MORPHOLOGICAL DIFFERENTIATION INDUCED BY GROWING SUBSTRATE AND SERUM DEPRIVATION ON OLN-93 CELLS

MIHAELA TEMELIE, MIHAELA BACALUM, C.C. MUSTĂCIOSU, M. RADU

"Horia Hulubei" National Institute of Physics and Nuclear Engineering – IFIN HH,
30, Reactorului St, P.O.BOX MG-6, Bucharest – Măgurele, Romania

Abstract. OLN-93 is a rat cell line frequently used as a model for oligodendrocytes, the glial cells which form the myelin sheath in the central nervous system. In this study we analyzed the morphological changes induced by serum deprivation and growing substrate on OLN-93 cells. Under normal growth conditions, in media with 10% fetal bovine serum (FBS) the cells have a bipolar, elongated morphology. At 1% FBS, OLNs adopt a more arborized morphology. The substrate also proved to have an important role, inducing morphological changes on OLN-93s even when the cells are grown at 10% FBS. On poly-lysine the proportion of highly branched cells increases and appears also a population of very large, flattened cells; the amount of branched cells is even higher on extracellular matrix (ECM). These changes suggest that ECM gel can be used as a better growing substrate for differentiation of OLN-93 cells, compared to poly-lysine. Combining substrate influence with serum deprivation provides an improved method to induce morphological differentiation of OLN-93 cells.

Key words: oligodendrocytes, OLN – 93, differentiation, morphology, ECM, poly-D-lysine.

INTRODUCTION

Myelination is a key process in the development of the nervous system, the myelin having a crucial role for the chemical and mechanical protection of the axons and for action potential propagation. The myelin sheet is formed by wrapping of glial cells around axons: the Schwann cells in the peripheral nervous system, respectively oligodendrocytes in the central nervous system. Demyelination disables saltatory conduction and leads to loss of neural functions, the unwrapped axons becoming at the same time more vulnerable to environmental stressors. Damages on the myelin sheet represent the cause of many degenerative diseases of the nervous system such as multiple sclerosis [18], Alzheimer [14], Parkinson [18].

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In multiple sclerosis demyelination is produced by extensive destruction of oligodendrocytes. Failure to remyelinate occurs at least partially because of the inability of precursor cells to differentiate into mature myelinating oligodendrocytes [20].

Oligodendrocytes are generated from early precursor cells (OPCs), cells with a simple bipolar morphology. The process of oligodendrocytes maturation involves several steps beginning with the OPC that develop into pre-oligodendrocyte, a simple multipolar mytotically active cell; the next stage is the immature oligodendrocyte distinguished by a progressive more complex morphology, followed by the premyelination oligodendrocyte and the final stage of mature myelination oligodendrocyte [2].

During maturation of OPC the cells extend a complex arborization required for the contact with the axons. After the contact with the neuron, oligodendrocytes form multiple wraps isolating the axon [3].

OLN-93 represents an immortalized cell line used as a model for oligodendrocyte, derived from spontaneously transformed cells in primary rat glial cultures. Under normal growth conditions, in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) most of the cells are bipolar, elongated cells [13] phenotype that corresponds to the OPCs [3]. In media with low serum concentration (FBS 0.5%), the cells slow the proliferation and expressed differentiation-like morphology, showing a more pronounced arborization of the cellular processes [19].

The growing substrate appears to have an important role in cell behavior, particularly in cells adhesion and differentiation. In many studies poly-lysine is used to increase the adherence of the cells in *in vitro* cultures [10] and has proved to stimulate morphological chances in OLN-93 cells [13].

Extracellular matrix (ECM) contains secreted molecules like laminin, type IV collagen, heparan sulfate proteoglycan [5]. The components of the ECM are implicated in cell signaling and differentiation. Even if the mechanisms are not fully understood, it seems that the functional interaction between laminin and the integrins expressed by oligodendrocytes have an important role in this process and changes in the ECM constituents are reflected by alterations in oligodendrocyte integrin expression [11]. The ECM gel has been used *in vitro* to increase cell adherence [6] or to promote differentiation in different types of cells – neuroblastoma (SH-SY5Y) [1], PC12 [15], adenoid cystic carcinoma [9], human salivary gland cell line (HSG) [17].

In the present study we tested the combined effect of serum deprivation and growth substrate – poly-lysine or ECM – on the morphology of OLN-93 cells.

MATERIALS AND METHODS

CELL CULTURES

The OLN-93 cells were provided as a gift from Prof. Marcel Ameloot (University of Hasselt, Belgium), with the agreement of Dr. C. Richter-Landsberg (Department of Biology, Molecular Neurobiology, University of Oldenburg, Germany). The cells were cultured in DMEM (Biochrom, Germany), supplemented with 10% FBS (Biochrom, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Biochrom, Germany), in a humidified incubator at 37 °C, 5% CO_2.

To promote differentiation, cells were seeded on round glass coverslips (10 mm), coverslips coated with poly-D-lysine (Sigma-Aldrich, Germany) or coverslips coated with ECM (Sigma-Aldrich, Germany), in DMEM with 1% FBS; as a control the cells were cultivated on the same growth substrates, but in DMEM with 10% FBS.

Poly-lysine coating: the coverslips were covered with 60 µL of poly-D-lysine (40µg/mL) and incubated at room temperature for 1 hour. The excess of poly-lysine was then removed, and the glass slides were left 10 minutes in the sterile hood to completely dry.

ECM coating: the coverslips were covered with ECM (0.24 mg/mL) diluted in cold DMEM without serum. 60 µL of this solution was added on each coverslip. The slides were incubated 2 h at room temperature, then the excess of ECM was removed, and the coverslips were left 10 minutes to dry.

The cells were seeded at a density of 500 cells per 10 mm coverslip, and left overnight for attachment. The next day the medium was replaced with DMEM 1% FBS, or renewed with DMEM 10% FBS, for controls. The medium was renewed after three days.

PHALLOIDIN STAINING

At 6 days after the first medium replacement, the cells were stained with phalloidin-FITC (Phalloidin, Fluorescein Isothiocyanate Labeled, Sigma-Aldrich, USA) a fungal toxin that binds the actin filaments of the cytoskeleton. The cells were gently washed with PBS (5 min, three times), fixed with 3% paraformaldehyde solution in PBS (5 minutes), washed in PBS, permeabilised with 0.1% Triton X 100 in PBS, washed, stained with a solution of phalloidin-FITC 40 µg/ml in PBS for 1 h at room temperature. The slides were then washed and mounted on glass slides with antifade mounting medium (ProLong Gold Antifade Reagent, Invitrogen, USA), and sealed with nail polish.

MORPHOLOGICAL ANALYSIS

Images showing the phalloidin binding to the F-actin fibers were acquired on a fluorescence microscope (Olympus IX 71, Germany) equipped with a 20X objective, and a CCD camera (iXON EM+ DU-897 E-CSO-UVB, Andor Technology, Ireland). The images were then analyzed with ImageJ software (*http://www.rsbweb.nih.gov/ij/*). The length of the processes was measured with the segmented line tool, and the area of the cell body using the polygon selection tool. The program was giving these data in pixels, so we made a conversion to micrometers or square micrometers, using a conversion factor that we calculated using a picture of a calibrated grid, taken in the same conditions.

Based on the morphological details the cells were classified into 5 categories: first category – cells with more than 4 branches, and frequently also with secondary branches, forming a dense network around cell body; second category – branched cells, with 4 or more branches, but without secondary branches; compared to first category the network is less dense; category 3 – round cell body, with a few processes (usually tripolar cells); category 4 – bipolar elongated cells; category 5 – flattened cells, with a very large cell body and few branches or none (Fig. 1).

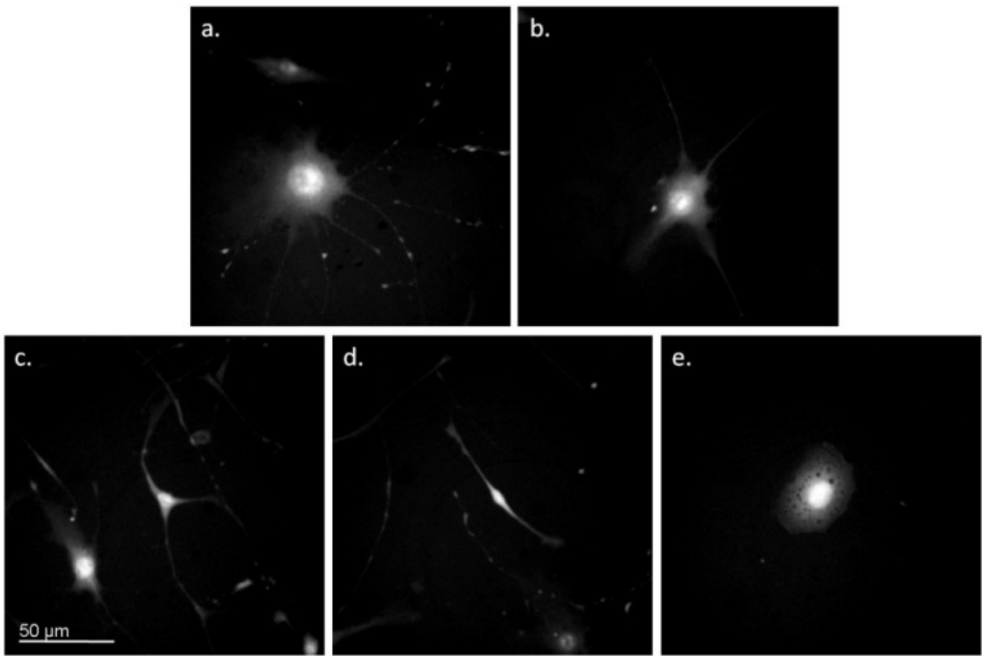

Fig. 1. Typical cell for each of the categories described based on the cellular morphology; a. category 1; b. category 2; c. category 3; d. category 4; e. category 5.

We analyzed at least 200 cells for each growth condition. For each cell we measured the length of the processes and the area of the cells body, at the same time classifying the cells according to the categories described above. We computed the means of the cell body area and of the processes length for each of the growth conditions, and the standard error of the mean; to establish if the differences between the growing conditions are statistically significant we performed the Student's t test.

RESULTS

In this study we analyzed the morphological changes induced by serum deprivation and growing substrate on OLN-93 cells. After the medium was changed from DMEM 10% FBS to DMEM 1% FBS the morphology of the cells started to modify. Considering the categories of cellular morphology presented above, on each growing variant a distribution of cells can be described given information about the ability of substrate to induce the evolution of OLNs from non-differentiated bipolar state to more specialized arborized morphology.

CATEGORY DISTRIBUTION

Under the usual growing conditions (on plastic or glass, with DMEM 10% FBS medium), most of the OLN-93 cells show an elongated, bipolar morphology corresponding to the 4th category described above; there can be also observed cells from the 5th category, very flattened, with few or no branches. As shown in Fig. 2, in these growing conditions none of the analyzed cells could be classified as belonging to category 1 (highly branched cells). In the serum deprived media (1% FBS) we also noticed that the proliferation of the cells is highly reduced, and after more than 7 days the cells become rounded, and the branches start to be thinner, showing early signs of apoptosis. This process was also observed by other authors, describing that after 48 hours in a medium with 0.5% FBS the cells start losing the processes and eventually die [16].

At 6 days of serum deprivation (DMEM 1% FBS) we may notice that the amount of branched cells (category 1–3) flattened cells (category 5) are increasing, and the proportion of bipolar cells is lowering (Fig. 3). The influence of the substrate is visible both on 1% FBS media, and also on DMEM 10% FBS.

On poly-D-lysine, at 10% FBS the proportion of cells from category 4 decreased compared to control and most of the cells belong to category 5. The amount of cells from the first 3 categories is also increased. On ECM the differences from the control are even higher, the proportion of cells from category 4 is decreasing even more, category 5 including the majority of cells. The third category also gains a larger proportion of cells. At 1% FBS, on poly-lysine most of the cells are flattened cells that belong to category 5; the proportion of bipolar cells

is lower compared to control, and the amount of branched cells is increased. The differences can be also observed if we compare the distribution on categories with the same substrate at 10% FBS.

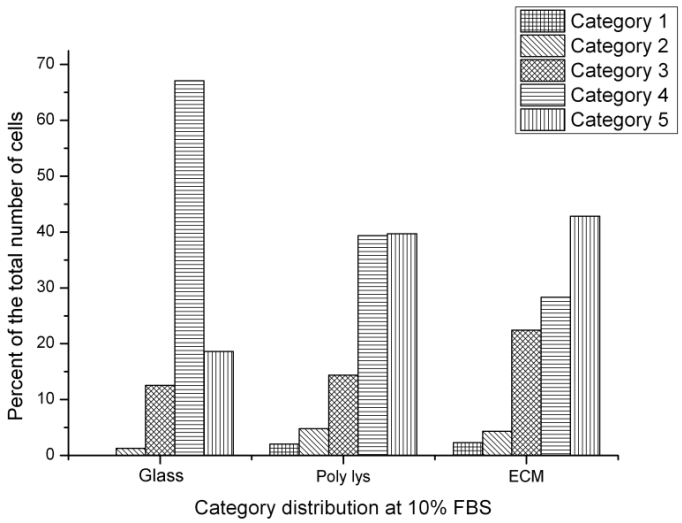

Fig. 2. Category distribution at 10% FBS on glass coverslips, poly-lysine or ECM coated coverslips.

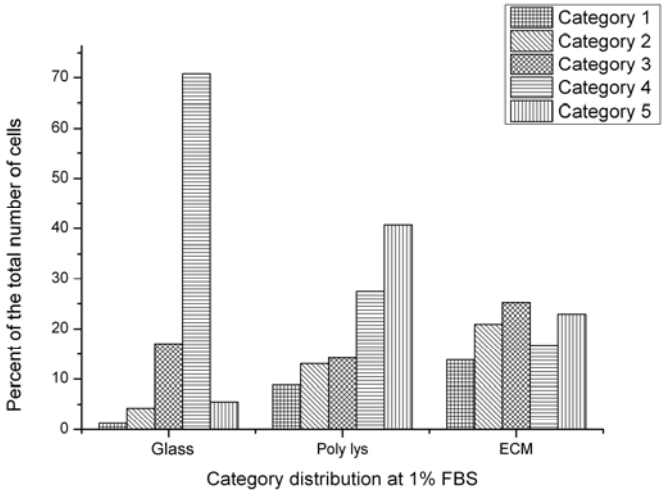

Fig. 3. Category distribution at 1% FBS on glass coverslips, poly-lysine or ECM coated coverslips.

On ECM at 1% FBS the amount of cells in categories 4 and 5 decreases, and proportion of cells in categories 1, 2 and 3, corresponding to arborized cells with long branches, is increased, the differences being notable both compared to the other growing substrates, or to the same substrate, but at 10% FBS.

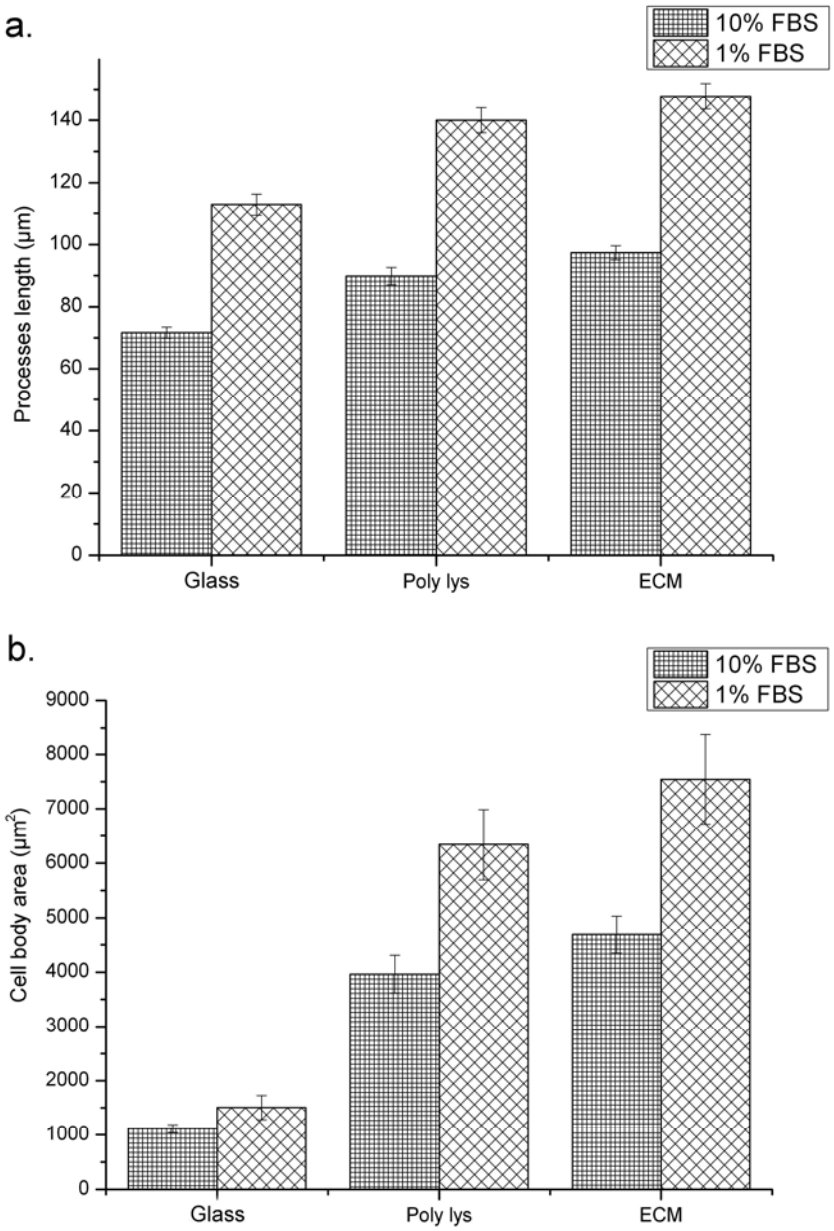

Fig. 4. a. Processes length; b. Cell body area. The means are calculated for at least 200 cells per growing variant, including cells from all the categories; bars represent ±SEM; Student's t-test revealed that the differences are statistically significant between the groups of cells analyzed from the different substrates, but also when we compared each of the variants at 1% FBS with the corresponding one at 10% FBS ($p < 0.001$).

PROCESSES LENGTH AND CELL BODY AREA

In the analysis of the images in ImageJ, we measured the cell body area, and the length of the cell processes, and we calculated the mean and the standard error for at least 200 cells distributed in all of the described categories. The length of the processes and the area of the cell body tend to increase when OLN-93 cells were cultured on poly-lysine, and even more when cultured on ECM coated coverslips, correlated with the changes in the morphology of the cells (Fig. 4). The differences are even more notable after the medium was changed from 10% FBS to 1% FBS.

DISCUSSION

In this study we investigated the morphological changes induced on OLN-93 cells when cultivated in low serum conditions, on different growing substrates. We choose as substrate glass coverslips as control, coverslips coated with poly-lysine, previously used in similar studies [4, 10], and coverslips coated with ECM gel, that has proved to be efficient in differentiation of other types of cells [1, 9, 15, 17].

Culturing the cells in serum deprivation conditions promoted morphology changes on OLN-93. These changes may be correlated with the fact that fibronectin, one of components of the serum decreases the ability of developing oligodendrocytes to produce long, branched processes [7]. Fibronectin binds to oligodendroglial αv integrins and modulates OPCs proliferation and survival, but also processes retraction. Process restriction is mediated by focal adhesion kinase (FAK) signaling, in the presence of fibronectin, and may contribute to failed differentiation in disease state where fibronectin levels are high [8].

The morphological changes observed in our study, on OLN-93 cells, after 6 days of growing in media with 1% FBS, are similar with those previously described. For example, in the study of Buckinx *et al.* after switching the media to DMEM 0.5% FBS the cells change the morphology from simple bipolar cells becoming flatted cells with multiple processes [4].

Growing substrate also has an important role in cell evolution. Poly-lysine is a polyaminoacid with a net positive charge. Cells do not normally have specific receptors for poly-lysine, so it is believed that this poly-aminoacid enhances cell adhesion by electrostatic interaction with anionic sites of the plasma membrane. However, poly-lysine coating is able to induce short-term cell adhesion molecule (CAM) reorganization, but the distribution of these molecules is restored after longer exposure time [12]. When cultured on poly-lysine OLN cells tend to become flattened and showed a more pronounced arborization of the cellular processes, a morphology that resembles a more mature phase of oligodendrocytes.

Smolders *et al.* described four categories of OLN-93 cells, when cultured on poly-L-lysine, in media with 0.5% FCS: flat cells, highly branched with many fine arborizations (category 1), cells that have lost the fine arborization, but still display elaborate branching (category 2), cells that have lost the branched morphology and started to becomes tri- or bipolar (category 3) and tri- or bipolar cells with the soma rounded up (category 4). According to this classification most of the cells were category 2 cells [16]. We also found an important proportion of cells corresponding to this description, but most of the cells were flattened cells with a very large cell body, described as a separate category in our study (category 5).

ECM was used to promote attachment of cells and proved to improve cell attachment when compared to poly-lysine, laminin or fibronectin. For example, most of the spinal cord explants plated on poly-L-lysine remained detached; on laminin or fibronectin-coared coverslips 50–60% of the explants attached after 3 days of culture, while on ECM after 12 hours 95% of the explants were attached; in this case three days after the culture the explant also showed extended neurites up to 1000 μm [6].

ECM components interact with integrin receptors that mediate signaling to govern various cellular processes [11]. The ECM gel was used in differentiation studies on various types of cells. For differentiation of SH-SY5Y cells into neurons, culturing on ECM gel and addition of different growth factors induced a neuritic distribution and also biochemical features of mature neurons [1].

Oligodendrocyte differentiation and maturation are also regulated by integrin through different signaling pathways. FAK signaling mentioned earlier is also involved in morphological maturation, in the presence of laminin-2 [8]. Laminin-integrin interactions regulate both processes length and processes branching. For example, when laminin receptor α6β1 integrin, normally expressed in developing oligodendroglia is blocked the cells differentiate improperly and have smaller myelin sheets. The expression pattern of the integrins expressed by these cells is regulated by the developmental state, but also by the surrounding environment [11]. Laminin mediated differentiation also involves interactions with dystroglycans [7].

Based on previous studies reporting the efficiency of ECM gel on cell differentiation, we choose to test this product as a growing substrate for the OLN-93 cells. Grown on this substrate the cells become even more branched compared to poly-lysine, that was previously used in studies regarding OLN-93 differentiation [4, 16, 13]. The morphological differentiation is higher in low serum media, where on ECM most of the cells were category 3 cells, but ECM proved to have an important role in inducing changes on OLN-93 even at 10% FBS. In this condition, on glass coverslips about 70% of the cells were simple elongated cells, on poly-lysine about 40% of the cells being bipolar, while on ECM this category includes less than 30% of the cells.

ECM gel coating also had a better effect in increasing the cell body area and the processes length compared to poly-lysine, probably involving a better interaction between the cells and the substrate.

These changes suggest that ECM gel can be used as a better growing substrate for differentiation of OLN-93 cells. The results indicate that combining the effect of the ECM coating with serum deprivation provides an improved way to induce the morphological differentiation of these cells.

CONCLUSIONS

Serum deprivation and growing substrate promote morphological differentiation of OLN-93 cells. Growth on poly-lysine the proportion of highly branched cells increases but appears also a population of very large, flattened cells; the amount of branched cells is even higher on ECM, where the population of large unbranched cells is smaller indicating ECM as a better growing substrate for OLN differentiation under FBS deprivation conditions. These morphological transformations may be correlated with biochemical and functional differentiation, but in order to confirm this process an analysis of the expression of some biochemical markers of differentiation must be done.

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