Effects of NGF on PC12 Neurite Growth

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***Abstract*— Pheochromocytoma cells, or PC12s, are a cell line used for neuroscience research. Even though these cells are derived from a rat adrenal medulla tumor, these cells mimic and behave similar to neurons. To further model these cells as neurons, PC12 are treated with nerve growth factor, or NGF. This process transforms the PC12s to differentiated PC12 which alters the cells morphologically to increase the number of neurite and length of neurites. Studies have use either 1% of 50 ng/mL or 100 ng/mL in the differentiation media, yet no study has tested varying densities. In this study, PC12 cells were cultured with varying concentration to determine what concentration led to the longest neurites.**

Keywords—PC12, differentiation, NGF, neurons

I. BACKGROUND

Different types of animal models are used for neurotoxicity research. Animal models are derived from the sacrifice of the animals for in vitro research. Sacrificing animals for research pose an ethical question, have high cost and labor, and often produce vague [7]. To combat this dilemma, a single cell clonal line was derived by Greene and Tischler in 1976 from a pheochromocytoma rat tumor [1]. The advantage of this cell line is its versatility, ease of culture, extensive background knowledge on proliferation and differentiation, and response to growth factors [6]. PC12s cultured without growth factors morphologically, physiologically, and biochemically behave similar to adrenal chromaffin cells [5]. PC12s cultured with nerve growth factor (NGF) differentiate, or transform, into ganglion neurons morphically and functionally [2].

Synapsin I is a protein marker of synaptic communication expressed in differentiated PC12s. Das [1] demonstrated the level of the protein expression depends on the level of cell differentiation. As more PC12s become differentiated, functional synapses are formed between the cells and induce neurite outgrowth. The expression of synapsin I is contributing factor for synaptogenesis and neuroplasticity [8]. Differentiated PC12s physically alter in two ways: length of axon and dendrites. The axon is long, thin part of the neuron which transmit signals while dendrites are shorter, numerous specialized to receiver signals. Differentiation of PC12s increase the length of neurites, or processes from the cell body of the neuron that includes dendrites and axons.

There is not one specific method to differentiate PC12s. For example, Kudo [4] differentiated PC12s using temperature-controlled repeated thermal stimulation (TRTS). Plated cells were placed on heating plate at three different temperatures: 39.5 °C, 42.5 °C, 37.6 °C. The latter temperature was used for the control of the experiment. The cell cultures were thermally stimulated for 6, 12, and 18 hours a day at the previously mentioned temperatures. The neurite outgrowth of the thermally stimulated cells was compared to the control and cells differentiated by BMP4. BMP4 are bone morphogenetic proteins (BMP) similar to nerve growth factors (NGF) which stimulates PC12s to differentiate. As the thermal stimulation time increased, the neurite-bearing cells increased with the 18 h stimulation with the highest. The BMP and 18 h stimulation had similar neurite-bearing cells signifying repeated thermal stimulation is a valid method of differentiation for PC12s. [4]

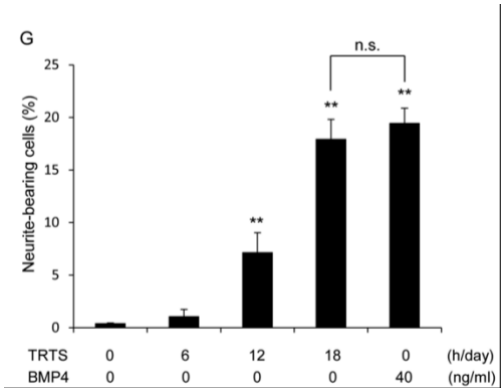

Fig. 1. Results of differentiation using temperature-controlled repeated stimulation [4]

Another method to differentiate PC12s is using piezoelectric materials. Piezoelectric materials generate electric charge as mechanical pressure is applied. Nanoparticles of piezoelectric material, boron nitride nanotubes (BNNTs), were used by Ciofani [3] to differentiate PC12s with ultrasound. A cell viability/cytotoxicity assay was performed to determine optimal density of BNNTs. BNNT did not induce significant oxidative stress to the PC12s even at high concentrations using immunofluorescent microscopy. This demonstrated the BNNTs did not adversely affect PC12s. PC12s cultured with BNNTs stimulated with ultrasound had higher neurite lengths compared to controls by 30%.

Another piezoelectric material used for differential is piezoelectric polymer polyvinylidene fluoride (PVDF). Hoop [9] used α-PVDF and β-PVDF, derivatives of PVDF. Both materials share the similar physical characteristics like crystallinity and Young's modulus, yet there is a key difference between both materials: dipole moments. α-PVDF contains no dipoles resulting in a nonpiezoelectric material unlike β-PVDF. The polymorph with highest piezoelectric property is β-PVDF. The nonpiezoelectric material was used to rule out surface chemistry influences and/or mechanical factors from differentiation. PC12s were cultured on α-PVDF, β-PVDF, and well plates with and without ultrasound stimulations for negative control. Ultrasound stimulation lasted for 10 minutes, five times a day. NGF induced differentiation cell cultures were used as positive control. Cells cultured in well plates (stimulated and unstimulated) and α-PVDF did not have neurites greater than 10 µm which was the threshold neurite consideration. Average neurites measured from the stimulated β-PVDF was 22.9 µm while

the average neurite measured from the NGF group was 34.5 µm. Even though the length of the neurites of the NGF differentiated cells was 1.5 times greater than the piezoelectric material, Hoop demonstrated the piezoelectric material is a viable option to differentiate PC12s. [9]

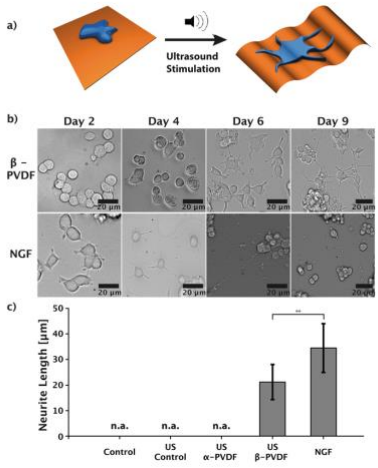

Fig. 2. Results of differentiation using piezoelectric material [9]

The previously mentioned studies used two concentrations of NGF: 50 or 100 ng/mL. From the studies reviewed, no specific study examines the neurite growth of varying NGF. The objective of this study to determine what concentration of NGF leads to the longest neurites.

II. Methods

A. Cell Line

PC12 cells were acquired from (American Type Culture Collection, Manassas, Virginia, USA Cat. No. CRL-1721) and stored at -80 °C. The cells were frozen in 2 ml microcentrifuge tubes containing 1.8 ml of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA Cat. No. 10569-010) and 0.2 mL of dimethyl sulfoxide (Thermo Fisher Scientific, Cat. No. D128-500). Frozen cells were defrosted and centrifuged for 4 mins at 900 RPM to form a pellet of cells. The supernatant was removed and replaced with fresh DMEM FULL media. Using a wide and small-bore transfer pipette, the pellet was broken and resuspended into the new media. Cells were plated at 20 x 10^3 cells/dish for the experiment.

B. Cell Culture Media

Two types of cell culture medias were for the experiments: DMEM FULL and differentiation media. Both cell culture media used DMEM for the base. DMEM supplemented with 10% horse serum (Atlanta Biological, Flowery Branch, Georgia, USA Cat. No. S12195H), 5% fetal bovine serum (Thermo Fisher Scientific, Cat. No. 10437-010), and 0.25% penicillin-streptomycin (Sigma-Aldrich, Saint Louis, MO, USA Cat. No. P4333) created the DMEM FULL culture media. Differentiation media contained DMEM supplemented with 1% horse serum, 1% penicillin-streptomycin, and 1% NGF (Alomone Labs, Jerusalem, Israel Cat. No. N-100). Concentration of the NGF was varied for the trails: 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 x 10^3 ng/mL, and 1 x 10^4 ng/mL. For the controls, the NGF portion of the differentiation media was replaced with 1x PBS. All medias were filtered through vacuum filters (Thermo Fisher Scientific Cat. No. S2GPU02RE) with a 0.22 µm pore size to ensure sterility of the media.

C. Cell Culture Plating

Cells were plated on non-coated 35 mm petri dishes (CELLTREAT, Pepperell, Massachusetts, USA Cat. No. 229638). The surface of the dishes is coated with 0.2 mL of 50 µg/mL poly-D-lysine (PDL) (Sigma Aldrich, Cat. No. P6407) and incubated at 37 °C and 10% CO_2 overnight. Next, the dishes were flooded with sterile deionized water and aspirated 10 minutes later. Flooding was repeated 3 times. Dishes were coated with laminin (Sigma Aldrich, Cat. No. CC095-M) and incubated at 37 °C and 10% CO_2. Laminin was aspirated after 1 hour and left to air dry in the biohood.

Fig. 3. Biohood

D. Cultuing Cells

After 3 days thawing PC12s, the cell culture media was replaced with differentiation media. A week is needed to differentiate PC12. During this week, the media was replaced every two days. The cells were imaged using an inverted microscope using a camera attachment (Edmund Scientific, Barrington, New Jersey, USA Cat. No. EO-0813C) and software uEye Cockpit.

E. Image Analysis

Digital images of the cells were taken after 1 week of differentiation. An average of 10 cells per field was used for each image. Differentiated cells were selected by visual inspection of the field counting cells with neurite length equaling the diameter of the cell body. NIH's software, ImageJ, was used with plugin, NeuronJ, of package Fiji to manually trace and measure the length of the neurites in pixels. Pixel length was converted into micrometer with a known pixel to distance ratio using a reticle.

III. Results

Visually and quantitative, higher concentration of NGF increased the length of the neurites which is expected. The smallest neurite was measured in the PC12 culture without NGF with length of 24.8 ± 2.1 µm while the largest neurite was measured in the PC12 culture with highest concentration of NGF at 127.2 ± 5.4 µm. Even though the cells were plated with a low density, cells clumped together which increased the difficult of counting neurites. Neurite length data was

analyzed using One-Way ANOVA (Analysis of Variance) where independent variable was the NGF density. Analysis was performed on IBM's SPSS Statistics software. The p-value calculated from the data was 0.07

TABLE I. AVERAGE NUERITE LENGTH

NGF Density [ng/mL]	Average Neurite Length [µm]	Error [µm]
0	23.3	2.1
1	28.9	1.5
10	28.1	4.1
100	35.9	3.0
1000	38	5.7
10000	52.2	5.4

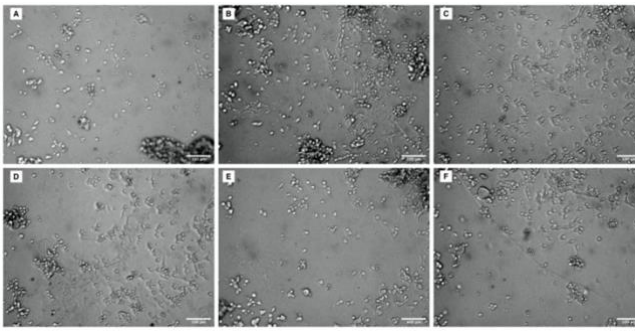

Fig. 4. Images captured of the PC12s at 10x magnification 1 week after differentiation. Clumping and overlapping cells are evident in these images. Labels A, B, C, D, E, F, correspond to 1, 10, 100, 1000, 10000, and 0 ng/mL of NGF, respectively.

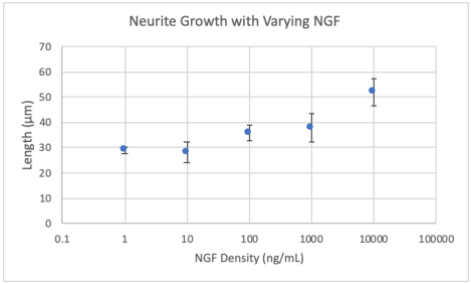

Fig. 5. Graph of neurite growth with varying densities of NGF.

IV. DISCUSSION

PC12s cells are a great alternative for neuroscience research compared to the sacrifice of a live animal for cells. Differentiating PC12s transforms the cells into ganglion neurons morphically and functionally that further model neuron from a live animal. NFG differentiation depends on a certain density of the neurotrophin used. Increasing the NGF density did increase the length of the neurites and changed the physical shape of the cell from circular to elongated. This study did not perform any neurotoxicity or cytotoxicity assay, so the effects of high densities of NGF was not studied. Further research is needed to determine the high densities of NGF. Das [1] observed neurite growth of roughly 200 µm after seven days which is 4 times greater than the neurite length measured in this study. A possible factor is the type of PC12 cell line used; the cell line used in this study is PC12. Das [1] does not specifically state which cell line was used. Finally, the clumping or overlapping of cell made the manual tracing of the neurites difficult. The clumping of cell indicates an overpopulated cell culture. Cell density should be reduced for replication studies. The calculated p-value approached the boarder of statistical significance.

V. ACKNOWLEDGEMENTS

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