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Neuritogenesis on antagonist surfaces

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Abstract

The nanoscale characteristics of the adhesion substrate and the composition of the culture medium are investigated as factors that have an influence on neuritogenesis and on the structure/morphology of the neurites. Poly-L-lysine (PLL) is known to produce a positively charged surface, as opposed to poly-L-ornithine (PLO) which produces a substrate that is very similar in its structure. Lamellipodia are observed all along neurites formed on a PLL-coated glass surface, but not on a PLO-coated glass surface. Collagen is a natural protein that enhances adhesion. Provided the matrix formed by collagen on a glass surface is thin enough, nanoroughness, rather than substrate compliance, is demonstrated to impact cell aggregates and neurite morphology. AFM and optical microscopy are used to illustrate the differences pertaining to PLL, PLO and collagen coated glass surfaces used as culture substrates for neuronal cell culture.

Keywords: Neurite outgrowth; Collagen; Poly-L-lysine; Poly-L-ornithine; Lamellipodia; Nanoroughness

1. Introduction

When the migrating neuroblast has found its destination in the nervous system, the neurons differentiate and make extensions, which form the axon and the dendrites. However, in this early phase, axons and dendrites are very similar and are called neurites. The growing end of a neurite is the growth cone that guides neurites outgrowth through specific directions, to a population of target cells, in order to establish the right connections during embryogenesis. Growth cones are able to re-orientate towards a diffusing source of nerve growth factor (NGF) *in vitro* [1]. This demonstrates that growth cones can detect gradients of diffusing molecules and respond to them with changes in the direction of growth.

Growth cones are also sensitive to the adhesion parameters of the extra-cellular matrix. Haptotaxis is the name given to cellular movements along an adhesive surface of substrate-bound molecules. The end of an exploratory growth cone is composed of flattened membrane sheets, widely known as *lamellipodia*. Thin expansions emerge from *lamellipodia*, the *filopodia*, that constantly stretch and shrink in order to explore the environment. Neurite outgrowth occurs when, instead of retracting, *filopodia* cling to the substrate surface and stretch the growth cone.

When an axon is severed, the distal segment degenerates, being no longer connected to the cell soma. The end of the severed axon attached to the soma will respond very quickly to injury by issuing growth cones. In the adult central nervous system, axonal growth is, however, quickly inhibited. What distinguishes neuron reactions in the central nervous system and in the peripheral nervous system are not the neurons themselves, but their respective environments, in other words: their substrates. The aim of this work is to contribute to clarify characteristics of physical cues of the substrates, and their effects on the arrangement of cell aggregates, on the initiation of neurites, and on the morphology of the extended neurites.

The interactions of cells, especially neurons, with controlled topography [2-5], and surface chemistry [6-8], were reported to be important parameters in controlling cell function. Another parameter, substrate stiffness, influences both neuritogenesis [9-12], and neurite branching rate [13]. In our previous studies [14, 15], we demonstrated the influence of nanoscale surface energy gradients on neuronal differentiation. We used PC12 cells, a well-known model line for neuronal differentiation studies [16]. PC12 cells, though not primary neuronal cells, express the transmembrane TrkA and p75 receptors to nerve growth factor (NGF) [17, 18], and differentiate into a neuronal phenotype when challenged by appropriate NGF concentrations [16].

Several key inducers of PC12 cell neuronal differentiation in NGF-free medium have been identified: PC12 cell neuritogenesis is observed on soft substrates composed of extra-cellular matrix (ECM) proteins such as collagen,

fibronectin and laminin [19], or of ECM derived from astrocytes [20]. We previously demonstrated the ability of PC12 1.1 cells to differentiate on rigid substrates and in NGF-free medium: when seeded on solid glass substrates covered with NH_2/CH_3-terminated alkylsiloxane self-assembled monolayers (SAMs) [14, 15]. These surfaces contained a nanoscale mixture of hydroxyl and amine/methyl groups which provided nanoscale chemical heterogeneities that were shown to promote PC12 cell adhesion and differentiation.

Conversely to glass-coated alkylsiloxanes SAMs, glass surfaces coated with collagen, poly-L-lysine (PLL) and poly-L-ornithine (PLO) are widely used as standard substrates for neuronal cell culture [21], and especially for PC12 cells [16, 22, 23]. In these conditions (forming a thin monolayer on a glass substrate) PLL and PLO provide a homogeneous surface for non-specific interactions. Using a low concentration (50 µg/mL in PBS) collagen is also supposed to give rise to a homogeneous substrate when coated on a glass surface. Nevertheless, collagen fibrils are supposed to provide a higher nanoroughness (i.e., vertical variations of a surface at the nanometer scale) than both PLL and PLO. In this study, we investigate the antagonistic surface effects of PLL, PLO and collagen-coated glass surfaces on the morphology of cell aggregates and that of the initiated neurites.

2. Experimental

2.1. Substrates preparation

Modified glass coverslips (30 mm-diameter and 100 µm-thick, Menzel-Glaser) were used for cell culture experiments. Prior to use, glass coverslips were cleaned, first by immersion in ultrasonic bath of chloroform for 20 min, second by immersion in piranha solution (3:1 (v/v) sulfuric acid:40% hydrogen peroxide) then thoroughly rinsed with deionized water and dried under a nitrogen stream *(caution: piranha solution is extremely corrosive and can react violently with organic compounds. Appropriate safety precautions including gloves and face shield should be used when handling).* For the self assembly, the cleaned glass substrates were immersed for 1 h at room temperature into solutions of the desired biopolymers: PLL (PLL solution, 0.01% in water, Sigma) or PLO (PLO solution, 0.01% in water, Sigma) or collagen (type I, 50 µg/mL in PBS). Substrates were then sterilized in a UV chamber, and either rinsed in sterile water prior to cell culture, or quickly rinsed in deionized water and dried under a nitrogen stream prior to air-imaging AFM experiments.

2.2. Cell culture

We used PC12 1.1 cells that derive from PC12 pheochromocytoma cells (ATCC CRL-1721). PC12 1.1 expresses the mammalian retinoid x-receptor [24]. One characteristic of PC12 1.1 cells is that they do not respond anymore to NGF treatment. In addition, they showed an aptitude to resist and survive to serum deprivation of the culture medium. Details of procedures used for cell seeding can be found in Ref. [14]. Briefly, cells were seeded using a standard DMEM culture medium supplemented with 10% (v/v) horse serum (HS) and 5% (v/v) fetal bovine serum (FBS). After 24 h of culture, this medium was removed and replaced by a HS-free medium containing only 0.5% (v/v) of FBS.

3. Results and Discussion

3.1. Cell culture on collagen I

After 5 days of culture in a serum-deprived culture medium (i.e. 6 days after seeding) PC12 1.1 cells extend neurites whose length may reach 100 µm (Figure 1). Though resistant to serum deprivation, PC12 1.1 cells are assumed to undergo some stress under these conditions. In addition to its assumed role in neurite initiation, this stress could also be responsible for a reduced affinity between the cells and the substrate. This behavior is observable on the left image of Figure 1, where some cells are observed exhibiting a high density per unit area (Figure 1, arrows) which might reflect that cells experienced poor adhesiveness with the substrate. This lack in adhesion to the solid surface would be compensated by cell self-clustering, which could explain the presence of scattered patches with a high density of cells.

The arrangement of cells on the substrate seems wholly different from what we observed in other experiences [14, 15]. When cells previously tended to form colonies that appeared circular (or at least elliptical) here they are aligned along specific directions (Figure 1). This arrangement suggests that PC12 1.1 cells are maintained in networks of interconnected channels, that force cells as well as the generated neurites to follow the directions taken by these channels. Actually, they are most probably the underlying collagen fibers that coat the glass substrate (Figure 2).

Collagen fibers provide a good substrate for PC12 cell culture [23]. The collagen matrix used here significantly differs from "standard" collagen matrices generally used in cell experiments. Its concentration is very low (~50 µg/mL in PBS solution, used to immerse the clean glass coverslip) therefore the generated matrix is assumed to be inelastic because the

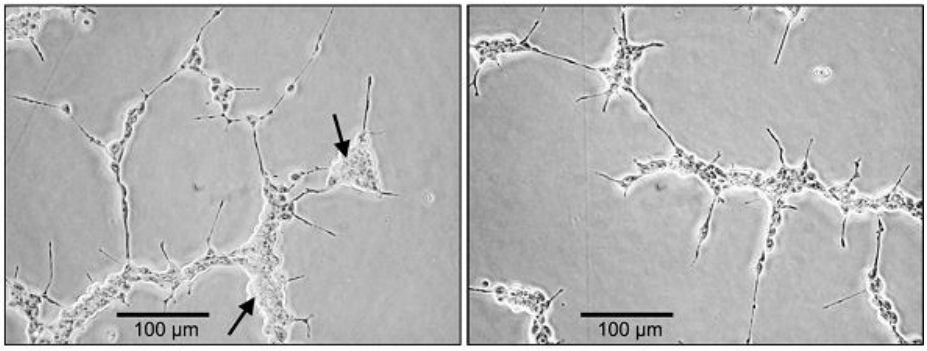

Figure 1. Generation of neurites by PC12 cells cultured on collagen in serum-free medium. Both cells and neurites seem to align along specific directions. Arrows indicate cell clusters, where cells look concentrated in high amounts. Observations were made using a phase-constrast optical microscope.

collagen layer on the clean glass is thin and characterized by a very low roughness (rms ≤ 3.5 nm). Here, we do not discuss neither the influence of chemical interactions between fibers and cells, nor the impact of serum deprivation from the culture medium on the initiation of neurites. Rather, we note the sensitivity of PC12 1.1 cells to the nanoroughness of the glass surface modified with collagen.

Although our previous results led us to neglect the roughness effects on neuritogenesis [15], it seems, at least in this work, that nanoroughness is able to influence cell morphology and that of the associated neurites. Surface roughness is characterized by the rms, an amplitude parameter which indicates vertical deviations of the roughness profile, as well as by morphological considerations. In other words, surfaces sharing the same rms value can exhibit different roughness characteristics, provided they display diverse topographies. PC12 cells grown on surfaces with diverse rms values (0.3 nm ≤ rms ≤ 1.4 nm) were not sensitive to rms variations [15], where topographical heterogeneities were evenly distributed all along the substrate. However, here the substrates considered display specific morphological features (i.e. the arrangement and the diameters of collagen fibers considered at the nanometer scale) which impact on cell and neurite growth [25], are presumably more important than the rms value.

3.2. Cell culture on glass-PLL and on glass-PLO substrates

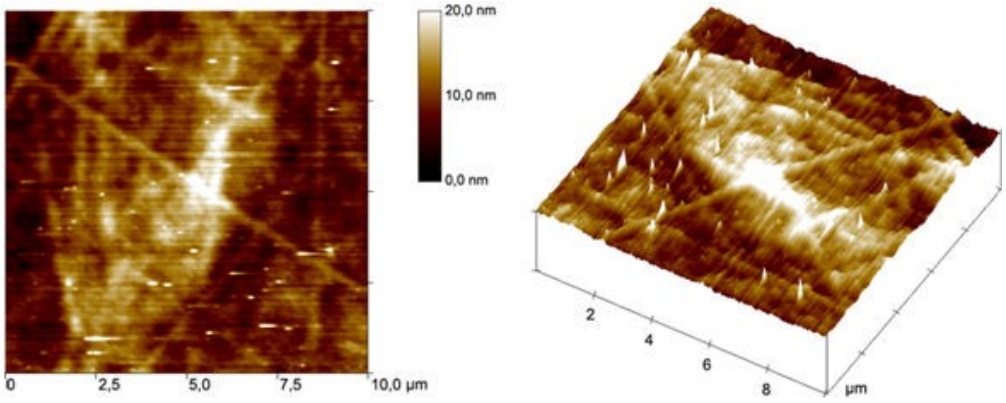

Figure 2. AFM images of collagen fibers adsorbed on clean glass. The root-mean-square (rms) roughness, evaluated by the AFM software Nanoscope, is roughly equal to 3.5 nm.

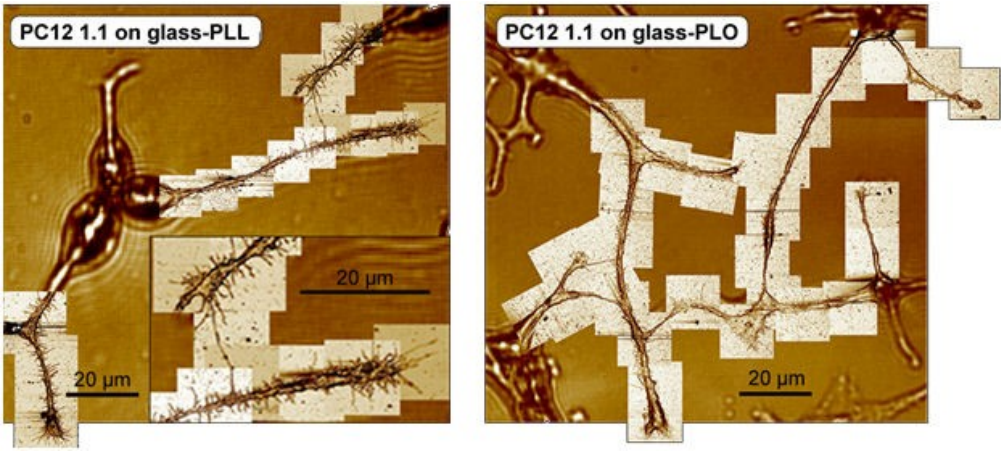

Figure 3. Generation of neurites after 6 days of culture by PC12 1.1 cells grown on glass-PLL and glass-PLO, in a serum-deprived and NGF-free medium. The images presented here display pictures taken with a phase-contrast optical microscope, combined with an assembly of images obtained by air-tapping mode AFM, after the cells have been fixed on the substrates using a solution of 2% glutaraldehyde in PBS.

Under the same conditions, as with collagen matrix, PC12 1.1 cells grow neurites on both PLL and PLO-coated glass surfaces (Figure 3) although these surfaces do not stimulate spontaneous neuritogenesis in PC12 cells [14, 15]. It has been shown that a substrate coated with PLL allows survival and maturation of primary neurons in serum-free medium [26]. According to the results presented above, serum deprivation of the culture medium is expected to trigger neuritogenesis. Nevertheless, it is possible that the progressive transformations (essentially due to different kinetics of proteins adsorption/desorption) experienced by the PLL/PLO matrices generate local gradients in energy of adhesion in these matrices, which could in turn stimulate neuritogenesis [14, 15], as well as serum deprivation. The deterioration of the matrix created by biomolecules that do not form covalent chemical bonds with the underlying substrate is observed in cultures that extend beyond three days [22, 23].

AFM imaging reveals the intense activity of filopodia and lamellipodia in growth cones (Figure 4, thick arrows) and all along the neurites (Figure 4, thin arrows). This observation indicates that the detection of the surrounding environment does not occur solely through the growth cone, in agreement with other reports [27-29]. Clearly, the initiation of branching

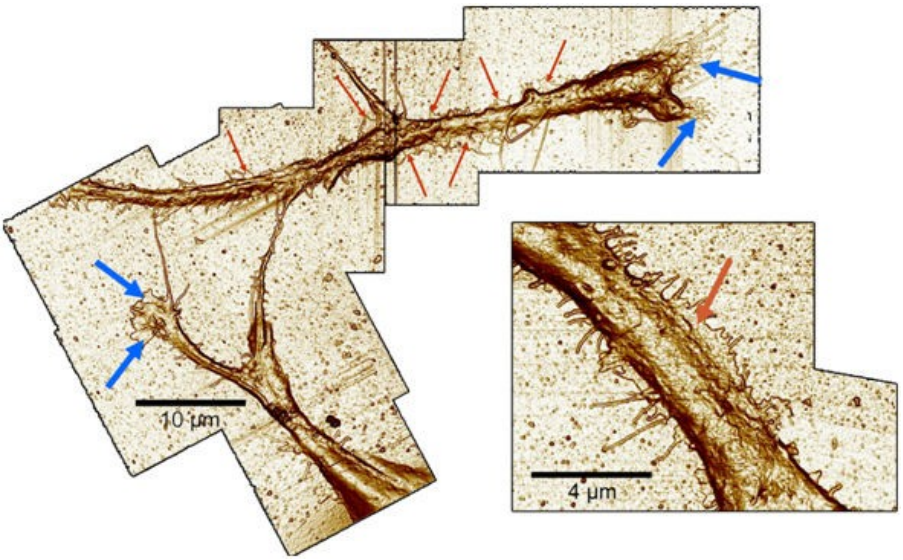

Figure 4. AFM images of filopodia and lamellipodia observed along the body of neurites on glass-PLO. The arrows indicate the presence of lamellipodia emerging from the growth cone (thick arrows) or from the body of neurites (thin arrows).

neurites and the diffusion of signaling molecules can also occur from the body of the neurites. Both PLL and PLO are assumed to coat a clean glass surface homogeneously. On a glass coverslip, these biomolecules will form thin monolayers of copolymers that are stabilized by steric interactions between their lateral amino-acid chains. Consequently, surface energy distribution of both PLL and PLO-coated glass is assumed to be highly homogeneous. Though, these substrates significantly differ because the PLL amino-acid chain is positively charged, while PLO amino-acid chain is not. Seeded on a PLO substrate, PC12 cells extend neurites which display lamellipodia (Figure 4) as opposed to neurites, including growth cones, initiated on a PLL substrate (Figure 3, inset box). Therefore, it might be inferred that the positively charged amino-acid group (NH_3^+) does not favor lamellipodia formation.

4. Conclusions

In this work we showed the importance of the characteristics of the substrate on the morphology of both the cells aggregates and of the initiated neurites. Indeed, on Poly-L-Lysine substrates, the structure of the lamellipodia is less developed than on Poly-L-Ornithine substrates. The neurites can get a smooth curvature; oppositely on collagen substrates the neurites follow the collagen fibrils, and cell bodies also regroup along these fibrils. The PLL surfaces are known to be positively charged, while PLO surfaces are not charged. This indicates that the surface charge can be responsible for the retention of the lamellipodia on PLL and the absence of the charge is favorable to the development of the lamellipodia.

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