Microstructured Topography Enhanced the Responsiveness of Voltage-gated Calcium Channels in H945RB.3 Human Neural Progenitor Cells

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***Abstract*—**A topographical substrate with a packed polystyrene microbead (1.98±0.20 μm in diameter) array was fabricated for the development of cell-based assay systems targeting voltage-gated calcium channels (VGCCs). We found that the microbead arrayed substrates enhanced the attachment and spreading of the cultured human neural progenitor cells (H945RB.3) as compared to the flat polystyrene surfaces. Microbead arrayed substrates also facilitated the development of higher VGCC responsiveness upon neuronal differentiation than flat substrates. The enhancement of both VGCC responsiveness and cell spreading were most significant until day 14 into differentiation.

Keywords- microbead; microfabrication; confocal; topography

I. INTRODUCTION

Surface topography is a major concern in the design of culture substrates for application in cell-based microdevices. For this purpose, microscale or sub-microscale topographical substrates have been designed to promote cell adhesion and even cell differentiation [1,2]. We have been interested in the development of a neuronal cell-based assay system with topographical scaffolds for early drug discovery targeting voltage-gated calcium channels (VGCCs) [3]. With the SH-SY5Y human neuroblastoma cells, we found that cells cultured on the topographical scaffolds, which were formed using arrayed Cytodex microbeads with a diameter of around of 170 μm, were less spreading, had fewer and shorter neuronal extensions, established more negative resting membrane potentials and showed a slower pace in the development of VGCC responsiveness than cells on flat collagen coated substrates [3]. This suggests a close relation between cell morphology or spreading and the functional response of ion channels for neuronal cells. In this study, we hypothesized that VGCC responsiveness of the neuronal cells could be enhanced by a substrate topography design that promotes cell spreading and attachment. Topographical substrates were fabricated using a packed polystyrene microbead array. H945RB.3 human neural progenitor cells were cultured and differentiated to neuronal lineage on both flat polystyrene substrates and microbead arrayed substrates. VGCC responsiveness was evaluated with Calcium Green-1 fluorescence staining combined with confocal microscopy in an attempt to understand the effects of microstructured topography on the functional establishment of VGCCs on neuronal cells.

II. MATERIALS AND METHODS

2.1. Preparation of the Cell Culture Substrates

Cell culture substrates were fabricated on 25-mm coverslips (Fisher Scientific). To prepare the microbead arrayed substrates, polystyrene microbeads of 1.98±0.20 μm in diameter (Cat. Code PS04N, Bangs Laboratories) were diluted to a concentration of ~1.7% (w/v) with distilled water and 30 μL microbead suspension was spread over the coverslip after a polystyrene coating step [3]. The whole set was tilted approximately 10˚ and dried in ambient condition for 30 minutes before packed microbead arrays were formed [3, 4]. Both the flat polystyrene substrates and microbead arrayed substrates were then etched with oxygen plasma (PLASMOD™, TEGAL Corporation) for 150 seconds to increase the surface roughness. The cell culture substrates, either flat or microstructured, were sterilized with 70% ethanol overnight before washing with distilled water. Substrates / cell culture dishes (FALCON) were bath coated with 40 μg/ml polyornithine (Sigma) and 5 μg/ml murine basement membrane laminin (Sigma) each for at least 2 hours.

2.2. Culture of the Neural Progenitor Cells

H945RB.3 human neural progenitor cells (neuroepithelial) were derived from WA09 human ES cells using methods previously described [5]. Derived neural progenitor cells were propagated in 35 mm Petri dishes with 2 mL proliferation medium in a 5% CO_2 humidified atmosphere at 37 ˚C. The proliferation medium was made with Neurobasal medium (Invitrogen) supplemented with 2 mM L-glutamine, 20 ng/ml bFGF, 1 μg/ml leukemia inhibition factor (LIF, Chemicon) and 1% B-27 supplements (Invitrogen). For cell differentiation experiments, progenitor cells of passage 25 to 42 were used in the present study. Cells were seeded in 35 mm Petri dishes containing either a flat or microbead arrayed substrate, in proliferation medium. On day 2 after plating, referred to as day 0 into differentiation hereafter, cell differentiation was initiated with starvation of bFGF, by changing medium to differentiation medium. The differentiation medium had the

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same composition as proliferation medium except bFGF was missing. The differentiation of neural progenitor cells into the neuronal lineage was confirmed by the progressive dominance of the neuronal marker Tuj expression and lack of the astral marker GFAP expression on days 7 and 14 into differentiation with immunofluorescence staining.

2.3. Scanning Electron Microscopy (SEM)

On days 0 and 14 into differentiation, cells on either flat or micorbead arrayed substrates were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 hour and post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for another 1 hour. Cells were then dehydrated with mounting concentrations of ethanol and dried in a SAMDRI-780A critical point drier. The samples were sputter-coated with gold for 90 seconds to achieve a coating thickness of about 23 nm. SEM images were captured with LEO 982 scanning electron microscope with an acceleration voltage of 5 kV. Cell morphological measurements for the SEM images were done with Simple PCI image software (Compix Inc.) by first tracking the contours enclosing the cell bodies, lamellipodia and neuronal extensions, measuring the cellular projection area and perimeter, and then calculating roundness. Roundness is defined as $4*\delta*\text{area}/(\text{perimeter})^2$.

2.4. Evaluation of VGCC Responsiveness

VGCC functionality was evaluated with the dynamics of calcium influx in response to high K^+ (50 mM) depolarization. The membrane permeable fluorescent dye, Calcium Green-1, Acetoxymethyl Ester (AM) (Molecular Probes), was used to visualize the calcium influx dynamics. On days 0, 7 and 14 into differentiation, cells on either flat or microbead arrayed substrates were loaded with 5 μM Calcium Green-1 AM in 1 ml of uncolored Neurobasal medium containing 2% B-27 supplements, 3% heat inactivated FBS and 0.02% Pluronic F-127 (Molecular Probes) for 1 hours at 37 ˚C in a 5% CO_2 humidified incubator. Cells were then washed with uncolored Neurobasal medium twice and incubated with 1 ml uncolored Neurobasal medium containing 2% B-27 supplements at 37 ˚C in the 5% CO_2 humidified incubator for another 45 minutes to allow dye de-esterification. Calcium Green-1 was excited with 488 nm argon laser and the emission was captured through a 515 nm long-pass filter. Confocal images were captured at a rate of 1 frame per 3 seconds. While images were being captured, cells were depolarized by adding 100 μL of uncolored Neurobasal medium containing high K^+ (500 mM) to achieve a final K^+ concentration of 50 mM. Functional VGCCs were demonstrated by cytosolic calcium concentration increase upon depolarization. This intracellular calcium dynamics was reflected by changes in relative intracellular Calcium Green-1 fluorescence intensities, which were plotted as gray level units against time.

2.5. Data Analysis and Statistics

Morphological parameters and fractional magnitudes of VGCC responsiveness were expressed as Mean±SD. Student's *t*-test was used for statistical comparisons of these parameters either between cells cultured on flat and microbead arrayed substrates or between cells on different days into differentiation. A level of $P<0.05$ was accepted as statistically significant.

III. RESULTS

3.1. Effects of Microstructured Topography on the Attachment and Spreading of Neural Progenitor Cells

Fig. 1 shows the SEM images for cells on days 0 (Figs. 1*a* and 1*b*) and 14 (Figs. 1*c* and 1*d*) into differentiation. Cell differentiation was initiated at a confluence of around 60% (Figs. 1*a* and 1*b*). Upon differentiation, the progenitor cells gradually stopped proliferating during the first week. Cell bodies became smaller and significant neuronal extensions appeared on day 7 into differentiation. On day 14 into differentiation, the neuronal extensions became even longer and more significant (Figs. 1*c* and 1*d*). SEM images also showed that before differentiation the progenitor cells were well attached and spread on both flat and microbead arrayed substrates. Upon differentiation, the progenitor cells began to lose their strong attachment to flat substrates. This is especially evident on day 14 into differentiation, where lots of cells were actually peeling off and clustering (Fig. 1*c*). In contrast, cells on microbead arrays maintained well attachment and spreading even until day 14 into differentiation.

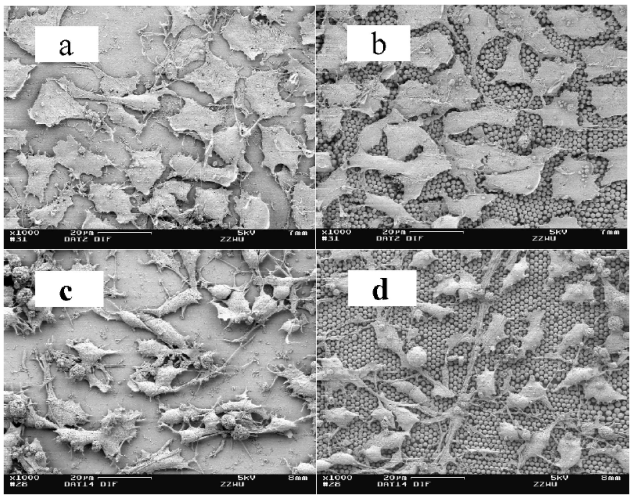

Figure 1. SEM images for progenitor cells growing on flat polystyrene surfaces (*a*, *c*) and microbead arrays (*b*, *d*) before differentiation (*a*, *b*) and on day 14 into differentiation (*c*, *d*). Bar = 20 μm.

The effects of the microstructured topography formed with bead array are further demonstrated by the quantitative analysis of cell morphology in terms of cell spreading or projection areas for the SEM images, as shown in Table 1. From Table 1, one can find that there was a significant decrease in cell projection area and roundness from day 0 to day 14 into differentiation for cells on both flat and topographical substrates ($P<0.01$). Before differentiation, cells on microbead array had larger perimeter and smaller roundness than cells on flat surfaces ($P<0.01$), whereas they had similar projection areas ($P>0.05$). On day 14 into differentiation, cells on microbead array had greater projection area ($P<0.05$) and perimeter ($P<0.01$) than those on flat surfaces. From day 0 to day 14 into differentiation, the perimeters for cells on flat surfaces did not increase significantly while this was

significant for cells on microbead array (P<0.05). Taken together, these results suggest that the microstructured topography formed with a packed microbead array promoted the attachment and spreading of neural progenitor cells, with the maximum promotion occurred on day 14 into differentiation.

TABLE I. ORPHOLOGICAL MEASUREMENTS FOR H945RB.3 NEURAL PROGENITOR CELLS ON FLAT SURFACES AND MICROBEAD ARRAYS

	Day 0 into Differentiation			**Day 14 into Differentiation**		
	Area (μ m^2)	***Perimeter (μm)***	***Roundness***	***Area (μ m^2)***	***Perimeter (μm)***	***Roundness***
Flat surface	262±108 (n=249)	84±25 (n=249)	0.48±0.13 (n=249)	120±48** (n=134)	87±31 (n =134)	0.23±0.10** (n=134)
Bead array	264±131 (n=263)	90±34## (n=263)	0.44±0.14## (n=263)	135±73#** (n=210)	98±50##* (n=210)	0.22±0.10** (n=210)

Compared with those for day 0 into differentiation: *P<0.05; **P<0.01. Compared with those for cells on flat substrates: #P<0.05; ##P<0.01. n: number of cells measured.

3.2. *VGCC Responsiveness for Neural Progenitor Cells*

Fig. 2. shows the confocal microscopic images of H945RB.3 neural progenitor cells loaded with 5 μM Calcium Green-1 before (*a*) and after (*b*) 50-mM K^+ depolarization. Fig. 3. shows a typical time course for the change in intracellular Calcium Green-1 fluorescent intensity upon 50-mM K^+ stimulation. A cell was only considered responsive when it showed an increase in Calcium Green-1 fluorescent intensity of 15% or higher over the basal fluorescent intensity level. We found that near 100% of the H945RB.3 neural progenitor cells on both flat and topographical substrates were responsive to 50 mM K^+ stimulation accordingly, indicating the expression of functional VGCCs. We have thus used the responding magnitude or the peak fractional increase over the basal Calcium Green-1 fluorescent intensity, as an index of the expression of VGCC functionality. Table 2 summarizes the response magnitudes for the increase of Calcium Green-1 fluorescent intensities of H945RB.3 neural progenitor cells upon 50-mM K^+ depolarization. From Table 2, one can find that the responding magnitudes for cells on microbead arrays (0.88±0.70) were not significantly different from those for cells on flat surfaces (0.86±0.46) before differentiation (P>2.5). For cells on flat substrates, these values remained at a similar level until day 7 into differentiation (0.90±0.43, P > 2.5 as compared to those before differentiation) and more than doubled by day 14 into differentiation (2.09±1.35, P<0.01 as compared to those for both day 0 and day 7). For cells on microbead arrays, the response magnitudes of Calcium Green-1 fluorescent intensities increased significantly on day 7 into differentiation (1.07±0.57, P<0.05 as compared to those for day 0) and even further quadrupled on day 14 (4.05±2.56, P<0.01 as compared to those for both day 0 and day 7). For either day 7 or day 14 into differentiation, the response magnitudes were higher for cells on microbead array than those on flat substrates (P<0.01). These results suggest that the microstructured topography thus formed promoted the development of VGCC responsiveness, especially on day 14 into differentiation.

TABLE II. VGCC RESPNSIVENESS OF H945RB.3 NEURAL PROGENITOR CELLS UPON STIMULATION WITH 50 mM K^+

	0 DID	7 DID	14 DID
Flat surface	0.86±0.46 (n=54)	0.90±0.43 (n=60)	2.09±1.35** (n=51)
Bead array	0.88±0.70 (n=73)	1.07±0.57#* (n=280)	4.05±2.56#** (n=102)

DID: Days into differentiation. Compared with those at day 0 into differentiation (before differentiation): *P<0.05; **P<0.01. Compared with those for cells on flat surfaces: #P<0.01. n: number of cells measured.

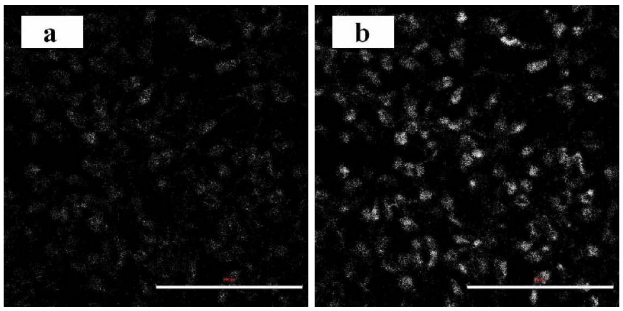

Figure 2. Confocal microscopic images for the evaluation of VGCC responsiveness of neural progenitor cells cultured on microbead arrays on day 14 into differentiation before (*a*) and after (*b*) stimulation with 50 mM K^+. Bar = 100 μm.

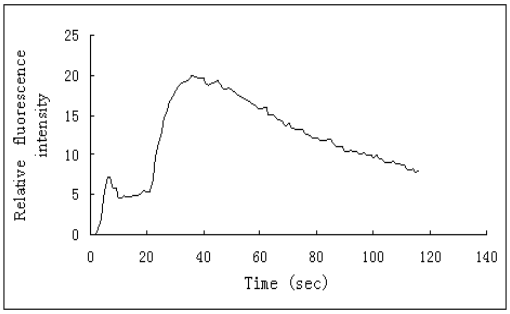

Figure 3. Typical time course for the change in cellular Calcium Green-1 fluorescent intensity upon 50-mM K^+ depolarization.

IV. DISCUSSION

We have adopted a simple tilting angle method [4] to fabricate microbead arrayed substrates to address the effects of surface topography on the functional development of VGCCs for neural progenitor cells. This fabrication method entails no specific instrumentation and enables easy mass production of the topographical substrates using tissue culture dishes for applications in cell-based assays for early drug discovery. The use of small microbeads (1.98±0.20 μm) in comparison to cells (approximately 10 μm) ensured no physical formation of three-dimensional cell aggregates in inter-bead spaces as previously reported for beads larger than cells [3]. Our cell culture experiments with H945RB.3 neural progenitor cells showed that the substrates were able to withstand the fluid mechanical forces in a culture period of more than 2 weeks. Analysis of the SEM images also showed that these topographical substrates

promoted neural progenitor cell attachment and spreading, especially on day 14 into differentiation, fulfilling our original design expectation.

Voltage-gated calcium channels are a type of plasma membrane ion channels that are related to a number of central nervous and cardiovascular diseases and thus form one of the most prospective screening targets in early drug discovery process [6]. Previous studies with SH-SY5Y human neuroblastoma cells showed that only small percentage of the cells developed VGCC responsiveness, suggesting that these tumor derived neuronal cells were probably not an ideal cell source for the development of cell-based assay systems targeting VGCCs [3]. In this regard, the use of stem cells may provide a prospective alternative for the development of such systems, due to their capabilities of unlimited expansion and differentiation to different neural cell types as well as the robust VGCC responsiveness as shown in the present study. Our study also showed that the microstructured topography formed with bead array enhanced the VGCC responsiveness of neural progenitor cells, and this provides additional novelty for using neural stem cells for establishing cell based assay systems targetting VGCCs. A comparison of the temporal profile for the spreading enhancement (Table 1) and the development of VGCC responsiveness (Table 2) of the neural progenitor cells suggests that the VGCC responsiveness is strongly related to cell morphological spreading, as both of which were most significantly enhanced on day 14 into differentiation. This is in keeping with studies with cardiac myocytes where changes in cell morphology strongly affected the properties of voltage-gated ion channels [7]. Our study thus provide the possibility for future design of topographic substrates or dishes to meet the challenge of low VGCC responsiveness found in early drug discovery, especially when cells of tumor origin are to be used as screening targets.

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