Neural Cells Respond to the Geometry of the Laminin Substrate

Camila Hochman Mendez\textsuperscript{1}, Madalena Sant’Anna Barroso\textsuperscript{2} and Tatiana Coelho Sampaio\textsuperscript{2}

\textsuperscript{1}Instituto de Biofísica Carlos Chagas Filho, IBCCF/UFRJ, camilahm2@gmail.com
\textsuperscript{2}Instituto de Ciências Biomédicas, ICB/UFRJ

ABSTRACT

The extracellular matrix glycoprotein laminin (LM) can self-polymerize both in vitro and in vivo and it is known as a key regulator of neural development. We have previously shown that biofilms of LM produced in acidic buffer (polyLM) display a homogeneous flat surface, while films produced in neutral buffer are rough and irregular. The present work was devised to investigate whether surface geometry contributed to the neural response to LM. Single cell suspensions were produced from developing rat cortex (E14) or retina (P1) and alternatively cultivated for 24 hours on polyLM, regular LM (rLM) or on filters of polyethersulfone mimicking the topology of the laminin biofilms and coated with non polymerized LM (EDTA). On rLM both cell types formed large clusters with internal neurites, whereas on polyLM cells did not clump and emitted long processes on the substrate. Similarly, the rough filter induced cell clustering and the smooth one favored neuritogenesis. Additionally, cultures were treated with inhibitors of MAPK (PD98059), PKA (H89) and PKC (Chelerythrine Chloride; CC). Treatment with H89 or CC decreased proliferation on polyLM and on its corresponding mimetic surface while the inhibitor of MAPK affected only the response to rLM and its mimetic surface. Our data suggest that the signaling properties of a laminin substrate are modulated by its topological organization.

Keywords: Laminin, proliferation, cell signaling.

INTRODUCTION

Laminin is a high molecular weight glycoprotein, composed of three different polypeptide chains. The 3-D structure of laminin trimers is particularly adequate to favor the assembly of a sheet-like polymer, where the three short arms simultaneously interact with each other within a single plan, while the long arm is left available to interact with the surface of contiguous cells. As a consequence of its structural properties, laminin can spontaneously self-polymerize in a test tube, only requiring a minimal protein concentration [1] or a decrease in the solution pH [2]. Knowing that the polymerization state contributes to the biological response to the protein [3-6], it becomes increasingly important to study laminin signaling by using its polymeric form. During retinal development laminin is expressed at different regions and the polymeric deposits can assume distinct shapes [7,8]. In the center of the P1 retina, from where maturation progresses, laminin does not present a regular structure, while in the periphery it is organized in a polygonal flat array [3]. In order to investigate the ability of neural cells to recognize the geometry of the laminin substrate, here we compared their responses to polyLM and rLM with those to two polyetherulfone filters (PES) of different topologies, chosen to mimic the surface of the polymeric protein.

MATERIALS AND METHODS

Culture of retinal cells. Cultures of retinal cells were prepared as previously described [9]. Embryonic (embryonic day 14, E14) or newborn rats (postnatal day 1, P1) were euthanatized, the cortex or retinas dissected and dissociated. Cells were seeded on 24-wells plates, where glass coverslips pretreated with laminin (polyLM or rLM) or PES filters had been previously placed. Cells were maintained in culture for 24 hours.

Preparation of substrates. The substrates were prepared by coating glass coverslips with laminin (laminin 111; Invitrogen, Carlsbad, USA) diluted in 20 mM sodium acetate (pH 4; polyLM) or 20 mM Tris-HCl (pH 7; rLM), both containing 1 mM CaCl\textsubscript{2}. The filters were incubated with laminin
solutions containing EDTA, which prevents the polymerization of the protein. Before cell plating, the glass coverslips or filters were washed three times with PBS 1X.

**Immunostaining.** After fixation with paraformaldehyde 4% for 20 min, cells were stained by indirect immunofluorescence. Glass coverslips were incubated with primary antibodies (Tuj1, marker of neural cells; 1:500, Santa Cruz Biotechnology ®; anti-LM, laminin, 1:30, Sigma-Aldrich Co., St. Louis, MO, and DAPI, DNA intercalation; blue). Cells were visualized with an epifluorescence microscope (Zeiss Axioplan).

**Scanning electron microscope.** Cells were fixated with Karnosky (4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) for 2 hours, washed three times with sodium cacodylate buffer 0.1 M, pH 7.2, dehydrated through increasing concentrations of ethanol and dried in Balzers CPD050 critical point. The samples were then coated with a thin layer of gold sputter (Bal-TecSCD 050) and viewed under a microscope Jeol 5310.

**Direct cell counting.** After 24 hours cells were released (0.05% trypsin) and counted in a Neubauer chamber. Since the ratio of dead cells was always below 2% in all cases we considered that the increase in cell number corresponded to an increase in proliferation.

**Statistical analysis.** All data were expressed as mean ± standard error (SEM) for a minimum of three experiments performed at least in triplicate. Statistical significance was first obtained by analysis of variance in a way (one-way ANOVA) (GraphPad Prism Program for Windows 4:00 GraphPad Software, San Diego, CA, USA). Values of P < 0.05 were considered statistically significant.

### RESULTS AND DISCUSSION

Laminin as well as collagen and fibronectin, forms aggregates in vivo. We hypothesized that the geometry of these supramolecular aggregates could modulate the spacing between the epitopes recognized by cell receptors. Using confocal microscopy, we compared the topographies of polyLM and rLM. Analysis of the images produced by superposition of optical cross sections revealed that while polyLM was thin and homogenous as a two-dimensional structure (Figure 1A and B), rLM was thicker and poorly organized, tending to a more three-dimensional structure (Figure 1C and D). These organizations seem to morphologically correspond to the previously reported differences between the peripheral and central retina, respectively. In order to directly assess the contributions of the different topologies to the behavior of neuronal cells, we chose two substrates with geometries similar to those arrays. To mimic the flat two-dimensional polyLM, we used a flat PES membrane and to mimic rLM we used a rough membrane of PES. The filters were coated with laminin in EDTA to prevent polymerization of the protein. Reconstructed matrices are shown as top (Fig. 1E, G) or side views (Fig. 1F, H). Comparison between the profiles observed in F and H with those in A and D, respectively, revealed that the two filters mimicked the topographies of rLM and polyLM. The filters were additionally characterized independently of immunostaining by using scanning electron microscopy (Fig. 1I, J). In this analysis it was possible to observe that the differences in roughness between the filters reflected the sizes of their constitutive pores.

Neurons from the cerebral cortex of embryonic rats at E14 and of P1 retina were cultured on the different substrates. The flat PES, as well as polyLM, induced pronounced neuritogenesis in both cell types (Fig. 2A, C and E). Conversely, on rough PES, as well as on rLM, cells tended to self organize in clusters and they did not extend neurites on direct contact with the substrate (Fig. 2B, D and F). In a previous study we had shown that polyLM stimulated proliferation of retinal cells in vitro and that such stimulation was mainly mediated by PKA [10]. On the other hand, rLM did not promote any increase in cell number. To further confirm the correspondences flat PES/polyLM and rough PES/rLM we compared cell proliferation in cultures established on the two PES filters in the presence of inhibitors of MAPK (PD98059), PKC (Chelerythrine Chloride, CC) or PKA pathways (H89). Figure 3 shows that cells proliferate more on the flat PES than on the rough filter, whereas such proliferation was majorly mediated by PKA as it was ca. 70% inhibited by H89. Cells did not
proliferate significantly on rLM and only the inhibitor of the MAPK pathway minimally decreased cell number.

**Figure 1: Characterization of substrates.** Top (A) and side views (C) of polyLM and rLM (B,D) immunostained with anti-laminin and analyzed by confocal microscopy. Side views of flat (E) and rough (F) PES coated with non-polymerized laminin and immunostained for laminin. (G,H) PES filters viewed by scanning electrical microscopy. Scale bars A,B (25µm); C-H (10µm).

**Figure 2: Morphology of cells growing on different substrates.** Panels A-D and F show retinal cells cultivated for 24 hours on polyLM (A), rLM (B), flat (C) or rough PES (D and F). Panel E
shows a culture of embryonic cortical cells growing on flat PES and panel F shows the internal 
structure of a cell cluster on rough PES. Scale bars A,B,E (50\(\mu\)m); C,D (100\(\mu\)m); F (10\(\mu\)m).

**Figure 3: Effect of kinase inhibitors on the total number of retinal cells plated on PES filters.** Control (No treatment, blue bars), inhibitor of pKA (H89, green bars), inhibitor of PKC (Chelerythrine Chloride, CC, purple bar) and inhibitor of MAPK (PD98059, red bar).

**CONCLUSIONS**

Our data indicate that the response of neuronal cells to laminin is modulated at least partially to the geometrical properties of the substrate.

**ACKNOWLEDGMENTS**

We thank CNPq, FAPERJ and CAPES for financial support.

**REFERENCE**