Neuroscience Letters 508 (2012) 61–66

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

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

The effects of different phenotype astrocytes on neural stem cells differentiation in co-culture

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ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form 1 December 2011
Accepted 13 December 2011

Keywords:
Protoplasmic astrocytes
Fibrous astrocytes
Neural stem cells
Differentiation
Co-culture

ABSTRACT

Astrocytes were reported to show neuroprotective effects on neurons, but there was no direct evidence for a functional relationship between astrocytes and neural stem cells (NSCs). In this experiments, we examined neuronal differentiation of NSCs induced by protoplasmic and fibrous astrocytes in a co-culture model respectively. Two types of astrocytes and NSCs were isolated from E13 to 15 cortex of rats. The neuronal differentiation of NSCs was examined after co-culture with two kinds of astrocytes. There were more neuronal marker β-tublin III positive cells from NSCs co-cultured with protoplasmic astrocytes. However the differentiated neurons, whether co-cultured with protoplasmic astrocytes or fibrous astrocytes, both expressed glutamate AMPA receptor subunit GluR2 protein and exhibited biological electrical reactivity after stimulated by glutamine. Therefore, these findings indicated that two types of astrocytes could induce the differentiation of NSCs and also possibly induce functional maturation of differentiated neurons, among which protoplasmic astrocytes have the ability to promote neuronal differentiation of NSCs compared with fibrous astrocytes.

1. Introduction

The injury of central nervous system (CNS) will cause damage to neurons, astrocytes and other cells in the tissue. To repair this damage requires suitable neural cell types in injured areas. Because neural stem cells (NSCs) have the ability to self-renew and differentiate into different types of neural cells, the discovery of NSCs has opened up a new avenue for CNS tissue repair [3,5].

A great deal of research indicated that internal and external signals affected proliferation and differentiation of NSCs. Promoting differentiation of NSCs towards desired phenotypes especially neurons and improving survival and integrating following transplantation has become a new researching field of CNS injury and repair [9]. However, an injured region or non-neurogenic region of adult CNS may provide a relatively non-permissive environment for transplanted NSCs in absence of exogenous neurotrophic support [4,9]. A method to provide sustained neurotrophic factor for transplanted cells may be advantageous in improving graft viability and long-term outcomes. As an alternative approach, provision of neurotrophic factors by co-grafting with supportive cells has been utilized in other neural transplantation paradigms and gained better survival of graft and functional rehabilitation [8,12,13,21].

As it is well known that astrocytes are one of the main component of CNS and have tight connection with neurons whether in embryonic development or adult stage. In addition, the conditioned medium of astrocytes showed a neuroprotective effect on survival of rat embryonic cortical neurons through secreting neurotrophic factors, such as GDNF, BDNF, CNTF, NT-3, NGF, IL-1, and IL-6 [1,15,19]. There are two types of astrocytes: protoplasmic astrocyte and fibrous astrocytes in CNS. However, the localization of the two types of astrocytes is different. Among which protoplasmic astrocytes are usually distributed in grey matter, but fibrous astrocytes are localized in white matter [17]. However, functional interaction between NSCs and two types of astrocytes is not clear. In this study, we planned to evaluate whether the two types of astrocytes can be serving as a source of sustained neurotrophic support to improve NSCs differentiation into neurons and to determine whether the differentiated cells under different conditions have physiological function. To examine a possible functional interaction, NSCs and two types of astrocytes from embryonic Wistar rat cortex were co-cultured and investigated.

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doi:10.1016/j.neulet.2011.12.019

2. Materials and methods

2.1. Cell preparation

All experiments were approved by the animal care and experimentation committee of the Third Medical Military University. Primary cultures of NSCs were derived from embryonic 13–15 days (E13–15) Wistar rats as described previously [3,10]. Briefly, the telencephalon was rapidly dissected and placed into 1.5 ml tubes containing 0.25% trypsin. The tissue was mechanically dissociated into single cell suspensions. Cell numbers and viability were assessed by staining a small volume of cell suspension with 0.4% trypan blue. Single-cell suspensions were then transferred to growth medium consisting of NB + 2% B27 supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, Gibico Invitrogen, USA), 20 ng/ml of epidermal growth factor (EGF, Gibico Invitrogen, USA) at 4×10^5 cells/ml. The cells were then planted into culture flasks and maintained under a humidified atmosphere of 5% CO_2 in air at 37 °C. After 5–6 d in vitro, the neurospheres were dissociated into single-cell suspensions and seeded onto 96-well plates at 1–2 cells per well. The neurosphere subcultures were digested and another passage was performed as before. The cell passage protocol was performed every 6 days to obtain neurospheres originating from a single primary cell. Secondary or tertiary neurospheres were used for subsequent experiments. For Brd-U labeling, NSCs were incubated in medium containing 10 μM Brd-U for 18 h prior staining.

Protoplasmic and fibrous astrocytes were prepared according to the following methods [7,20]. Cortex tissues were collected from embryonic 13–15 days Wistar rats and triturated to produce a single-cell suspension. The cells were planted at a density of 5×10^5/ml on poly-L-lysine treated culture flasks with DMEM/F12 plus 10% fetal bovine serum. After 14 days 10 mmol/L L-leucine-methyl-ester was added into the media about 1 h to kill microglials, then the cells were quaked with 180 rpm rate for 16 h to separate O-2A progenitor. The protoplasmic astrocytes were left in the flask. Cells were planted at an initial concentration of 5×10^5 cells/ml in 75 cm^2 cell culture flasks. The O-2A cells were plated in the DMEM plus 10% fetal bovine serum and induced to fibrous astrocytes. After 2 weeks of expansion, protoplasmic astrocytes and fibrous astrocytes were replanted on poly-L-lysine-treated 35 mm culture petri dishes at a density of 1×10^4 cells/dish for co-culture with NSCs respectively.

For co-culture of different astrocytes and NSCs, transwell dishes were used to produce a cell co-culture environment. NSCs were plated in poly-L-lysine pretreated six-well culture dishes (Nunc, Denmark) and astrocytes were cultured in the insert (Millipore, USA) which had a porous membrane of 0.4 μm pore size inhibiting migration of cells and preventing direct contact between NSCs and astrocytes. Co-cultured cells were maintained for 7 days in NB + 2%B27 medium with medium being half-exchanged every 3 days. At 7 days post co-culture the differentiated cells from NSCs co-culture with two types of astrocytes were fixed and immunochemically stained with neuronal specific marker β-tublin III (mouse anti-rat IgG, 1:800, Sigma–Aldrich, USA) and astrocytes specific marker glial fibrous acidic protein (GFAP, rabbit anti-rat IgG, 1:400, Sigma–Aldrich, USA).

2.2. Immunocytochemistry identification of two types of astrocytes, NSCs and differentiated neural cells

After 30-min fixation in 4% paraformaldehyde, the cells were air-dried for 10 min, treated with 2 N HCl for 10 min to denature DNA, and then neutralized with 0.1 M sodium borate for another 10 min. Samples were washed three times with PBS. 0.5% Triton-X-100 and 1%BSA prior to an overnight incubation with the primary mouse anti-rat Brd-U antibody (1:800, Sigma–Aldrich, USA), mouse anti-rat Nestin (1:200, Chemicon, USA) for NSC and rabbit anti-rat GFAP for two types of astrocytes respectively. In order to identify the multiple differentiation of NSC, the differentiated cells from NSCs were double stained with β-tublin III and GFAP antibodies. After 3× 5 min washed, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (1:100; Chemicon, MA, USA) was used for staining β-tublin III and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary antibody (1:100; Chemicon, MA, USA) was staining for GFAP respectively. Subsequently the samples were counterstained with 300 nM of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma–Aldrich) for 3 min and then washed three times in PBS prior to scanning with laser con-focal microscope (Leica, SP-2, Germany) [16].

2.3. The glutamine receptor protein subunit GluR2 expression of differentiated neurons

7 days after NSCs/protoplasmic astrocytes or fibrous astrocytes co-cultures, the differentiated cells were fixed with 4% paraformaldehyde in PBS for 20 min. After 3× 5 min washed with PBS, the cells were incubated an overnight with mouse monoclonal anti-rat GluR2 antibody (1:600, Santacruz, USA) and β-tublin III respectively. After 3× 5 min washed with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (1:100; Chemicon, MA, USA) was used for staining β-tublin III and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse secondary antibodies (1:100; Chemicon, MA, USA) was staining for GluR2.

2.4. Calcium activity in differentiated neurons

The differentiated neural cells in co-culture medium were incubated with Fura-3AM (Sigma–Aldrich, USA) in Ca^{2+}-free D-Hanks medium for 30 min. After washed with PBS, the medium was replaced with NB + 2%B27 medium. Then the differentiated neurons were observed timely with laser confocal scanning microscope at xyt model to examine the changes of free Ca^{2+} concentration in differentiated neurons stimulated by 2000 nM glutamine.

2.5. Statistical analysis

The percentage of positive cells in relation to the total cell number was determined in 5 random fields under a 40× objective for each group in three independent experiments. All data were presented as mean ± SD. Statistical analysis of data was performed using a one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. NSCs have the ability to proliferate and differentiate into different types of neural cells

On the second day after primary cultures, the cells growing as spheres in suspension are NSCs and express the neural stem cell specific marker Nestin (Fig. 1A and B). Detection of DNA replication using Brd-U immunostaining in spheres confirmed that these cells undergo proliferation (Fig. 1C). At the same time, the NSCs have a multi-differentiation potential which can differentiate into neurons and astrocytes after withdraw of bFGF (Fig. 1D).