NGF IMPROVES NEURITE FASCICULATION OF EMBRYONIC RAT CEREBRAL CORTICAL NEURAL STEM CELLS ON EVAL SUBSTRATES UNDER SERUM FREE CONDITIONS

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ABSTRACT

The behaviors of multipotential neural stem cells are regulated by several factors, including culture substrates, soluble factors, and cell-cell interactions. The purpose of this study was to explore the effects of cell-cell and cell-substrate interactions on developmental potential of neural stem cells from embryonic rat cerebral cortex at neurosphere level in the presence of nerve growth factors (NGF). The results suggested that, in combination with NGF, EVAL substrates could induce neurite fasciculation of differentiated forming-neurosphere cells under serum free conditions. Quantitative analysis of process growth reveals that, under serum free conditions, when NGF was present the process breadth was significantly larger than that of neurospheres cultured without NGF. In contrast, when serum were added in the medium, regardless of the addition of NGF or not, the neurospheres were induced into typically an extensive cellular substratum of protoplasmic cells upon which process-bearing cells spread. It indicated that, when serum was present, the differentiated GFAP-positive astrocytes layer could serve as a supported architectural for growth and survival of process-bearing cells. At this time, neurites were failed to fasciculate. Here, we proposed that the mediation of cell-biomaterial interaction by cell-cell interaction should be taken into account for regulation of neurites fasciculation. Moreover, the pattern of neural stem cell growth on EVAL substrates with or without NGF in the absence or presence of serum in this studies suggested that neurons are more adherent to glial cells than to other neurons but are more adherent to other neurons than to EVAL substrates.

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1. INTRODUCTION

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The discovery of neural stem cells in the central nervous system (CNS) and their capacity to regenerate functional neural cells have raised hopes for once untreatable diseases and injuries to the brain and spinal cord. In vitro culture system, the plasticity of neural stem cells was thought to be controlled and manipulated by the effects of extrinsic and intrinsic signals coming from substrates, medium components, and several complex interactions among cells [1-2]. These multipotential neural stem cells can divide and expand to form free-floating neurospheres when grown

in defined medium supplemented with basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), or transforming growth factor α (TGF- α) [1,3-4]. 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 [5-6]. 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 [2,7]. However, when exposed to suitable substrates in the absence of these mitogens, these cells attach and differentiate into neurons, astrocytes, and oligodendrocytes. However, numerous natural and synthetic polymers have been used as substrates or scaffolds for peripheral and central nerve regeneration in vitro or in vivo [8]. It is reasonable to assume that biomaterials may stimulate or inhibit the proliferation and differentiation of neural stem cells.

According to our previous studies, EVAL and PVA substrates would exert different influences on the behavior of neural stem cells under serum free conditions [9]. 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, the effect of nerve growth factors (NGF) on growth and differentiation of embryonic rat cerebral cortical stem cells on EVAL substrates under the same conditions was explored. NGF plays an important role in the developments and survival of certain neurons in the CNS and could improve better neurites growth. In addition, it has also been suggested that the regulation of neurite fasciculation was determined by the affiliative competition between neuron-neuron and neuron-substrates [10]. Thus, this study proposed that, in combination with nerve growth factors, EVAL substrates could induce neurite fasciculation of differentiated cortical neural stem cells under serum free conditions. The information should be useful for the development of biomaterials to regulate the morphology and differentiation of neural stem cells and their eventual manipulation to replace lost or dysfunctional neurons following trauma or disease.

2. MATERIALS AND METHODS

2.1 Fabrication of EVAL Membranes

EVAL (E105A, Kuraray, Japan, containing ca. 56mole % vinyl alcohol) membranes were prepared by using the dry process of the phase inversion method [11]. Circular membranes (1.5 cm in diameter) suitably sized for 24-well tissue culture polystyrene plates (Corning, New York, USA) were cut from the prepared

membranes, and were placed in wells by placing a silicon rubber ring on top of each membrane. Subsequently, membranes were sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively with phosphate-buffer saline (PBS) for culturing cortical neural stem cells.

2.2 Isolation and Culture of Cortical Neural Stem Cells

Cortical neural stem cells were prepared from pregnant Wistar rat embryos on day 14-15 according to a protocol detailed previously [1-2]. 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 $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.6 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 g/ml insulin, 100 g/ml human apotransferrin, 20 nM progesterone, 30 nM sodium selenite, pH 7.2) [12]. 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, New York, USA) at a density of 50,000 cells/cm² 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% CO₂. After 1 to 3 days in vitro, cells were undergoing cell division and the proliferating cells formed clusters of cells, termed 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/cm² in the fresh culture medium containing the same concentration of bFGF. These cells grew into new spheres in the subsequent 2-3 days, that is, single cells proliferated and formed new neurospheres. The procedure of subculture was repeated again to achieve the purified cortical neural stem cells and proliferating neurospheres. The plasticity of these purified cortical neural stem cells was then identified by the method of immunocytochemistry with anti-nestin, anti-NSE, and anti-GFAP. Subsequently, these purified neurospheres were seeded on EVAL substrates at 125 neurospheres/cm² in the presence or absence of NGF (20 ng/ml) under serum or serum-free conditions after bFGF withdrawal. 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 Length and Breadth

Digital photomicrographs were taken of random fields of neurospheres at indicated experimental points. Quantification of process growth was evaluated by the end-to-end distance of extensive processes. The lengths of 10 longest processes (neurites from neurons or processes from glial cells) per neurosphere were estimated from the edge of the neurospheres to the tip of the processes in linear form. And the process breadth was measured from five random cross-distances of 5 fasciculated processes per neurosphere. Process lengths and breadths were measured by tracing the processes using NIH Image software (ImageJ). The length and breadth of processes of twelve independent neurospheres were calculated at each experiment, and the means and standard error of mean (SEM) were also calculated [13-14].

2.4 Immunocytochemistry

For immunocytochemical characterization, cultured cells were fixed in ice-cold 4% paraformaldehyde in PBS for 20 min and washed three times in PBS at indicated time points. 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), rabbit anti-microtubule-associated protein 2 polyclonal antibody (anti-MAP-2; 1:500; Chemicon, Temecula, CA), mouse anti- β -tubulin-III monoclonal antibody (anti- β -tubulin-III; 1:200; Chemicon, Temecula, CA), rabbit anti-glial fibrillary acidic protein polyclonal antibody (anti-GFAP; 1:500; Chemicon, Temecula, CA) [15-18]. 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).

3. RESULTS AND DISCUSSION

The plasticity of proliferation and differentiation of neural stem cells was identified by the method of immunocytochemical stainings with anti-nestin, anti-MAP-2, and anti-GFAP, which are immunoreactive for proliferating neural stem cells, differentiated neurons and astrocytes, respectively. Figure 1 shows that most of neurosphere-forming cells were immunoreactive for nestin, an intermediate filament protein specifically found in neuroepithelial stem cells [15], and demonstrates that these neurospheres remain the ability of proliferation. Next, to identify the differentiating ability of neural stem cells, neurospheres were seeded onto poly-D-lysine-coated (50 ng/ml) glass cover slips after bFGF withdrawal to allow cell attachment and differentiation. After 3 d of culture, neurospheres had attached onto poly-D-lysine-coated glass cover slips and neurites extended and cells migrated out from neurospheres were observed; see Figure 2(a). Because these cultures might contain a heterogeneous population of cells at different stages of neuronal and glial development, antibodies to different marker proteins were used to determine their phenotypes immunocytochemically. MAP-2- (neurons), β -tubulin-III-, and GFAP- (astrocytes) immunoreactive cells were present; see Figure 2(b), (c), and (d). These results confirm previous studies suggested that cells isolated from embryonic rat cerebral cortex proliferate to produce clusters of cells with antigenic properties of neuroepithelial stem cells [1-2], and these neurosphere-forming cells can be induced to differentiate into neurons and astrocytes under appropriate conditions.

Fig. 1. The phase-contrast image of a neurosphere, generated from embryonic rat cerebral cortex, indicating cells were immunoreactive for nestin, an intermediate filament protein specifically found in neuroepithelial stem cells. Scale bar=100 μ m.

Fig.2. Neurospheres cultured on poly-D-lysine-coated glass cover slips. (a) Neurites extended and cells migrated out from neurospheres after 3 d of culture. (b) MAP-2- (neurons), (c) β -tubulin-III- (neurons), and (d) GFAP- (astrocytes) immunoreactive cells were present after 3 d of culture. Scale bar=200 μ m.

After isolation and purification of the embryonic cortical neural stem cells, these bFGF-responsive neurospheres were then seeded onto EVAL substrates under serum-free conditions without the addition of NGF. As shown in Figure 3(a), after 3 d of culture, neurospheres had attached onto EVAL substrates and several straight and thin processes were extended out from the neurospheres. After all, when NGF were added to above culture conditions, as shown in Figure 3(b), processes were not only extended out from the neurospheres but also fasciculated to form neurofilament bundles after 3 d of culture in vitro. It is noticed that very few of these neurosphere-forming cells migrated out from their original aggregated under serum-free conditions, regardless of addition of NGF. Figure 4 shows the quantitative analysis of process growth by measuring process length and breadth on EVAL substrates in the presence or absence of NGF under serum or serum-free conditions. The process length and breadth on EVAL substrates without NGF

Fig. 3. Neurospheres cultured on EVAL substrates (a) with or (b) without the addition of NFG under serum-free conditions. Scale bar=200 μ m.

Fig. 4. Quantification of process growth of neurospheres on EVAL substrates under serum-free conditions at 125 neurospheres/cm² after 3 d of culture. Values represent means $\pm$ SEM of 10 independent neurospheres. Asterisk denotes significant differences ($p < 0.05$) as determined by Student's t-test.

under serum-free conditions were $213.6 \pm 12.3 \mu\text{m}$ and $3.1 \pm 1.2 \mu\text{m}$, and were $207.6 \pm 13.1 \mu\text{m}$ and $21.6 \pm 3.2 \mu\text{m}$ when NGF were added. Under serum-free conditions, the process length of differentiated neurospheres on EVAL substrates without the addition of NGF had no significant difference from that in the presence of NGF. However, under the same conditions, when NGF was added in the above culture system the process breadth was significantly larger than that of neurospheres cultured without NGF. It is noted that no obvious protoplasmic cells were observed in the serum-free medium.

Next experiment, we want to see whether NGF has the same effects on regulation of the process growth and differentiation of neurosphere-forming cells on EVAL substrates if the serum proteins were present in the culture system. As shown in Figure 5 (a), regardless of the addition of NGF or not, the neurospheres were induced into typically an extensive cellular substratum of protoplasmic cells upon which process-bearing cells spread. The protoplasmic cells, exhibiting large, flat cell bodies with very few processes, would migrate far away from the neurospheres and the process-bearing cells grown on these protoplasmic cell layers. These protoplasmic cell layers were characterized as GFAP-immunoreactive astrocytes; see Figure 5(b). Therefore, when serum was present, the differentiated GFAP-positive astrocytes layer could serve as a supported architectural for growth and survival of process-bearing cells. It is notice that a great number of cells migrated out far

(a)

(b)

Fig. 5. (a) Photomicrograph illustrates the morphologic growth of neurospheres cultured on EVAL substrates with or without NGF. (b) These protoplasmic cell layers were characterized as GFAP-immunoreactive astrocytes. Scale bar=200 μm .

away from their original aggregates and no obvious neurite fasciculation was found under serum conditions, regardless of the presence or absence of NGF. Moreover, under serum conditions, the process length and breadth of differentiated neurospheres cultured on EVAL substrates with or without the addition of NGF have no significant difference (data not shown).

The purpose of the present study is to focus on the effects of cell-biomaterial and cell-cell interactions on the developmental potential of stem cells from embryonic rat cerebral cortex. In order to interpret the phenomenon further, we proposed the mediation of cell-biomaterial interaction by cell-cell interaction should be taken into account. In this study, under

serum free conditions, although straight and thin differentiated processes were found, the amount or maturity of differentiated neurons were not enough to form neurites fascicles without the addition of NGF. These differentiated processes from neurosphere-forming cells shows relatively little tendency to fasciculate. Since the NGF are capable to improve better neuronal growth and neurites elongation, when the NGF was present in the above culture system, more neurons were differentiated from forming-neurosphere cells, as a consequence, neurite fasciculation were observed. The behavior of fasciculation of neurites may be due to the affinitive competitions between neuron-neuron and neuron-substrate. In this case, because the neuron-neuron affinity is stronger than neuron-EVAL affinity, neurites outgrowth was primarily in large fascicles in the presence of NGF. In contrast, when serum proteins were added in the medium, many forming-neurosphere cells were induced to differentiate into glial cells and is more adherent to EVAL substrates than neurons. At this time, process-bearing cells were grown on astrocyte layers, and neurites did not fasciculate. It may be due to that the neuron-glia interaction is stronger than neuron-neuron interaction resulting in neurites failed to fasciculate. In addition, these glia layers may form a barrier between neurons and EVAL substrates and inhibited the interactions between them. These results confirmed previous studies [10], which suggested that neurons aggregated and neurites fasciculated on nonglial layer but not on glial layer. In this study, EVAL substrates serve as nonglial layer while serum-inducing differentiated astrocytes serve as glial layer. The influence of glial and nonglial layers on the morphology of neuronal growth could theoretically be due to either to cell surface interactions or to soluble factors which could cause alternations in neurite adherence to the substrates or to the neurites. Finally, these pattern of neural stem cell growth on EVAL substrates with or without NGF in the absence or presence of serum in this studies also suggested that there exist hierarchies of adherence in the nervous system such that CNS neurons are more adherent to glial cells than to other neurons but are more adherent to other neurons than to EVAL substrates.

4. CONCLUSION

In summary, the present study provide evidence that EVAL can be a potent controller of cell interactions involving cell-cell and cell-substrate contacts. We proposed that, in combination with nerve growth factors, EVAL substrates could induce neurite fasciculation of embryonic rat cortical neural stem cells under serum free conditions These in vitro results

are very encouraging since this information should be useful for the development of strategies for regulating the differentiation and fate determination of neural stem cells.

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