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Exploration of physical and chemical cues on retinal cell fate

Marina Castro Zalis [a], Sebastian Johansson [a], Fredrik Johansson [b], Ulrica Englund Johansson [a,*]

[a] Lund University, Faculty of Medicine, Department of Clinical Sciences Lund, Ophthalmology, Lund, Sweden
[b] Lund University, Department of Biology, Section of Functional Zoology, Lund, Sweden

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ABSTRACT

Identification of the key components in the physical and chemical milieu directing donor cells into a desired phenotype is a requirement in the investigation of bioscaffolds for the advancement of cell-based therapies for retinal neurodegeneration.

We explore the effect of electrospun poly-ε-caprolactone (PCL) fiber scaffold topography and functionalization and culture medium, on the behavior of mouse retinal cells. Dissociated mouse retinal post-natal cells were seeded on random or aligned oriented fibers, with or without laminin coating and cultured with either basic or neurotrophins enriched medium for 7 days.

Addition of laminin in combination with neurotrophins clearly promoted cell– morphology, fate, and neurite extension. Nanotopography *per se* significantly affected cell morphology, with mainly bipolar profiles on aligned fibers and more multipolar profiles on random fibers. Laminin induced a remarkable 90° switch of neurite orientation. Herewith, we demonstrate that the chemical cue is stronger than the physical cue for the orientation of retinal neurites and describe the requirement of both neurotrophins and extracellular matrix proteins for extended neurite outgrowth and formation of complex retinal neuronal networks. Therefore, tailor-made PCL fiber mats, which can be physically and chemically modified, indeed influence cell behavior and hence motivate further retinal restorative studies using this system.

1. Introduction

The introduction of tissue engineering in ocular research has played a fundamental role for the recent advancements in the exploration of restorative therapies for retinal neurodegeneration. Replacement of photoreceptors using donor cells for late stages of diseases such as retinitis pigmentosa, age-related macula degeneration and retinal ganglion cells (RGCs) in glaucoma, has gained lots of attention (Johnson et al., 2011; Lavik et al., 2005; Luo et al., 2014). However, in early preclinical retinal transplantation studies, poor cell survival and integration was found. Providing cells with physical three-dimensional support and biochemical signals to construct cell scaffolds may solve these problems (McHugh et al., 2013). In the organism many of these cues are provided by the extracellular matrix (ECM), which consists of water, fibrous proteins and proteoglycans (Järveläinen et al., 2009; Schaefer and Schaefer, 2010) and form highly organized meshworks in the nano- and micrometer range (Cassidy, 2014; Lee and Livingston Arinzeh, 2011; Lu et al., 2011; Tam et al., 2014; Taylor et al., 2015; Wang et al., 2013).

The ideal scaffold material for retinal repair must be biocompatible- and degradable, not cause an immunogenic response and preferable enable very thin constructs, so it can be implanted with minimum invasive technique (Zorlutuna et al., 2013). Electrospinning is a well-described method for production of suitable fiber sheets, mimicking the fibrous nature of native ECM, with regard to fiber diameter, porosity and large surface areas (Agarwal et al., 2009; Vasita and Katti, 2006).

Pioneering work demonstrate that electrospun fibrous scaffolds can promote cell survival, influence differentiation and guide neurite growth, and furthermore facilitate delivery and integration to the retina (Chen et al., 2011; Hertz et al., 2014; Hynes and Lavik, 2010; Redenti et al., 2008; Steedman et al., 2010; Trese et al., 2012). For photoreceptor replacement, electrospun fiber substrates of poly-ε-caprolactone (PCL), a flexible, biocompatible and biodegradable polymer was shown permissive for mouse- and human retinal progenitor cells (RPCs) differentiation, and facilitated integration with the recipient retina *in vitro* and *in vivo* (Cai et al., 2011; Lawley et al., 2015). Moreover, directed neurite growth and survival of post-natal mouse RGCs after transplantation was reported using functionalized aligned PCL fiber substrates *in vitro* (Kador et al., 2014, 2013).

Dissociated retinal post-natal cells (RPNCs) *in vitro* can mimic *in vivo* tissues and possess a greater potential to form synaptic connections,

Abbreviations: AB, AlamarBlue; CNS, Central Nervous System; DIV, Days *in vitro*; ECM, Extracellular matrix; FFT, Fast Fourier Transform; PCL, Poly-ε-caprolactone; PLL, Poly-L-lysine; PLLA, Poly-L-lactic acid; PN4, Post-natal day 4; RPNCs, Retinal post-natal cells; RGCs, Retinal ganglion cells; RPC, Retinal progenitor cells; RT, Room temperature; SD, Standard deviation of a sample; SEM, Scanning electron microscope; SEM, Standard error of mean.

* Corresponding author.
E-mail addresses: marina.castro_zalis@med.lu.se (M.C. Zalis), atn09sjo@student.lu.se (S. Johansson), per_fredrik.johansson@biol.lu.se (F. Johansson), ulrica.englund_johansson@med.lu.se (U.E. Johansson).

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compared to corresponding progenitors, with the host retina, making this cell type an appropriate control assay and hence a tool for basic studies in retinal restoration (Hertz et al., 2014).

Fabrication of tailor-made cell-scaffolds for retinal restoration is yet in an early phase, mostly due to the retina being a complex and well-organized laminar neural circuit. Each retinal cell type would most likely profit from different topographies; therefore it is currently challenging the fabrication of versatile scaffolds that would resemble its natural surroundings. Studies made so far, haven't lead to any clear solution. Hence, we believe that with systematic work on basic effects of the main physical- and chemical factors that affect cell behavior, important pieces will be added to the overall puzzle that may put previous studies in a better context.

Here, we wanted to investigate the effects that: a) electrospun PCL submicron fibrous with different organization (aligned and random fiber orientations), b) functionalization (±laminin coating) and c) different culture media may have on the overall adhesion, polarization of nuclei- and neurites, morphological formation and phenotypic marker expression of RPNCs. The effects were quantified by detailed immunocytochemical- and morphometric analysis.

2. Material and methods

2.1. Electrospinning of fiber substrates

A 15 wt% solution of PCL (80 kDa, Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving PCL pellets in acetone at 50 °C. A 1 mL syringe with a 22 G blunt needle was filled with the PCL solution and put into a stand with a syringe pump, which was set to a continuous feed. For the randomly aligned fibers a rectangular metal plate (135 × 90 mm) was used as a collector, whereas for the parallel fibers a rotating disk with a diameter of 145 mm was used. To produce randomly aligned fibers the flow rate was 1.5 mL/h, the strength of the electrical field was 20 kV and the syringe-target distance was 20 cm. For parallel-aligned fibers production, the flow rate used was 3.0 mL/h, the strength of the electrical field was 17.5 kV, the distance to the rotating disk was 20 cm and the rotation speed of the disk was 1500 rpm. PLLA plastic film was used as the collector film for all the fibers. The substrate construct (50 μm plastic film + 6 μm spun fibers) was cut into pieces of approximately 10 × 10 mm, sterilized with 70% ethanol and dried at room temperature (RT) before use for cell cultures.

2.2. Fiber characterization using scanning electron microscope (SEM)

Fibers were sputtered with gold palladium (AuPd) and then analyzed using a scanning electron microscope (SEM, SU3500, Hitachi) for high-resolution images. Diameters of at least 50 fiber filaments per sample (n = 3 per fiber type) were measured and the mean diameter was calculated. To measure the alignment of the fibers, the FFT function (Fast Fourier Transform) of ImageJ and an oval profile plug-in was used (Bill O'Connel, http://rsbweb.nih.gov/ij/plugins/oval-profile.htmL).

2.3. Poly-L-lysine (PLL) and laminin coating of PCL nanofibers

Random and aligned fibers were coated with a 10 μg/mL PLL (Sigma Aldrich Sweden AB) solution for 1 h at RT and subsequently washed with Millipore water and air-dried. An overnight incubation at RT on a shaker was after performed with 10 ng/mL laminin solution (Sigma Aldrich Sweden AB). Succeeding washing steps in tris-buffer saline (TBS) were performed.

2.4. Isolation and dissociation of post-natal day 4 mouse retinal tissue

Animal handling was performed in accordance with approved guidelines of the Ethics Committee of Lund University, the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals), and the ARVO statement for the use of animals in ophthalmic and vision research. Retinas were isolated from post-natal day 4 mice (PN4). A single cell suspension of the retinal tissue was prepared by incubating 8 retinas in a 1 mg/mL (18 units/mL) papain solution (DMEM/F-12, 5 ng/mL DNase I (Sigma-Aldrich, St. Louis, MO, USA), 0.3 g/mL L-cysteine (Sigma-Aldrich, St. Louis, MO, USA)) for 30 min at 37 °C and then mechanically dissociated with a burned tip glass Pasteur pipette. Single cell suspension was centrifuged for 5 min at 480 g and resuspended in culture medium. Two different culture mediums were used in the study, *i.e.* basic neuronal medium (DMEM/F-12, 2% B27 supplement (Invitrogen, Ltd., Paisley, UK) and 2% L-glutamine + Penicillin/Streptomycin (Invitrogen, Ltd., Paisley, UK)) and the enriched Full-SATO medium (Neurobasal supplemented with insulin, sodium pyruvate, penicillin/streptomycin, *N*-acetyl cysteine, triiodo-thyronine, forskolin, SATO supplement, B27 and BDNF and CNTF growth factors at previously published concentrations (Barres et al., 1988; McKerracher et al., 1989). Cells were counted with an automated cell counter TC20 from BioRAD and then seeded onto uncoated or PLL-laminin coated PCL nanofiber substrates at cell densities of 1×10^5 cells/cm^2 and incubated for 4 h (0 DIV), 7 days or 18 days at 37 °C in a humidified atmosphere of 5% CO_2. In addition, RPNCs were also seeded on PLL coated chamber slides, to serve as comparison in the nuclei orientation analysis. Medium was changed every 2–3 days throughout the experiments. At least three independent seeding for each experimental group, including three replicates, were performed.

2.5. AlamarBlue proliferation and viability assay

Viability of the cultured retinal cells on fibers and glass flat controls for 4 h, 7 days and 18 days was assessed with AlamarBlue (AB) assay (Invitrogen Ltd., Paisley, UK). Two cell seedings were performed for this assay. Protocol was followed according to manufacturer's instructions. In brief, medium from cell cultures was removed on the day of the assay and replaced with fresh medium containing AlamarBlue (1:10) and re-incubated at 37 °C, 5% CO_2 for 22 h. Remaining medium with AB was also incubated and used as no-cell (negative) control. At the end of the incubation time, 100 μL of the cultured medium was transferred to a 96-well plate containing 50 μL of 3% SDS, which stops the reaction and stabilizes fluorescence. Fluorescence was measured in a spectrophotometer at 560 nm excitation wavelength and 590 nm emission wavelength. Average fluorescence values of the culture medium background (negative controls) were subtracted from all fluorescence values of each experimental well. Normalization of the data was performed relative to the control group flat + basic medium.

2.6. Immunocytochemistry

Cell cultures on the nanofibers substrates were fixed in a solution of 4% paraformaldehyde (PFA) in Sørensen's buffer for 10 min at RT, washed three times with phosphate buffered saline (1× PBS), pH 7.2. Cultures were blocked and permeabilized using a solution of PBS, 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), and 0.25% Triton X-100 for 30 min. Blocking was followed by overnight incubation at 4 °C with primary antibodies diluted in blocking solution. Washing steps were performed before and after 1 h incubation with the secondary antibodies at RT in the dark. Samples on fiber scaffolds were then arranged on glass slides, mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Inc. Burlingame, CA, USA) for nuclei counterstaining before adding the coverslip. Full lists of the primary and secondary antibodies used are presented in appendices Tables A.1 and A.2, respectively.