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An *in vitro* study of non-aligned or aligned electrospun poly(methyl methacrylate) nanofibers as primary rat astrocytes-loading scaffold

Haijian Xia*, Yongzhi Xia

Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

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ABSTRACT

After spinal cord injury (SCI), functional regeneration of neurites is hard to achieve due to the existence of glial scar, meanwhile astrocytes are believed important for post injury neuroregeneration, therefore how to handle the contradictory roles of astrocytes remains a problem for better neurogenesis. In this report, aligned electrospun poly(methyl methacrylate) (PMMA) nanofibers were assessed as an astrocytes-loading scaffold *in vitro*. We found that cell adherence and cell expansion of astrocytes could be supported by PMMA nanofibers, which topographic features could obviously influence the growth manner of astrocytes, and cells on aligned nanofibers finally formed longer and highly aligned processes along the axis of substrate fibers compared with cells cultured on film and non-aligned nanofibers. Regarding the relationship between astrocytes and substratum nanofibers, different topographic feature of substrate nanofibers showed varying degree of impact on cell expansion. On non-aligned nanofibers astrocytes expanded along the orientation of nanofibers early, while on aligned nanofibers astrocytes complied with the cues of nanofibers gradually with time. The results strengthen the rationale that aligned nanofibers could serve as the candidate of implantable scaffold after SCI, and it may relieve the stress of proliferated astrocytes by manipulating the growth pattern of astrocytes through its topographic features.

1. Introduction

After spinal cord injury (SCI), functional regeneration of neurites is hard to achieve due to the existence of physical growth barrier of glial scar, which is mainly made up of overactive astrocytes and begins to form during the subacute phase [1–4]. Because the proliferated astrocytes converts from a radial, lengthwise direction into an orientation perpendicular to the long axis of the spinal cord to a great extent, the glial scar presents obvious obstruction to any potential regeneration of the principal projection axon pathways [2]. Inhibiting the overactivation of astrocyte is one of the important ideas in the attempting of neurogenesis after SCI [3,5,6]. At the same time, astrocytes have been found serving a variety of important functions for neuroregeneration after SCI, such like releasing kinds of growth factors, restarting the hematoencephalic barrier and providing support and guidance for axonal growth [4,7,8]. Therefore, how to handle the contradictory roles of astrocytes remains a serious problem for better neurogenesis after SCI. Since the abnormal topological arrangement of proliferated astrocytes accounts for the failure of regeneration primarily, after SCI it remains a target to manipulate the growth manner of astrocytes by using biomedical scaffolds with definitive surface characteristics [9–11].

Polymer nanofiber-based scaffolds have drawn increasing attention in the field of tissue engineering. [12–16]. It is not only because of the facile and low-cost implementation of electrospinning, the easiness of creating complex nanostructures and tailoring components and compositions [17–19], but also the similar properties of electrospun nanofibers to the natural extracellular matrix (ECM), *e.g.*, thin, long fibers, well-marked surface-to-volume ratios, and abundant variable sized pores between the fibers [12,13,15,16]. Cell morphology and expansion capacity have be found to be substantially influenced by the topographic features of electrospun fibers [12,20]. In our previous study, poly(methyl methacrylate) (PMMA) nanofibers with different topographic features were fabricated as scaffold for culturing rat dorsal root ganglion neurons (DRGn), and the results indicated nanofibers having strong contact guidance for neurites expansion. Compared with cells cultured on film and non-aligned fibers, DRGn on aligned nanofibers formed longer and aligned neurites along the orientation of substrate nanofibers, and had a higher chance of colocalization with Schwann cells, which may be beneficial for the following myelination [20,21].

Because astrocytes played critical roles in the neuranagenesis after SCI, which are also the most likely cell types to contact the nanofiber after *in vivo* implant [2], whether and how PMMA electrospun

* Corresponding author at: Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, Youyi Road 1, Yuzhong District, Chongqing 400016, China.
E-mail address: 81479184@qq.com (H. Xia).

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nanofibers influence the growth pattern of astrocytes need to be evaluated. In this study, we intend to show the detailed growth manner of rat primary astrocytes when cultured on non-aligned or aligned nanofibers *in vitro*, which findings could be based for future *in vivo* transplantation study after SCI.

2. Material and methods

2.1. Ethics statement

This study was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Chongqing Medical University (CQMU-2008-127).

2.2. Material

PMMA, poly-L-lysine (PLL), trypsin, soybean, DNase I, and 5-bromo-2-deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St Louis, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin mixture, and normal goat serum were purchased from Invitrogen (Carlsbad, USA). GFAP and DAPI antibodies were purchased from Abcam (Cambridge, UK), BrdU antibody was from Dako (Cambridgeshire, UK), and Alexa Fluor second antibodies were from Molecular Probes (Carlsbad, USA). Silicone isolators were obtained from Grace (Oregon, USA) and vacuum grease was from Dow Corning (Vale of Glamorgan, UK). The experimental animals were provided by the Experimental Animal Center of Chongqing Medical University (Chongqing, China).

2.3. PMMA nanofiber fabrication and chamber construction for astrocyte culture

Nanofibers were fabricated by electrospinning 6.5% (w/v) PMMA suspended in dimethylformamide in a bacteria-free operating environment [20,22]. The PMMA suspension was delivered through a 30-G spinneret (Hongken Industry, Shanghai, China)at 0.3 ml/h using a syringe pump, with the spinneret located a distance of 20 cm or 12 cm from the collector to produce aligned or non-aligned fibers respectively. An electrospinning voltage of 10 kV was applied through a high-voltage power supply (Wisman, Shanxi, China). Fibers were gathered on 15 × 15 mm glass coverslips, which had been fit into grooves formed by steel bars on the four sides of the brick-shaped collector, and secured by metal strips along their edges. By a high-speed motor the collector was rotated at 1200 or 400 rpm to produce aligned or non-aligned fibers, respectively. 200 μl of 6.5% (w/v) PMMA suspension in dimethylformamide was applied on top of a 15 × 15 mm coverslip to make a PMMA film.

The PMMA materials were prepared at a temperature of 22–24 °C and under an atmosphere of < 60% humidity, and dried subsequently *in vacuo* at room temperature.

The culture chamber was designed to fasten a coverslip coated with PMMA nanofibers or a film as described before [20], so that the coverslip formed its bottom wall. The coverslip was attached onto a silicone isolator by coating its edges with vacuum grease with the PMMA coating facing inward, thus the silicone isolator formed the vertical walls of the culture chamber, which had a 500-μl capacity and13-mm diameter.

The chamber was sterilized after assembly, using a UV light (250 nm) irradiation (UV Light Technology Limited, Birmingham, UK) for 15 min in the laminar flow hood, followed by three times washing with phosphate-buffered saline. Before astrocytes culture, the chambers were coated with 50 μg/ml PLL for 1 h, then washed twice with sterile water. PLL-coated film served as controls.

2.4. Characterization of the fabricated PMMA nanofibers

The morphology of the PMMA nanofibers was examined by a scanning electron microscope (JEOL 820, JEOL Ltd., Tokyo, Japan) and an optical microscope (TE2000-S, Nikon, Tokyo, Japan). Under electron micrographs the nanofiber diameters were measured using ImageJ (National Institutes of Health, MD, USA). Averagely seven fields with 50 fibers per field per substrate were surveyed. By averaging the fiber diameters from five trials, data was analyzed.

To present the alignment of the nanofibers as a function of orientation, Fast Fourier transform (FFT) was performed by the FFT function of ImageJ [23,24].

2.5. Lentiviral vector construction

cDNA which encode green fluorescent protein (GFP) was cloned into a pRRL transfer vector (QIAGEN, Hilden, Germany). The lentiviral vector was constructed using the protocol of Dull and colleagues [25]. By transduction of HEK293 cells with three 100-fold serially diluted viral solutions after first diluting the solution 1000-fold, the titers were determined. The original titer of the GFP-encoding lentivirus construct (GFP/LV) was 7.2×10^{10} titer units/ml.

2.6. Astrocytes purification and culture on PMMA fibers

From the cerebral cortex of 2 day old Wistar rats, astrocytes were purified according to the protocol of McCarthy and colleagues [26]. Dissected brains were removed off meninges and blood vessels in the serum free DMEM, then were chopped and incubated with 0.1% trypsin in DMEM for 30 min at 37 °C in a humidified incubator with 5% CO_2. The mixture was triturated in triturating solution which contained 100 μg/ml soybean trypsin inhibitor, 0.5 μg/ml DNase I, and 10% FBS in DMEM, then the cells were centrifuged and resuspended in the culture medium containing 10% (v/v) FBS and 1× penicillin/streptomycin mixture in DMEM. Cells were plated in 75 cm^2 tissue culture flasks coated with PLL (10 μg/ml) at a concentration of 1.5×10^7 cells in 10 ml of medium, and incubated in a moisturized incubator with 5% CO_2 at 37 °C with flask lid loosened. Afterwards every 48–72 h the medium was changed. The medium was changed to remove microglias floating in the medium after 6–7 days of culture. Phase-dark and process-bearing cells were observed at the end of 7–9 days to approximate a confluent bed layer of cells. After culture medium changed again, flasks were placed on a shaking platform in a horizontal position with lids closed tight, and were shaken at 200 rpm at 37 °C for 6 h, in order to separate the oligodendrocytes from the astrocytes. After medium changed, the flasks were shaken for another 18 h to discard oligodendrocytes, macrophages and dividing astrocytes. Then the flasks were shaken at 100 rpm with fresh culture medium replenished, until under a 10× objective fewer than 10 phases–dark cells per field of view were observed. Then the multiplied cells were ready for immunocytochemistry and culture experiment, with fresh medium replenished every 2 days. Immunostaining for the astrocyte marker GFAP was conducted to assess the purity of all primary astrocyte cultures. (Fig. 1) Highly purified cultures passed no more than three times (> 90% astrocytes) were used.

The viability of astrocytes was evaluated through BrdU assay. Freshly transferred cells were plated on PMMA film and non-aligned fibers at a density of 50 cells mm^{-2} in the fresh medium of the same composition as described above, which also involved 10 μM BrdU. Bare coverslips treated with PLL served as the control. For image data processing, seven fields per substrate averagely under a 10× optical objective were imaged. The number of astrocytes adhered to the substrate stained with 4′,6-diamidino-2-phenylindole (DAPI) was counted, and the number of BrdU positively stained cells was counted as well. The ratio of BrdU positive cells to the total number of cells (represented as DAPI positive cells), was calculated. The calculations were averaged for