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Directed growth and selective differentiation of neural progenitor cells on micropatterned polymer substrates

Jennifer B. Recknor[a], Donald S. Sakaguchi[b], Surya K. Mallapragada[a,*]

[a]Department of Chemical and Biological Engineering and The Neuroscience Program, Iowa State University, 2114 Sweeney Hall, Ames, IA 50011, USA
[b]Department of Genetics, Development and Cell Biology and The Neuroscience Program, Iowa State University, 503 Science II, Ames, IA 50011, USA

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Abstract

Directional growth and differentiation of adult rat hippocampal progenitor cells (AHPCs) were investigated on micropatterned polymer substrates in vitro. Astrocytes or AHPCs cultured on micropatterned polystyrene substrates chemically modified with laminin exhibited over 75% alignment in the groove direction. AHPCs co-cultured with astrocytes preferentially acquired neuronal morphology, with nearly double the percentage of cells expressing class III β-tubulin on the micropatterned half of the substrate, as opposed to the planar half of the substrate, or compared to those growing in the absence of astrocytes. This indicates that substrate three-dimensional topography, in synergy with chemical (laminin) and biological (astrocytes) guidance cues, facilitates neuronal differentiation of the AHPCs. Through multi-dimensional cell–cell interactions, this environment provides spatial control selectively enhancing neuronal differentiation and neurite alignment on topographically different regions of the same substrate. Integrating these cues is important in understanding and controlling neural stem cell differentiation and designing scaffolds for guided nerve regeneration.

Keywords: Neural stem/progenitor cell; Astrocyte; Co-culture; Micropatterning; Nerve tissue engineering

1. Introduction

Axonal regeneration in the central nervous system (CNS) is limited by inhibitory influences of the glial and extracellular environment [1–3]. Experimental strategies employing neural stem cell (NSC) transplantation hold great promise for repairing the injured and diseased CNS. NSCs are self-renewing and multi-potent producing differentiated, functional progeny including neurons and glial phenotypes [4,5]. NSCs, as well as neural progenitor cells (NPCs), which have more limited capacities in terms of growth and differentiation, have been isolated and expanded from the developing and adult CNS in a variety of mammalian species, including humans [5–7].

Efforts have been made in vitro to elucidate the stem cell microenvironment, or 'niche', controlling cell fate in vivo [4,8,9]. Studies have demonstrated that the fate of differentiating stem cells is strongly influenced by direct cell-to-cell and cell-to-extracellular matrix (ECM) contacts involving a complex "cocktail" of growth factors, signaling molecules and ECM proteins [10–12]. Furthermore, astrocytes have been studied as influential components of an 'instructive' stem cell niche [13] and can induce neurogenesis in NSCs [8,13–16].

Another promising strategy providing instructive environments for axonal regeneration and restoration of function involves scaffolds or bridging substrates [17,18]. Such substrates can be designed with specific microarchitectures that allow optimal structural support for axonal regrowth and affect cellular orientations and the presentation of ECM proteins to the cells. These guidance channels have been used to study NSC behavior for application in the treatment of spinal cord injury and neurodegenerative diseases of the CNS [17–27]. Together, these studies support the notion that synthetic materials can provide mechanical scaffolds supporting NSC growth and differentiation. However, to our knowledge, the synergistic effects of multiple stimuli involving physical or topographical cues, in conjunction with chemical and biological

*Corresponding author. Tel.: +1 515 294 7407; fax: +1 515 294 2689.
E-mail address: suryakm@iastate.edu (S.K. Mallapragada).

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cues on NPC differentiation have not been explored, and the mechanisms by which this combination of cues affects cellular differentiation are unknown.

Combining physical, chemical and biological guidance cues that enable spatial control over NPC differentiation on topographically different regions of the same substrate can potentially generate a supportive environment for eliciting regeneration and restoring function in the injured or diseased CNS. Integrating multiple stimuli to direct the lineage of endogenous or engrafted CNS-derived precursor cells on specific substrate regions offers opportunities to mimic the natural in vivo environment and elucidate the mechanisms behind efficient stem cell-mediated repair of the CNS that cellular transplantation or scaffolds alone do not provide. In the present study, the effects of these cues on directing alignment and spatially controlling the differentiation of adult rat hippocampal progenitor cells (AHPCs) were investigated. Postnatal rat type-1 astrocytes [28] and AHPCs extended axially along the grooves of micropatterned polystyrene (PS) substrates chemically modified with laminin. The biological influence of astrocytes was integrated with the physical (micropatterned substrate) and chemical (laminin) guidance cues, and the synergistic effects of these cues on AHPC differentiation was compared to cells not exposed to physical and/or biological guidance cues. This research provides insights into mechanisms of NSC differentiation and a foundation for a promising regeneration strategy for guided CNS repair.

2. Materials and methods

2.1. Micropatterned substrate fabrication

PS was chosen for substrate fabrication, as it is a biocompatible polymer that is used extensively in cell culture experimentation. Conventional photolithographic techniques and reactive ion etching were used to fabricate silicon wafers with the desired micropatterns that were then transferred to the polymer substrates using solvent casting [28]. The patterns used were described by: groove width (μm)/groove spacing (or mesa width) (μm)/groove depth (μm). To study the physical guidance of AHPCs on micropatterned PS substrates, the pattern dimensions used for these experiments were 16/13/4 μm.

The solvent cast polymer substrates were fabricated from an 8% (w/v) PS (MW 125,000–250,000) (Polysciences, Inc., Warrington, PA) solution in toluene. Substrate thicknesses of approximately 50–70 μm were achieved using solvent casting techniques. After casting and drying for a minimum of 24 h, the PS substrate was removed by soaking in deionized (DI) water and then sterilized with 70% ethanol. The micropatterned substrates were imaged using scanning electron microscopy (SEM). After mounting, the samples were sputter coated (SEM Coating Unit E5100, Polaron Instruments, Inc., Watford Hertfordshire, UK) with gold and imaged using a JEOL JSM-840A at an accelerating voltage of 20 kV, a 50 μm diameter aperture and a vacuum level of 1×10^{-6} Torr.

Cell growth chambers were constructed using PTFE (Teflon®) o-rings (Small Parts, Inc., Miami Lakes, FL), glass coverslips and PS substrates (1 cm^2 in area) as described previously [28]. The PS substrates were coated with poly L-lysine (PLL; Sigma, St. Louis, MO) solution at a concentration of 100 μg/ml and/or laminin (LAM, Sigma) at a concentration of 10 μg/ml in Earle's Balanced Salt Solution (EBSS; Gibco, Grand Island, NY).

2.2. Astroglial cell isolation and purification

All animal procedures were conducted in accordance with and had the approval of the Iowa State University Committee on Animal Care. A population of purified cortical astrocytes was obtained from neonatal rat pups as described in Recknor et al. [28] Briefly, cerebral hemispheres were freshly dissected from 1–3-day-old Sprague–Dawley rat pups and treated with papain solution (20 IU/ml; 37 °C, 5% CO_2/95% air, 1 h) (Sigma). After subsequent treatment with trypsin inhibitor solution (10 mg/ml; Sigma), the tissue was mechanically dissociated in modified minimal essential culture medium (MMEM). The cultures were grown to confluence in 25 cm^2 tissue culture flasks (T-25; Falcon) at 37 °C in a humidified 5% CO_2/95% air atmosphere. The culture medium, MMEM, consisted of minimum essential medium (MEM; Gibco) supplemented with 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate and 14 mM $NaHCO_3$, penicillin (100 IU/ml) and streptomycin (100 μg/ml) with 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT), pH 7.35.

Enriched type-1 astrocyte cultures were prepared as previously described [28]. After the cultures reached confluency (~8 days), the cells were shaken twice on a horizontal shaker at 260 RPM at 37 °C, first for 1.5 h and then for 18 h. The remaining adherent cells were enzymatically detached with trypsin (0.1% in EBSS; Sigma), pelleted (100*g*, 10 min), resuspended in MMEM and passaged into 25 cm^2 tissue culture flasks. Cultures were fed every 3 days and were not passaged more than eight times. Over 90% of the cells cultured under these conditions were selectively labeled by GFAP antibody (data not shown) confirming their astrocyte identity.

2.3. Adult hippocampal progenitor cell culture

AHPCs were originally isolated from the brains of adult Fischer 344 rats as reported by Palmer and colleagues [6]. The expanded cultures of single clones were infected with retrovirus to express enhanced GFP [6,29]. The AHPCs were maintained in plastic tissue culture flasks (T-75, Fisher Scientific, Pittsburgh, PA) coated with poly L-ornithine (10 μg/ml; Sigma) and mouse-derived laminin (5 μg/ml; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The AHPCs were maintained in complete medium containing Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/ml basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and L-glutamine (2.5 mM L-glu; Gibco BRL, Gaitherburg, MD). For in vitro analysis on PS substrates, the AHPCs were detached from the T-75 flask using ATV solution (Gibco BRL, Gaithersburg, MD), harvested and collected by centrifugation at 1000*g* for 5 min. The pellets were resuspended in the culture medium stated above without FGF (referred to as differentiation medium) or co-culture medium (described below) and triturated gently. The cells were then plated on the micropatterned PS substrates coated with PLL (100 μg/ml in borate buffer) and laminin (10 μg/ml EBSS) (PS-LAM substrates) at initial densities of 10,000 to 15,000 cell/cm^2. Cells were maintained at 37 °C in a 5% CO_2/95% air atmosphere for 6 days in culture medium.

2.4. Co-culture of astrocytes and AHPCs

Purified astrocytes were seeded onto PS-LAM substrates inside growth chambers and cultured for 2 days to generate near confluent monolayers. AHPCs were plated on top of the astrocyte monolayer at approximately 15,000 cells/cm^2. The co-cultures were maintained in a mixed medium that consisted of astrocyte MMEM (without FBS) in a 1:1 mixture with AHPC differentiation media (referred to as co-culture media). As controls, AHPCs and astrocytes were plated in the same co-culture medium at the same density. AHPC–astrocyte co-cultures, AHPCs and astrocytes were grown for 6 days and then fixed with 4% paraformaldehyde in 0.1 M PO_4 buffer (pH = 7.4).