2004, Cunningham et al. 2005). Under physiological conditions the MMPs activity is precisely controlled; however, excessive MMP production and activation is thought to be a key feature of many inflammatory and neurodegenerative diseases. MMPs play important roles in neuronal development, where they are likely to regulate cell migration, neurite outgrowth and myelination (reviewed by Milward et al. 2007).

Numerous clinical conditions associated with hypo/demyelination could be treated with oligodendrocyte progenitor transplantation. Cells should be obtained in unlimited amounts from the low immunogenic sources for the purpose. Unfortunately, the proposed therapy is hampered by the lack of accessible and yet efficient source of progenitors for autologous/allogenic transplantations.

In the presented work we describe the HUCB-NSC line as a suitable source for generation of considerable amounts of human oligodendrocytes. The neural stem cells of HUCB-NSC line are untransformed and display the stable karyotype (Bużańska et al. 2006). The obtained data show that ECM along with MMPs activation may provide a signal for the oligodendroglial lineage commitment.

METHODS

Cell culture

Neural-like stem cell line derived from human umbilical cord blood (HUCB-NSCs) (Bużańska et al. 2002) was cultured as a mixed population of committed adherent progenitors and free-floating undifferentiated cells in F12/DMEM + 2% FBS+ITS medium (Gibco). The pooled fractions of adherent and floating HUCB-NSCs were seeded at 2×10^4 cells/cm^2 density on glass plates coated either with poly-L-lysine (PLL) or one of ECM-components: laminin (20 μg/ml in PBS), fibronectin (10 μg/ml in PBS) and collagen (10 μg/ml in PBS), respectively. Prior to seeding, laminin, fibronectin and collagen remained on culture dishes overnight at 4°C without air drying and then the substrates were removed and plates were rinsed by warm PBS. The cells spontaneously differentiated on uncoated glass plates served as a control. The HUCB-NSCs were cultured for the following 12 days at 37°C, 5% CO_2 in fully humidified atmosphere. The F12/DMEM + 2% FBS+ITS medium was changed every 2–3 days. After 12 DIV the standard medium was replaced with the serum-free equivalent and the cells were cultured for the following 48 h.

In situ zymography

HUCB-NSCs cultured on glass cover slips were incubated with gelatin conjugated with FITC in 50 μg/ml concentration (Sigma) in activation buffer, for 3 h in a dark humid chamber at 37°C. Gelatin-FITC cleavage by MMPs releases quenched fluorescence representative of net proteolytic activity. To define the localization of MMPs activity cells were fixed in 4% paraformaldehyde in PBS for 20 minutes. The amount of cells expressing MMPs activity was estimated under fluorescence microscope Axiovert 25. Statistical analysis of the results obtained in control experiments (uncoated plates) and with addition of selected factors (PLL and ECM components) was done by using one-way ANOVA followed by two-tailed Student's *t*-test for grouped data.

Immunostaining

Blocking solution, containing 10% normal goat serum in PBS, was applied for 1 h at 25°C (RT). Immunoreaction with the primary antibodies was carried out by overnight incubation at 4°C. After rinsing with PBS, the cells were incubated for 1 h at RT with appropriate secondary antibody conjugated to Alexa Fluor-546 (1:1000, Molecular Probes). Controls for specificity of immunostaining were processed with excluding either the primary or the secondary antibody. The markers for two stages of oligodendrocyte development were used: monoclonal anti-mouse against O4 (1:200, Sigma) for immature oligodendrocytes and GalC (1:200, Chemicon) for differentiated, mature cells. The mouse monoclonal IgM secondary antibody was applied to detect O4 and mouse IgG1 was used as a secondary antibody for GalC. Cell nuclei were visualized by 30 min incubation (RT) with 5 μM Hoechst 33258 (Sigma). The labeled cells were examined under fluorescence microscope Axiovert 25 and confocal microscope LSM 510 (Zeiss). Images were captured on the Videotronic CCD-4230 camera and processed by image analysis system Axiovision (Zeiss).

RESULTS

The neural stem cells of the HUCB-NSC line are multipotent and in the presence of low serum are able to differentiate spontaneously into neurons and glia. Oligodendrocytes constitutes however the very minor fraction. In the presented study we show, that addition of extracellular matrix components results in nearly 3-fold increase in the oligodendrocyte number (11.8 ± 0.88% on laminin *versus* 4.9 ± 1.9% in controls) (Figs 1, 2). In control conditions, the single O4-positive, pre-mature cells are present (Fig. 1A). The adhesion to laminin results in formation of the frequently observed groups of several $O4^+$ oligodendrocytes (Fig. 1B). Similarly, the mature GalC-expressing cells with the very characteristic *in vitro* morphology are sparsely distributed in controls (Fig. 1C), while those cultured on laminin are often grouped and significantly more numerous (Fig. 1D).

Active forms of MMPs can be detected by *in situ* zymography in the nuclei of the less developed, immature O4-positive cells (Fig. 3A-C). Further, co-localization studies (*in situ* zymography followed by the immunocytochemistry) show that only a marginal (1 ± 0.43%) (Fig. 4) fraction of control cells (cultured on the uncoated glass plates) differentiated into GalC-positive oligodendrocytes express similarly active forms of MMP-2 and MMP-9, however with preferential extranuclear

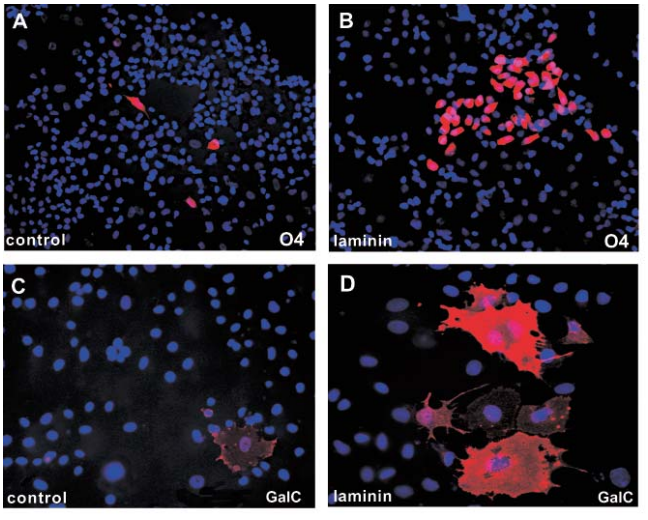

Fig. 1. Oligodendrocytes differentiated from HUCB-NSC line during 7-day culture on either the uncoated (A, C) or the laminin-coated glass plates (B, D). (A, B) O4-positive immature cells; (C, D) GalC-stained mature oligodendrocytes with characteristic *in vitro* morphology. The cells are often observed in groups of several oligodendrocytes when cultured on the plates coated with the extracellular matrix components.

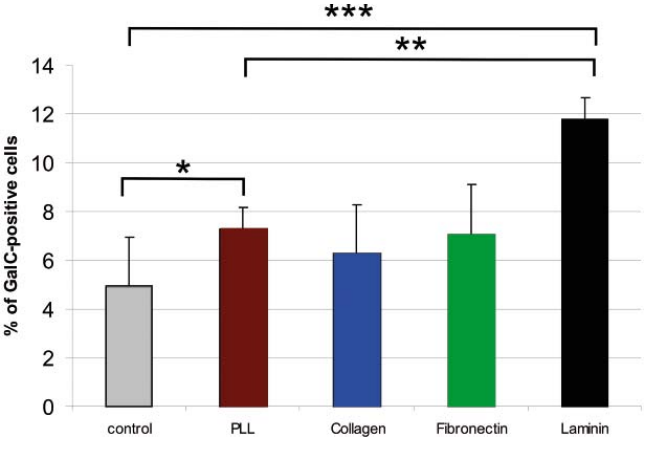

Fig. 2. GalC-positive oligodendrocytes differentiated for two weeks from HUCB-NSC line on coated glass plates. Asterisks mean significant differences (*$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$).

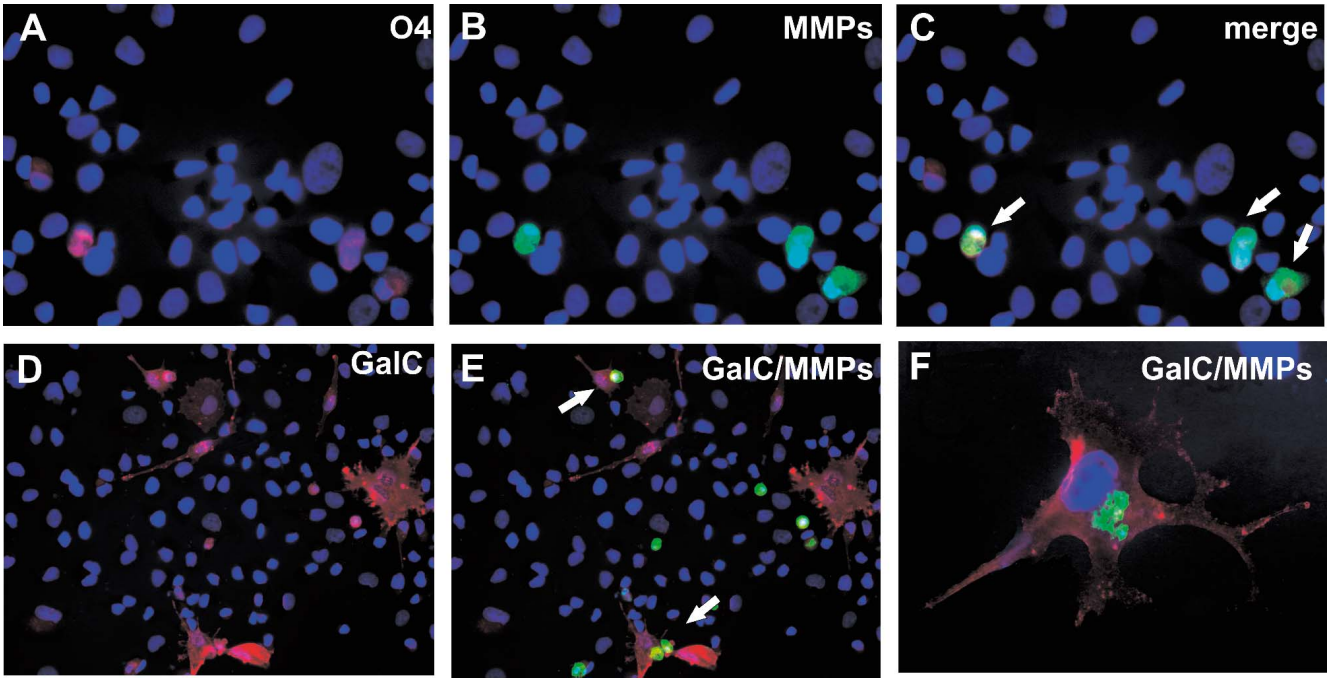

Fig. 3. Co-localization of active forms of MMPs (detected by *in situ* zymography) with oligodendrocytes (visualized by immunocytochemistry), spontaneously differentiated on laminin-covered surface: (A–C) Phenotypically immature O4-positive cells (red) and active MMPs (green), an arrow points to the $O4^+/MMPs^+$ cells; (D–E) phenotypically mature, GalC-expressing oligodendrocytes (arrows: $GalC^+/MMPs^+$ cells); (F) extranuclear localization of active MMPs in differentiated, $GalC^+$ oligodendrocyte.

localization. Moreover, the $GalC^+/MMPs^+$ cells are usually smaller and morphologically less differentiated than the neighboring $GalC^+/MMPs^-$ cells (Fig. 3D,E). Nonetheless, the extranuclear localization of the active forms of MMPs could still be found in the clusters of mature, GalC-expressing oligodendrocytes, showing the characteristic *in vitro* morphology (Fig. 3F). In the cells cultured on the extracellular matrix components the MMPs expression is increased nearly 4 fold (3.6 ± 0.23% on laminin and 2.05 ± 0.38% on fibronectin, as compared to less than 1% of total cell number in controls) (Fig. 4), pointing towards a link between the extracellular matrix components and the activity of metalloproteinases in maturating oligodendrocytes.

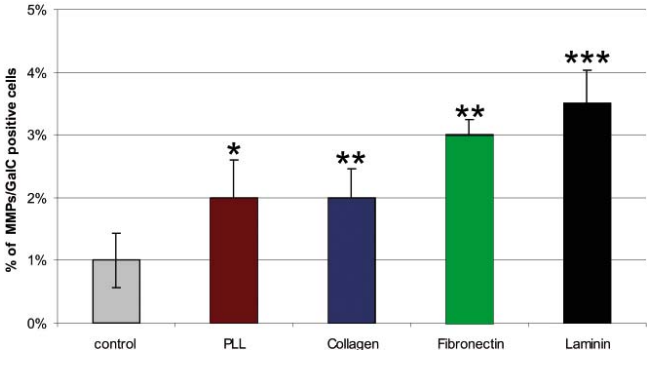

Fig. 4. Co-localization of MMPs activity with GalC-positive oligodendrocytes differentiated for two weeks from HUCB-NSC line on glass plates coated with extracellular matrix-components. Asterisks mean significant differences compared to control (*$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$).

DISCUSSION

Here we describe the enhanced generation of considerable amounts of human mature oligodendrocytes by means of applying the extracellular matrix components into *in vitro* culture. Concomitantly we show a transient expression of intracellular gelatinases in maturating OLs with intra- or extranuclear localization. It suggests that the extacellular matrix signaling might be involved in oligogliogenesis.

Oligodendrocyte origin and development

Oligodendrocyte progenitors (OPC) are derived from the restricted area of the ventral neuroepithelium.

In the developing neural tube, the notochord and the floor plate express the opposing gradients of morphogenic signals and establish the domains where different neural cell lineages are generated (Bongarzone 2002). The induction of neural stem cells to become OPC depends on the Sonic Hedgehog (Shh) concentration, Notch signaling, the expression of several transcriptional (e.g. Olig1, Olig2, Sox 10) and trophic factors (e.g. PDGF). After the developmental fate is determined, the precursors migrate, sometimes over considerable distances, and populate the rest of forming CNS. A number of cell surface and extracellular matrix (ECM) molecules play an instructive role in the control of OPC migration, for instance the glycoprotein Tenascin-C (Garwood et al. 2004) or extracellular metalloproteinases MMP-9 (Uhm et al. 1998, Larsen et al. 2006) and MMP-12 (Larsen and Yong 2004). A participation in the regulation of process extension is an additional function of MMP-9 (Uhm et al. 1998, Oh et al. 1999) and MMP-12 (Larsen and Yong 2004) in oligodendrocyte development. MMP-9 has been found in demyelinating lesions, where it has promoted oligodendrocyte maturation, leading to a partial remyelination (Larsen et al. 2003).

The proper response to extracellular signals, which plays a crucial role both in cell proliferation and migration, is possible due to an array of integrin receptors (reviewed by Baron et al. 2002). Activation of different intracellular signaling pathways (PI3K, MAPK) triggered by the binding of ECM (laminin-2, fibronectin, collagen, vitronectin) to integrins and limiting levels of trophic factors (PDGF, neuregulins) regulates the number of oligodendrocytes: excess cells that are unable to contact target axons are eliminated (predominantly by apoptosis), in this way precisely matching OLs to axons (Baron et al. 2002, Watkins and Barres 2002, Fu et al. 2007, Finzsch et al. 2008, Hu et al. 2008). Moreover, the morphology of the survived oligodendrocytes is controlled by the dynamic regulation of integrin activation, evoked by both the intracellular and extracellular signals. For instance, due to laminin(s) present on axons and integrin glial receptor $\alpha 6\beta 1$, the OL morphology and myelinogenesis might be regulated independently in every OL process (Olsen and Ffrench-Constant 2005).

In our work we show that oligogliogenesis increases nearly 3-fold by neural stem cells being adhered to laminin. Actually, the presence of other matrix-specific components that facilitate adhesion promotes oligodendroglial fate-decision as well, however to a lesser extent.

The cell development is associated with the activity of metalloproteinases, as it was shown in the co-localization experiments. The MMPs have the well established role in the neural progenitor migration, when the cells are still proliferative (Mitra et al. 2005, Suzuki et al. 2006, Bovetti et al. 2007), but less is known about their involvement in differentiation process, especially of the oligodendroglial lineage. The oligodendrocyte recruitment and development is governed by mutual interaction of extracellular components and cell-derived enzymes. Laminin is well known to promote the migration of oligodendroglial precursors (Colognato et al. 2005, 2007). MMPs expressed in the growing tips of migrating, developing precursors might cleave the ECM components (especially laminin, fibronectin) thus influencing the migratory pathways. Once they have reached their final destination, the precursors loose their proliferative and migratory abilities and differentiate into mature, myelinating cells.

In vivo, the process of Ols maturation is known to depend on the presence of thyroid hormones (Fernandez et al. 2004, Schoonover et al. 2004). *In vitro*, numerous trophic factors and neuromorphogenes promote OPC maturation and survival: triiodothyronine, progesterone, insulin, transferrin, PDGF, bFGF and many others. Certain cytokines and growth factors, as for instance IGF, are the MMPs substrates. The MMPs activity can also modulate the bioavailability of the particular factors in various developmental stages, as well as might regulate the spatial distribution of myelinating cells.

In response to the microenvironmental signals, the differentiating progenitors change their morphology from small bipolar cells to a few process-bearing immature oligodendrocytes. The maturation is characterized by the increase in number of processes, as well as in their length and complexity. Their function is to recognize, to ensheathe and to myelinate nerve axons (Sherman and Brophy 2005). Again, the process outgrowth seems to be modulated by ECM components and changes in expression and activity of metalloproteinases. Laminin, fibronectin and collagen, either present in extracellular matrix or expressed by the adjacent cells, might play a crucial role in the process guidance. In turn, the role of the active metalloproteinases (e.g. MMP-9/gelatinase B) might be the clearance

of pathways for the process elongation (Uhm et al. 1998, Oh et al. 1999, Hoshina et al. 2007).

While the activity of the secreted, extracellular forms of MMPs could be described in context of the utilization of ECM components, a very little is known about the cellular localization of metalloproteinases. In the present study we have used stage-specific markers: O4 for the so called prooligodendroblasts and GalC for the mature oligodendrocytes. Here for the first time we show the intracellular, sub-nuclear activity of MMPs (indicated by in situ zymography) in a small fraction of both phenotypically immature (O4-positive) and differentiated ($GalC^+$) oligodendrocytes. Moreover, the observed activity is correlated with the ECM components, especially with laminin, indicating a mutual role in the oligodendrocyte development.

MMPs have been found in nuclei of different cell types, e.g. cardiac myocytes (Kwan et al. 2004), hepatocytes (Si-Tayeb et al. 2006) and chondrocytes (Eguchi et al. 2008). They have been shown to be involved in an apoptotic response and play a role as transcription factors. These findings along with the observation of TIMP-1 (tissue inhibitor of metalloproteinases) accumulating in the cell nuclei in dependence on the cell cycle (Zhao et al. 1998) might explain their involvement in various distinct developmental processes, like migration, proliferation or differentiation.

In the present study we show that the oligodendrocyte recruitment from neural stem cells and their differentiation might be modulated by extracellular compounds. The possibility of increasing the fraction of mature cells means also the enlargement of the pool of early progenitors. Recently, many studies have focused on the oligodendroglia-committed NG2 progenitors: their multipotentiality and ability to repair damaged tissue (Belachew et al. 2003, Aguirre and Gallo 2004, Kulbatski et al. 2008, Sypecka et al. 2009).

The successful results obtained after the NG2 progenitor transplantation in laboratory animals (e.g. Archer et al. 1997, Watanabe et al. 2002, Windrem et al. 2004, Givogri et al. 2006) enables us to think ahead about the therapeutic perspectives. However a question arises about a potential, efficient, easy-to-handle and ethically uncontroversial source of stem/progenitor cells for autologous transplantation in humans.

Human umbilical cord blood neural stem cells (HUCB-NSC): donor source for oligodendrocytes

In an attempt to find an accessible source of either neural stem cells or committed progenitors suitable for autologous transplantation, various types of mesenchymal tissues have been investigated. Recently, it has been reported, that oligodendrocytes could be generated from bone marrow stromal cells (Kabos et al. 2002, Lu et al. 2008). Here we propose the HUCB-NSC line as another alternative source of human oligodendrocytes. The line has been derived from a $CD34^-$ (nonhematopoietic) adherent subpopulation of human cord blood cells (Bużańska et al. 2002). The HUCB-NSC line has been shown to exhibit the feature of multipotential neural progenitors, as well as the clonal and indefinitely self-renewing properties *in vitro* (Bużańska et al. 2006). When cultured in the presence of 2% serum, the cells spontaneously differentiated into neurons, astrocytes and oligodendrocytes. Increased oligogliogenesis forced in population of neural stem cells by application of ECM components, presents the possibility of obtaining an optimal composition of donor cells for transplantation. The fraction of the committed oligodendrocyte progenitors represents cells of the phenotype desired in the de/dysmyelinated areas. The undifferentiated donor stem cells could modify the microenvironment (thus possibly reducing the inflammation, providing neuroprotection or/and stimulation of endogenous stem cells) or/and further differentiate to replace the injured cells (Pluchino et al. 2005, Vendrame et al. 2005, Zhang et al. 2006). The complex composition of neural stem cell grafts consisting of both neuronal and glial precursors might be useful for the regeneration of CNS structures in various degenerative diseases (Reier 2004, Martino and Pluchino 2006). Taken together, such composition of donor cell groups is likely to provide the most efficient transplant integration and tissue repair. This promising therapeutic perspective is further supported by an optimal source of donors offered by the neural stem cells derived from human cord blood.

CONCLUSION

The HUCB-NSCs are low-immunogenic, ethically uncontroversial and prone to stimulation by biological compounds. Application of laminin promotes oligogliogenesis and therefore HUCB-NSC could be considered also as a source of oligodendrocytes.

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