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Inducing functional radial glia-like progenitors from cortical astrocyte cultures using micropatterned PMMA

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ABSTRACT

Radial glia cells (RGC) are multipotent progenitors that generate neurons and glia during CNS development, and which also served as substrate for neuronal migration. After a lesion, reactive glia are the main contributor to CNS regenerative blockage, although some reactive astrocytes are also able to de-differentiate *in situ* into radial glia-like cells (RGLC), providing beneficial effects in terms of CNS recovery. Thus, the identification of substrate properties that potentiate the ability of astrocytes to transform into RGLC in response to a lesion might help in the development of implantable devices that improve endogenous CNS regeneration. Here we demonstrate that functional RGLC can be induced from *in vitro* matured astrocytes by using a precisely-sized micropatterned PMMA grooved scaffold, without added soluble or substrate adsorbed biochemical factors. RGLC were extremely organized and aligned on 2 μm line patterned PMMA and, like their embryonic counterparts, express nestin, the neuron-glial progenitor marker Pax6, and also proliferate, generate different intermediate progenitors and support and direct axonal growth and neuronal migration. Our results suggest that the introduction of line patterns in the size range of the RGC processes in implantable scaffolds might mimic the topography of the embryonic neural stem cell niche, driving endogenous astrocytes into an RGLC phenotype, and thus favoring the regenerative response *in situ*.

1. Introduction

Despite the presence of multipotent neural stem cells (NSC) in the adult central nervous system (CNS), their ability to regenerate after an injury is very limited and there is currently no effective treatment to improve CNS healing. The formation of a glial scar is one of the most important causes of the lack of spontaneous CNS regeneration. Reactive astrocytes, fibroblasts and other glial cells within the scar produce inhibitory molecules for axon growth [1,2]. However, current evidence indicate that astrocytes also play beneficial effects for CNS recovery, as they restart the hemato-encephalic barrier, secrete neurotrophic factors and provide support and guidance for axonal growth [3–5]. Moreover, a pool of early reactive astrocytes changes their phenotype adopting many of the molecular traits of embryonic radial glia and NSC [6,7]. Therefore, a promising strategy can be enhancing the beneficial astrocytic response to obtain a permissive glial environment for neural growth and the re-establishment of functional connections after an injury.

Cell-based approaches to CNS regeneration have had little success, partly because of the limited survival and integration of the implanted cells, which is probably due to the absence of biochemical and topographical cues normally present at the NSC niche. To overcome this problem, the tissue engineering approach aims to provide instructive information to regenerative capable cells through the implantation of intelligent materials that mimic the natural NSC microenvironment. There is increasing evidence that surface topography can modulate the cell response by changing cell morphology and differentiation state. The most

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recent findings and theories have been elegantly reviewed by Bettinger et al. [8] and Hoffman-Kim [9], the latter focusing specifically on nerve regeneration. Among the results they mention, micropatterned or aligned fibers of poly-caprolactone enhanced the differentiation of retinal progenitor cells into neurons and glia [10] or Schwann cells maturation [11], respectively, while the diameter of electrospun fibers influenced NSC differentiation [12]. Furthermore, mature astrocytes have been shown to de-differentiate *in vitro* under certain specific conditions. For instance, induction of the polycomb transcription factor Bmi1 and exposure to FGF-2 or sulfated hyaluronan can induce stem cell-like features in quiescent astrocytes [13,14]. Astrocytes may also de-differentiate by physical methods, such as freeze-thawing [15] or by mechanical and scratch insults [7]. These findings suggest that differentiated glial cells retain a certain plasticity that might be manipulated to evoke a reparative response to damage by presenting the appropriate signals.

Several recent studies have used micropatterned polymer substrates to direct the growth and differentiation of NSC [16]. However, glial cells and, in particular, astrocytes are the most likely cell types to contact the material after *in vivo* implant. To identify instructive cues that could induce astrocytes to adopt a radial glia-like phenotype permissive for neural growth, we analyzed *in vitro* the differentiation of glial cells in response to linear patterns imprinted on a substrate of poly(methyl methacrylate) (PMMA).

PMMA is a transparent synthetic material that, thank to its thermoplastic nature, can be structured by hot embossing with high resolution [17–19]. It has been used for nerve tissue engineering *in vitro* [20,21] and has recently been successfully employed for rat sciatic nerve regeneration *in vivo* [22]. Here therefore, we used uncoated PMMA films carrying line topographies of different dimensions in order to determine whether the introduction of topographical cues might bias glial differentiation toward a supportive phenotype for neuronal growth.

2. Materials and methods

2.1. PMMA characterization and microstructuring

Characterization of PMMA wettability was achieved via contact angle measurements using an OCA 20 system (Dataphysics, GmbH, Germany). Advancing contact angle measurements were taken using 3 μL Milli-Q water. Four substrates and at least four different measurements were performed on each. Z-potential measurements were carried out using a SurPASS apparatus and VisioLab software (Anton Paar Ltd. - UK). All the measurements were performed four times at the pH of the electrolyte (KCL 1 mM, pH 5.5) after 2h of equilibration using the Adjustable Gap Cell for small samples (20 mm × 10 mm).

Micropatterns were introduced on 125 μm thick PMMA sheets (Goodfellow Ltd., UK) by nano-imprinting lithography (NIL) (Obducat AB, Sweden) and following the protocol described by Mills et al. [17]. Micropatterns consisted of 2 μm and 10 μm wide lines (ln2 and ln10), all 1 μm deep/tall and 1.5″ length. The silicon molds were provided by AMO GmbH (Aachen, DE) and consisted of 1.5″ × 1.5″ silicon squares. For cell culture, PMMA films were sterilized with 70% ethanol for 15 min and cut to fit in 60 mm ø tissue culture dishes. The characterization of patterned PMMA films was achieved by white light interferometry (WYKO NT1100 apparatus and the software Vision 32 V2.303 (Veeco Instruments, Inc, USA)).

2.2. Cell culture

All animal housing and procedures were approved by the Institutional Animal Care and Use Committee in accordance with Spanish and EU regulations. Glial cells were derived from brain cortex of postnatal mice as described elsewhere [23]. Briefly, P0 brain cortices were dissected out free of meninges in dissection buffer (PBS 0.6% glucose (Sigma), 0.3% BSA (Sigma)) and digested with trypsin (Biological Industries) and DNAse I (Sigma) for 10 min at 37 °C. The tissue was dissociated in Dulbecco's Modified Eagle Medium (DMEM, Biological Industries) 10% normal horse serum (NHS, GIBCO), 1% penicillin-streptomycin (Pen-Strep, Biological Industries), and 2 mM L-glutammine (Biological Industries), referred to in this text as growing medium (GM). After centrifugation and resuspension, cells were plated and grown to confluence at 37 °C, 5% CO_2 (approximately 25–30 days *in vitro*, DIV). All the experiments were performed using glial cells from the first passage (Ps1).

To assess the influence of different line topographies on glial cell morphology and differentiation state, Ps1 cells were cultured at a density of 2×10^5 cells/cm^2 for 5 DIV in Neurobasal™ (NB), 3% NHS, 1% Pen-Strep, and 2 mM L-glutamine (experimental medium = EM) on PMMA ln2, ln10 or flat (non patterned = NP). To avoid any confusion caused by the heterogeneity of glia culture types described in the literature, we defined three reference conditions within the *in vitro* system used here to compare biochemical changes of glial cells on PMMA. Control glia were Ps1 glial cells cultured on non-coated culture plastic (for Western blotting) or glass (for ICC) under the same conditions as for PMMA. Reactive/mature glia were obtained by culturing Ps1 glial cells for 8 DIV in EM and with EM supplemented with dibutyril cyclic AMP (dcAMP, 500 mM, SIGMA) during 7 more days [24,25]. Progenitor glia were obtained by culturing Ps1 glial cells for 24h in EM and then in NB supplemented with G5 (GIBCO) for 7 DIV. G5 supplement contains mitogens, such as FGF-2 and EGF, and it is used to maintain neural stem cells in culture; it significantly promotes the proliferation of neuronal precursor cells, radial glial cells and astrocytes *in vitro* [15,26]. The choice of culture conditions for progenitor and reactive glia, and of the EM, was made after numerous preliminary studies using different culture mediums, supplements, serum types and concentrations.

Neurons were obtained from embryonic brains. Brain cortices from E16 mice were isolated in dissection buffer, digested with trypsin-DNAse I, dissociated and preplated for 30 min in preplating medium (CO_2-equilibrated Neurobasal™ supplemented with 5% NHS, 1% Pen-Strep, 0.5 mM L-glutammine, 5.8 μl/ml $NaHCO_3$ (Sigma–Aldrich, Saint Louis, MO)). The supernatant was then collected, centrifuged and resuspended in serum-free neuronal culture medium (NB, 1% Pen-Strep, 0.5 mM L-glutamine, 1x B27, 5.8 μl/ml $NaHCO_3$). Neurons were plated at a density of 2.5×10^5 cells/cm^2, directly on top of 5DIV glial cell cultures, and then cultured for 5 more days in neuronal culture medium.

Explants were obtained from the cerebral cortex of E16 actin-GFAP transgenic mice. Brains were isolated and then cut into 350 μm thick slices with a McIlwain Tissue Chopper (Camden Instruments, UK). Explants of approximately 300 μm diameter were obtained by microdissection, incubated in preplating medium for 1h and seeded on top of glial layers in neuronal culture medium for 2 DIV.

2.3. Western blot

Total extract proteins were separated by SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Membranes were first blocked in 5% non-fat milk and then incubated with primary antibodies overnight at 4 °C, followed by their corresponding secondary HRP-conjugated antibodies (1:3000, Santa Cruz Biotechnology, San Diego). Protein signal was detected using the ECL chemiluminescent system (Amersham, Buckinghamshire, UK). Densitometric analysis, standardized to actin as a control for protein loading, was performed using ImageJ software (National Institutes of Health, USA).

For quantification, triplicate samples were analyzed.

2.4. Immunocytochemistry and primary antibodies

For immunofluorescence, fixed samples (4% PFA for 1h at RT) were incubated with primary antibodies and appropriate Alexa488 or Alexa555 secondary antibodies (1:500, Molecular Probes, Eugene, Oregon). Phalloidin was used to stain F-actin (1:2000, Sigma–Aldrich, Saint Louis, MO) and To-Pro-3 iodide (1:500, Molecular Probes, Eugene, Oregon) to stain nuclei. Finally, the preparations were coverslipped with Mowiol (Calbiochem, San Diego) for imaging.

The following primary antibodies were used: rabbit anti-GFAP (mature and reactive glia marker, 1:500-1:8000, Dako), mouse anti-Vimentin (reactive glia marker 1:1000, Santa Cruz Biotechnology, INC), rabbit anti-EAAT-2 (mature glia marker 1:500, Cell Signaling) rabbit anti-BLBP (radial glia marker, 1:1000-1:8000, Chemicon), mouse anti-Nestin (progenitor and radial glia marker, 1:250, Abnova Corporation), goat anti-Actin (cytoskeleton Marker, 1:2000, Santa Cruz Biotechnology, INC), mouse anti-Tuj-1 (neuronal marker 1:10000, Covance) and rabbit anti-PH3 (proliferation marker, 1:250, Millipore), goat anti-Pax6 (neurogenic radial glia marker, 1:250, Santa Cruz Biotechnology, INC) and rabbit anti-TBR2 (neurogenic intermediate progenitor cells marker, 1:500, Abcam), rabbit anti-NG2 (oligodendrocytes precursor cells marker, 1:200, Millipore), rabbit anti-Ki67 (proliferation marker, 1:500, Abcam), mouse anti A2B5 (glial precursor cell marker, 1:100, Miltenyi Biotec) and goat anti-Tuj-1 (neuronal marker 1:1000, Covance).

2.5. Flow cytometry analysis

The absolute number of living and dead cells was determined at 1-4 DIV by flow cytometry using a FACScalibur apparatus (Becton Dickinson). For absolute live/dead cell number counts we used propidium iodide (PI, Sigma, 5 μg/mL) to label dead cells and CountBright™ absolute counting beads (Molecular Probes, Invitrogen), according to the protocol suggested by the provider. Ps1 cells were cultured at a density of 2×10^5 cells/cm^2, then trypsinized at 1-4 DIV, and resuspended in 1 ml of PBS. Ten thousand CountBright™ counting beads (Molecular Probes, Invitrogen) were recorded and cell populations were determined based on cell size (FSC) and granularity of the cytoplasm (SSC). The cell suspensions were analyzed after PI incubation. Percentage of positive cells and sample relative fluorescence were