Neuroscience Letters 562 (2014) 69–74

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

A fast and simple differentiation protocol to study the pro-neurogenic activity of soluble factors in neurospheres

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HIGHLIGHTS

- Neuronal differentiation in SVZ neurospheres occurs within hours.
- Assay to measure differentiation largely independent of cell death and cell proliferation.
- Neuronal differentiation in SVZ neurospheres drops with increasing passage number.
- Inhibition of EGFR-, Erk1/2-, Akt-, and Shh-signaling induces neurogenesis.

ARTICLE INFO

Article history:
Received 11 November 2013
Accepted 2 January 2014

Keywords:
Neurosphere assay
EGF receptor
Erk1/2
Akt
Shh

ABSTRACT

Sphere-forming assays are widely used for the propagation, characterization and manipulation of adult brain-derived stem- and progenitor cells. However despite the broad application of this cell culture system in neural stem cell- and brain tumor research, no standardized protocols exist. Variations in experimental procedures not only concern the use of media components but also cell density, the number of passages the cells are propagated before analysis and, in cases where the neurogenic or gliogenic potential of the cells is investigated, the duration that the cells are allowed to differentiate. The latter deserves consideration because the proportion of differentiated cells obtained at the endpoint of the experiment depends not only on the absolute number of cells that differentiate at a given time, but also on the number of cell divisions prior to differentiation and the rate of cell death in the cultures. In the present study we describe a fast and simple differentiation protocol to investigate the pro-neurogenic potential of soluble factors added to subventricular zone (SVZ)-derived neurospheres. The assay relies on the use of primary neurospheres and very short differentiation times, thereby largely excluding the contribution of cell proliferation and cell death to the results. We use this modified assay to test the consequence of pharmacological inhibition of the EGF receptor-, Erk1/2-, Protein Kinase B/AKT-, and Sonic Hedgehog-pathways on neuronal differentiation of SVZ-neurosphere cultures.

1. Introduction

The subventricular/subependymal zone (SVZ/SEZ), a thin layer of cells located between the lateral walls of the lateral ventricle and the striatum, is a major stem cell niche in the uninjured, adult mammalian brain. SVZ neural stem cells, also known as type B-cells according to the nomenclature proposed by Doetsch and Alvarez-Buylla [1], exhibit astroglial characteristics and retain the capacity to proliferate and self-renew over extended periods. They produce an intermediate population of fast dividing progenitor cells (transient amplifying progenitors, 'TAPs' or type C-cells), which can differentiate into neurons, astrocytes and oligodendrocytes, the three primary neural lineages in the mammalian brain [2,3].

An important step toward reaching general acceptance of the idea of ongoing neurogenesis in the adult mammalian brain was the discovery that cells from the adult rodent forebrain can be cultured over extended periods in a serum-free culture system [4]. Under non-adherent conditions and in the presence of defined growth factors, which included epidermal growth factor (EGF), these cells form free-floating spheres of actively proliferating cells, termed neurospheres. In principle, the neurosphere assay requires the mechanical or enzymatic dissociation of the tissue of choice into a single cell suspension and plating them under non-adherent, serum-free conditions. Although widely used, the results obtained with the neurosphere assay have, however, to be interpreted with caution [5–7]. In particular, sphere numbers and sphere size obtained in the neurosphere assay have been used as indicators

Abbreviations: aNS, adult; SVZ, derived neurosphere(s); EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; OB, olfactory bulb; RMS, rostral migratory stream; Shh, Sonic Hedgehog; SVZ, subventricular zone.

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http://dx.doi.org/10.1016/j.neulet.2014.01.003

for the presence of operationally defined stem cells in a given tissue sample. Yet neurospheres are rather heterogeneous structures and contain activated stem cells and rapidly proliferating progenitors (TAPs) as well as differentiated cells. Both, activated stem cells and TAPs, are EGF-responsive. In addition, cell density in the culture critically affects sphere formation, because neurosphere cells release soluble factors into the culture medium, which affect cell proliferation and survival in an autocrine or paracrine manner. Another complication arises from the fact that spheres are highly motile in culture and tend to fuse, which obscures the intrinsic sphere-forming capacity of individual cells.

Despite these shortcomings, the neurosphere assay proved itself as straightforward and easy-to-carry-out cell culture system to study the biology of stem- and progenitor cells in acutely dissociated brain samples. A particular strength of the neurosphere assay is that it may allow to separate different physiological parameters that affect neurogenesis in vivo. Specifically, the absolute number of terminally differentiated cells that are generated over a particular time depends not only on the ability of the respective progenitor cells initiate differentiation programs, but also on the rate of cell proliferation prior to differentiation and cell survival. This is particularly important for in vivo approaches, such as intracranial injection of viruses or the delivery of pharmacological agents by micro-osmotic pumps, where the results are usually analyzed several days after the manipulation.

A simple way to separate differentiation–proliferation- and survival-effects would be to shorten the time allowed for differentiation to a minimum, which facilitates the onset of neuronal differentiation in vitro while largely eliminating any contribution of cell division or cell death to the results. In the SVZ, activated, proliferating stem cells (type B1-cells) divide with a cycle of approximately 17–18 h, whereas TAPs (type C-cells) divide every 18-25 h [8]. These data correspond well to an estimated cell cycle length of 14 and 17 h for adult SVZ-derived stem-/progenitor cell cultures growing in the presence of EGF and FGF2 [9]. By contrast, only few studies have measured the time course of cell death in neurosphere cultures upon growth factor withdrawal. One report observed activation of caspase-3 approximately 25 h after growth factor withdrawal from free-floating neurosphere cultures obtained from the E14.5 mouse striatum [10]. Based on these reports, we hypothesized that a differentiation time of well under 10 h may be sufficient to quantify the onset of neuronal differentiation with minimal contribution of cell proliferation and cell death to the results.

We here describe a variation of the commonly used neurosphere assay protocol to separate the effect of pharmacological treatments on neuronal differentiation of adult SVZ derived stem-/progenitor cells from any accompanying effects of these drugs on cell proliferation and survival. The assay requires the use of primary SVZ-derived neurosphere cultures and a standardized treatment protocol. Under these conditions, differentiation times of few hours are already sufficient to lead to robust and reproducible neuronal differentiation. As a fist test of this assay, we show that inhibition of the EGF receptor-, PKB/Akt-, Erk1/2- and Sonic Hedgehog pathways leads to rapid induction of neuronal differentiation in SVZ-neurosphere cultures in the presence of EGF and FGF2.

2. Material and methods

2.1. Neurosphere culture

Neurosphere cultures from the SVZ of 7–12-week old C57/BL6 mice were prepared as described [11] and grown in the presence of 2% v/v B-27 supplement (GIBCO), 10 ng/ml fibroblast growth factor-2 (FGF2, human recombinant; Peprotech, Rocky Hill, NJ, USA) and 20 ng/ml epidermal growth factor (EGF, human recombinant; Peprotech) at 37 °C, 5% CO_2. After 4 days, the neurospheres were split in the presence of accutase and plated at a density of 60,000 cells per cm^2 on glass coverslips coated with 100 μg/ml poly-D-lysine (in DPBS, 90 min at 37 °C; Sigma–Aldrich) followed by 1 μg Laminine per cm^2 (in DPBS; 90 min at 37 °C; Roche, Mannheim, Germany). After 16 h, different inhibitors were added as detailed below. For comparison of different passage neurospheres, spheres were split every 4 days until the passages indicated. The first spheres that formed in the cultures after tissue dissection were considered 'primary neurospheres' or P1. 'Tertiary'/P3 neurospheres accordingly had been split twice and 'quinary'/P5 neurospheres four times prior to growth factor withdrawal and differentiation. Differentiation times varied between 4 h and 3 days.

2.2. Pharmacological inhibition of signaling pathways

Primary neurosphere cells growing over night as adherent monolayer on poly-D-lysine/Laminin coated coverslips were treated with AG1478 (100 nM; LC- Laboratories, Woburn, MA; Cat# T-7310), AZD6244 (10 μM; Selleckchem, Munich, Germany; Cat# S1008), Akt Inhibitor VIII (2 μM; Merck Millipore, Billerica, MA; Cat# 124018), or Cyclopamine (10 μM; Calbiochem, San Diego, CA; Cat# 239803). Initially, different durations of treatment between 2 h and 8 h and different inhibitor concentrations were tested. The minimal duration of treatment and minimal concentration was then used for further experiments. Cells treated with DMSO (vehicle) alone for identical times served as controls. All assays were performed in triplicates. Standard deviation was calculated between technical replicates, error bars represent S.E.M. Values were normalized to DMSO treated control cells obtained from the same cell culture. Comparison between two groups was performed with paired student's *t*-test (Prism 5.01, Graph Pad). Statistical significance was assumed when $p < 0.05$, indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.3. Antibodies and immunhistochemical analysis

Cells were fixed in 2% PFA, washed three times in PBS and stained over night at 4 °C with a monoclonal antibody against neuronal β-tubulin, isotype III, (TuJ1; Covance, Princeton, NJ, USA) at 1:1000 in 5% v/v Chemiblock (Chemicon/Millipore Bioscience Research Reagents, Billerica, MA, USA) and 0.5% Triton X-100 (AppliChem, Gatersleben, Germany). Secondary antibody was Alexa 488-conjugated anti-mouse (Dianova, Hamburg, Germany). Cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei, embedded in Aqua Poly Mount (Poly Science, Inc.; Warrington, PA, USA) and analyzed with a 80i fluorescence microscope (Nikon, Tokyo, Japan).

3. Results

Adult stem- and progenitor cells are known to shift their character from neurogenic to gliogenic upon prolonged exposure to EGF [12,13]. To directly compare the proportion of neurons that could be generated from a defined neurosphere culture upon extend culture periods, we serially passaged SVZ-derived neurosphere cultures for up to five passages as free-floating spheres in the presence of EGF and FGF2. From the primary (P1), tertiary (P3) and quinary (P5) spheres cells were dissociated and identical numbers of single cells were plated in the absence of growth factors on poly-D-lysine/Laminine coated surfaces and allowed to differentiate for three days. (Fig. 1A). A three day differentiation time was chosen, because after this time neuronal differentiation is occurring at a high rate (but is still far from complete), whereas cell death is still rather low. Primary neurosphere cultures generated neuronal