Development of Neurites from SH-SY5Y Neuroblastoma Cells *in Vitro*

Thesis

Presented to the Graduate Council of

Southwest Texas State University

in Partial Fulfillment of the Requirements

for the degree of

Master of Science

by

Tracy L. Glass, B.S., B.A.

San Marcos, Texas

August, 2000

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ACKNOWLEDGMENTS

I thank my mentor, Dr. Joseph Koke, for providing me an opportunity to discover that graduate school is not only a place where I would learn about science, but also a place where I would learn about myself. Thanks for having enough faith in me as an undergraduate to provide a place for me in your lab to grow and learn, make a lot of mistakes, learn from those mistakes, and make a few discoveries. I appreciate all the guidance you have given throughout the last three years.

I thank my additional committee members, Drs. Timothy Raabe and Dana García, for sacrificing their time to take an active role in the development of my thesis. Dr. Raabe, I greatly appreciate the long drives from San Antonio to be a part of this committee. I know that wasn't always easy on your schedule. Your expertise in the area of neurobiology was highly valued. Dr. García, I value your critical opinions of my research. Thanks for always expecting more of your students and never settling for less.

I also thank my family, who has sacrificed much along the way of my education. I dedicate this manuscript to my parents, Keith and Laura Merkel, who are two of my biggest fans. Thanks for the love and encouragement during some of the more difficult times. Your friendships will outlast any other gift you have ever given me. I thank my husband, Greg, who all too often had to tolerate the mood swings associated with thesis work. Thanks for the many sacrifices you didn't have to make, but did

anyway. You are more of a husband than I deserve. I thank my Lord and Savior, Jesus Christ, who has given me the strength to make it through graduate school and so much more (Phil 3:13).

Thanks to the "Koke Crew" for their constant support, encouragement, advice, and input. You have all helped to make this experience an enjoyable one.

This work has been supported in part by grants from the National Science Foundation (NSF DUE # 96-50654), Texas Higher Education Coordinating Board (003658-0193-1999), and the Faculty Research Enhancement Program and Department of Biology of Southwest Texas State University.

ABSTRACT

The ability to cause cells to differentiate *in vitro* presents an opportunity to develop model systems for the study of neural differentiation and regeneration. Culturing cells in a gel composed of extracellular matrix (ECM) components, as opposed to standard culture on substrates of glass or plastic, has powerful effects on cell behavior and differentiation. Previous studies of primary neural cultures in ECM have demonstrated the ability of the gel to promote axonal growth. The following two questions were asked: 1) does the morphogenic effect result from soluble growth factors present in the ECM, and 2) in comparison to *in situ* axons, do these neurite-like structures emanating from gel cultured cells express neurite specific antigens? The behavior of cells grown in normal and growth factor-reduced ECM gels was compared, and no significant morphological differences were apparent. Cells grown in ECM were found to express neurite-specific antigens in amounts comparable to that of mature rat brain tissue. These proteins were not detectable by western analysis in cells cultured without ECM, but were observed by immunohistochemistry in apparently lesser amount in the processes of cells cultured without ECM. It therefore appears that SH-SY5Y processes are neurites and that growth in ECM promotes and accelerates expression of neurite specific antigens.

INTRODUCTION

The ability to cause cells to differentiate *in vitro* presents an opportunity to develop model systems for the study of neural differentiation and regeneration. Many studies have shown that culturing cells in a three-dimensional gel composed of extracellular matrix (ECM) components, as opposed to standard culture on substrates of glass or plastic, has powerful effects on cell behavior and differentiation (Taub *et al.*, 1990; Schor *et al.*, 1982; Haber *et al.*, 1988; Schwarz *et al.*, 1990; Bao and Hughes, 1995). One source of these ECM components is the matrix derived from the Engelbreth-Holm-Swarm (EHS) murine sarcoma. The high molecular mass components of the EHS-ECM preparation are laminin (60-80%), collagen type IV (10-20%), heparin sulfate proteoglycan (1-5%), and entactin (10-20%). Small amounts (<1% each) of transforming growth factor beta (TGF-β), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor one (IGF-1), and platelet derived growth factor (PDGF) are also present in the ECM gel (Kleinman *et al.*, 1993).

Previous studies of primary neural cultures in ECM have demonstrated the ability of the EHS-ECM gel to promote neurite outgrowth. For example, Tonge *et al.*, (1997), using peripheral nerve-dorsal root ganglion (DRG) preparations from adult mice and amphibia, showed that neurite growth in the EHS-ECM was much more profuse when compared to growth in gels of type I collagen. When preparations of mouse DRG were cultured in environments containing individual components of the EHS-ECM gel and type I collagen, regenerating neurite were strikingly longer in cells grown in the presence of laminin and

type IV collagen than in the control cells grown on type I collagen alone. They also demonstrated that a diluted EHS-ECM gel (diluted to provide the same concentration of laminin) was more effective at promoting neurite growth than laminin or collagen IV alone, suggesting that in addition to these two molecules, other components within the ECM gel may be necessary to support optimal neurite growth.

The intent of the work reported here is to characterize the morphological responses of cells grown in an ECM environment with a long term goal of developing an *in vitro* model of axon repair using cultured SH-SY5Y neuroblastoma cells. In previous reports, marked behavioral and morphological differences between SH-SY5Y neuroblastoma cells cultured on coverslips and cultured in the EHS-ECM gel have been described (Hahn *et al.*,2000). Cells grown in an EHS-ECM gel formed into ganglion-like clusters which generated what appeared to be bundles of neurites that targeted other "ganglia," whereas the same cells cultured on coverslips, with or without collagen, did not cluster, did not generate bundled neurites-like structures, and appeared as fibroblasts in culture (Price *et al.*, 1993).

To begin to understand these effects, I investigated the following two questions: 1) does the previously observed morphogenic effect from the gel result from the growth factors present in the EHS-ECM gel, and 2) in comparison to *in situ* axons, do these neurite-like structures emanating from gel cultured cells express the same cytoskeletal proteins and neurite specific antigens?

To answer the first question, I compared the behavior of cells grown in normal and growth factor-reduced ECM gels. Results from this study revealed no significant morphological differences. The cells grown in growth factor-reduced gels formed similar ganglia-like clusters and neurite-like extensions as observed with cells grown in the

complete EHS-ECM.

To determine if the processes extending from cells cultured in the EHS-ECM gel were, in fact, neurites, I compared the expression of cytoskeletal proteins and neurite specific antigens in cells grown on coverslips, cells cultured in the EHS-ECM gel, and in *in situ* axons found within the rat brain. I labeled phosphorylated neurofilaments using the SMI-31 antibody that recognizes phosphorylated neurofilaments H and M (Sternberger and Sternberger, 1983). In mammals, neurofilaments are heterotrimeric intermediate filaments made up of light (NF-L), medium (NF-M), and heavy (NF-H) chain subunits. The subunits are expressed differentially during development, with NF-H and NF-M abundant in mature axons and dendrites (Nixon and Shea, 1992). Previous studies have also indicated that developmentally, NF-M and NF-L are expressed in early embryonic stages while NF-H is generally expressed only after the axon is fairly well established and synaptogenesis has occurred (Carden *et al.*, 1987).

I labeled the SNAP-25 presynaptic membrane protein because of its known neurite specificity on the cytoplasmic face of the plasma membrane in synaptic terminals, throughout the axon, and with secretory vesicles (Hodel, 1998). SNAP-25 forms an exocytosis complex with syntaxin and synaptobrevin and is responsible for vesicle docking and fusion with the plasma membrane; therefore, localization of SNAP-25 at contacts between SH-SY5Y "neurites" and "ganglia" would provide evidence of functional differentiation and synapse formation.

These markers were detected by confocal microscopy. Using quantitative electrophoresis and western analysis, I determined that cells grown in EHS-ECM express both the SMI-31 and the SNAP-25 antigens in amounts comparable to that of mature rat

brain tissue. However, these proteins were not detectable by western analysis in cells cultured without EHS-ECM. Surprisingly, the processes of some cells cultured on coverslips were positive for the neurite markers, although expression appeared reduced in comparison to gel-cultured cells. It therefore appears that the bundled processes extended by the SH-SY5Y ganglia-like clusters are neurites according to the criteria described above.

MATERIALS AND METHODS

A. Cell line and matrix factors

SH-SY5Y cells are a subclone of the SK-N-SH human neuroblastoma cell line derived from neoplastic neural crest cells (Ross *et al.*, 1983) and were donated by Dr. Dandan Sun, University of Wisconsin Medical School. This cell line has been previously shown to provide an excellent model in which to study neuronal cell differentiation and function (review, Pahlman *et al.*, 1990; Hynds and Snow, 1999).

The gel forming material used was an ECM preparation derived from Engelbreth-Holm-Swarm murine sarcoma (EHS) (Sigma Chemical Co., #E1270, St. Louis, MO) as described by Kleinman *et al.* (1993). The major constituents of the EHS-ECM preparation are described above (Introduction). It should be noted that EHS-ECM gel and Matrigel® ECM are derived from the same tumor, but manufactured by different companies. According to the manufacturers, there are no differences among the constituents of these two products.

Growth Factor Reduced-Matrigel ® Matrix (GFR-M) (Becton-Dickinson Labware, #40230, Bedford, MA) was used during investigations requiring a reduction in the level of growth factors present in the EHS-ECM gel. This preparation of EHS-ECM was reduced in all soluble growth factors, with the exception of TGF-β, which may be bound to collagen IV and sequestered in a latent form during the purification process (Table 1; Paralkar, *et al.*, 1991).

Table 1. *Comparative values of growth factors present in Matrigel and Growth Factor Reduced-Matrigel.

Parameter	Matrigel Matrix	Growth Factor Reduced Matrigel Matrix
bFGF (pg/ml)	0-0.1	0-0.1
EGF (ng/ml)	0.5-1.3	<0.5
IGF-1 (ng/ml)	15.6	5
PDGF (pg/ml)	12	<5
NGF (ng/ml)	<0.2	<0.2
TGF-β (ng/ml)	2.3	1.7

*Becton Dickinson, 1998

B. Preparation and Maintenance of Cells Cultured on Uncoated Coverslips

SH-SY5Y cells were maintained in 25 cm^2 culture flasks in Minimum Essential Medium (MEM, Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 2 mM L-glutamine and 10,000 U penicillin/10,000 mg/ml streptomyocin, at 37°C in an atmosphere containing 5% CO_2. Cells were harvested at confluency using a sterile cell scraper and then pelleted at 2000 rpm using a clinical centrifuge. The supernatant was discarded and the pellet was resuspended in 2 ml of fresh MEM. Next, 25 µl of the freshly prepared cell suspension were added to 10 X 100 mm petri dishes containing 6-8 18 mm, square #1 uncoated coverslips and 10 ml MEM. The cells were allowed to grow in culture for 168 hours.

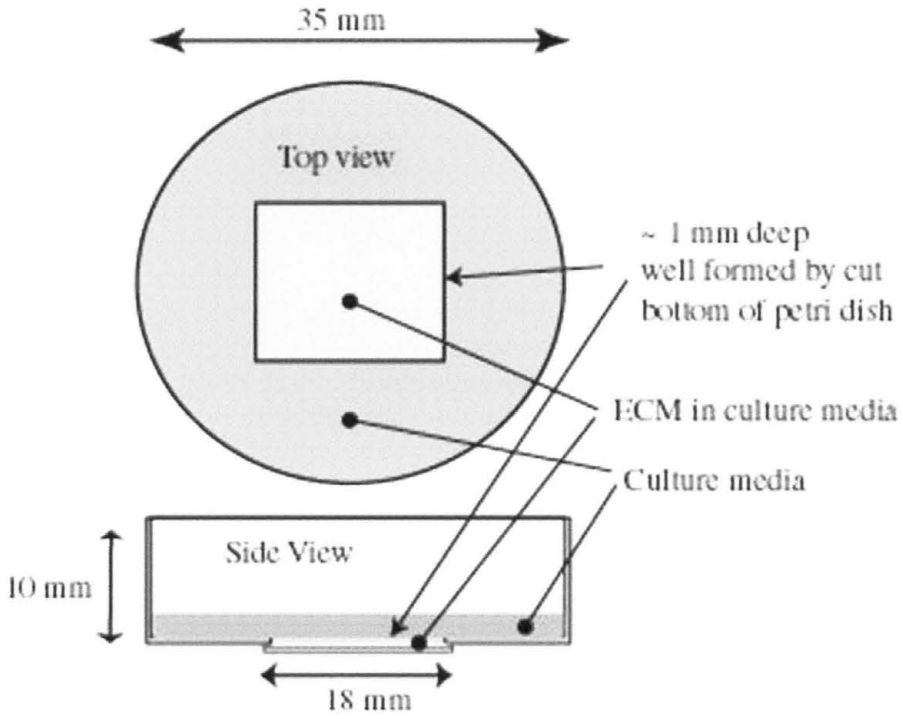
C. Preparation and Maintenance of Cells Cultured in the EHS-ECM gel

Three-dimensional cultures were created in specially designed 35 X 10 mm petri dishes as described by Hahn (Hahn *et al.*, 2000; Figure 1). These containers were made by melting a square hole in the bottom of the dishes, and attaching an 18 mm, square #1 coverslip using a thick layer of silicone sealant around the edges. The dishes were allowed to dry and then placed into a 70% ethanol solution for sterilization until subsequent use. This design not only provided a reservoir for the ECM gel, but the use of a thin coverslip allowed for a shorter working distance and allowed for the use of a higher magnification lens during confocal microscopy.

Cells were first grown under the conditions previously described in the preparation of two-dimensional cultures. Cells were harvested at confluency and then pelleted at 2000 rpm using a clinical centrifuge. The supernatant was discarded and the pellet was

Figure 1

A diagram showing the dimensions of the modified culture dish used for laser scanning confocal microscopy of cells cultured in EHC-ECM. See text for details.


35 mm
Top view
~ 1 mm deep
well formed by cut
bottom of petri dish
ECM in culture media
Culture media
Side View
10 mm
18 mm

resuspended in 2 ml of fresh MEM. Manipulations with the EHS-ECM gel were carried out at 4°C to maintain the EHS-ECM gel's liquid state. Prior to use, the EHS-ECM gel was diluted 1:1 with ice cold MEM. Next, 25 µl of the previously prepared cell suspension were added for every 200 µl of the diluted gel. Immediately following, 200 µl of the cell/gel suspension were added to each petri dish well. The gel solutions were then placed into a 37°C incubator for 30 minutes to allow for polymerization. Once solidified, the dishes were flooded with 2 ml of warm MEM, and the cells were cultured for 7 days.

Preparation of cell cultures using GFR-M followed the same procedure as described above for the EHS-ECM gels. Investigations of cells grown in GFR-M as compared to EHS-ECM gels were carried out over a period of time ranging from 48 hours to 240 hours.

D. Fixation and Staining for Immunofluorescence Microscopy

Cells grown in EHS-ECM gels were washed briefly in a phosphate-buffered saline (PBS) solution (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and then fixed at room temperature in a 4% formaldehyde (PFA) solution (freshly made from paraformaldehyde [Electron Microscopy Sciences, Ft. Washington, PA] and buffered with PBS) containing 0.5% gluteraldehyde (Electron Microscopy Sciences, Ft. Washington, PA). After 60 minutes, the cells were washed three times in PBS containing 0.2% Tween-20 (PBST) for 15-30 minutes each. All sections were blocked in 10% non-fat powdered milk in PBST for 1 hour at room temperature followed by three 5-minute washes in PBS. The sections were then labeled with the appropriate monoclonal antibody (see Table 2) for 16-24 hours at 4°C. The sections were washed three times in PBST (5 minutes each) and labeled with the anti-mouse antibody

conjugated to Oregon Green (see Table 2). The sections were allowed to incubate at room temperature for 2 hours and then washed three times in PBST. Preparations were then stained at room temperature with 200 µl of Texas Red-conjugated phalloidin to reveal filamentous actin (see Table 2), and 50 µl of TO-PRO-3 to reveal nuclei (see Table 2). After 1 hour, cells were washed two times in PBST for 30 minutes each. Negative controls for all immuno-preparations were prepared by use of the appropriate normal mouse serum in place of the primary antibody.

Cells cultured on uncoated coverslips were fixed and stained following the same protocol as described above for the EHS-ECM cultured cells. The coverslips were mounted in 90% glycerol in p-phenylenediamine.

All procedures involving the use of animals were approved by the Southwest Texas State University Institutional Animal Care and Use Committee (#00-05). Brain tissue was obtained from a rat euthenized by carbon dioxide asphyxiation followed by vascular perfusion into the left ventricle with 4% formaldehyde and 0.5% glutaraldehyde in PBS. The brain was removed and sectioned mid-sagittally. After a brief wash in PBS, the sample was placed into 30% sucrose in PBS for 48 hours. Tissue was embedded in TissueTek O.C.T. (Fisher Scientific, Houston, TX) and cryosectioned into 25 µm sections using a Zeiss Microm HM 505 N refrigerated microtome. All brain sections were stained following the same protocol as described above for the EHS-ECM cultured cells. All preparations for light microscopy were mounted in 90% glycerol in PBS containing 1 mg/ml p-phenylenediamine.

E. Microscopy

For fluorescence and confocal microscopy, an Olympus IX-70 fitted with a Bio-Rad MRC 1024 confocal scanhead via the Keller port was used. Image acquisition and initial processing were done using Bio-Rad LaserSharp software running on a Compaq PC; final processing and printing of images was done using Adobe Photoshop software running on a Power Macintosh and driving an Epson Stylus 800 color ink-jet printer.

F. Preparation of SH-SY5Y and rat brain extracts

In previous electrophoresis studies conducted on EHS-ECM, it was observed that components within the matrix (presumably heparin sulfate proteoglycan) caused a smeared effect within the lanes making it difficult to resolve individual bands. Therefore, I developed the following procedure to retrieve the neuroblastoma cells from the EHS-ECM gel after the 7 day growth period. Gels containing the SH-SY5Y cells from the specialized petri dishes were removed and placed in a sterile, ice-cold 15 ml centrifuge tube. The matrix was then diluted with ice-cold PBS and incubated at 4°C for 24 hours to cause the gel to liquefy. The liquefied EHS-ECM was centrifuged at 2000 rpm using a clinical centrifuge to separate the neuroblastoma cells from the matrix components. The cells were then resuspended in extraction buffer which contained 1.0 mM ethylene glycol-*bis* (*b*-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), 100 μg/ml phenylmethylsulphonylfluoride, and 1% (v/v) Triton X-100, all in PBS. After incubation for 3 hours at 4°C on a rocker plate, the extract was centrifuged at 10,000 x *g* for 30 minutes. For subsequent analysis by electrophoreses and western blotting, the pellet was resuspended in PBS and the supernatant was retained. Protein concentrations were

determined by measuring ultraviolet absorbance at 260 nm and 280 nm according to Layne (1957) and Peterson (1983). We chose this assay because measuring UV absorbance has little damaging effect to the extract and our sample volumes were small.

SH-SY5Y cells grown in uncoated plastic cell culture flasks were harvested after a seven day growth period using a sterile cell scraper, pelleted, and placed in extraction buffer (see above) for 6 hours at 4°C, then processed as described above.

Brain tissue samples were obtained from rats euthanized by carbon dioxide asphyxiation. The tissue was placed in 5 ml of ice-cold extraction buffer (see above) and broken up using a tissue homogenizer. The suspension was stirred at 4°C for 6-8 hours and then filtered through two layers of cheesecloth. The resulting filtrate was centrifuged at 10,000 x *g* for 30 minutes and then processed as described above.

G. Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method originally described by Laemmli (1970). Samples were adjusted so that known amounts of protein were loaded in each well of the gels. One volume of 5x sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue) was added to 4 volumes of sample, and the resulting solution was boiled and then briefly centrifuged. Samples were applied to pre-cast polyacrylamide linear gradient gels (8%-16% separating, 4% stacking; #161-1222, Bio-Rad, Hercules, CA; Table 3). The gradient was selected because of the wide difference in molecular mass (M_r) among the phosphorylated neurofilaments and the SNAP protein markers.

Gels were prepared in duplicate. Electrophoresis was carried out at 200 V until the dye front reached the bottom of the gel. One of each pair of gels was then stained and fixed in Coomassie blue dye (1.2 mM Coomassie blue R-250, 45% methanol, 10% acetic acid) for 10 minutes and destained in 10% acetic acid and 10% methanol in water for 12 hours. The duplicate gel was placed in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol; pH 8.3) and prepared for electrophoretic transfer onto a nitrocellulose membrane (#162-0115, Bio-Rad, Hercules, CA). The transfer was performed at 100 V for 1 hour at 4°C. The identities of the proteins bound to the nitrocellulose membrane were revealed by western analysis using an alkaline phosphatase conjugated secondary antibody (Table 2; Price *et al.*, 1993).

Gels were recorded and quantified and by placing them directly on a digital scanner (Apple Color One) and scanning at 600 dpi. Gel images were opened with NIH Image and quantitative density histograms of appropriate lanes prepared. The matching western blots were used to identify specific bands in the Coomassie blue stained gels. Since the protein concentration of each extract and the amount of protein loaded in each well of the gels was known, the amounts of the marker proteins (NF and SNAP-25) could be compared among the gel cultured cells, cells cultured on coverslips, and the rat brain tissue. Images for printing were prepared from the same scans using Adobe PhotoShop.

Table 2. Probes and Antibodies used.

Probe/ Antibody to (Catalog #)	**mAB/pAB**	**Conjugated to**	**Dilution**	**Supplier**
SNAP-25 (# NB09)	mAB IgG1 (mouse)	-	1:5000 in PBS	Oncogene Research Products, Cambridge, MA
Phosphorylated neurofilament probe: SMI-31	mAB IgG1 (mouse)	-	1:5000 in PBS	Sternberger Monoclonals, Inc. Lutherville, MD
Mouse IgG (# G-7527))	pAB	5 nm colloidal gold	1:50 in PBS	Sigma Chem. Co, St. Louis, MO
Mouse IgG (# O-6380)	pAB	Oregon Green	1:100 in PBS	Molecular Probes, Eugene, OR
Mouse IgG (# A-0162)	pAB	Alkaline Phosphatase	1:1000 in PBS	Sigma Chem. Co., St. Louis, MO
Actin binding probe: Phalloidin (# T-7471)	-	Texas Red	Stock 300 units/ml in methanol, 1:40 in PBS	Molecular Probes, Eugene, OR
Nucleic Acid binding probe: TO-PRO-3 iodine (# T-3605)	-	-	Direct	Molecular Probes, Eugene, OR

RESULTS

A. Comparison of cells cultured in growth factor reduced-Matrigel (GFR-M) to cells cultured in EHS-ECM gels

To determine if the additional growth factors present in the EHS-ECM gel contributed to the observed morphological differences between cells grown on coverslips and cells grown in a matrix environment, I compared cells grown in normal and growth factor reduced ECM gels. The morphology of the neuroblastoma cells grown in the GFR-M was surprisingly similar to those grown in the EHS-ECM gel. Under both culture conditions, the cells maintained a rounded, more normal neuronal shape not observed in cells grown on coverslips. Both GFR-M and EHS-ECM cultured cells extended neurite-like processes and formed ganglion-like clusters similar to those seen in primary neuronal cultures (Carey *et al.*, 1986). Texas Red-phalloidin revealed filamentous actin throughout the cell as well as in the neurites. TO-PRO-3 revealed the location of the nuclei (Figure 2).

B. Localization of SNAP-25 by immunofluorescence microscopy

To determine if gel cultured SH-SY5Y neuroblastoma cells express the same neurite specific antigens as observed in *in situ* axons, I used the SNAP-25 mouse IgG1 monoclonal antibody to detect the human pre-synaptic protein, SNAP-25. The SNAP-25 protein is located on the cytoplasmic face of the plasma membrane in synaptic terminals, with secretory vesicles, and throughout the axon (Hodel, 1998).

When SH-SY5Y cells were cultured in the EHS-ECM gel for 7 days and labeled with the SNAP-25 antibody, antibody binding was found throughout the cell bodies in a

distinct punctate distribution, suggestive of secretory vesicles (Figure 3B; Hodel, 1998; Goodall *et al.*, 1997). SNAP-25 colocalized with F-actin in the distal portion of neurites of cells grown in the EHS-ECM gel (Figure 3A).

A similar staining pattern was observed in neuroblastoma cells grown on coverslips for seven days (Figure 4). Co-localization between SNAP-25 and F-actin was observed around the periphery of the cell and in some distal portions of neurites. Regions labeled in the cell body revealed a punctate staining pattern, while regions in the neurite were intense and distinct.

Labeling was observed in rat brain sections stained for SNAP-25. Labeling in rat brain was punctate (data not shown) as seen in cells grown in the EHS-ECM gels and on coverslips. Controls for staining techniques were done using normal mouse serum in place of the anti-SNAP-25 monoclonal antibody. No labeling was detected in the control cells.

C. Localization of phosphorylated neurofilaments H and M by immunofluorescence microscopy:

To determine if gel cultured SH-SY5Y neuroblastoma cells express the same cytoskeletal proteins as observed *in situ*, I used the SMI-31 mouse IgG1 monoclonal antibody to detect the phosphorylated epitopes in NF-H and NF-M 1992). When SH-SY5Y cells, cultured in the EHS-ECM gels, were grown for 7 days and labeled with the SMI-31 antibody, localization appeared primarily in nuclei and in the neurite processes. Antibody binding was also detected in the cell bodies, but localization was not as well defined. Nuclear staining was diffuse and not distinct as in the processes (Figure 5).

Cells that were cultured on coverslips showed similar labeling patterns; however,

labeling was less well developed within the processes and only a little more than half of the observed cell clusters showed positive labeling in the neurite extensions. Diffuse nuclear staining was present, and the staining within the cell body region was more common as compared to gel-cultured cells (Figure 6). Some co-localization occurred between phosphorylated neurofilaments and F-actin in areas that resemble centrosomal regions. This co-localization was not observed in gel cultured cells, or in rat brain sections.

When rat brain sections were stained with the SMI-31 antibody, binding occurred only in neuronal nuclei and processes (Figure 7). Negative control preparations for SMI-31 had no detectable staining (data not shown).

D. Electrophoresis and western analysis

SDS-PAGE and western analysis revealed the presence of SNAP-25 in rat brain tissue and gel-cultured cells (Figure 8) and NF-H and NF-M proteins (Figure 9). However, some differences were found in the amounts of these proteins present relative to the total amount of protein loaded. In particular, densitometric analysis of the SNAP-25 and NF bands, as identified by their respective antibodies, indicated that gel cultured cells expressed a comparable amount of SNAP-25 but only half as much NF-M and NF-H, as compared to mature rat brain tissue. Neither SNAP-25 nor SMI-31 was detectable by western analysis in cells cultured in the absence of EHS-ECM.

Figure 2

Confocal micrographs prepared by digital projection of SH-SY5Y neuroblastoma cells grown in Growth Factor Reduced-Matrigel (GFR-M) (panel A) or EHS-ECM gel (panel B). Microfilaments were revealed by Texas Red-conjugated phalloidin (red signal),and the nuclei were revealed by the DNA-specific dye, TO-PRO-3 (blue signal). Cells pictured in both micrographs were grown for 168 hours. Cells cultured in GFR-M (panel A) retained the same ganglia-like clusters, and neurite-like projections as observed with cells grown in the EHS-ECM gel (panel B).

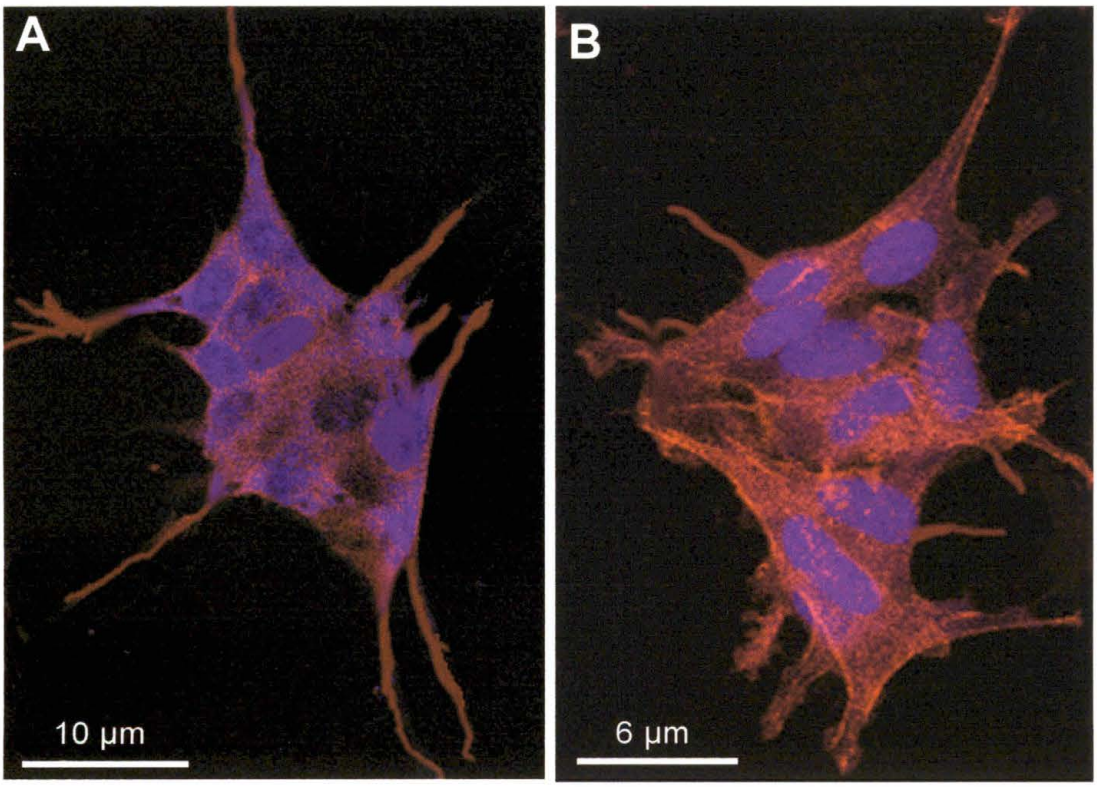

A
10 µm
B
6 µm

Figure 3

A confocal micrograph projection of multiple image planes showing SH-SY5Y cells grown in EHS-ECM gel and labeled to show the location SNAP-25. Texas Red-conjugated phalloidin was used to reveal F-actin (red signal). SNAP-25 was revealed using a primary SNAP-25 antibody followed by an Oregon Green-conjugated secondary antibody (green signal; see Table 2). TO-PRO-3, was used to reveal the location of nuclei (blue signal). In micrograph A, SNAP-25 is seen co-localized (yellow) with F-actin in the distal portion of the neurites of these cell clusters (arrows). Rotation of the confocal section revealed this to be true co-localization and not super position of signals from multiple optical sections (data not shown). The green signal observed between the two cell clusters in micrograph A is autofluorescenee of the collagen found within the matrix. Autofluorescence surrounding the cells was removed digitally using Photoshop software (see Material and Methods)

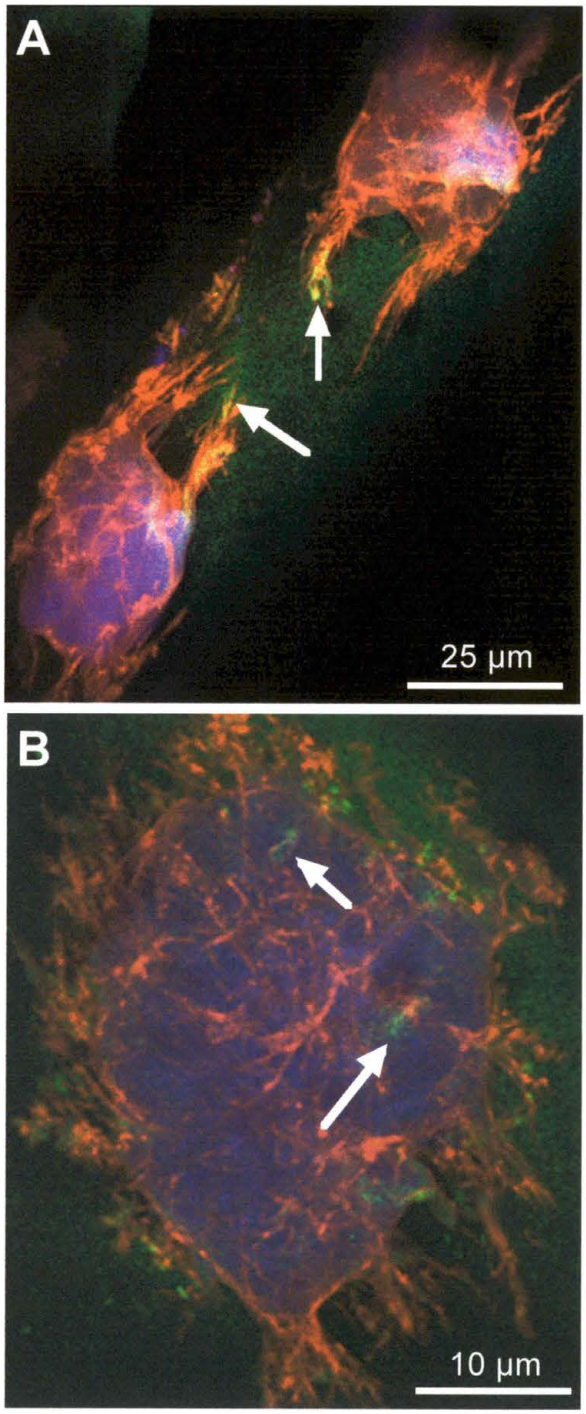

A
25 µm
B
10 µm

Figure 4

A confocal micrograph prepared by projection through multiple image planes of SH-SY5Y cells grown on coverslips and labeled with the SNAP-25 antibody. The Texas Red-conjugated phalloidin probe (red signal) revealed microfilament staining, and the SNAP-25 antigen was stained with a primary SNAP-25 antibody followed by an Oregon Green-conjugated secondary antibody (green signal). Cells grown on coverslips in the absence of the EHS-ECM gel showed a similar punctate distribution of SNAP-25 (arrows) as compared to cells cultured in the EHS-ECM gel. As with matrix cultured cells, SNAP-25 labeling is also observed at the distal portions of the neurite extensions.

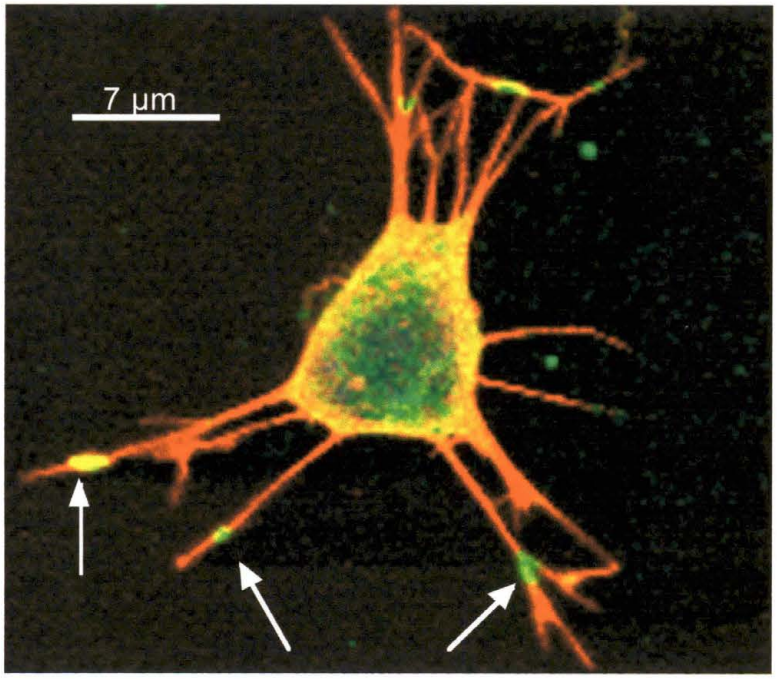

7 µm

Figure 5

A confocal micrograph prepared by digital projection through multiple image planes of SH-SY5Y cells grown in the EHS-ECM gel and labeled with the SMI-31 antibody to reveal phosphorylated neurofilaments H and M. Microfilaments were revealed by Texas Red-conjugated phalloidin (red signal), and phosphorylated neurofilaments were labeled using the SMI-31 antibody followed by an Oregon Green-conjugated secondary antibody (green signal). Detection of phosphorylated neurofilaments is observed primarily in the nuclei and within the neurite extensions (arrows). Some staining is observed within the cell body region as well.

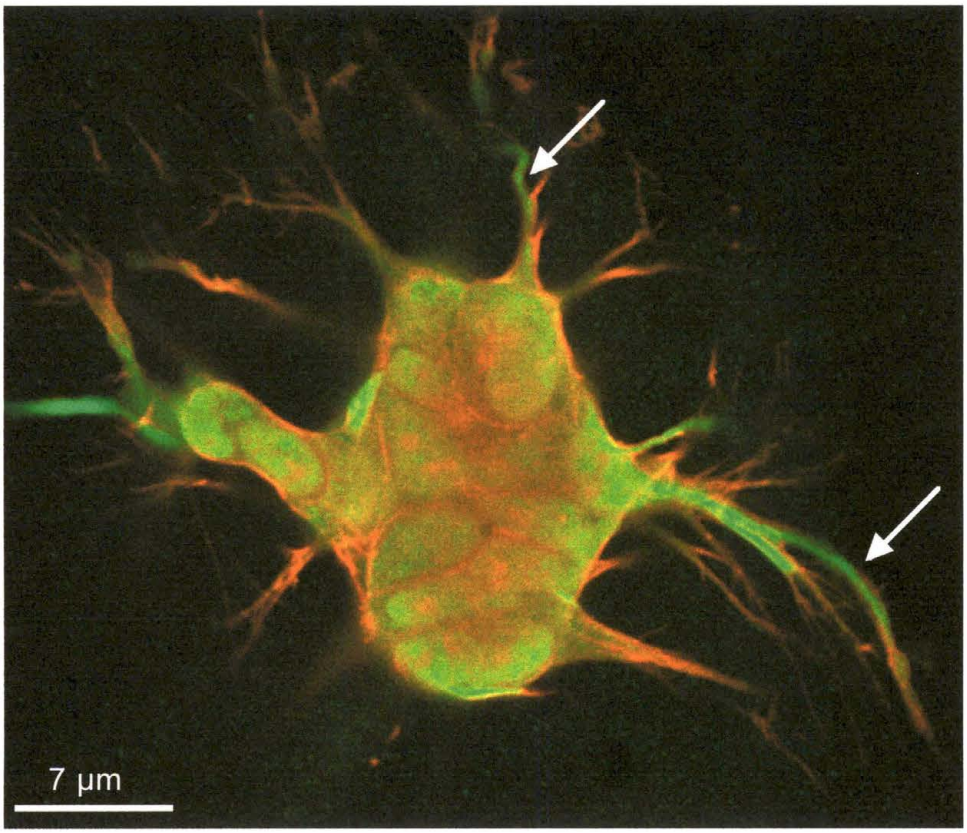

7 µm

Figure 6

Confocal projections of SH-SY5Y cells grown on coverslips and labeled with the SMI-31 antibody to reveal phosphorylated neurofilaments H and M. Microfilaments were revealed by Texas Red-conjugated phalloidin (red signal; panel A), and phosphorylated neurofilaments were labeled using the SMI-31 antibody followed by an Oregon Green-conjugated secondary antibody (green signal; panel B). Phosphorylated neurofilaments were observed throughout the cell body and nuclear regions, as well as in the neurite processes (arrow). Panel C is an overlay of panels A and B with the inclusion of a third stain, TO-PRO-3, to reveal the nuclei (blue signal).

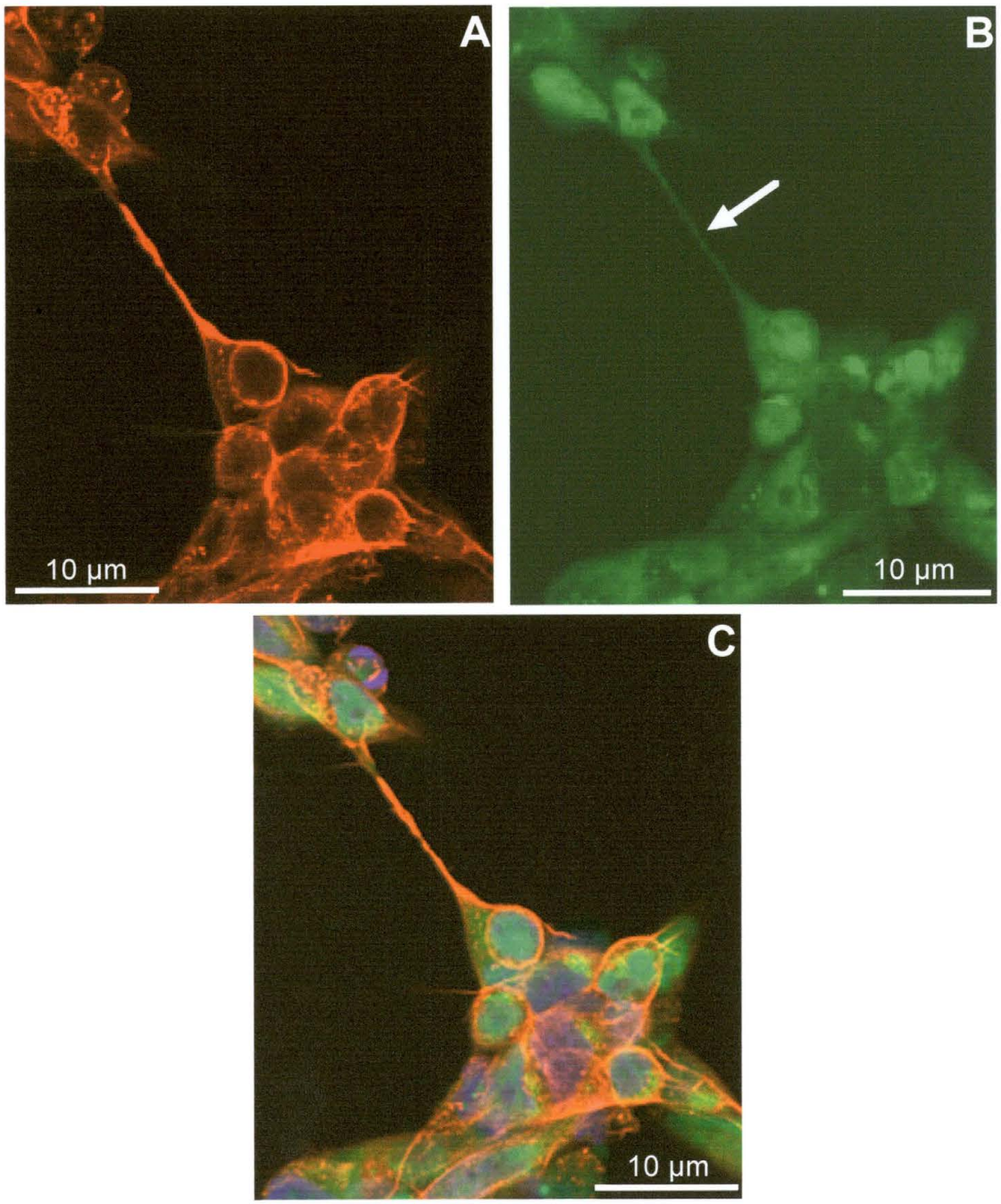

A
10 µm
B
10 µm
C
10 µm

Figure 7

A confocal projection of rat brain tissue revealing the localization of phosphorylated neurofilaments (NFH and NFM) by the SMI-31 antibody. Phosphorylated neurofilaments were observed throughout the brain. Note that nuclear staining is evident as well as the expected staining of neuronal processes.

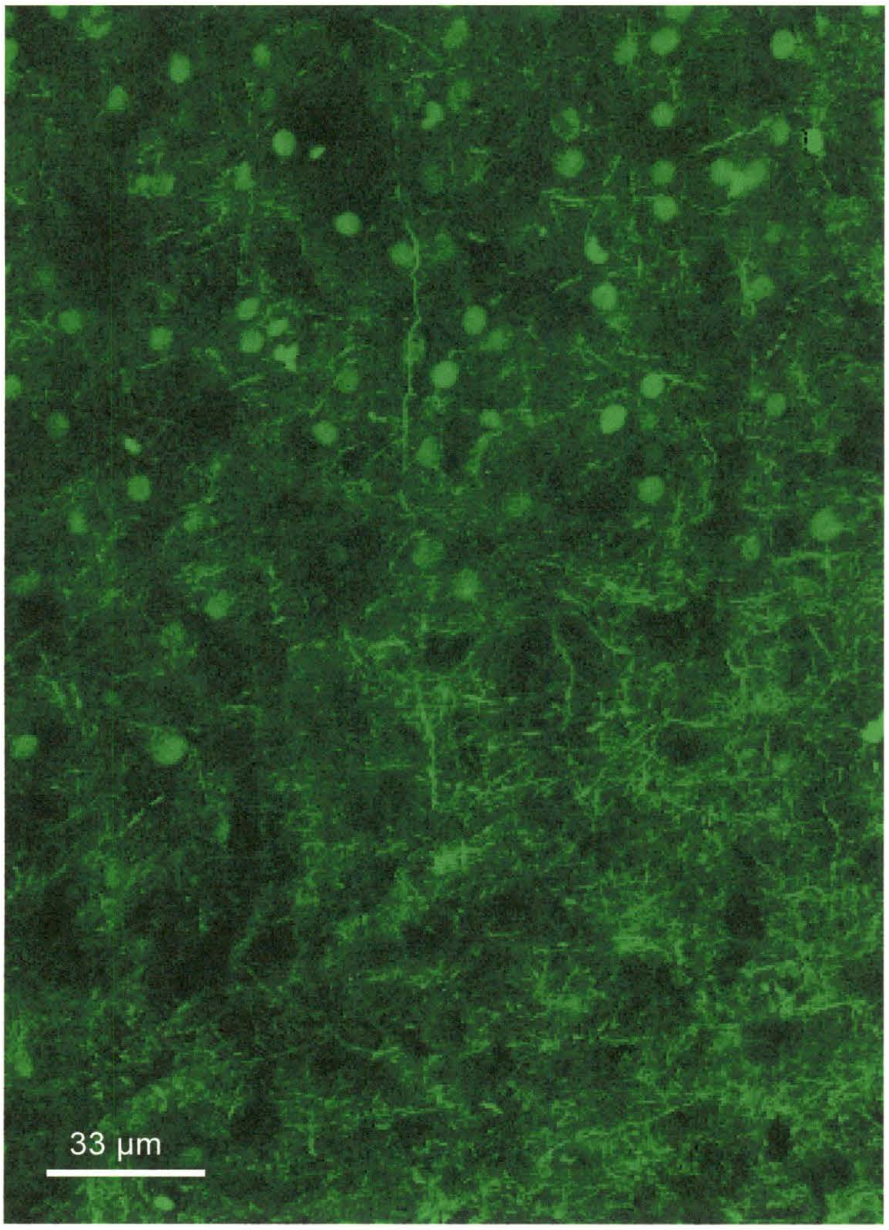
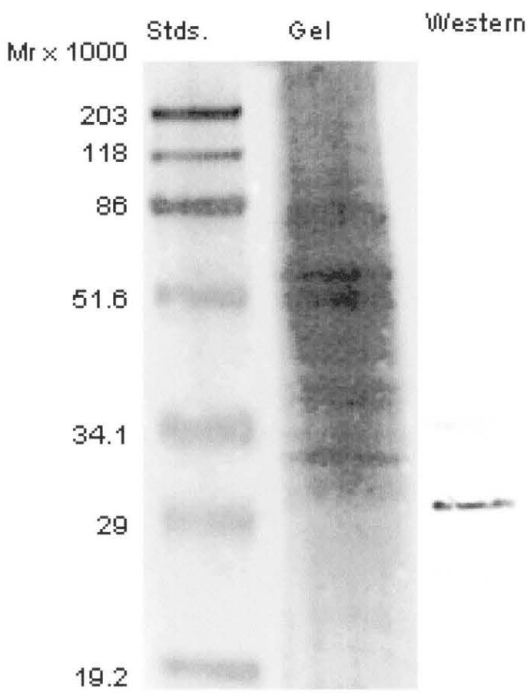

33 µm

Figure 8

SDS-PAGE and western analysis revealing the presence of SNAP-25 in EHS-ECM-cultured cells and rat brain tissue. The M_r values were estimated by comparison to the standards shown and were estimated to be 29,500 to 30,000 in gel-cultured cells and 28,700 in rat brain tissue. Western analysis failed to detect SNAP-25 in cells grown in the absence of EHS-ECM (data not shown).

Matrix Cultured Cells



Rat Brain Tissue

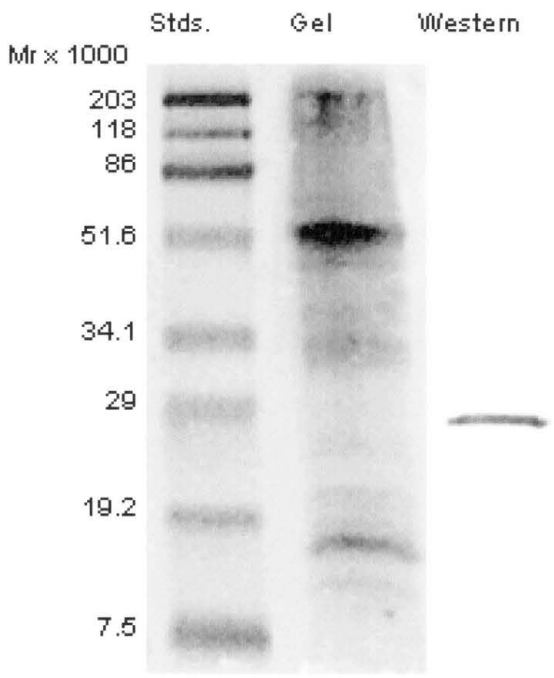
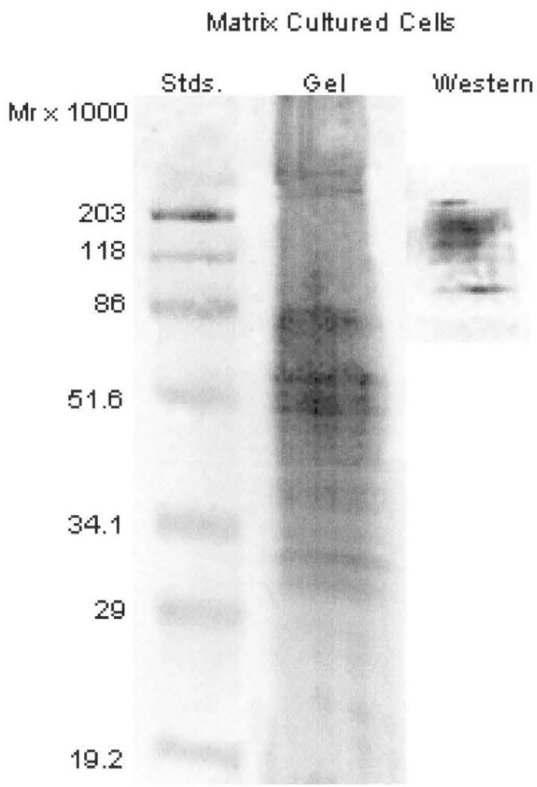
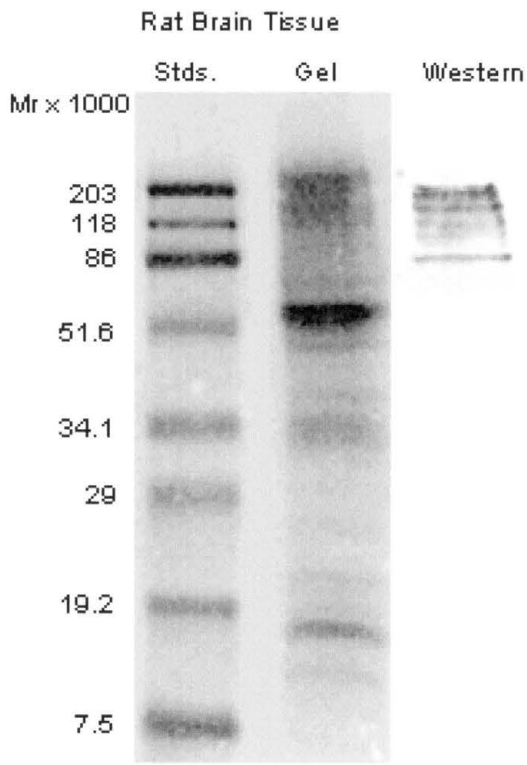


Figure 9

SDS-PAGE and western analysis revealing the presence of phosphorylated NF-H and/or NF-M in EHS-ECM-cultured cells and rat brain tissue. Western analysis failed to detect phosphorylated NF in cells grown in the absence of EHS-ECM (data not shown). The multiple bands shown in the western likely result from multiple levels of phosphorylation (see Discussion).


Matrix Cultured Cells
Stds.
Gel
Western
Mr x 1000
203
118
86
51.6
34.1
29
19.2


Rat Brain Tissue
Stds.
Gel
Western
Mr x 1000
203
118
86
51.6
34.1
29
19.2
7.5

DISCUSSION

A. Does the observed morphogenic effect from the gel result from the growth factors present in the EHS-ECM gel?

SH-SY5Y neuroblastoma cells grown under growth factor reduced conditions appeared morphologically identical to cells grown in EHS-ECM gels. GFR-M-grown cells, like EHS-ECM grown cells, formed ganglion-like structures and extended neurite-like processes. These results suggest that some factor other than the additional growth factors present in the EHS-ECM gel is responsible for the morphogenic differences previously observed between cells cultured on uncoated coverslips and cells cultured in the EHS-ECM gel.

Although previous studies have indicated the importance of growth factors in the regulation of cellular differentiation during development (Taub *et al.*, 1990), our earlier results suggest that other factors are involved in the development of neurites from SH-SY5Y cells. Prior work summarized by Lelievre *et al.* (1996) describes a "solid state" signaling pathway dependent primarily on laminin-integrin interactions that is responsible, in part, for initiating and maintaining the differentiated state of cells in tissues. Integrin-laminin receptors that mediate neurite outgrowth from SH-SY5Y neuroblastoma cells have been identified by Choi *et al.* (1994). They have shown that both untreated SH-SY5Y cells and those treated with NGF express integrins $\alpha 1/\beta 1$, $\alpha 2/\beta 1$, and $\alpha 3/\beta 1$, but the differentiated NGF-treated cells expressed a 5 fold higher level of $\alpha 1/\beta 1$ and a 2 fold higher level of $\alpha 2/\beta 1$ and $\alpha 3/\beta 1$. When monoclonal antibodies were used to block the function of individual integrins, they found that despite the presence of

multiple integrins, $\alpha 1/\beta 1$ mediated the majority of neurite outgrowth in these neuroblastoma cells.

B. Do the neurite-like structures emanating from gel cultured cells express the neurite specific antigen SNAP-25?

The SNAP-25 antibody revealed a punctate staining pattern in SH-SY5Y neuroblastoma cells throughout the cell body and processes. Some diffuse staining was also evident in the cell body. This punctate labeling pattern has been described as characteristic of secretory vesicles in a study by Goodall *et al.* (1997), in which they labeled proteins involved in movement, docking, and fusion of vesicles in SH-SY5Y neuroblastoma cells grown under two-dimensional culture conditions.

I intended to use SNAP-25 to identify possible synaptic zones in the SH-SY5Y neurites, but instead found widely distributed labeling located within the cell bodies and throughout the neurite. It has been reported that neurons differentially express SNAP-25 in two isoforms: SNAP-25a, and SNAP-25b. SNAP-25a is expressed early in development, while SNAP-25b expression does not occur until synaptogenesis (summarized by Hodel, 1998). The staining pattern I observed is consistent with what has been reported for SNAP-25a in cells early in development.

On the basis of these results, the processes extended by the gel-cultured cells appear to be neurites as labeled by SNAP-25. SDS-PAGE and western analysis confirmed the presence of SNAP-25, and these cells contained comparable amounts of SNAP-25 as a percentage of total protein as did mature rat brain tissue. Cells cultured on uncoated coverslips also appeared to contain SNAP-25 as demonstrated by immunofluorescence,

but at much lower levels that were undetectable by western analysis. This could reflect lower basal rates of expression or slower differentiation, as one of the consistent effects of the EHS-ECM has been to accelerate development of pseudotissues from a variety of cell lines (Hahn *et al.*, 2000). However, it should also be noted that the lack of detection by western analysis could be the result of possible morphological differences between cells cultured in uncoated plastic cell culture flasks (used in the SDS-PAGE/western analysis studies) and cells cultured on uncoated coverslips (used in the immunofluorescence studies).

C. Do the neurite-like structures emanating from gel cultured cells express phosphorylated neurofilaments as detected by SMI-31?

Phosphorylated NF-M and NF-H, as revealed by the SMI-31 antibody both by immunocytochemistry and western analysis, were found in SH-SY5Y neuroblastoma cells cultured in the EHS-ECM gel. Mature brain tissue was found to contain about twice as much of the heavy NF forms (as a percent of total protein) when compared to gel cultured cells. This is consistent with the observations of Carden *et al.* (1987) who showed that NF-M and NF-L are expressed in early embryonic stages while NF-H is expressed after the axon is well established.

By immunocytochemistry, processes of SH-SY5Y cells cultured on uncoated coverslips were also labeled although the antigen was undetectable by western analysis. Again, it should be noted that the lack of detection by western analysis could be the result of possible morphological differences between cells cultured in uncoated plastic cell culture flasks (used in the SDS-PAGE/western analysis studies) and cells cultured on uncoated coverslips (used in the immunofluorescence studies).

In addition, and in contrast to previous reports (Schilling *et al.*, 1988), I found SMI-31 labeling in the nucleus and perinuclear regions of all cultured cells and in mature rat brain. The staining pattern was diffuse and less intense as compared to the staining pattern observed in the neurite processes, suggesting that the SMI-31 antibody labeled phosphorylated neurofilament subunits or dimers, not the filamentous structure indicative of assembled neurofilaments (Figure 6). Phosphorylated neurofilaments have been previously observed to localize in the perikarya (rat, mouse, gerbil, rabbit, pig and chicken; Klosen *et al.*, 1994) and the axons and dendrites of mature neurons (mouse NB2a/d1 neuroblastoma cells; Shea *et al.*, 1988). Schilling *et al.* (1988) identified phosphorylated neurofilament epitopes in the nuclear regions of embryonic neural cells of fetal rats, noting that they were not observed in adult neural cells.

The presence of intermediate filament proteins and IF associated proteins in nuclei has been previously reported in astrocytes (Herrera *et al.*, 1995; Stevenson, 1996) and in adipocytes (Traub *et al.*, 1983). In astrocyte nuclei, the antigen appears to be a phosphorylated form of GFAP (Stevenson, 1996). This finding is similar to the finding of phosphorylated NF epitopes in neuronal nuclei reported here.

D. Conclusions and further studies

It is evident from these results that SH-SY5Y neuroblastoma cells extend neurites in the EHS-ECM gel as determined by the detection of neurite specific antigens and cytoskeletal proteins. Surprisingly, processes produced by cells grown on uncoated coverslips also were determined to be neurites by the same criteria, though expression of these markers was much reduced in comparison to the EHS-ECM cultured gels of the

same age.

Although cells cultured on uncoated coverslips produced neurites, it should be noted that these cells still failed to form ganglia-like cluster and the neurites produced did not appear to bundle together as seen in cells grown in the EHS-ECM gel. Cells grown in the EHS-ECM gel assumed a rounded neuronal cell shape, while those on coverslips were flattened.

To further assess the use of this model for nerve regeneration studies, an important observation would be to determine if these neurites are generating and propagating action potentials. This could be accomplished using a voltage sensitive dye such as RH-795 (Molecular Probes, Eugene, OR) to detect changes in membrane potential. If action potentials occur between clusters of cells and it can be determined where axons are found in a cell cluster, then this model could be used to compare the cytoskeletal proteins in uninjured and injury axons.

BIBLIOGRAPHY

Bao, Q., and Hughes, R.C. (1995). Galectin-3 expression and effects on cyst enlargement and tubulogenesis in kidney epithelial MDCK cells cultured in three-dimensional matrices *in vitro*. *J Cell Sci* **108**: 2791-2800.

Carden, M.J., Trojanowski, J.Q., Schlaepfer, W.W., and Lee, V.M. (1987). Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J Neurosci* **7**: 3489-3504.

Carey, D.J., Todd, M.S., and Rafferty, C.M. (1986). Schwann cell myelination: Induction by exogenous basement membrane-like extracellular matrix. *J. Cell Biol.* **102**:2254-2263.

Choi, E.S.-H, Rettig, W.J., Wayner, E.A., Srour, M.L., and Clegg, D.O. (1994). Functional Identification of Integrin Laminin Receptors that Mediate Process Outgrowth by Human SY5Y Neuroblastoma Cells. *J Neurosci Res* **37**: 475-488.

Goodall, A.R., Danks, K., Walker, J.H., Ball, S.G., and Vaughan, P.F.T. (1997). Occurrence of Two Types of Secretory Vesicles in the Human Neuroblastoma SH-SY5Y. *J Neurochem* **68**: 1542-1552.

Haber, S., Finklestein, S.D., Benowitz, L.I.., Sladek, Jr., J.R., and Collier T.J. (1988). Matrigel enhances survival and integration of grafted dopamine neurons into the striatum. *Prog Brain Res* **78**: 427-433.

Hahn, M., Glass, T., and Koke, J. (2000). Extracellular matrix effects on a neuroblastoma cell line. *Cytobios* **102**: 7-19.

Herrera J. J., *Taylor T. A., Malhotra S. K. and Koke J. R. 1995. *In vitro* astrogliosis: chronology of J1-31 antigen expression in reactive 9L astrocytes. *Molecular Biology of the Cell*, **6**(suppl.): 748a

Hodel, A. (1998). SNAP-25. *Int J Biochem Cell Bio* **30**: 1069-1073.

Hynds, D.L. and Snow, D.M. (1999). Neurite Outgrowth Inhibition by Chondroitin Sulfate Proteoglycan: Stalling/Stopping Exceeds Turning in Human Neuroblastoma Growth Cones. *Exp Neurol* **160**: 244-255.

Kleinman, H.K., Kibbey, M.C., Schnaper, H.W., Hadley, M.A., Dym, M., and Grant, D.S. (1993). Role of basement membrane in differentiation. In "Molecular and Cellular Aspects of Basement Membranes," Rohrbach, D.H. and Timpl, R., eds. Academic Press (San Diego), pp. 309-326.

Klosen, P., and Van Den Bosch De Aguilar, P. (1994). Phosphorylated neurofilament

epitopes in neuronal perikarya in the septum, mesencephalon, and dorsal root ganglia of mammals and birds. *J Neurocyto* **23**: 297-311.

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227**: 680-685.

Layne, E. (1957). Spectrophotometric and turidimetric methods for measuring proteins. *Methods Enzymol* **3**: 447-454.

Lelievre, S., Weaver, V.M., and Bissell, M.J. (1996). Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation. *Rec Prog Horm Res* **51**: 417-432.

Nixon, R.A., and Shea, T.B. (1992). Dynamics of Neuronal Intermediate Filaments: A Developmental Perspective. *Cell Motil Cytoskeleton* **22**: 81-91.

Pahlman, S., Mamaeva, S., Meyerson, G., Mattsson, M.E.K., Bjelfman, C., Ortoft, E., and Hammerling, U. (1990). Human Neuroblastoma Cells in Culture: a Model for Neuronal Cell Differentiation and Function. *Acta Physiol Scand Suppl* **592**: 25-37.

Paralkar, V.M., Vukicevic, S., and Reddi, A.H. (1991). Transforming Growth Factor β

Type 1 Biunds to Collagen IV of Basement Membrane Matrix: Implications for Development. *Dev Biol* **143**: 303-308.

Peterson, G.L. (1983). Determination of total protein. *Methods Enzymol* **91**:95-119.

Price, K.A.S., Malhotra, S.K., and Koke, J.R. (1993). Localization and characterization of an intermediate filament-associated protein. *Cytobios* **76**: 157-173.

Ross, R.A., Spengler, B.A., and Biedler, J.L. (1983). Coordinate morphological and biochemical interconversion of human neuroblastoma cells. *J. Natl. Cancer Inst.* **71**(4):741-749.

Stevenson, P. 1996. Biochemical Characterization of a Possible Precursor to Glial Fibrillary Acidic Protein in Reactive Astrocytes, Thesis, Southwest Texas State University.

Schilling, K., Duvernoy, C., Keck, S., and Pilgrim, C. (1989). Detection and Partial Characterization of a Developmentally Regulated Nuclear Antigen in Neural Cells In Vitro and In Vivo. *J Histochem Cytochem* **37**: 241-247.

Schor, S.L., Schor, A.M., Winn, B., and Rushton, G. (1982). The Use of Three-Dimensional Collagen Gels for the Study of Tumour Cell Invasion *in vitro*: Experimental Parameters Influencing Cell Migration into the Gel Matrix. *Int J*

Cancer **29**: 57-62.

Schwarz, M.A, Mitchell, M., and Emerson, D.L. (1990). Reconstituted Basement Membrane Enhances Neurite Outgrowth in PC12 Cells Induced by Nerve Growth Factor. *Cell Growth Diff* **1**: 313-318.

Shea, T.B., Majocha, R.E., Marotta, C.A., and Nixon, R.A. (1988). Soluble, phosphorylated forms of the high molecular weight neurofilament protein in perikarya of cultured neuronal cells. *Neurosci Letters* **92**: 291-297.

Sternberger, L.A., and Sternberger, N.H. (1983). Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc Natl Acad Sci* **80**: 6126-6130.

Taub, M., Wang, Y., Szcesney, T.M., Kleinman, H.K., and Martin, G.R. (1990). Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in Matrigel cultures in serum free medium. *Proc. Natl. Acad. Sci. USA* **87**:4002-4006.

te Velde, A.A., Ladigges, N.C.J.J., Flendrig, L.M., and Chamuleau, R.A.F.M. (1995). Functional activity of isolated pig hepatocytes attached to different extracellular matrix substrates. Implication for application of pig hepatocytes in a bioartificial liver. *J. Hepatol.* **23**:184-192.

Tonge, D.A., Golding, J.P., Edbladh, M., Kroon, M., Ekstrom, P.E.R., and Edstrom, A. (1997). Effects of extracellular matrix components on axonal outgrowth from peripheral nerves of adult animals *in vitro*. *Exp. Neurol.* **146**:81-90.

Traub, P., Nelson, W. J., Kuhn, S. and Vorgias, C. E. 1983. The interaction *in vitro* of the intermediate filament protein vimentin with naturally occurring RNAs and DNAs. *J. Biol. Chem.* **258**: 1456