Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomaterials 26 (2005) 4291–4299
Biomaterials
www.elsevier.com/locate/biomaterials

Behavior of embryonic rat cerebral cortical stem cells on the PVA and EVAL substrates

Tai-Horng Young*, Chih-Huang Hung

Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, #1, Sec. 1 Jen-Ai Road, Taipei 100, Taiwan, R.O.C.

Received 27 June 2004; accepted 29 October 2004

Abstract

Cell behavior is determined by intrinsic programs and complex interactions among cells, medium components and substrates. Several previous reports have demonstrated the usefulness of extrinsic signals coming from soluble growth factors and cell–cell contact for regulating the proliferation and differentiation of neural stem cells. At present, the effects of substrate on neural stem cells are not known. In this study, the behavior of neural stem cells, isolated from embryonic rat cerebral cortex, was observed and compared on the polyvinyl alcohol (PVA) and poly (ethylene-*co*-vinyl alcohol) (EVAL) substrates in the presence of the mitogenic effect of basic fibroblast growth factor (bFGF) in the serum-free medium. It was found that PVA and EVAL exerted different influences on the fate of neural stem cells. The behavior of neural stem cells on the EVAL was independent of cell density at the single-cell level. Single neural stem cells seemed to remain dormant on the EVAL. Conversely, the development of cell clusters, termed neurospheres, was in a density-dependent manner on the EVAL. Neurospheres continuously proliferated under high-density culture condition, but differentiated into neurons and astrocytes under low-density culture condition. However, regardless of single cells or neurospheres, cultured cells could not survive on the PVA. Therefore, it is reasonable to assume that biomaterials may stimulate or inhibit the proliferation and differentiation of neural stem cells. These in vitro results are very encouraging since this information should be useful for the development of strategies for regulating the preservation, proliferation and differentiation of neural stem cells.

Keywords: Neural stem cells; Proliferation; Differentiation; EVAL; PVA

1. Introduction

The discovery of neural precursors with the ability to generate progenitors for neurons, astrocytes and oligodendrocytes in vitro provides a source of cells for tissue engineering that may in the future lead to novel therapeutic approaches for treating neuronal loss associated with neurodegenerative conditions [1,2]. A central focus of neuron regeneration is to understand the role of extracellular signals in regulating proliferation and differentiation of neural stem cells. Previous work has explored the potential of neural stem cells in culture by inducing proliferation and differentiation in the presence of various soluble growth factors [3,4]. For example, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) [5] and hepatocytes growth factor [6] have been shown to induce the proliferation of embryonic precursors of the central nervous system, leading to the formation of cell clusters, termed neurospheres [1,2,7]. In addition to the diffusible factors, signal coming from cell contact also has an effect on the transition of a multipotential precursor to post-mitotic neurons of different types [8]. However, what determines the choice of the differentiation pathway taken by neural stem cells on the different substrates remains unexplored. It is possible that neural stem cells respond

*Corresponding author. Tel.: +886 2 23123456x1455; fax: +886 2 23940049.
E-mail address: thyoung@ha.mc.ntu.edu.tw (T.-H. Young).

0142-9612/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2004.10.043

to different substrates, and their fate determination depends on the chemical properties of the substrate.

Recently, our group has been interested in developing the polyvinyl alcohol (PVA) and poly (ethylene-*co*-vinyl alcohol) (EVAL) membranes as the cell substrates to regulate cellular events [9–14]. These two polymers have rather different properties. EVAL contains both hydrophilic vinyl alcohol segments and hydrophobic ethylene segments but PVA has hydrophilic segments only. Since neural stem cells can proliferate in suspension above the substrate [1,2,7], the purpose of this study is to investigate whether the effects of PVA and EVAL on neural stem cells, isolated from embryonic rat cerebral cortex, are different from those on anchorage-dependent cells, and to analyze the lineage relationship between substrate-responsive cells. Our hypothesis is that identification of substrates to induce or inhibit the proliferation and differentiation of neural stem cells may allow for their eventual manipulation to replace lost or dysfunctional neurons following trauma or disease.

2. Experimental methods

2.1. Preparation of substrates

Commercially available PVA (BF-17, Chang Chun, Taiwan) and EVAL (E105A, Kuraray, Japan, containing ca. 56 mol% vinyl alcohol) were used as substrate materials. The substrate used in the form of a membrane with dense structure was prepared by using the dry process of the phase inversion method [10]. Circular membranes (1.5 cm in diameter) suitably sized for cell culture wells were cut from the prepared membranes, sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively with phosphate-buffered saline (PBS). Subsequently, membranes were placed in 24-well tissue culture polystyrene plates (Corning, New York, USA) by placing a silicon rubber ring on top of each membrane for cell culture.

2.2. Culture of primary neural stem cells

Neural stem cell culture was prepared from pregnant Wistar rat embryos on day 14–15 according to a protocol detailed previously [8,15]. Briefly, embryonic rat cerebral cortices were dissected, cut into small pieces and mechanically triturated in cold Hank's balanced salt solution (HBSS) containing 5.4 mM KCl, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.6 mM $MgSO_4 \cdot 7H_2O$, 137 mM NaCl and 5.6 mM D-glucose. The dissociated cells were collected by centrifugation and resuspended in a serum-free medium containing DMEM-F12, 8 mM glucose, glutamine, 20 mM sodium bicarbonate, 15 mM HEPES and N2 supplement (25 μg/ml insulin, 100 μg/ml human apotransferrin, 20 nM progesterone, 30 nM sodium selenite, pH 7.2) [16]. The number of live cells was counted by trypan blue exclusion assay in a hemocytometer.

Cerebral cortical cells were cultured in T25 culture flasks (Corning, New York, USA) at a density of 50,000 cells/cm^2 in the above culture medium in the presence of bFGF at a concentration of 20 ng/ml. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/ 5% CO_2. After 1 day of culture, suspended cells underwent cell division. Cell division continued for an additional 2 days, after which proliferating cells formed neurospheres. Subsequently, adherent cells were discarded and neurospheres were collected by centrifugation, mechanically dissociated and replated as single cells in a new T25 culture flask at a density of 50,000 cells/cm^2 in the fresh culture medium containing the same concentration of bFGF. These single cells proliferated and grew into new spheres in the subsequent 2–3 days. The procedure of subculture was repeated again and then cells were collected for investigating the effects of PVA and EVAL on them.

2.3. Immunocytochemistry

At indicated time points, cultured cells were fixed in ice-cold 4% paraformaldehyde in PBS for 20 min and washed three times in PBS. After fixing, cells were incubated with primary antibodies diluted in PBS containing 0.3% triton-X-100 and 10% bovine serum albumin for 2 h at 37 °C. The primary antibodies and their dilution used in this study were mouse anti-nestin monoclonal antibody (anti-nestin; 1:1000; Chemicon, Temecula, CA), mouse anti-neuron specific enolase monoclonal antibody (anti-NSE; 1:250; Chemicon, Temecula, CA) and rabbit anti-glial fibrillary acidic protein polyclonal antibody (anti-GFAP; 1:500; Chemicon, Temecula, CA). FITC- and rhodamine -conjugated secondary antibodies were used to visualize the signal by reacting with cells for 1 h at room temperature. The secondary antibodies and their dilution were FITC-conjugated goat anti-mouse IgG (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon, Temecula, CA), FITC-conjugated donkey anti-rabbit IgG (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon, Temecula, CA) and rhodamine-conjugated goat anti-mouse IgG. (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon, Temecula, CA). Cell morphology was viewed with a photomicroscope (Zeiss LAMBDA 10-2, Germany). Immunostained cells were visualized by indirect fluorescence under the fluorescent microscope (Axiovert 100TV, Germany). The number of cells showing different immunoreactivity was determined by counting the immunopositive and the total number of cells in three to seven visual fields (10–30 cells per field). The means and SEM of at least