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Research paper

Immunophenotype of mouse cerebral hemispheres-derived neural precursor cells

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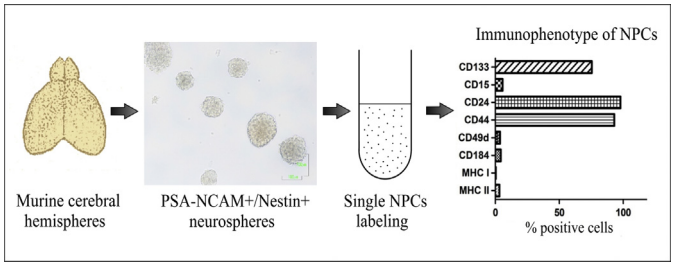
HIGHLIGHTS

- Postnatally isolated NPCs (piNPCs) differentiated mainly into glial cells, *in vitro*.
- CD24 and CD44 though not CD49d were among the expressed cell adhesion molecules.
- Absence of CD184 and MHC expression indicate the piNPCs low antigenicity profile.

GRAPHICAL ABSTRACT



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ABSTRACT

Postnatally isolated neural precursor cells (piNPCs) from mouse cerebral tissue have been studied in cell-based therapeutic approaches for Experimental Autoimmune Encephalomyelitis (EAE). Transplantation experiments in EAE rodents revealed that piNPCs manage to integrate into the host tissue and ameliorate clinical symptoms. When cultured *in vitro*, mouse cerebral piNPCs form neurospheres consisting of immature cells positive for polysialylated neural adhesion molecule (PSA-NCAM) that differentiate mainly towards glial cells, but also neurons. Herein, we have characterized piNPCs immunophenotype, with flow cytometry. NPCs were positive for CD24, CD44, and CD133 though negative for CD15, CD184 and CD49d. This immunophenotype, determined for the first time, among cells isolated from neonates might be useful for the identification of NPC population aiming at the development of transplantation protocols.

1. Introduction

Neural precursor cells (NPCs) constitute a valuable cell population of the central nervous system (CNS) due to their ability to self-renew and differentiate into neurons, astrocytes and oligodendrocytes, both *in vivo* and *in vitro* [1]. During embryonic development and adulthood, NPCs are located in the neurogenic niches of the mammalian CNS, particularly the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus [2]. These features render NPCs a useful tool for studying the molecular mechanisms involved in self renewal and differentiation enabling the development of CNS-targeted regenerative therapies.

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Several neurological conditions such as cerebral palsy (CP), spinal cord injury (SCI), and multiple sclerosis (MS) are characterized by demyelination. More specifically, in MS and its animal model, experimental autoimmune encephalomyelitis (EAE), CNS is characterized by multifocal inflammation resulting in myelin and axonal loss [3]. Thus, cell therapies of MS are focused on the enhancement of axonal remyelination and consequently the restriction of axonal dysfunction and disruption. Therefore, NPCs are studied as a potential source to replace oligodendrocytes and remyelinate damaged structures.

Keirstead et al. first proposed a protocol for the *in vitro* culture of postnatally isolated NPCs (piNPCs) from mouse cerebral tissue, which express the polysialylated neural adhesion molecule (PSA-NCAM) [4]. piNPCs are characterized as multipotent neural precursors which generate mainly glial cells, when cultured *in vitro* or transplanted in shiverer (dysmyelinating) mice [5]. Subsequent transplantation studies of piNPCs in EAE rodents, demonstrated that they migrate to the inflammatory foci and ameliorate symptoms, although the mechanism of action is not clear yet [6,7].

Several markers expressed by NPCs have been proposed for their identification, yet they are not useful for isolation of live cells due to their cytoplasmic or nuclear localization. In order to overcome this restriction, recent studies have focused on the analysis of the profile of cell surface antigens, allowing the rapid identification and selection of a specific cell population for use in downstream applications. Cluster of differentiation (CD) antigens are cell surface markers that have been used for the identification of NPCs [8–10].

This is the first report on the characterization of cell surface markers in piNPCs. In particular, the aim of the current study is to investigate the expression of widely used CD markers and establish the immunophenotype of piNPCs in order to develop a rapid and quantitative method for their identification and selection.

2. Materials and methods

2.1. Cell cultures

Animal handling was conducted according to the guidelines of the National Institute of Health, the Greek Government and the local ethics committee after receiving approval from Veterinary Directorate of Thessaloniki (36684/202). piNPCs were isolated from cerebral hemispheres of newborn C57Bl/6J mice as previously described [11,12]. Cells from the same culture were used for immunofluorescence staining and flow cytometric analysis. Bone marrow-derived mesenchymal stem cells (MSCs) [13], adult SVZ NPCs (aNPCs) [14], murine Neuro2a (N2a) cells [15] and mouse splenocytes were used as cellular controls. Further information is provided as Supplementary material.

2.2. Immunofluorescence staining

Neurospheres (150–250 spheres/slide) adhered and differentiated in pre-coated 2-well chamber slides. Primary antibodies were used and detected with fluorescent-labeled secondary antibodies (Alexa Fluor 568 and 488, Invitrogen). Ten microscopic fields from each slide were studied. Details are provided as Supplementary material.

2.3. Flow cytometry

Neurospheres were enzymatically dissociated. Single cell suspensions from piNPCs, adult NPCs, MSCs, N2a cells, splenocytes and freshly isolated SVZ cells were stained for flow cytometry analysis. Data were acquired using FACS Calibur cytometer (BD bioscience) and analyzed with FlowJo software (Tree star Inc.). Details are provided as Supplementary material.

2.4. Statistical analysis

Experiments were performed in triplicate. Immunofluorescence and flow cytometry data were analyzed with GraphPad Prism 5.0 software and are presented as mean percentage of positive cells ± standard deviation (SD).

3. Results

3.1. piNPCs express stemness markers and retain their multipotentiality, in vitro

In our *in vitro* study piNPCs were expanded as floating cell clusters with a diameter of 50–150 μm, according to the protocol described by Einstein et al. [11,12,16]. The undifferentiated state of the isolated cells was confirmed with immunofluorescence upon growth factor withdrawal (day 0). Neurospheres expressed the neural precursor marker PSA-NCAM (89.42 ± 5.97%) and the progenitor marker nestin (94.6 ± 2.17%) (Fig. 1B), while the glial precursor marker neural/glial antigen 2 (NG2) was not expressed (1.09 ± 0.31). We then investigated whether piNPCs have the ability to differentiate towards the CNS lineages. On day 2, piNPCs expressed PSA-NCAM (66.9 ± 2.18%) and nestin (70.25 ± 4.67%). $NG2^+$ glial progenitor population increased dramatically (17.91 ± 2.22%), whereas the percentage of $GFAP^+$ cells was low (8.92 ± 0.73%). Five days after the onset of differentiation, a small population of differentiating piNPCs expressed the neuronal marker NeuN (3.01 ± 0.4%). On the contrary, approximately one-third of total piNPCs expressed the oligodendrocyte marker O4 (30.72 ± 2.23%) and nearly half the cells expressed the astrocyte marker GFAP (48.34 ± 4.87%) (Supplementary Fig. 1). Our results showed that piNPCs cultured *in vitro* under these specific conditions retain the potential to give rise predominantly to glial cells, but also to neurons.

3.2. Undifferentiated NPCs express a unique profile of surface antigens

We further investigated the identity of piNPCs analyzing the expression of several surface markers by flow cytometry. The selection of the method to dissociate neurospheres is crucial as it affects cell survival and maintenance of cell surface proteins. We used enzymatic dissociation with Accutase, a mild treatment for single cells propagation with low impact on cell surface molecules and cell viability [17,18].

Single cells from undifferentiated neurospheres (day 0) were examined for the stem cell markers CD15 and CD133, present on rodent multipotent NPCs [18]. Double staining showed that a relatively large subpopulation of piNPCs expressed CD133 (73.74 ± 4.28%), while CD15 was not expressed at significant levels (5.68 ± 1.58%) (Fig. 1B). Of note, all cells expressing CD15 antigen were also positive for CD133 marker.

Moreover, undifferentiated piNPCs were labeled for CD44 antigen and signal transducer CD24, which are cell adhesion molecules also present on mammalian NPCs [19–21]. Almost all undifferentiated piNPCs expressed CD24 (98.22 ± 1.79%) and CD44 (91.07 ± 5.1%) (Fig. 1C–E). Double labeling revealed that $CD133^+$ piNPCs also express CD24 and CD44 molecules (Fig. 1D and E). We also explored the presence of the chemokine receptor CD184 and CD49d, which are found on NPCs surface providing migratory potential [20]. CD184 and CD49d were present on a small subset of piNPCs (4.66 ± 1.24% and 4.51 ± 1.73%, respectively) (Fig. 1F and