

Gallium nitride induces neuronal differentiation markers in neural stem/precursor cells derived from rat cerebral cortex

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Abstract

In the present study, gallium nitride (GaN) was used as a substrate to culture neural stem/precursor cells (NSPCs), isolated from embryonic rat cerebral cortex, to examine the effect of GaN on the behavior of NSPCs in the presence of basic fibroblast growth factor (bFGF) in serum-free medium. Morphological studies showed that neurospheres maintained their initial shape and formed many long and thick processes with the fasciculate feature on GaN. Immunocytochemical characterization showed that GaN could induce the differentiation of NSPCs into neurons and astrocytes. Compared to poly-D-lysine (PDL), the most common substrate used for culturing neurons, there was considerable expression of synapsin I for differentiated neurons on GaN, suggesting GaN could induce the differentiation of NSPCs towards the mature differentiated neurons. Western blot analysis showed that the suppression of glycogen synthase kinase-3 β (GSK-3 β) activity was one of the effects of GaN-promoted NSPC differentiation into neurons. Finally, compared to PDL, GaN could significantly improve cell survival to reduce cell death after long-term culture. These results suggest that GaN potentially has a combination of electric characteristics suitable for developing neuron and/or NSPC chip systems.

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1. Introduction

Stem cells have three important fundamental abilities: to self-renew, to produce functional differentiated progeny, and to maintain these features over a long period of time [1,2]. For example, neural stem/precursor cells (NSPCs) can not only self-renew but also can differentiate into neurons, astrocytes and oligodendrocytes [3–6]. Therefore, the discovery of NSPCs in the central nervous system has raised hopes for a wide range of clinical applications to treat neuronal loss associated with neurodegenerative conditions in the brain and spinal cord. Previous studies have suggested that the proliferation and differentiation of NSPCs were determined by the effects of extrinsic and intrinsic signals coming from medium components [3–8].

For example, basic fibroblast growth factor (bFGF) has been shown to induce the proliferation of NSPCs [7,8].

Numerous natural and synthetic polymers have been used as substrates or scaffolds for culture of neurons. For example, coating poly-D-lysine (PDL) on the substrate has been used to promote neuron adhesion and survival [9,10], though this is a non-permanent modification, which may be lost by washing steps [11]. In our previous studies, gallium nitride (GaN), which has unique physical, chemical and electric properties [12,13], has been shown not only to promote neuron survival and neuritic growth, but also to maintain neuronal function after long-term culture [14,15]. However, the novel function of the so-called III–V compound semiconductor material, GaN applied to culture NSPCs has not been established. Of course, it is possible that NSPCs respond to different substrates, and their fate determination depends on the chemical properties of the substrates [5].

In the present study, GaN was used to culture NSPCs to examine its effect on NSPC survival and differentiation.

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For comparison, commercial tissue culture polystyrene (TCPS) and PDL-coated TCPS were also used to culture NSPCs. It was found that NSPCs were able to attach to the GaN surface and to exhibit a dense fiber network from neurospheres. Immunocytochemical characterization and Western blot analysis showed that GaN induced the differentiation of NSPCs toward the mature differentiated neurons with the expression of the dendrite and axon markers. Moreover, our data show that low activity of glycogen synthase kinase-3 (GSK-3 β) plays an important role in regulating cell survival and neuronal plasticity on GaN [16–18], which is consistent with the high levels of synapsin I [19,20]. Even after incubation for 21 days, GaN still could provide a neuron-favorable environment for NSPC adhesion and differentiation by immunocytochemistry and lactate dehydrogenase (LDH) release from damaged cells. Therefore, GaN, combined with its electric characteristics, is a possible candidate for use in neurochips, which requiring the integration of biological systems and semiconductor materials.

2. Materials and methods

2.1. Sample preparation

The GaN substrate used in this study was obtained from Huga Optotech Inc. (Taiwan). All samples with a size of approximately 1 cm² and a thickness of 1 ± 0.1 mm were cleaned by sonication in water and 70% alcohol, sterilized with autoclave and then rinsed extensively with phosphate buffer solution (PBS). Subsequently, samples were placed in 24-well TCPS plates (Corning, New York, USA). The empty and PDL-coated wells were used as controls. PDL was purchased from Sigma and diluted to 50 ng ml⁻¹ in PBS. Before cell culture, wells were covered with 1 ml of PDL diluted solution and incubated for 4 h, after which time excess solution was removed by suction and dried for another hour.

2.2. Isolation and culture of NSPCs

NSPCs were prepared from pregnant Wistar rat embryos on day 14–15 according to a protocol detailed previously [21,22]. 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 Na₂HPO₄·7H₂O, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 0.5 mM MgCl₂·6H₂O, 0.6 mM MgSO₄·7H₂O, 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 mg ml⁻¹ insulin, 100 mg ml⁻¹ human apotransferrin, 20 nM progesterone, 30 nM sodium selenite, pH 7.2) [23]. 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) 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⁻¹. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO₂. 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, neurospheres (120 neurospheres cm⁻²) were seeded on PDL, GaN and TCPS in the serum-free medium containing 20 ng ml⁻¹ bFGF to investigate the effects of substrates on NSPCs. At indicated time points, morphologies of cultured neurospheres were observed under a phase-contrast microscope (Zeiss LAMBDA 10-2, Germany).

2.3. Immunocytochemistry

For immunocytochemical characterization, cultured cells were fixed in ice-cold 4% paraformaldehyde in PBS for 25 min and incubated with 0.5% Triton X-100 for 5 min. After fixing, cells were incubated with primary antibodies diluted in PBS containing 10% bovine serum albumin overnight at 4 °C. The primary antibodies and their dilution used in this study were mouse anti-nestin monoclonal antibody (1:500; Chemicon), rabbit anti-gial fibrillary acidic protein polyclonal antibody (anti-GFAP, 1:500; Chemicon), rabbit anti-microtubule associated protein 2 polyclonal antibody (anti-MAP2, 1:500; Chemicon), and mouse anti-synapsin I protein monoclonal antibody (1:500; BD). 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 dilutions were FITC-conjugated donkey anti-rabbit IgG (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon) and rhodamine-conjugated goat anti-mouse IgG (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon). These immunostained cells were visualized with a confocal scanning laser microscope (Leica TCS SP5).

2.4. Western blot analysis

Cells were collected by gentle shaking of the wells and washed twice with PBS. Cell lysates were prepared with ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM NaF, 1 mM Na₃VO₄, 1:200 dilution of Protease Inhibitor Cocktail II; Calbiochem) for 30 min and then were sonicated at 4 °C for 15 s. Lysates were clarified by centrifugation at 10,000 rpm for 30 min at 4 °C and the resulting supernatant was saved for protein analysis and Western blot analysis. Protein concentration was measured by using the commercial protein assay reagent (Bio-Rad, Hercules, CA). For Western blotting, the supernatant was added to an equal volume of Laemmli sample buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β -mercaptoethanol) and heated to 95 °C for 5 min. Pro-

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