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Characterization of PC12 cell proliferation and differentiation-stimulated by ECM adhesion proteins and neurotrophic factors

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Among the various elements which influence axonal outgrowth *in vivo* is the physicochemical interaction of actively outgrowing nerve fibers with the various substrata they encounter during differentiation. Several experiments have explored the role of the extracellular matrix (ECM) in the control of neuronal differentiation. The nature, however, of the interactions between neurons and components of the ECM during regeneration and development are largely a matter of speculation. Although previous studies have already explored the influence of a number of ECM adhesion proteins and neurotrophic factors on neurite outgrowth, none have been carried in a systematic approach that allows for the simultaneous comparison of different surface conditions in relation to different neurotrophic factors. Motivated by the necessity of establishing controlled environments that allow for the rational design of stable neuronal/biomaterial interfaces, the long-term effects of NGF and FGF-2 on the behavior of PC12 cells plated on collagen and laminin modified surfaces were evaluated. A pheochromocytoma cell line derived from transplantable rat adrenal medulla, PC12 cells have been commonly employed as an instructive model for studying the underlying mechanisms of neuronal differentiation.

Long-term characterization of PC12 proliferation and neuronal differentiation for an experimental duration of 7–22 days was achieved by both qualitatively and quantitatively assaying for cell count, neurite number, neurite mean length, and neurite stability. Neurite stability was determined in terms of resistance to loss after neurotrophic factor (NGF/FGF-2) withdrawal. The present findings demonstrate that ECM adhesion proteins collagen and laminin are equally effective in promoting PC12 proliferation. It was noted as well that NGF supplemented collagen cultures are significantly more efficient in providing long-term support to PC12 differentiation in terms of neurite number, mean length, and stability.

Introduction

Cellular growth and differentiation, in 2D cell cultures as well as in 3D space of developing tissues, require the presence of a structured environment with which the cells can interact [1]. The extracellular matrix (ECM) is composed of different macromolecules whose structure and function are critical to the maintenance of normal tissue architecture. Identified to exert a multitude of influences on cell activities including adherence, spreading, migration proliferation, and differentiation, a number of molecules such as ECM adhesion proteins and neurotrophic factors are identified with playing a central role in neurite outgrowth.

Collagen is the predominant component of mammalian connective tissue, accounting for approximately 30% of human body protein. At least 14 different forms are identified in vertebrate tissues, with each being dominant in a specific tissue type. The most abundant form is type I collagen, found in high concentrations in many tissues such as tendon, skin, bone, and fascia [2]. Because of its abundance and its unique physical and biological properties, type I collagen has been used extensively in the formulation of biomedical materials [3, 4]. Due to its phylogenetically well-conserved primary sequence and its helical structure, collagen is found mildly immunoreactive [5, 6].

Laminin (LN), a heterodimeric glycoprotein predominantly found in tissue basement membranes has been shown to play a pivotal role in cell proliferation, differentiation, migration, and death [7]. Laminin is recognized for its role in induction and maintenance of cell differentiation and in control of cell proliferation [8].

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In vitro evidence implies a neurite outgrowth promoting effect for laminin [9]. Likewise, in the central nervous system (CNS), laminin has been shown to mediate neuronal differentiation, migration, and regeneration, characterized by neurite formation and extension [7].

Neurite promoting factors are agents that stimulate fiber outgrowth from neurons and are often involved in both neuronal maintenance and differentiation [10]. Nerve growth factor (NGF) holds a unique place in the study of those substances, as it was the first to be identified and characterized at the molecular level [11]. Although NGF was originally named a growth factor, there is very limited evidence that it acts as a mitogen [12]. Two types of NGF proteins isolated from mouse submandibular gland, the traditional source of NGF purification, are high molecular weight 7S NGF complex and low molecular weight β NGF or 2.5S NGF [13]. NGF is identified with its property in directing outgrowing or regenerating axons of sensory and sympathetic neurites along its concentration gradient [12]. The neurotrophic family, particularly NGF has been extensively used in the study of their effects on neurodegenerative diseases of the central and peripheral nervous systems (PNS). Examples of such diseases are Parkinson's [14], Alzheimer's [15], Huntington's [16], and memory disorders.

Originally determined as peptides containing mitogenic activity for fibroblasts, the fibroblast growth factor (FGF) family is now identified as not limited to a single target cell or one biological activity as might be inferred. Compatible with their wide cross species distribution, both basic FGF (FGF-2) and acidic FGF (FGF-1) have a broad range spectrum of activity [17]. Found to promote both the survival and differentiation of nerve cells derived from either the hippocampal region or the cortex, FGF-2 is hypothesized to play a role in the early development of the nervous system [18]. The effect of FGF-2 on cell differentiation is likely due to its ability to control the synthesis and deposition of various ECM components that are known to affect cell surface polarity and gene expression. These ECM components include collagen, fibronectin and proteoglycans [19].

PC12 is a pheochromocytoma cell line that can be stimulated to differentiate into sympathetic-like neurons in the presence of neurotrophic factors. Originally developed by Greene and Tischler in 1976, PC12 cells derived from transplantable rat adrenal medulla have been commonly employed as an instructive model for studying the underlying mechanisms of neuronal differentiation. When cultured in serum containing medium, PC12 cells exhibit several of the phenotypic properties associated with adrenal chromaffin cells and their neoplastic counterparts, pheochromocytoma. In the presence of serum, PC12 cells adopt a round morphology, and proliferate to high density. In the presence of neurotrophic factors, however, PC12 cells undergo dramatic changes in phenotype as they acquire many of the properties characteristic of sympathetic neurons. The cells cease division, sprout neurites, and become electrically excitable [20].

In this report, a systematic approach was designed to simultaneously investigate the behavior of PC12 cells on different adhesion substrata in relation to different neurotrophic factor conditions. Long-term characterization of PC12 proliferation and neuronal differentiation for an experimental duration of 7–22 days was achieved by both qualitatively and quantitatively assaying for cell count, neurite number, mean length, and stability.

Materials and methods

Tissue culture

Three sets, each containing $(n = 3)$ $100 \times 20\,\text{mm}^2$ dishes of different culture conditions were prepared. Each set comprised of tissue culture grade polystyrene dishes, polystyrene dishes with a uniform application of rat tail collagen type I and polystyrene dishes with a uniform application of mouse laminin (Becton Dickinson, Franklin Lakes, NJ). Within each set, the $(n = 3)$ dishes contained RPMI1640 + 10% FBS (complete growth medium), complete growth medium containing 50 ng/ml 2.5S mouse NGF (Alomone Labs, Jerusalem) and complete growth medium containing 5 ng/ml recombinant human fibroblast growth factor basic (FGF-2) solution respectively (Research Diagnostics, Flanders, NJ). PC12 cells (ATCC, Manassas, VA) passages 3–5 were plated at a density of $1–2 \times 10^6$ cells, maintained at 37 °C in 5% CO_2 and re-fed every 2–3 days.

Cell proliferation assay

The measurement of change in number of undifferentiated PC12 cells attached to the non-neurotrophic factor supplied dishes was determined at days 1, 3, 5, and 7. After the designated incubation times, the culture surfaces were rinsed twice with phosphate buffered saline (PBS) to remove loosely adherent cells. PC12 cells were then detached by washing the culture surface several times with fresh complete medium. The PC12 cell suspension was then centrifuged at 850 rpm for 5 min. PC12 single cell suspensions were then counted twice with the use of a hemacytometer.

Image analysis

Neurite samples chosen for the number, length, and stability assay measurements were those extending from single cells and from aggregates present within the field of view. Five randomly chosen visual fields with at least 200 randomly selected cells were scored using an Olympus Oly-750 digital camera mounted on an Olympus BX60 image analysis microscope system (Melville, NY). Using Image Pro Plus software, PC12 neurite number and mean length assays were quantified every other day for 14 days, with mean lengths determined by an average end to end measurement of at least 30 random neurites. On day 14, the neurotrophic factor treated dishes were washed out several times and re-fed with complete media in absence of the added neurotrophic factors. Long-term maintenance of neurite stability subsequent to neurotrophic factor withdrawal was assayed by measuring the retraction in neurite lengths over an additional 8-day experimental duration, with quantification again taking place at two day timed intervals.

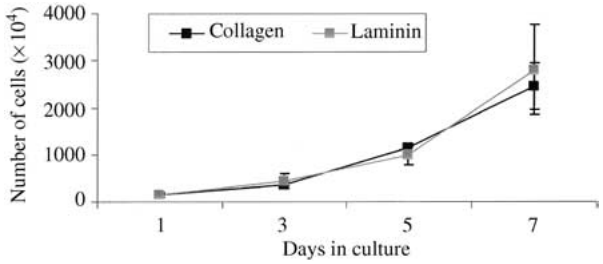

Figure 1 PC12 proliferation on ECM adhesion proteins collagen and laminin in absence of neurotrophic factors. Reported are average values of experiments performed with 3 × standard deviations.

A paired two sample for means *t*-test was employed, with $n = 5$ and 4 degrees of freedom. A 95% confidence interval was used to determine statistically significant differences, with a *p*-value less than 0.05 considered statistically significant.

Results

PC12 proliferation

Fig. 1 shows the change in number of adherent PC12 cells onto the collagen and laminin coated dishes, determined at days 1, 3, 5, and 7, due to cell proliferation, detachment and/or death. Reported are average values of duplicate $(n = 3)$ trials with standard deviations per dish. PC12 cells were found to attach more readily and efficiently to the ECM proteins collagen and laminin than to polystyrene (not shown), causing an exponential increase in PC12 cell number. Exponential regression analysis revealed proliferation equations of $y = 641114e^{0.9234x}$ with an R value of 0.997 and $y = 665809e^{0.9259x}$ with an R value of 0.999 for collagen and laminin, respectively. There was no statistically significant difference in terms of PC12 proliferation between the collagen and laminin coatings as they both appeared equally effective in promoting PC12 attachment. The polystyrene (control) substrates failed to support PC12 attachment and proliferation as cells continuously detached from the surface (not shown).

Neurite number assay

PC12 cells cultured on collagen and laminin plates began to extend neurites within only 48 h of treatment with neurotrophic factors NGF and FGF-2. Differentiation was absent from within the non-trophic factor-supplemented collagen and laminin dishes (data not shown). After 14 days of PC12 culture under differentiation supportive conditions, Image Analysis revealed a statistically significant increase in the number of PC12 neurite outgrowths observed within NGF supplemented Collagen (Fig. 2). The greatest linear increase (slope = 20) in neurite extension number occurred under NGF supplemented collagen culture. There was a smaller linear increase in the number of neurites over the first 12 days in both the FGF-2 supplemented collagen (slope = 4) and NGF supplemented laminin (slope = 5) cultures (Fig. 2). Collagen + NGF reached a maximum at day 12, after which there was a minor decline suggesting reaching plateau (Fig. 2). Over the duration of the experiment, collagen + FGF-2 and laminin + NGF plated PC12 cells showed a slight increase in the number of neurite extensions, yet no statistical significance could be attributed to this apparent increase (Fig. 2). There was no observed increase in the number of neurite extensions on laminin + FGF-2 plated PC12 cells. In fact, visual analysis showed a slight decrease in the number of neurite extensions associated with laminin + FGF-2 plated PC12 cells (Fig. 2). Day 14 experienced a statistically significant $(p < 0.01)$ difference between collagen + NGF and all other culture conditions (Fig. 2). As the polystyrene dishes failed to support PC12 attachment, no differentiation was observed.

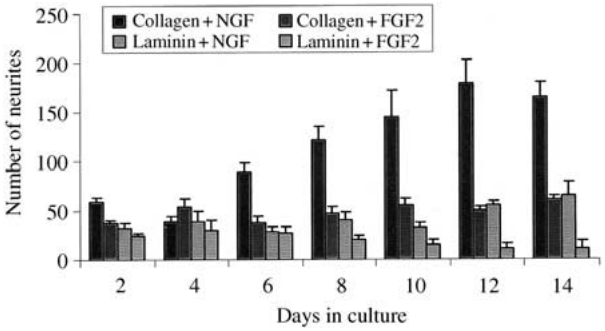

Figure 2 Time course for the appearance of neurites in cultures of PC12 cells following treatment with NGF and FGF-2.

Neurite length assay

The mean length of PC12 neurite extension was quantified using Image Analysis. Overall, there was an increase in the mean length of neurite extensions for three of the four culture conditions. Collagen supplemented NGF $(p < 0.01)$, and FGF-2 $(p < 0.05)$ and laminin supplemented NGF $(p < 0.01)$ dishes displayed a significant increase in mean neurite length at day 14 (Fig. 3). Except for laminin + FGF-2 $(p < 0.05)$, there was no statistically significant difference in terms of mean neurite length between collagen + NGF and all other dishes. Between days 10 and 12, all dishes experienced a maximum of neurite length, followed by a slight decline by day 14 (Fig. 3). Taken together with the data acquired from the neurite length assay, collagen plated dishes supplemented with NGF had the most substantial effect on the differentiation of PC12 cells (Fig. 3).

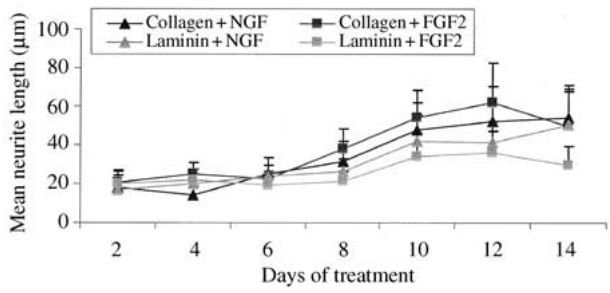

Figure 3 Time course for neurite elongation in cultures of PC12 cells following treatment with NGF and FGF-2.

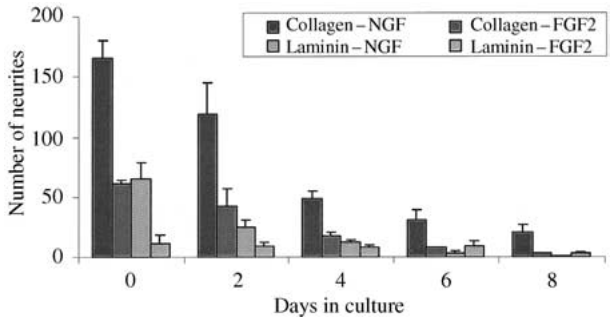

Figure 4 Stability of PC12 neurites following treatment with neurotrophic factors NGF and FGF-2 for 14 days and subsequent removal of these factors. (Notice: Day 0 coincides with day 14 in Fig. 2).

Neurite stability assay

Following treatment with neurotrophic factors NGF and FGF-2 for 14 days and subsequent removal of these factors, all PC12 cultures experienced a temporary neurite outgrowth, marked by a considerable reduction in neurite outgrowth number over an 8-day period. All cultures, except for FGF-2 supplemented laminin, experienced an exponential decay in the stability of neurite extensions (Fig. 4). FGF-2 supplemented laminin also showed decay in neurite stability, yet both the size and rate of decay were insignificant considering the lack of neurite outgrowths initially produced (Fig. 4). Cells cultured on collagen plates previously treated with NGF maintained the highest stability as they sustained the greatest number of neurite outgrowths in absence of the added neurotrophic factor (Fig. 4). The greatest decline in neurite stability appeared to take place within the first 4 days of culture, after which maintenance of neurite stability seemed steady.

Discussion

The effective attachment and exponential increase in PC12 cell number on the ECM proteins collagen and laminin coated substrates are attributed in part to the cell binding motif Arg–Gly–Asp (RGD) contained in collagen and laminin, resulting in covalent, ionic, and/or hydrophobic interactions at the cell–surface interface [21, 22]. Comparably, the poor attachment of PC12 cells to polystyrene is consistent with other findings, where PC12 cells seeded to plastic have been observed to grow mostly as floating cell aggregates [23]. Some PC12 strains exhibit short extensions of only a few microns prior to the onset of differentiation. Other groups using those PC12 cell lines have therefore been inclined to consider extensions as neurites only if longer in length than twice the cell body diameter [24]. Alternatively, we used a PC12 cell line that was rounded and exhibited no extensions prior to the onset of differentiation. Consequently, we adopted a technique in which all extensions from representative aggregates are regarded as countable neurite outgrowths [25]. The role of cell substratum adhesion in the initiation, elongation, and branching of neurons has been the focus of numerous studies [26, 27]. Our study showed that PC12 cells preferably differentiated on collagen as compared to laminin modified substratum. This result is consistent with other findings in which the mean neurite length of PC12 cell suspensions in collagen gels at 4 days of exposure to NGF did not deviate significantly from collagen gels modified with laminin, indicating that laminin did not contribute considerably to the neurite outgrowth process [28]. A different study, however, carried by culturing Dorsal Root Ganglion (DRG) cells has shown it to grow extensive networks of fibers on substrata containing fibronectin, collagen (types I, III, IV), and especially laminin. The same study has shown DRG cells to extend neurites in absence of NGF only on laminin as opposed to fibronectin, collagen, or poly-lysine-based substrates [29]. Although marked differences in neuronal response are suggested by the studies mentioned above, it should be kept in mind that variations in cell type make cross study comparisons difficult to evaluate.

A study by Rydel *et al*. noted that the only responses for which b-FGF and NGF consistently showed quantitative differences where in the rates of neurite initiation and elongation. Furthermore, the network of outgrowth elicited by NGF at any given time of treatment was always of greater density [30].

Another study that examined the interactions of PC12 cells with ECM proteins found that the cells adhered readily to laminin or collagen IV but poorly to fibronectin. The ability of PC12 cells to attach to laminin or collagen IV was observed as concentration dependent [31].

In this study, the presence of NGF did not only prove essential to the onset of differentiation, but also to the survival and maintained stability of neurite outgrowth. A variety of cells, including PC12 cells have been reported to produce NGF like substances [32], raising the possibility that the demonstrated neurite stability in the absence of NGF may be mediated by an endogenous source [33]. In our study, the presence of NGF proved essential for the onset of differentiation as well as for the survival and maintained elongation of neurite outgrowth.

Conclusions

The realization that the ECM is important in the regulation of cellular shape, fate, and metabolism and consequently tissue and organ structure and function, has led to need for a detailed understanding of the components of the ECM and their interactions with each other [1]. The findings reported in this paper are of importance in establishing optimal conditions for maintenance of differentiated PC12 cells which have been extensively used as a model for understanding the physiology of neurodegenerative diseases, particularly Parkinson's and Alzheimer's. Moreover, such knowledge is critical for the development of stable biomaterial substrates and neural tissue engineering constructs. In an attempt to examine the interaction between the various components present in the ECM in combinations which may be reflective of their association *in vivo*, we have undertaken to evaluate the long-term effect of nerve growth factor and fibroblast growth factor basic on the differentiation of PC12 cells plated on collagen- and laminin-coated surfaces.

Long-term characterization of PC12 proliferation and

neuronal differentiation for an experimental duration of 7–22 days was achieved by both qualitatively and quantitatively assaying for cell count and neurite number, mean length, and stability. The present findings demonstrate the interaction of PC12 cells with multiple ECM constituents with the inference that NGF supplemented collagen cultures are significantly more efficient in providing long-term support to PC12 differentiation in terms of neurite number, mean length, and stability.

Acknowledgments

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