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The effect of chitosan and PVDF substrates on the behavior of embryonic rat cerebral cortical stem cells

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Abstract

In this study, the behavior of neural stem cells from embryonic rat cerebral cortex were compared on the chitosan and poly(vinylidene fluoride) (PVDF) substrates at single-cell and neurosphere level. It was found that chitosan and PVDF substrates inhibited the proliferation and differentiation of single neural stem cells. It seemed that single-cell cultures on both substrates show cells remained dormant. However, neurospheres could exhibit different or similar behavior on these two substrates, which is dependent on the presence or absence of serum. More cells migrated outside from the neurospheres and longer processes extended from differentiated cells on chitosan than on PVDF when neurospheres were cultured in the serum-free medium. On the contrary, when serum was added to the culture system, chitosan and PVDF could induce the neurosphere-forming cells into an extensive cellular substratum of protoplasmic cells upon which process-bearing cells spread. In addition, based on the immunocytochemical analysis, the percentages of differentiated cell phenotypes of neurospheres cultured on chitosan and PVDF substrates became similar in the presence of serum. Therefore, it is reasonable to suggest that biomaterials may stimulate or inhibit the proliferation and differentiation of neural stem cells according to the complex environmental conditions. The information presented here should be useful for the development of biomaterials to regulate the preservation, proliferation, and differentiation of neural stem cells.

Keywords: Chitosan; Poly(vinylidene fluoride) (PVDF); Neural stem cells

1. Introduction

The discovery of neural stem cells in the central nervous system (CNS) and their capacity to regenerate functional neural cells has raised hopes for treating neural diseases and injuries. In vitro, these neural stem cells or neural progenitor cells are endowed with different stemness properties to exhibit the potential to differentiation to neurons, astrocytes, and oligodendrocytes [1–4]. Previous studies suggested that the proliferation and differentiation of embryonic cortical neural stem cells were determined by the effects of extrinsic and intrinsic signals coming from medium components and several complex interactions among cells [1–11]. For example, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and hepatocytes growth factor (HGF) have been shown to induce the proliferation of embryonic precursors of the CNS, leading to the formation of cell clusters, termed neurospheres [3,7,8]. In addition, ciliary neurotrophic factor (CNTF) and insulin-like growth factor-1 (IGF-1) have also been reported to function as key cues in regulation of development of neural stem cells [9,10]. Besides the diffusible factors, signal coming from cell–cell contact also has an effect on the transition of multipotential neural stem cells to postmitotic cells of different types [6,11].

Numerous natural and synthetic polymers have been used as substrates or scaffolds for peripheral and central nerve regeneration in vitro or in vivo [12–15], but little is known what determines the choice of the differentiation pathway taken by neural stem cells on different substrates [11]. It is possible that neural stem cells respond to different substrates, and their fate determination depends on the chemical properties of the substrates. In this study, neural stem cells, isolated from embryonic rat cerebral cortices,

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were cultured on chitosan and poly(vinylidene fluoride) (PVDF) substrates at single-cell and neurosphere level. Chitosan, a deacetylated product of chitin, is a non-toxic material for the human body [16,17] and a potential candidate for use in nerve regeneration [18]. PVDF is an acid resistant, chemically inert, and mechanically strong polymer, which also may be used for the application of nerve regeneration [19–22]. Therefore, the aim of the present study was to evaluate in vitro whether chitosan or PVDF may be useful as a source of substrate to support survival of neural stem cells and differentiation towards desired phenotypes.

2. Materials and methods

2.1. Preparation of chitosan and PVDF substrates

In this study, chitosan (Sigma C-3646) and PVDF (Elf Ato Chem Kynar 740 type) were used in the form of membranes, prepared by the dry process of the phase inversion method [23]. The solvents used for chitosan and PVDF were 0.5 N acetic acid and 0.5 N triethylene phosphate, respectively. The concentrations for chitosan and PVDF were 2 and 20 wt%, respectively. First, the polymer solution was spread on a glass plate in the thickness of 500 μm and was evaporated in a convection oven at 60 °C over 24 h to form solid membranes. Subsequently, the residual solvent in the nascent membrane was removed by a series of washing steps. Finally, circular membranes (1.5 cm in diameter) were placed in 24-well tissue culture polystyrene plates (Corning, NY, USA), sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively with phosphate-buffer saline (PBS).

2.2. Isolation and culture of cortical neural stem cells

Cerebral cortical neural stem cells were prepared from pregnant Wistar rat embryos on days 14–15 according to a protocol detailed previously [5,6]. Briefly, rat embryonic 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 were 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) [24]. The number of live cells was counted by trypan blue exclusion assay in a hemocytometer.

Cerebral cortical neural stem cells were purified and cultured in T25 culture flasks (Corning, NY, 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 1–3 d in vitro, cells underwent cell division and the proliferating cells formed neurospheres, which were suspended in the medium. Subsequently, adherent cells were discarded and suspended neurospheres were collected by centrifugation, mechanically dissociated and subcultured 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 cells again grew into new spheres in the subsequent 2–3 d. The procedure of subculture was repeated to achieve the purified cortical neural stem cells. The plasticity of these purified cortical neural stem cells was identified by the method of immunocytochemistry with anti-nestin, anti-NSE, and anti-GFAP, which has been reported in a previous publication [11].

Subsequently, single cells (50,000 cells/cm^2) and neurospheres (350 neurospheres/cm^2) were seeded on chitosan and PVDF substrates in the serum-free medium in the presence of 20 ng/ml bFGF for investigating the effects of substrates on neural stem cells. In addition, the effect of 10% fetal bovine serum (FBS) on the differentiation of neurospheres on chitosan and PVDF substrates were also examined in this study. At indicated time points, morphologies of cultured neurospheres were observed under a phase contrast microscope (Zeiss LAMBDA 10-2, Germany).

2.3. Quantification of process growth

Digital photomicrographs were taken from random fields of neurospheres cultured at indicated time points. The length of 10–15 longest processes on each neurosphere was measured from the edge of the neurospheres to the tip of these processes, i.e., the end-to-end distance. Process lengths were measured by tracing the processes using NIH Image software (ImageJ). The length of processes of 20 independent neurospheres was calculated at each experiment, and the means and standard error of mean (SEM) were also calculated [25,26].

2.4. MTT assay

The cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide; Sigma) colorimetric assay [27,28]. The culture medium was removed at the indicated time points, and the cells were incubated with 0.1 ml MTT solution (2 mg/ml in PBS) for 3 h at 37 °C. After incubation, the MTT solution was aspirated and the formazan reaction products were dissolved in dimethyl sulfoxide and shaken for 20 min. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK) at 570 nm.

2.5. Immunocytochemistry

For immunocytochemical characterization, cultured cells were fixed in ice-cold 4% paraformaldehyde in PBS for 20 min and washed three times in PBS after 5 d of culture. After fixing, cells were incubated with primary antibodies diluted in PBS containing 0.3% Triton X-100 and 10% bovine serum albumin (BSA) 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), rabbit anti-microtubule-associated protein 2 polyclonal antibody (anti-MAP-2; 1:500; Chemicon, Temecula, CA), rabbit anti-glial fibrillary acidic protein polyclonal antibody (anti-GFAP; 1:500; Chemicon, Temecula, CA), and mouse anti-oligodendrocyte marker O4 monoclonal antibody (anti-O4; 1:250; Chemicon, Temecula, CA) [29–32]. FITC- and Rhodamine-conjugated secondary antibodies were used to visualize the signal by reacting with cells for 30 min 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). Immunostained cells were visualized by indirect fluorescence under the fluorescent microscope (Axiovert 100TV, Germany).

2.6. Bromodeoxyuridine (BrdU) incorporation

For the analysis of the distribution of cells in proliferation, neurospheres cultured on chitosan and PVDF substrates under serum-free conditions were labeled with BrdU (20 μM) for 24 h to permit later identification. Afterwards, indirect immunocytochemical staining with anti-BrdU was performed to label the cells that maintain the ability of proliferation in culture. Briefly, cells were fixed with ice-cold 4% paraformaldehyde in PBS for 20 min at 4 °C after 5 d of culture. After fixing, each well was rinsed with ice-cold PBS for 5 min at 4 °C. Cells were then incubated with anti-BrdU (1:500) diluted in PBS containing 0.3%