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| 13. ABSTRACT (Maximum 200 Words) Further studies documenting the molecular mechanisms responsible for Schwann cell proliferation in NF1-derived Schwann cell lines have been carried out. We have documented that the absence of neurofibromin leads to the presence of the tyrosine kinase receptors cKit and PDGF receptor in these Schwann cell lines. C-Kit is expressed early in normal Schwann cell development, where it does not play a role in proliferation, differentiation, or acting as a co-mitogen. Ongoing studies have shown that the c-Kit receptor acts to prevent apoptosis. Activation of cKit in normal Schwann cells leads to the activation of the transduction molecule Akt. In contrast, activation of NF1-derived Schwann cells leads to the activation of both Akt and MAP kinase. It was documented that transformed Schwann cells expressed additional isoforms of adenylyl cyclase and additional prostaglandin receptors relative to normal human Schwann cells. The cKit mediated pathways, which lead to the prevention of apoptosis in NF1-derived Schwann cells are under investigation.. In summary, these studies have further documented some of the molecular mechanisms responsible for the proliferation of Schwann cell NF1 tumors. | | | |
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INTRODUCTION

A. Research Subject

The subject of this investigation is Type 1 neurofibromatosis (NF-1), which is an inherited disease that occurs at a frequency of approximately 1 in 3,000. Two types of tumors are evident in this disease: relatively benign neurofibromas, which occur as subcutaneous tumors, and neurofibrosarcomas (NFS), the more invasive and aggressive type tumors that can potentially be life-threatening. The major cell type that proliferates and forms either of these tumors is the Schwann cell. Our laboratory has obtained and developed a number of different Schwann cell lines derived from neurofibrosarcomas and/or neurofibromas, which we are currently using to study this disease. In addition, we have obtained benign Schwann cell tumors (Schwannomas) from patients who are not affected by NF-1 to serve as controls for the neurofibromatosis-derived Schwann cells.

B. Purpose

The purpose of this investigation is to understand the molecular mechanisms that are responsible for the abnormal proliferation of Schwann cells, which characterizes NF-1. In particular, we would like to understand how the absence of neurofibromin leads to changes in intercellular signaling, which ultimately leads to a sustained proliferation of Schwann cells.

C. Scope of Research

Our approach to understanding the abnormal proliferation of Schwann cells is to evaluate growth receptor expression in NF-derived and non-NF-derived Schwann cells. The presence of additional growth factor receptors is closely related to increased proliferative potential of Schwann cells. In the presence of the appropriate ligand to activate the receptor, these additional receptors could be responsible for the abnormal proliferation, which is observed in NF-derived Schwann cells. As outlined in the original proposal, we proposed to manipulate receptor expression in order to create Schwann cells that mimic cells obtained from NF-1 patients. We also extended our investigation to ask whether or not other growth factor receptors were overexpressed in these cells and what intercellular metabolic pathways could be responsible for increased growth factor expression.

We are also documenting signal transduction pathways activated by growth factor receptor stimulation as well as the functional consequences of such activation. In addition, we are characterizing for the first time the types of synthetic enzymes, adenylyl cyclases and prostaglandin receptors, which may play key roles in maintaining the abnormal growth factor receptor expression and is characteristic of Schwann cells derived from neurofibromas and neurofibrosarcomas.

D. Body

This section of the progress report will include experimental methods, results, and discussion in relation to the statement of work outlined in the award proposal, which contains three specific aims. Progress in achieving the specific aims and associated tasks will be reported. In addition, we will document further research that has amplified and extended the original tasks. This additional research is entirely supportive of the original specific aims. The statement of work and specific tasks from the previously funded proposal are indicated below:

STATEMENT OF WORK

Specific Aim 1:

Effect of decreased neurofibromin expression on human Schwann cell proliferation

Task 1: Culture and expansion of human Schwann cells; determination of basal proliferation levels.

Task 2: Transfection of human Schwann cells with oligonucleotide antisense to neurofibromin; determination of the optimal conditions for viability and efficiency of transfection; Northern blot analysis; proliferation assay.

Specific Aim 2:

Role of Kit expression in NF-1 derived Schwann cell proliferation

Task 3: Establishment of cultures from frozen stocks. Verify basal doubling time.

Task 4: Determination of optimal conditions for transfection for each NF1-cell line; generation of clonal transfected cell lines.

Task 5: Collection of RNA for determination of DN-Kit transcript level of expression; proliferation assay.

Task 6: Northern blot; quantitation of DN-Kit expression; correlative analyses DN-Kit expression/level of proliferation.

Specific Aim 3: Effect of simultaneous Kit expression and neurofibromin alteration on human Schwann cell proliferation.

Task 7: Determination of optimal conditions for transfection of Kit constructs into human Schwann cells.

Task 8: Establishment of clonal cell lines; determination of levels of Kit expression in various clones; proliferation assay.

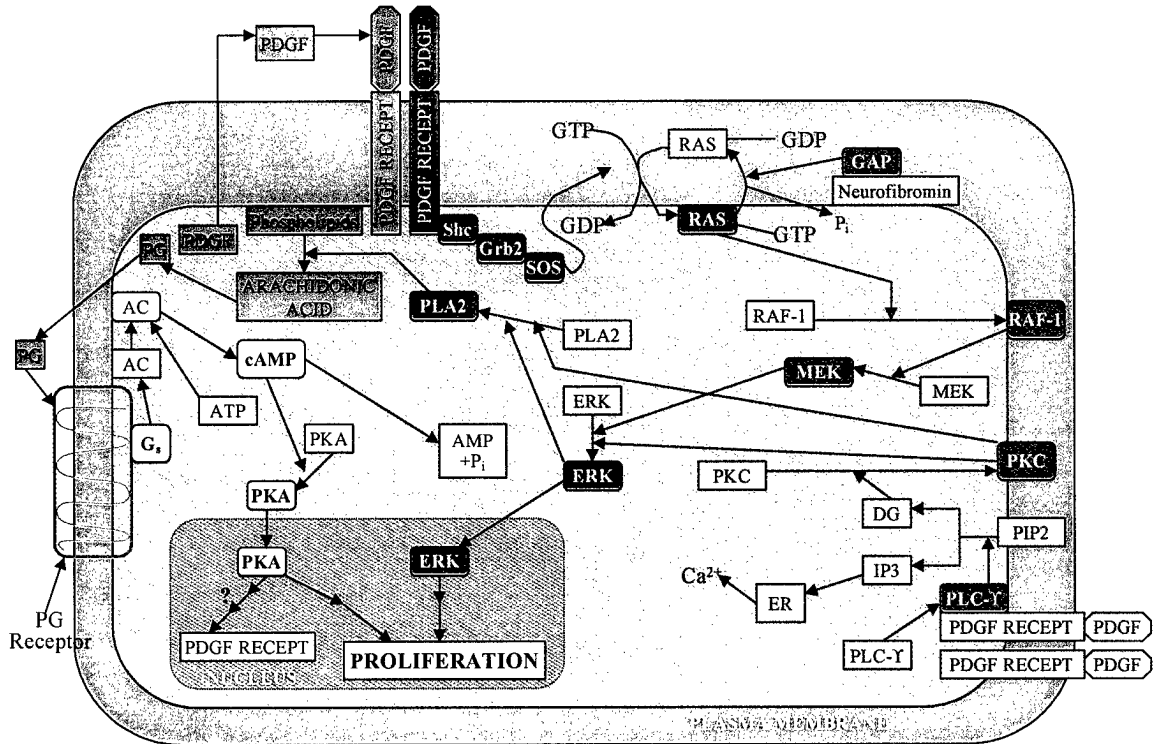
Task 9: Transfection with antisense oligonucleotide against neurofibromin of Kit expressing human Schwann cells selected in Task 8, using optimal conditions determined in Task 2. Proliferation assay; collection of RNA for Northern Blot.

Task 10: Northern blot on double transfected Schwann cells. Correlative analysis level of Kit/neurofibromin expression and proliferation.

In the past year we have continued to concentrate on specific aims 1 and 2; during the final year we will concentrate on Specific Aim 3. We have expanded our theoretical

basis for the molecular mechanism of Schwann cell proliferation in NF-1 as outlined in the color diagram below.

PATHWAYS TO PROLIFERATION IN NF-1



This figure is not meant to be all inclusive but only serves to highlight the major pathways which will be investigated in this proposal. For example p53 is known to be involved in the etiology of this disease (Vogel et al, 1999) but will not be investigated. We have focussed on key transduction molecules depicted in this diagram. The pathways are divided into three separate color schemes, which correspond to the three specific objectives of this proposal. Before delving into the individual transduction pathways, there are several overall features that need to be appreciated. In the first place, as shown in the nucleus of the stylized cell in the figure, there are two pathways to proliferation that will be considered: the ERK pathway and the cAMP-stimulated pathway. Activated ERK is translocated to the nucleus where it can stimulate proliferation while cAMP activates protein kinase A (PKA) causing a translocation of the catalytic subunit to the nucleus where it also can stimulate proliferation. One major question addressed by this proposal is: Does the loss of neurofibromin cause these transduction pathways to be abnormally active in NF-1, and if so, how are these pathways for proliferation sustained in neurofibromatosis?

The cAMP transduction mechanisms are shown in light yellow in the figure. Note that intracellular cAMP levels are attained by a balance between synthesis (ATP to cAMP) and degradation by phosphodiesterases (cAMP to AMP plus phosphate). The cAMP activates protein kinase A (PKA), which causes a translocation of the catalytic subunit, or PKA, to the nucleus. In this location the kinase phosphorylates and activates transcription factors involved in proliferation. In addition the elevated cAMP can lead to increased expression of PDGF receptors (Weinmaster and Lemke, 1990; Badache and De Vries, 1998) possibly via nuclear PKA as indicated in the diagram. The loss of neurofibromin impacts this pathway via increased RAS activation, ERK activation, phospholipase A2 activation and prostaglandin (PG) secretion. In turn, PG stimulates and increases cAMP which drives proliferation.

Transduction mechanisms, including MAP kinase or the ERKs as they are commonly termed, are depicted in blue in the figure. The activation ERKs via MEK leads to two consequences: first the activated ERK can be translocated to the nucleus where like PKA it can phosphorylate transcription factors leading to proliferation; secondly the activated ERK can phosphorylate and activate phospholipase A2 (PLA2) leading to the release of arachidonic acid from cellular phospholipids. In turn the arachidonic acid is a precursor for the production of prostaglandins. Note that there are two pathways which converge on and activate ERK: the PDGF receptor initiated RAS pathway (PDGF receptor-upper part of diagram) and the PDGF receptor initiated phospholipase C- γ (PLC- γ) pathway (PDGF receptor lower right of diagram). In the first pathway activation of the PDGF receptor leads to activation of the transducer molecules Shc, Grb2 and SOS which in turn can activate RAS. Activated RAS transduces its signal to and activates Raf which then activates MEK which finally activates ERK. In the second pathway the activated PDGF receptor activates PLC- γ which acts on PIP2 to produce diglyceride and IP3. The diglyceride then activates Protein Kinase C which in turn will phosphorylate and activate ERK. Of course each PDGF receptor can simultaneously activate both pathways but for the sake of simplicity we have shown two separate receptors each activating a single pathway. In summary the consequence of activation of the PDGF receptor is the dual activation of ERK. As shown in the diagram activated ERK can stimulate two pathways: it can be translocated to the nucleus and stimulate proliferation and it can phosphorylate and activate PLA2. As stated above, the released arachidonic acid can serve as a precursor for PG. The loss of neurofibromin causes overactivation of this pathway via increased ERK activity.

The extent to which secreted PDGF and PG can stimulate the activation of their own receptors and in turn further exacerbate the proliferation of the Schwann cells, which is depicted in orange in the figure. We have preliminary evidence for both the secretion of PG as well as the presence of appropriate PG receptors for the secreted PG (Dang and De Vries, unpublished observations). In addition Schwann cells which are transformed are known to secrete PDGF (Eccelston et al, 1990). Therefore it is reasonable to suppose that these secreted factors play a key role in the proliferation of NF-derived Schwann cells. The loss of neurofibromin impacts this pathway via increased PG synthesis and secretion due to the activation of PLA2. The specific effect of the loss of neurofibromin on the secretion of PDGF is not known.

It is important to recognize the cyclical and self-sustaining nature of these interacting transduction pathways. Intact neurofibromin contains a domain, which can inactivate RAS (GAP domain). Therefore, the lack of neurofibromin leads to overactivation of RAS, which in turn ultimately can lead to overactivation of PLA2 and PG production. This overactivation, via a receptor-mediated G-protein linked mechanism, stimulates an increase in intracellular cAMP. In turn, this mediator can stimulate proliferation via PKA in addition to possibly increasing and sustaining the elevated PDGF receptor levels. This scheme suggests how the loss of neurofibromin can lead to a sustained proliferative response on the part of the Schwann cells. We realize that this scheme is only a working hypothesis and could well be modified as we obtain the relevant data and we are open to this possibility. However, based on our published data and

preliminary observations, these pathways give us a working hypothesis on which to base our experiments.

Our investigations during the second year of funding of this research have yielded a wealth of data, which is included in this progress report. In turn, this data will form the basis for the papers listed below. The data has been presented at a number of national meetings, as noted in the abstracts that have been published. In addition, the principal investigator was recently invited to present this data in axonal presentation at a meeting sponsored by the National Neurofibromatosis Foundation in the summer of 1999. The presentation was entitled "Neurofibrosarcoma-derived Schwann Cells have elevated PGE2 Secretion and intracellular cAMP." The data was also presented this year at the International Consortium on NF-1 and NF-2 in a poster entitled "Molecular Mechanisms of Proliferation in NF-1 Derived Schwann Cells."

Papers in preparation

Dang, I. and DeVries, G.H. (2000), Developmental Role of c-Kit in Schwann Cell function, *J. Neurosci Res*, in preparation.

Dang, I. and DeVries, G.H. (2000), cAMP is elevated in NF-derived Schwann Cells, *Oncogene*, in preparation.

Dang, I. and DeVries, G.H. (2000), Prostaglandins are elevated in NF-derived Schwann Cells, *Oncogene*, in preparation.

Farrer, R. Farrer, J. and DeVries, G.H. (2000), cKit Transduction pathways in NF-derived Schwann Cell Lines, *J. Biol. Chemistry*, in preparation.

Klein, K., and DeVries, G.H., Phenotypic Characterization of Human Schwann Cell Lines Derived from Malignant Peripheral Nerve Sheath Tumors, in preparation.

Abstracts:

Muja, N., and DeVries, G.H., (2000) Recovery of ATP-evoked calcium responses in Schwann cells following chronic treatment with either prostaglandins or neuregulins, *Soc. for Neurosci Abstracts*, in press.

Muja, N., and DeVries, G.H., (2000) Prostaglandins Stimulate cAMP Mediated Signaling Events in Schwann Cells, *Journal of Neurochemistr*, Vol. 74, suppl. S81B.

Dang, I. and DeVries, G.H., (2000) Neurofibrosarcoma-Derived Schwann Cells have Elevated PGE2 Secretion and Intracellular cAMP Levels in Response to PDGF BB., *Journal of Neurochemistry*, Vol. 74, suppl. S81C.

Muja, N. and G.H. DeVries (1999) Prostaglandins elevate intracellular cAMP levels in Schwann cells. *Soc. Neurosci. Abst.*, 25:205.

Dang, I., S. Suy, and G.H. DeVries (1999) Neurofibrosarcoma-derived Schwann cells have decreased Ras-GAP and elevated cAMP levels. *Soc. Neurosci. Abst.*, 25:2085.

Blackman, S.C., N. Muja, G.C. LeBreton and G.H. DeVries (1999) Identification and functional characterization of thromboxane A2 receptors in Schwann cells. *J. Neurochem.* (Suppl.)

BODY:

**Results with Respect to Specific Aim One:
Effect of Decreased Neurofibromin on Human Schwann Cell Proliferation**

Our data has supported the fact that the absence of neurofibromin in Schwann cells leads to a number of metabolic consequences. We have already demonstrated that tyrosine kinase receptor cKit is overexpressed in these cell lines. Since the receptor for the growth factor PDGF is closely related to cKit, we decided to see if there was a relationship between the expression of neurofibromin and the expression of the PDGF receptor. In this experiment, 25µg of total homogenate from NF derived cell lines were separated by polyacrylamide electrophoresis followed by probing for either neurofibromin or PDGFR, the PDGF receptor. The results are shown below.

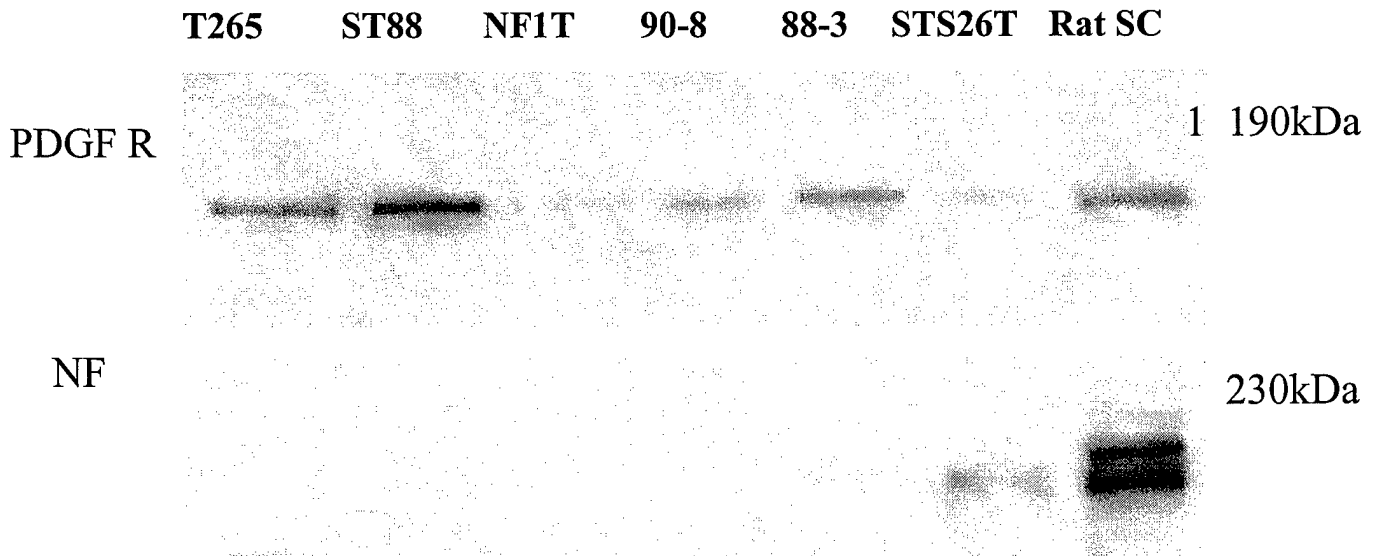


Figure 1: Immunoblot analysis of PDGF Receptor β and neurofibromin expression in NF Schwann cells and normal Schwann cells. Cells were lysed and 25ug of proteins were analyzed by immunoblotting with PDGF Receptor β and neurofibromin antibodies.

Note that there is a clear relationship between the absence and the expression of PDGFR. The expression of this receptor is particularly high in the T265 and ST88 cell lines. Expression of PDGF receptor is somewhat less but still significant in the 90.8 and 88.3 lines. Of particular interest is that the STS26T line, which contains neurofibromin, does not show much expression of PDGF at all. Thus, we have extended our original observations from expression of cKit receptor to include the PDGF receptor and have conclusively demonstrated that the absence of neurofibromin is associated with the overexpression of the receptor for the growth factor PDGF.

We were then interested in determining the type of signal transduction pathways used by these overexpressed receptors. Therefore, using PDGF-BB to activate the PDGF receptor, we looked at the activation of a key transduction molecule, MAP Kinase. As a consequence of stimulation of a prototype NF1-derived Schwann cell line, the T265 line, by PDGF-BB. The results are shown in Figure 2 below.

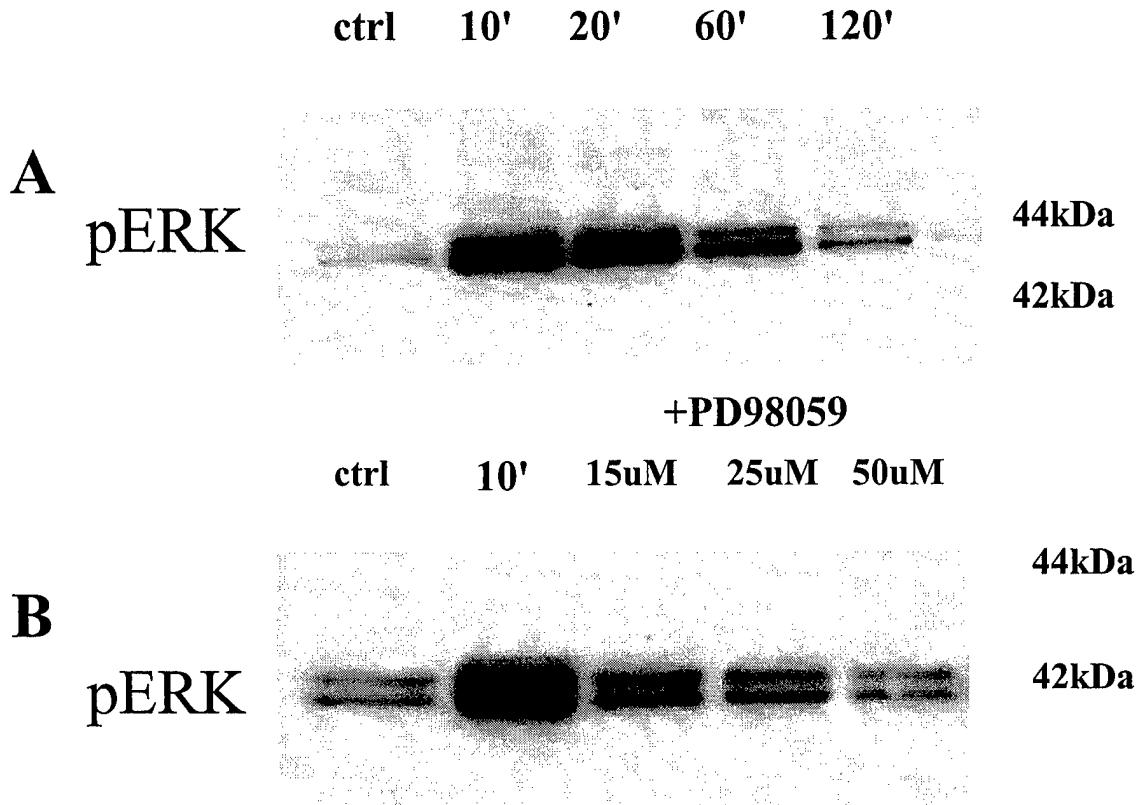


Figure 2. PDGF BB induces phosphorylation of ERK in T265 cells. T265 cells were cultured with 20ng/ml of PDGF BB for up to 120 minutes. Cells were lysed and 25ug of proteins were analyzed by immunoblotting with a phospho specific ERK antibody (A). Cells were incubated with PD98059 for 2 hours prior to adding 20ng/ml of PDGF BB for 10 minutes (B).

As shown in the top panel, after ten minutes there was a strong activation of MAP Kinase as a consequence of stimulation with PDGF-BB. This activation was diminished by 120 minutes. To prove the specificity of this response, we used the drug PD98059 and found that this drug could block the MAP kinase activation observed on the Western-blot, proving that the protein species that were analyzed were, indeed, MAP Kinase.

We previously demonstrated that PDGF-BB is a strong mitogen for these cells. We wanted to understand something about the signal transduction pathways from the overexpressed receptor, which lead to proliferation. Therefore, we carried out an experiment in which the prototype for an NF1-derived Schwann cell line, the T265 line, was evaluated for proliferation in the presence and absence of the inhibitor for MAP Kinase, known as PD98059. The results are shown in Figure 3 below.

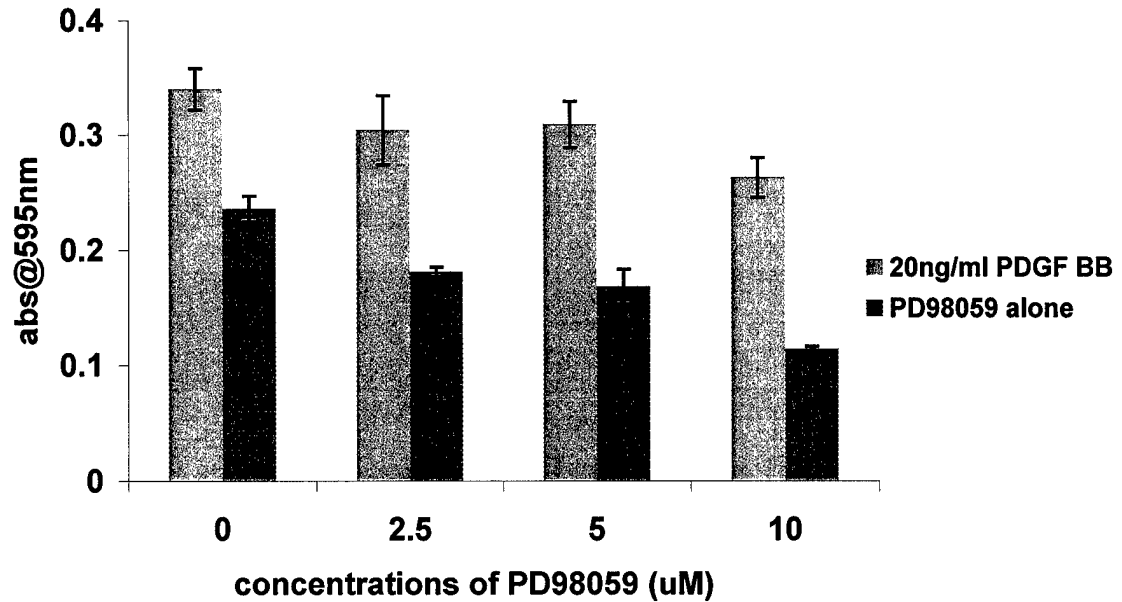


Figure 3. PDGF induced proliferation of NF Schwann cells is not mediated by ERK. T265 cells were cultured in serum free medium in the presence of increasing concentrations of PD98059 lone or with PDGF BB (20ng/ml) for 72 hours. Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. Values are expressed as mean +/- standard deviation of 3 replicates from a representative experiment repeated at least 3 times.

Note at the higher concentrations of the drug, which inhibited MAP Kinase, there was modest inhibition of the proliferation of these Schwann Cells, which could not be ascribed to the non-specific effects of the drug. In these experiments, as demonstrated in the green bar, the effect of drug alone.

In this experiment, one set of cells was treated with vehicle alone to ensure that any differences we saw in the proliferation rate as measured by the MTT proliferation assay were due to specific effects of the drugs and not cell death induced by the drug. Note that at the higher concentrations of drug, there was a tendency toward decreased cell number when the cells were stimulated in the presence of the drug. This experiment supports the view that MAP Kinase does not play a role in the proliferation stimulated by the PDGF-BB. We next asked the question about other signal transduction molecules, namely the PI3 Kinase. The results are shown in Figure 4 below.

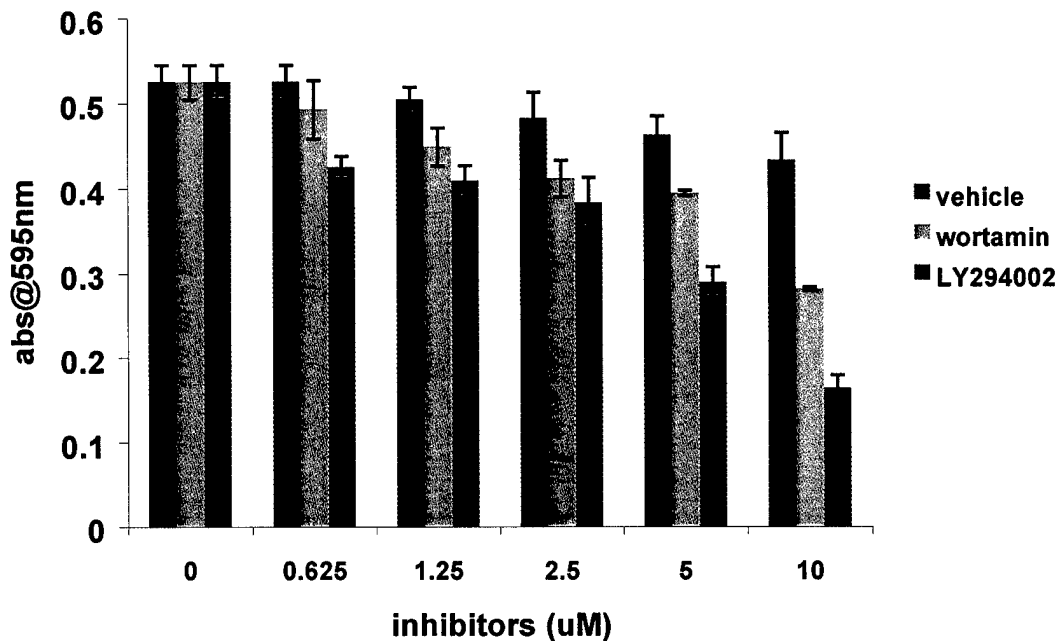


Figure 4. PDGF BB induced NF Schwann cell proliferation is mediated by PI3K. T265 cells were cultured in serum free medium in the presence of 20ng/ml of PDGF BB with vehicle or increasing concentrations of PI3K inhibitors, wortamin and LY294002 for 72 hours. Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. Values are expressed a mean+/- standard deviation of 3 replicates.

Controls with vehicle only (blue bars) were evaluated to ensure that any effect we saw on cell number was not due to the vehicle rather than inhibition by the drug itself. We utilized two inhibitors for PI-3 Kinase, Wortamin and LY294002. It appeared that the LY24002 was a more effective inhibitor of PI-3 Kinase, which resulted in a greater decrease in proliferation relative to the Wortamin. These experiments indicated that PI- 3 Kinase does play a role in the PDGF-mediated cell proliferation.

We were still intrigued with the reason for increased expression of the PDGF receptor. The only indication that we have of how this receptor expression is controlled comes from the experiments of Weinmaster and Lemke (1990), who demonstrated that by elevating cAMP, PDGF receptor was upregulated in rat neonatal Schwann Cells. Therefore, we reasoned that perhaps cAMP, for some reason, is overexpressed in the NF1-derived Schwann cells and that this was the reason for the sustained overexpression of PDGF receptor in these cells. Therefore, we utilized an ELISA assay for cAMP and analyzed the basal levels of cAMP in NF1-derived and normal Schwann cells. Results are shown in Figure 5 below.

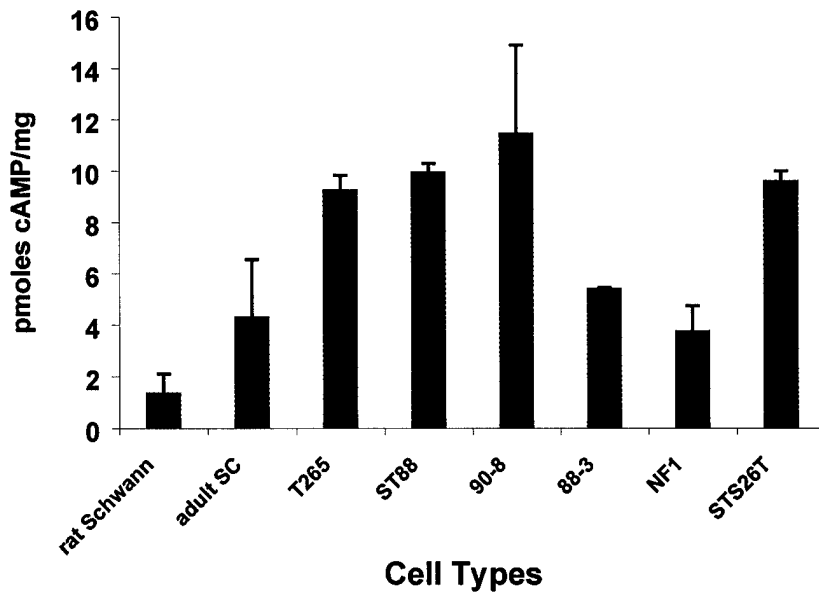


Figure 5. NF Schwann cells have higher basal cAMP levels than normal Schwann cells. Schwann cells were incubated in serum free medium with 200uM IBMX for 1 hour. Concentrations of cAMP were determined from cell lysates by ELISA in duplicate. Values are expressed as mean +/- standard error of the mean.

Note that relative to the normal Schwann cells, there is a very significant elevation of cAMP. However, this is not specific to Schwann cells lacking neurofibromin as the STS26T also showed elevation. Perhaps this is a consequence of abnormal proliferation not necessarily linked to the NF1-derived Schwann cell proliferation. Further studies to understand why there is elevation of cAMP are in progress. We then asked the next logical question, why is cAMP elevated? One well-known metabolite, whose receptor is linked to increased expression of cAMP, is prostaglandin. Therefore, we looked at prostaglandin levels in these cells to see if for some reason these metabolites were elevated. Going back to the diagram "Pathways of Proliferation in NF-1," note that phospholipase A2 is on the pathway for prostaglandin production and overstimulation of this enzyme by other transduction molecules, such as MAP Kinase, could lead to abnormal production of arachidonic acid leading to prostaglandin secretion. Therefore, it was reasonable to suppose that there may be overexpression of prostaglandins in these cells. The results of our investigation are shown in Figure 6 below.

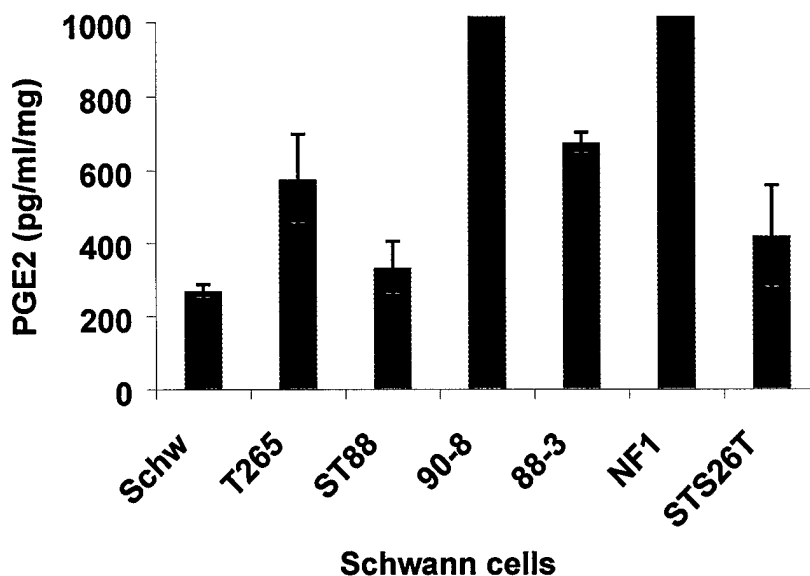


Figure 6. NF Schwann cells secrete higher levels of PGE2 than normal and nonNF Schwann cells. Cells were incubated for 1 hour in serum free medium. Conditioned media were collected and concentrations of PGE2 were measured by ELISA in duplicate. Values are expressed as means \pm standard error of the mean.

It is clear that these cells all secrete large amounts of prostaglandins. Therefore, it is reasonable to suppose that the prostaglandins via receptor activation could be driving the expression of cAMP, which in turn is responsible for the continued elevation of PDGF and cKit receptor. The question then arises: What would happen to cAMP under conditions in which the PDGF receptor was stimulated? The laboratory of Ratner (1997) has reported that tyrosine Kinase receptors, such as the Erb receptors could lead to elevations of cAMP. Therefore, we carried out an experiment in which PDGF-BB was added to the cultured Schwann cells, and cAMP was evaluated. The results are shown in Figure 7 below.

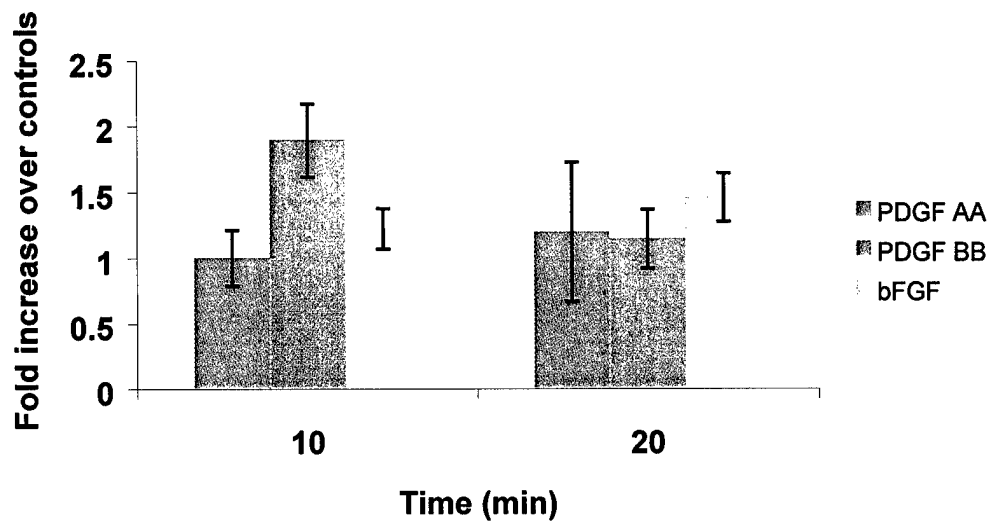


Figure 7. PDGF BB increases intracellular cAMP in NF cells. NF Schwann cells were incubated in serum free medium with 1mM IBMX and growth factors (50ng/ml) for up to 20 minutes. At the time indicated in the text, concentrations of cAMP from the cell lysates were measured in duplicate by ELISA. Values are expressed as a mean standard error of the mean of 3 different experiments.

Note that there was a several fold elevation of cAMP as a result of stimulation with PDGF, particularly PDGF-BB. PDGF-AA had no effect, nor did the bFGF, which we had previously shown to be a weak mitogen for these cells. We were also interested in evaluating whether or not the stimulation of the PDGF-BB receptors could lead to overexpression of the prostaglandins (PGs), which in turn could lead to the elevation of cAMP. Therefore, we carried out an experiment in which we stimulated the T265 cells with PDGF-BB and evaluated the levels of PGE2. The results are shown in Figure 8 below.

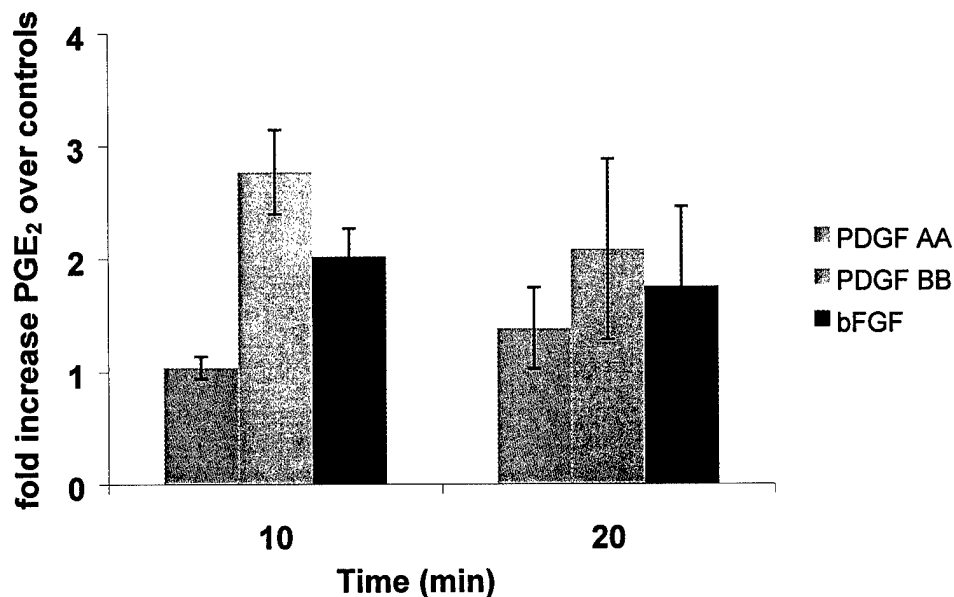


Figure 8. PDGF BB increases PGE₂ levels in NF cells. Cells were incubated in serum free medium with 50ng/ml of growth factors. At the time indicated in the text, conditioned media were collected and concentrations of PGE₂ were measured in duplicate by ELISA. Values are expressed as mean +/- standard error of the mean of 3 different experiments.

Note that PDGF-BB was able to stimulate the secretion of PGE₂, whereas bFGF being a weaker mitogenic stimulus was not able to elevate PGE₂ to the same degree as PDGF. The results of the following two experiments indicate using the T265 as a prototype of the NF1-derived Schwann cells, there is a vicious cycle that begins when the PDGF receptors are overexpressed. Activation of these receptors will aid in sustaining their own expression via increased production of prostaglandins and, in turn, elevation of cAMP. We then investigated the ability of this prostaglandin pathway to stimulate proliferation. What would happen to proliferation if one were to stimulate the PDGF receptor in the presence of drugs that are known to inhibit the expression of prostaglandins? The results are shown in Figure 9.

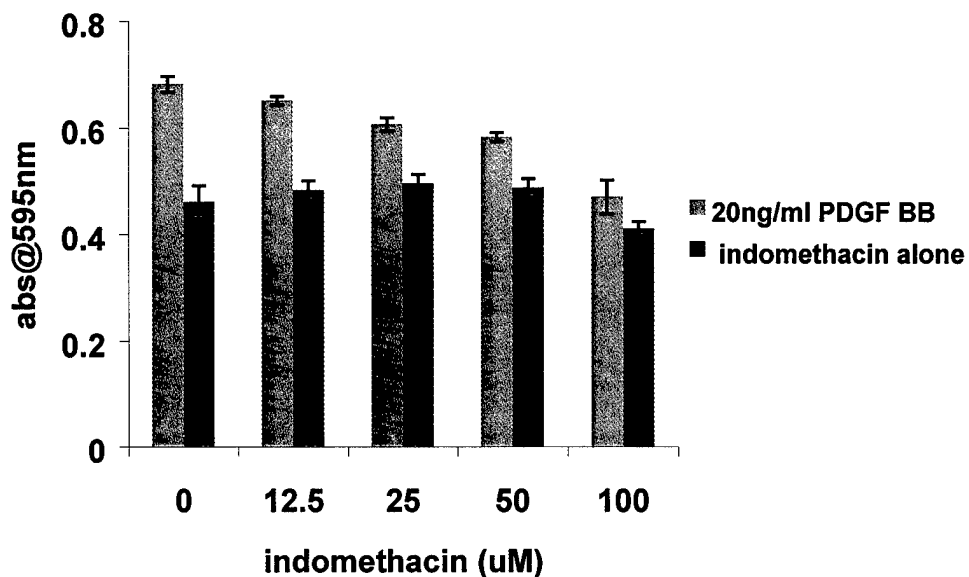


Figure 9. PDGF BB induced proliferation of NF cells is mediated by cyclooxygenase 1 and 2. T265 cells were cultured in serum free medium in the presence of increasing concentrations of indo methacin alone or in combination with 20ng/ml of PDGF BB for 72 hours. Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. Values are expressed as mean +/- standard deviation of 3 replicates from a representative experiment repeated at least 3 times.

Note once again, the blue bars indicate a control, showing that there was no effect on cell number with the drug alone until the drug reached a concentration of 100µM. When stimulated with PDGF, there was a clear drug-dependent decrease in the proliferation, which was related to the degree to which prostaglandin synthesis was inhibited. This data indicates that at least for the PDGF proliferative response, prostaglandins played a key role. We then asked about the role of MAP Kinase in the PDGF-stimulated proliferation. The results are shown in Figure 10.

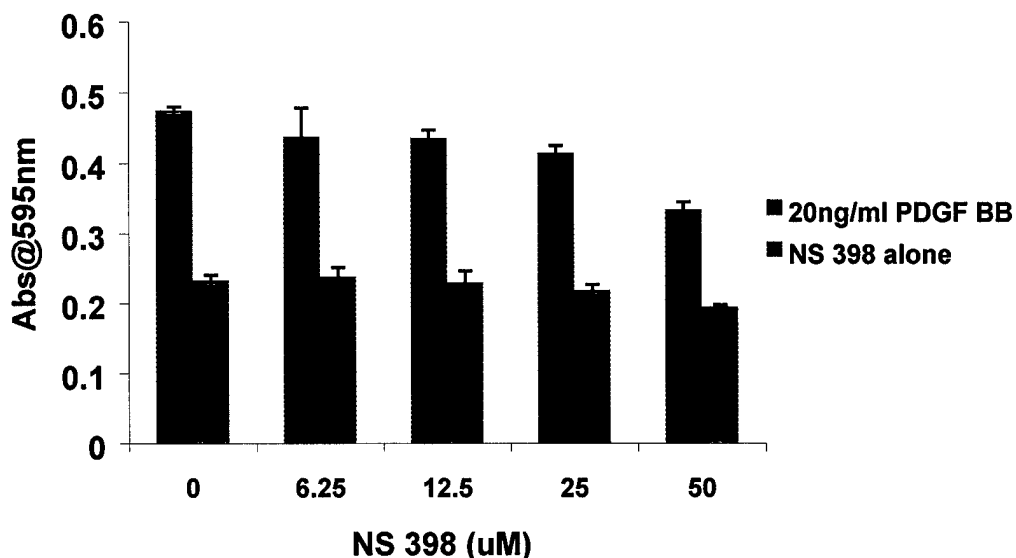


Figure 10. PDGF BB induced proliferation of NF cells is mediated by cyclooxygenase 2. T265 cells were cultured in serum free medium in the presence of increasing concentrations of NS 398 alone or in combination with 20ng/ml of PDGF BB for 72 hours. Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. Values are expressed as mean +/- standard deviation of 3 replicates from a representative experiment repeated at least 3 times.

The blue bars represent cells treated with drug alone and show that there is no drug effect on cell number at the concentrations of the drug utilized. However, there was a drug dose dependent decrease in the proliferation as noted in the green bars. These results indicate that the PDGF-BB proliferative response, in addition to being dependent on prostaglandin synthesis, is also dependent on activation of MAP Kinase for its activity. Since we had shown that cAMP is elevated in these cells, it was reasonable to suppose that protein Kinase A was also playing a role in the proliferation. Therefore, we carried out an experiment in which the protein Kinase A was inhibited with the drug H89. Results are shown in Figure 11 below.

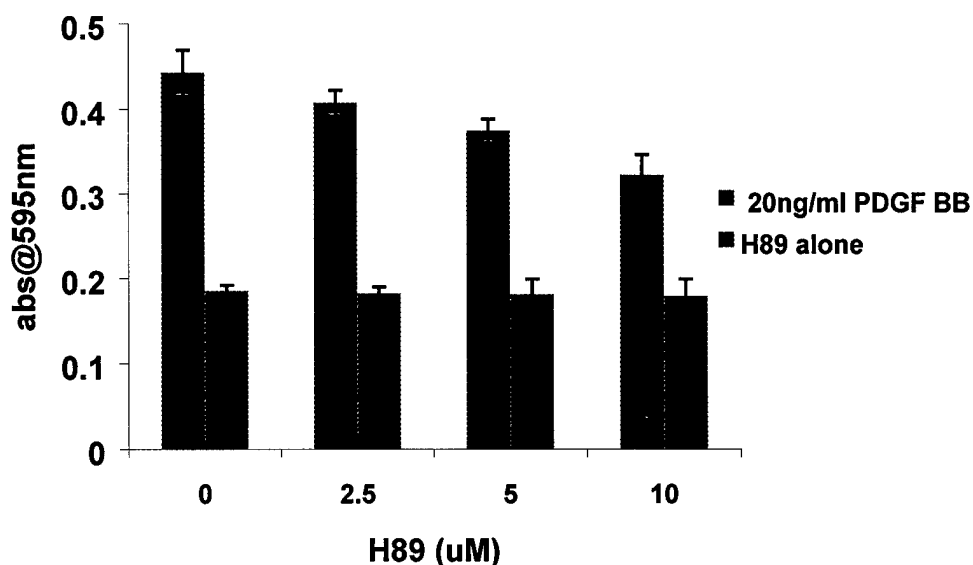


Figure 11. PDGF BB induced proliferation of NF cells is mediated by PKA. T265 cells were cultured in serum free medium in the presence of increasing concentrations of H89 alone or in combination with 20ng/ml of PDGF BB for 72 hours. Cell number was evaluated by the colorimetric MTT assay after 72 hours in culture. Values are expressed as mean +/- standard deviation of 3 replicates from a representative experiment repeated at least 3 times.

Once again the blue bars indicate the drug control experiment and show there was no dose dependent effect on cell number induced by the drug alone. However, when stimulated with PDGF-BB there was a clear and dose dependent decrease in proliferation indicating that protein Kinase A played a key role in the transduction of mitogenic signals from the PDGF receptor to the nucleus of the T265 NF1-derived Schwann cells.

Next we wanted to ask some questions about the types of adenylyl cyclases that were present in these Schwann cells. Adenylyl cyclase is present in a number of different isoforms and they have never been characterized in either normal Schwann cells or NF1-derived Schwann cell lines. To do this, we designed primers to the adenylyl cyclase and looked for the type of isoforms of this enzyme that could be present in the T265 cells. Results are shown in Figure 12 below.

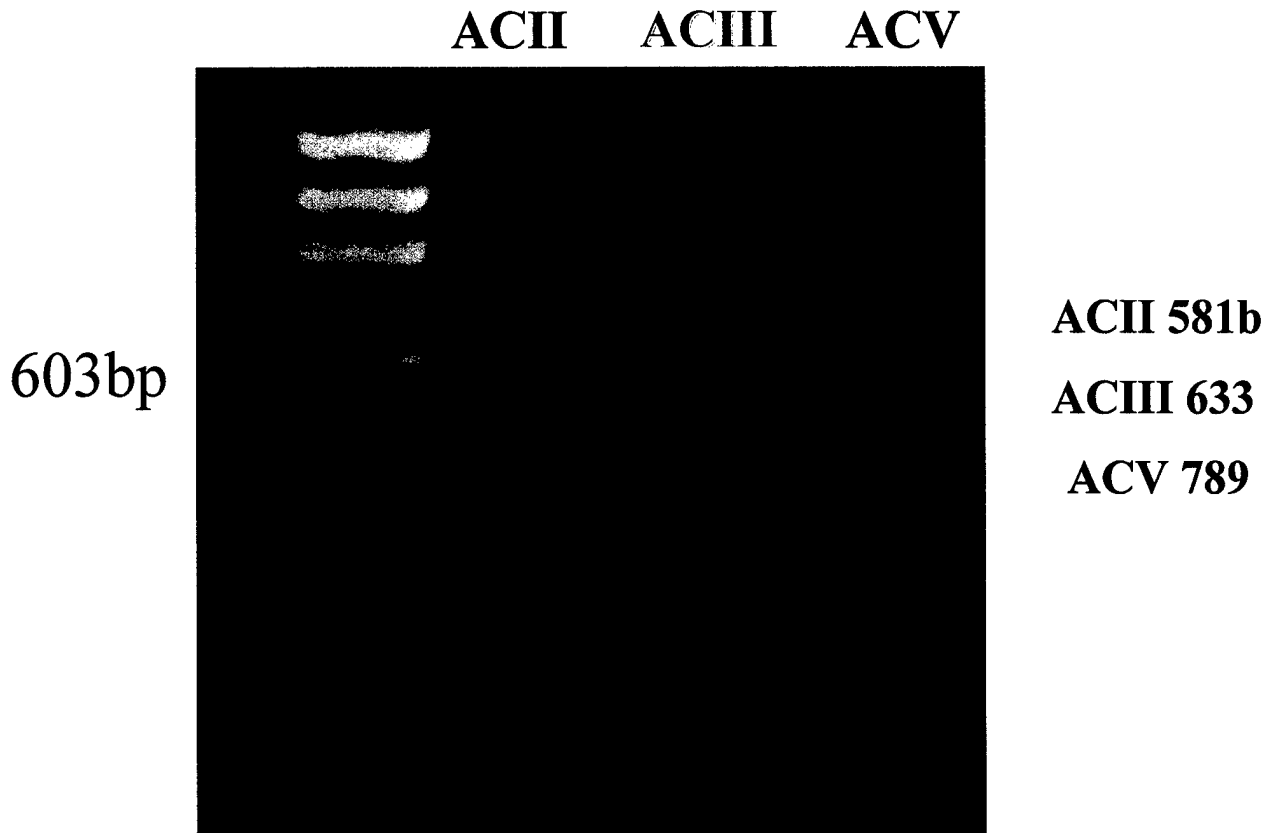


Figure 12. Normal human adult Schwann cells express adenylyl cyclases(AC) III. RT/PCR amplification products for ACIII, 633 base pairs, were separated on a 2% agarose gel stained with ethidium bromide. Products for ACIII and ACV were not detected. Molecular weight markers are derived from Hae III digest of $\phi\chi$ -174 viral DNA as shown in lane 1.

Note that we do not yet have the conditions or the primers exactly right for AC3 and AC5 since the predicted product size in the case of AC3 was 633 base pairs and only one of the two bands certainly appears to have that molecular weight. For AC5 the predicted size was 789 base pairs and no band of this size is evident. New primers are being designed and new conditions for stringency to further evaluate the presence of these receptors. However, preliminary results indicate that there is an AC2, AC3 present in the NF1-derived T265 cells. These results only have significance when compared to normal human Schwann cells. What type of adenylyl cyclates are present in normal Schwann cells? We were fortunate to get normal adult human nerve from Dr. Pat Wood at the Miami Project and prepared adult human Schwann cells and analyzed them by PCR for the presence of mRNA for adenylyl cyclases. The results are shown in Figure 13 below.

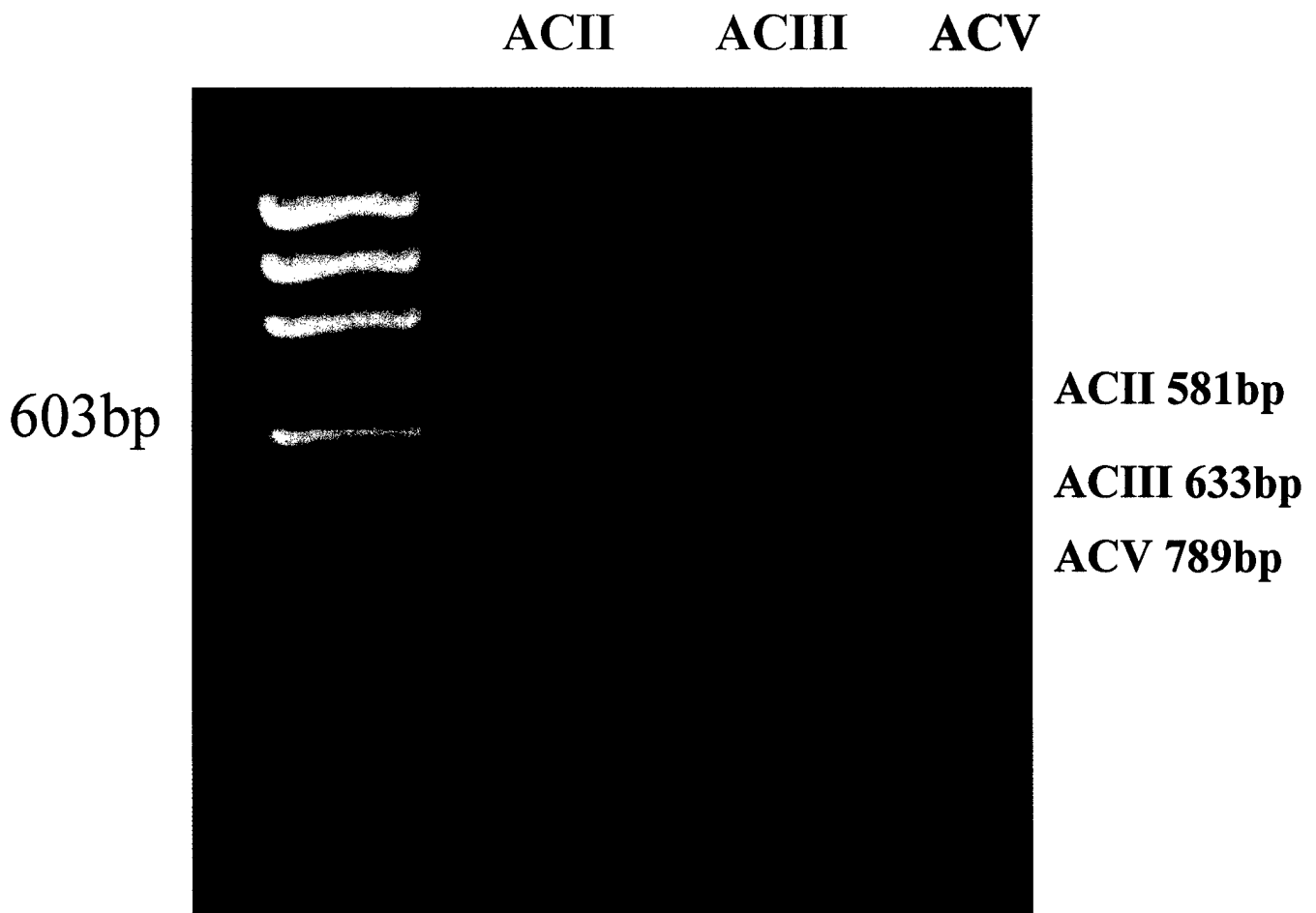


Figure 13. NF Schwann cells express adenylyl cyclases(AC) type II and III. RT/PCR amplification products for ACII, 581 base pairs, and for ACIII, 633 base pairs, were separated on a 2% agarose gel stained with ethidium bromide. The product for ACIII is not of the right size. Molecular weight markers are derived from Hae III digest of $\phi\chi$ -174 viral DNA as shown in lane 1.

Note that with the caveats mentioned for Figure 12, it appears that the Type 2 and Type 5 isoforms of adenylyl cyclase are not present. With only the Type 3 adenylyl cyclases mRNA being present in human Schwann cells. This data indicates another metabolic abnormality in the transformed Schwann cells in that they begin to express a form of adenylyl cyclase not seen in the normal untransformed Schwann cells. Activation of this additional adenylyl cyclase can contribute to the overall elevation of cAMP observed in these cells. We then wanted to ask similar questions about the prostaglandin receptors in normal and transformed Schwann cells. The results and analysis of the T265 cells for the prostaglandin receptors is shown in the Figure 14 below.

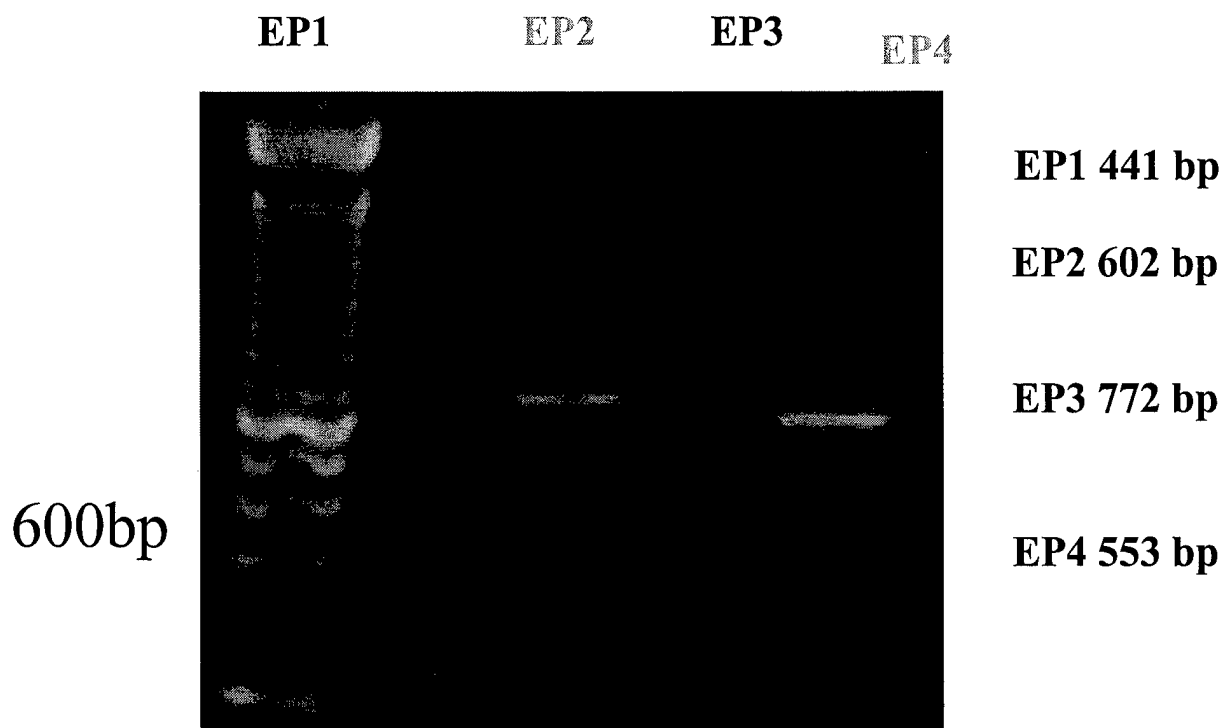


Figure 14. NF Schwann cells express EP2 and EP4 receptors. RT/PCR amplification products for EP2, 602 base pairs, and for EP4, 533 base pairs, were separated on a 2% agarose gel stained with ethidium bromide. Products for EP1 and EP3 were not detected. Molecular weight markers are derived from Hae III digest of $\phi\chi$ -174 viral DNA as shown in lane 1.

Note that the two receptors that are clearly present are EP2 and EP4, with no EP1 or EP3. This would be reasonable in the light of the stimulations that were observed with prostaglandin E-2, which is known to activate these two receptors. These two receptors are also known to be coupled to increases in cAMP. We then analyzed a cell line lacking neurofibromin, (the STS26T), for its prostaglandin receptor profile. The results are shown in Figure 15 below.

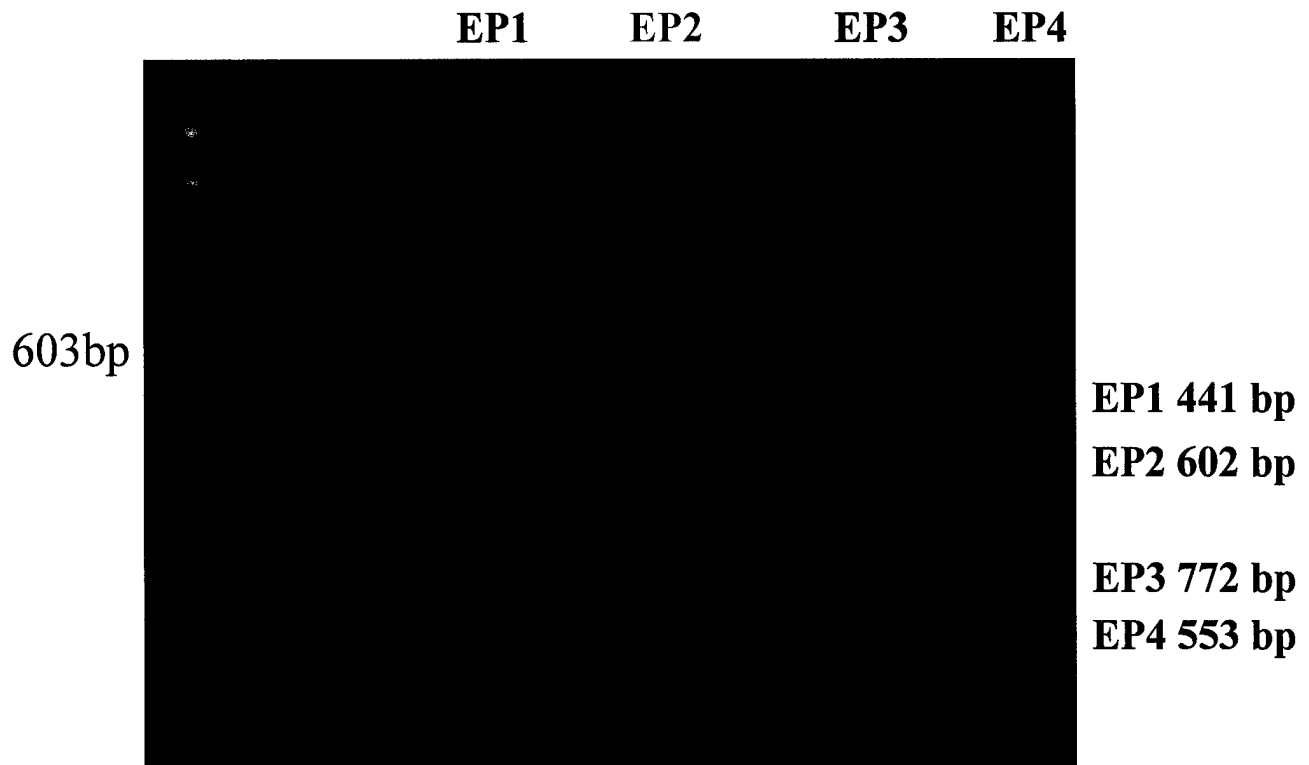


Figure 15. Non-NF Schwann cells express EP2 receptors. RT/PCR amplification product for EP2, 602 base pairs, was separated on a 2% agarose gel stained with ethidium bromide. Products for EP1, EP3, and EP4 were not detected. Molecular weight markers are derived from Hae III digest of $\phi\chi$ -174 viral DNA as shown in lane 1.

Note that in this case, there was no EP4 receptor in contrast to the NF1-derived Schwann cells. In order to put both of these findings in the context of normal human Schwann cell biology, we analyzed normal adult human Schwann cells for the presence of prostaglandin receptors. Using human peripheral nerve obtained from Dr. Pat Wood at the Miami project, we prepared normal human Schwann cells from this source. The results of analyzing these cells for the prostaglandin receptor are shown below.

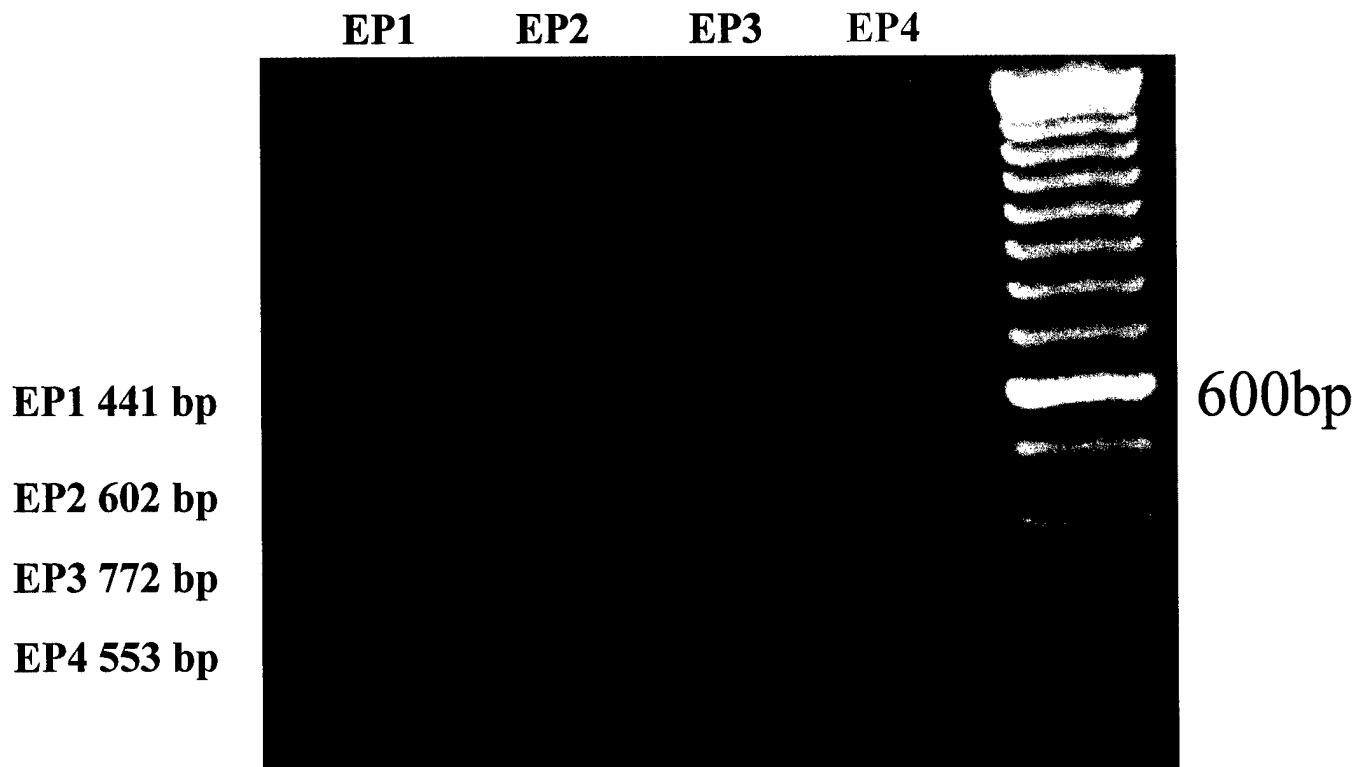


Figure 16. Normal adult human Schwann cells express EP4 receptors. RT/PCR amplification product for EP2, 553 base pairs, was separated on a 2% agarose gel stained with ethidium bromide. Products for EP1, EP2, and EP3 receptors were not detected. Molecular weight markers are derived from a 100 base pairs ladder.

Clearly, the adult human Schwann cells express only the EP4 receptor. This has led to the concept that the expression of EP2 is associated with a malignant transformation. Both the STS26T and the NF1-derived T265 cells uniquely show the expression of EP2. Additionally, in contrast, the cells lacking neurofibromin continue to express the normal human Schwann cell, EP4. This type of receptor profile allows the transformed Schwann cells to very effectively respond to any prostaglandin in their vicinity, leading to an elevation of cAMP, and in turn, abnormal expression of growth factor receptors.

We then investigated the role that PGE2 could have on elevating cAMP in the NF1-derived and non-NF1-derived Schwann cell lines. The results are shown in Figure 17 below.

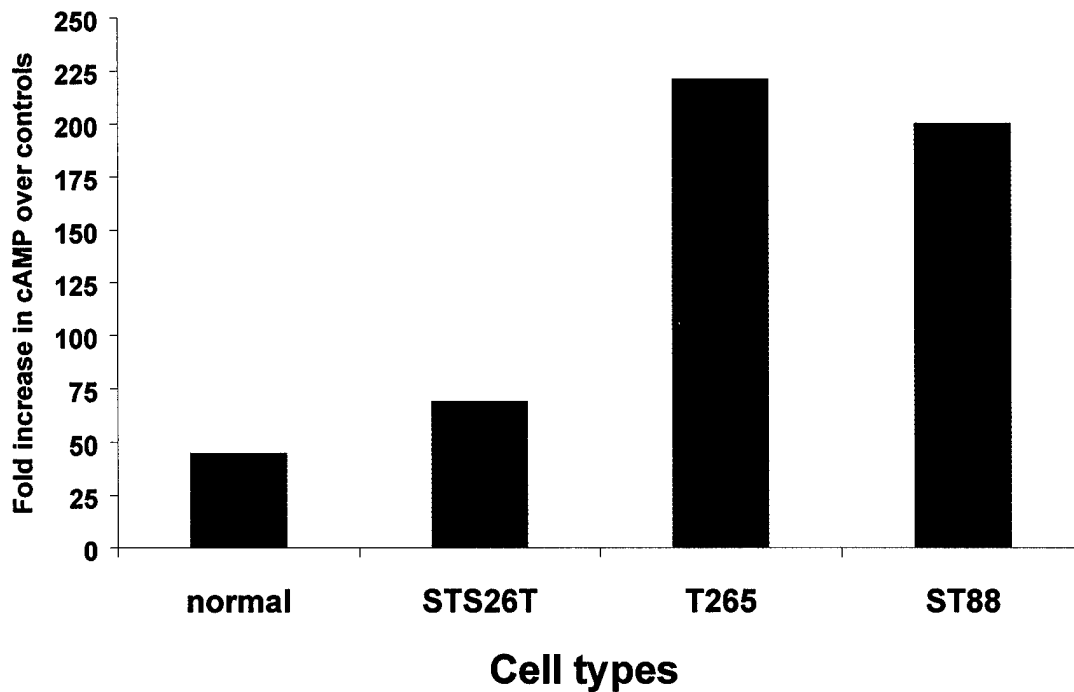


Figure 17. Effects of 1uM PGE2 on cAMP levels in NF, non-NF and normal adult Schwann cells. Cells were incubated with 1uM PGE2 and 200uM IBMX. After 15 minutes, concentrations of cAMP from cell lysates were measured in duplicate by ELISA. The levels of cAMP were normal to unstimulated cells.

Note that the addition of prostaglandin E2, as expected from the receptor profile, leads to a profound increase in cAMP in the T265 and ST88 cell lines. As expected, since the STS26T has less receptors, only expressing EP2, its response in terms of elevating cAMP is somewhat less. This indicates that the prostaglandins, if found in the vicinity of the tumor, could lead to an increased intracellular cAMP, which in turn could lead to the chronic elevation of growth factor receptors.

Results and Progress made with Respect to Specific Aim 2: Role of Kit expression in NF-1 derived Schwann Cell Proliferation:

We were intrigued with over expression of the c-Kit in the NF derived Schwann cell lines. We first looked at c-Kit expression in the 90.8 and 88.3 cell lines, which we had not previously analyzed for c-Kit expression. The results of this are shown in Figure 18 below.

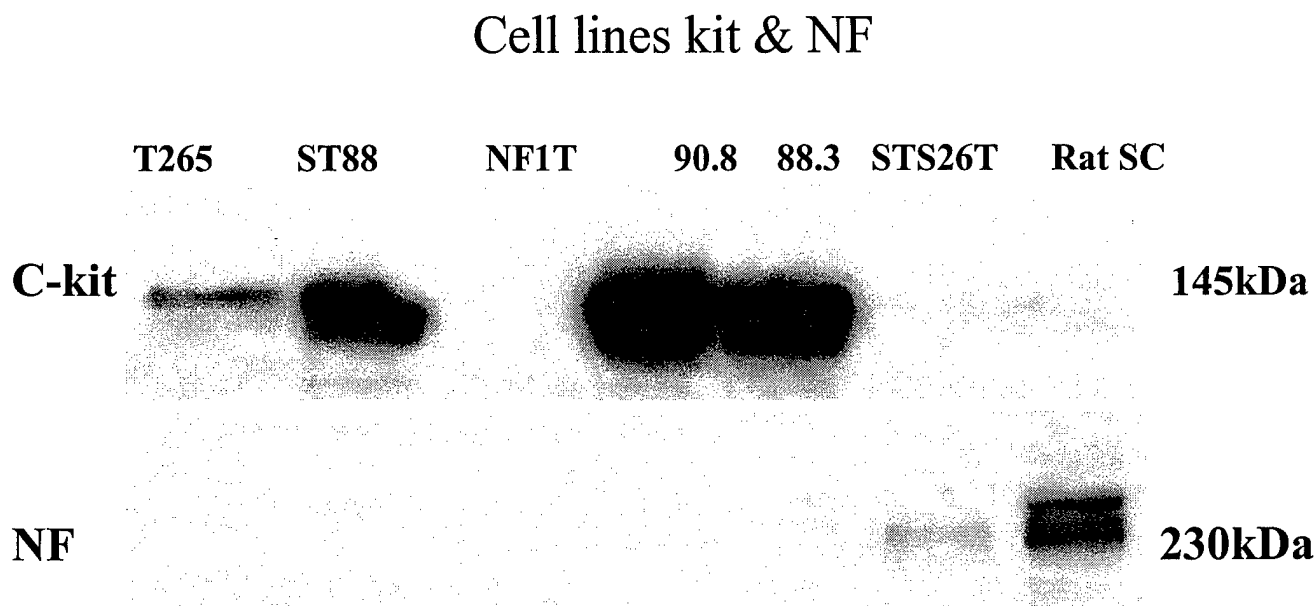


Figure 18: Western-blot for c-Kit and Neurofibromin in NF-1 derived Schwann cells and normal Schwann cells

Note that the 90.8 and 88.3 NF1-derived Schwann cell lines strongly expressed c-Kit as we had previously noted for ST88 and T265 cell lines. Note that cell lines that have no expression of NF express c-Kit, whereas the STS26T cell lines, which expresses NF, does not express c-Kit. This extended our confidence that this change in receptor expression was specific for neurofibromatosis.

A theme in cancer biology is that in malignant transformation, molecules are abnormally expressed in the adult, which have an important role to play in development. Therefore, we asked the question whether or not Kit had a role to play during neuronal development. To do this, we analyzed either neonatal Schwann cells or sciatic nerves obtained at different developmental time points. Our intent was to look for a role for Kit developmentally, with the expectation that early in development it would be expressed and in the adult it would not be expressed. We utilized the well-established myelin associated marker P0 to study normal development of myelination in the peripheral nerve. The results of this investigation are shown in Figure 19 below.

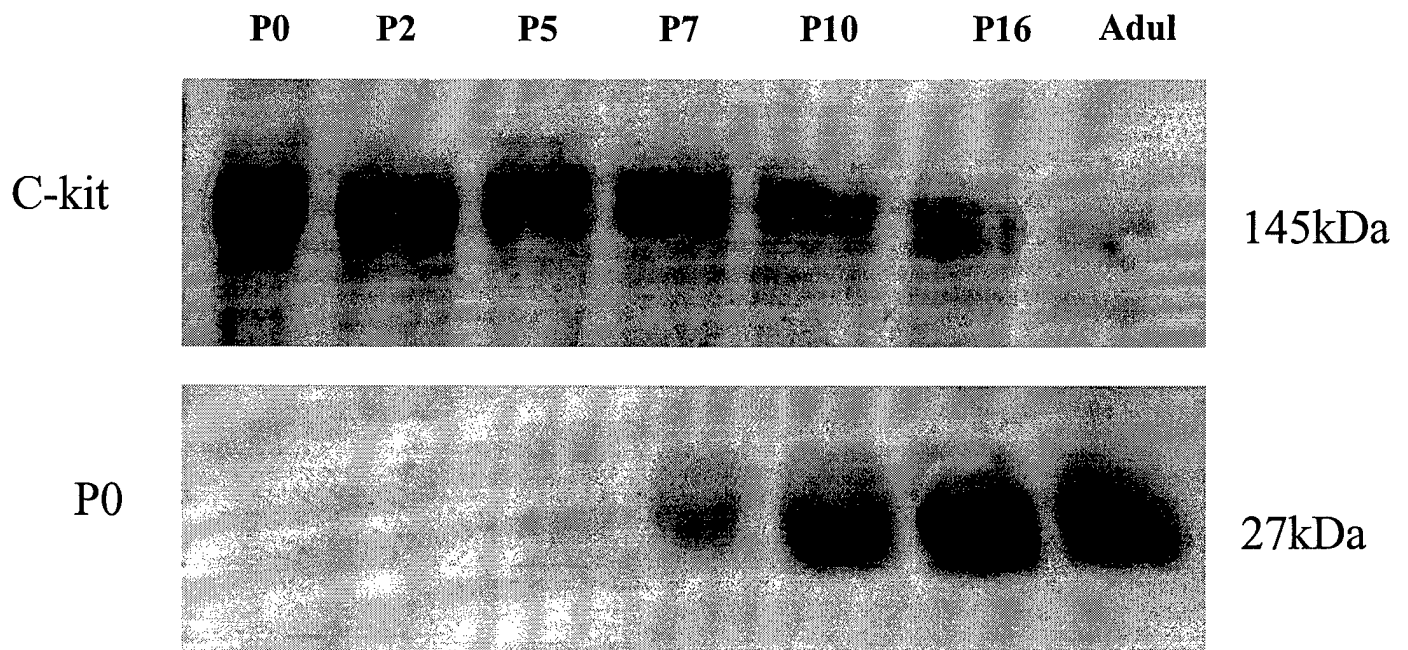


Figure 19. Immunoblot analysis of c-kit and P0 protein in rat sciatic nerves, postnatal day 0, postnatal day 2 (P2), postnatal day 5 (P5), postnatal day 7 (P7), postnatal day 10 (P10), postnatal day 16 (P16), and adult. Rat sciatic nerves were homogenized with lysis buffer, and 100ug of proteins were analyzed by immunoblotting with c-kit and P0 antibodies.

Note that c-Kit is strongly expressed in the P0 sciatic nerve and expression decreases with time, whereas P0 as expected is not expressed in the neonatal period, but during the period of active myelination from P7-P16 and continues to be strongly expressed in the adult nerve. These results are quantitatively represented after densitometry in Figure 20 below.

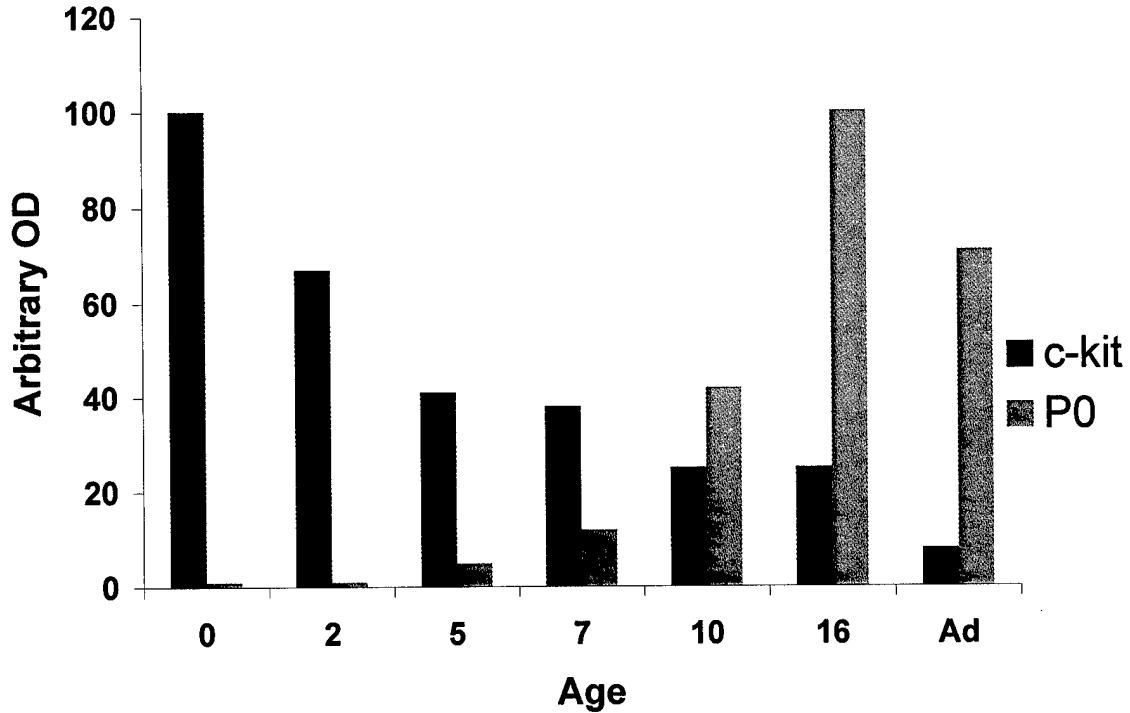


Figure 20. C-kit and P0 expression are inversely related during development. Relative densitometric analysis of c-kit and P0 protein expression in rat sciatic nerves during development.

This figure very nicely illustrates the inverse relationship between the expression of c-Kit and P0; c-Kit is highest at Day 0 and decreases linearly and markedly with development until its expressed at very low levels in the adult. The myelination marker, P0, starts to be expressed at age 7 days and continues its expression into the adult. These results indicated that indeed there was a developmental role for c-Kit in normal Schwann cells. Next we looked at the expression of the mRNA for cKit in neonatal Schwann cells as revealed by PCR. The results are shown in Figure 21 below.

603bp



513 bp

Figure 21. Neonatal rat Schwann cell express c-kit mRNA. RT/PCR amplification of c-kit mRNA and GAPDH in cultured neonatal rat Schwann cells. Photograph of c-kit products, 513 base pairs (lane 2 and 3), and GAPDH, 892 base pairs (lane 4), separated on a 2% agarose gel stained with ethidium bromide. Molecular weight markers are derived from Hae III digest of $\phi\chi$ -174 viral DNA as shown in lane 1.

Note that the c-Kit mRNA as revealed by PCR has the expected molecular weight. We also carried out extensive sequencing of this PCR band and found it had the normal c-Kit sequence (Dang and DeVries, unpublished observations).

We are still puzzled by the role of c-Kit in normal Schwann and rat Schwann cell development. Therefore we asked, first, if it had a role in proliferation. Stem cell factor was added at increasing concentrations to neonatal rat Schwann cells and the effect on proliferation was observed. Results are shown in Figure 22 below.

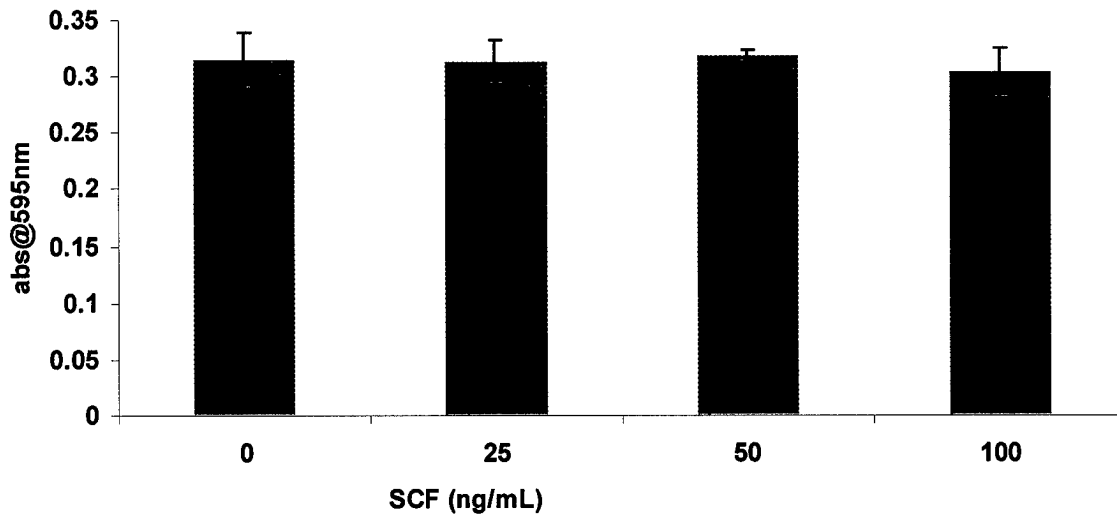


Figure 22. SCF does not induce neonatal rat Schwann cell proliferation. Schwann cells were cultured in the presence of increasing concentrations of SCF for 96 hours. Cell number was evaluated by the colorimetric MTT assay after 96 hours in culture. Values are expressed as mean +/- standard deviation of six replicates from a representative experiment repeated at least three times.

Note that over a wide range of stem cell factor concentrations, there was no increase in proliferation. Therefore, it is clear that whatever the role of c-Kit, it was not related to proliferation. However, it was possible that the stem cell factor was acting as a co-mitogen and would synergize with other mitogens and increase Schwann cell proliferation. Therefore, we utilized one of the strongest mitogens known for Schwann cells, namely neu differentiation factor, or NDF, at a concentration of 25 ng/ml and stimulated cells for four days in the presence of increasing concentrations of stem cell factor. The results are shown in Figure 23 below.

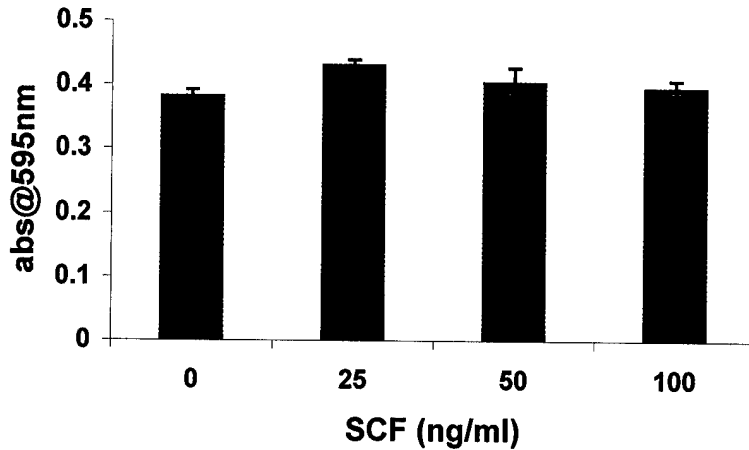


Figure 23. SCF does not synergize with NDF to increase Schwann cell proliferation. Schwann cells were cultured in the presence of 20ng/ml of NDF and increasing concentrations of SCF for 96 hours. Cell number was evaluated by the colorimetric MTT assay after 96 hours in culture. Values are expressed as mean +/- standard deviation of 6 replicates from a representative experiment repeated at least 3 times.

Note that there was no increase proliferation as a result of the presence of stem cell factor, indicating at least in the case of NDF, there was no role for stem cell factor as a co-mitogen. The other attractive possibility was that stem cell factor was acting as a differentiation factor and starting the differentiation process to allow the cells to prepare for myelination. In order to evaluate this possibility, we utilized primers for the myelin-specific major glycoprotein, P0, and analyzed neonatal rat Schwann cells for the message for P0 at different times after stimulation with stem cell factor. We utilized the GAPDH mRNA to normalize and insure that the loading and amplification was similar for all time points for the P0. The results are shown in Figure 24 below.

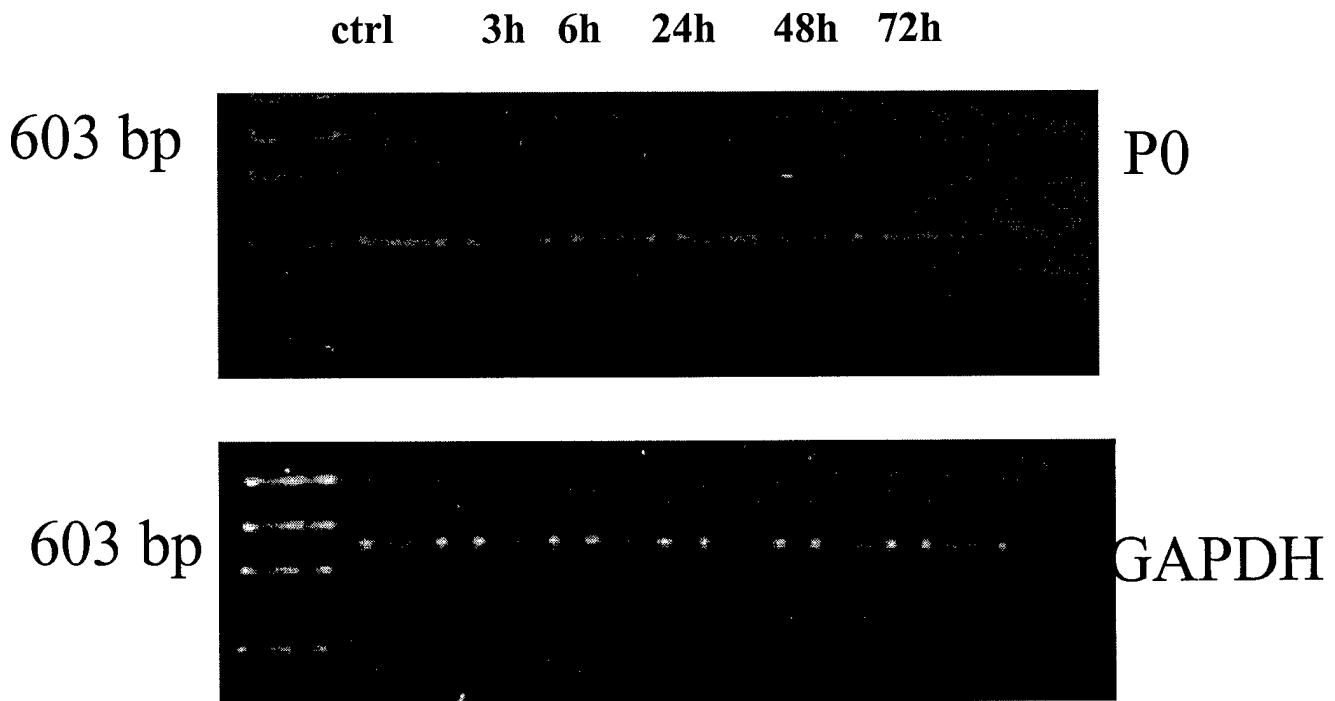
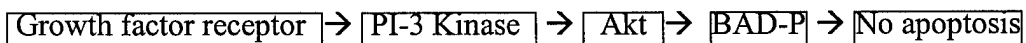


Figure 24. SCF does not induce Schwann cell differentiation. Schwann cells were cultured in the presence of 100ng/ml of SCF up to 72 hours. RT/PCR amplification of P0 and GAPDH mRNA were performed for each time point. Photograph of P0 products, 603 base pairs, and GAPDH, 892 base pairs, were separated on a 2% agarose gel stained with ethidium bromide. Molecular weight markers are derived from Hae III digest of $\phi\chi$ -174 viral DNA as shown in lane 1.

Note that there was no increase in the mRNA for P0 as a result of stimulation with stem cell factor, ruling out a profound role for stem cell factor in stimulating differentiation.

The only other major role that stem cell factor could have then would be in preventing apoptosis. As an indication of this role, we evaluated the potential of stem cell factor to activate a key transduction molecule AKT, which is known to be on the pathway and leads to the prevention of apoptosis as indicated by the following pathway:



Akt is a kinase (also referred to as protein Kinase B), which phospholates a factor called BAD and thus prevents it from binding to and inactivating BCL, which is the major factor that is required to prevent apoptosis. The results of a time course of activation of Akt by SCF is shown in Figure 25 below.

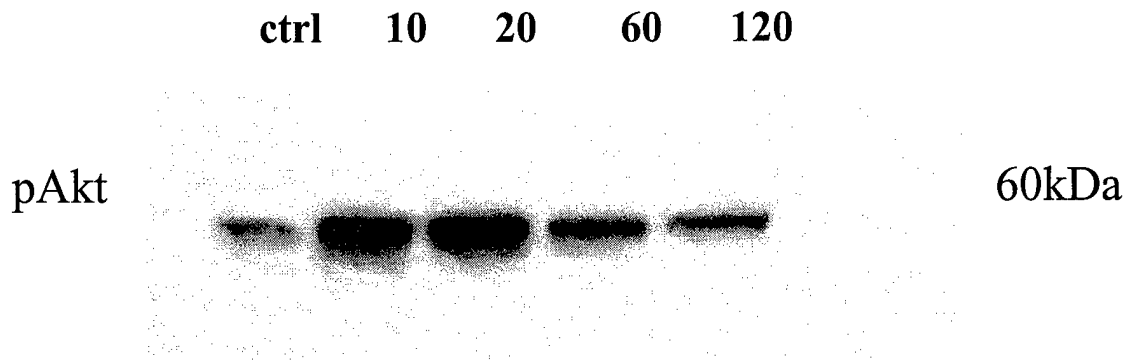


Figure 25. SCF induces Akt phosphorylation in neonatal rat Schwann cells. Schwann cells were cultured with 100ng/ml of SCF for up to 120 minutes. Schwann cells were lysed and 25ug of proteins were analyzed by immunoblotting with a phosphorylated specific Akt antibody.

Note that there is a rapid sustained increase in the activation (phosphorylation) of Akt, which then decreases by 120 minutes. It should also be noted that there was negligible activation of MAP Kinase in primary Schwann cells (Dang and DeVries, unpublished observations). These results demonstrate that SCF definitely is linked to activation of Akt and supports the view that SCF may play a role in preventing apoptosis in rat Schwann cells. Further studies of the anti-apoptotic effect of SCF on neonatal rat Schwann cells are in progress.

Having established a potential role for SCF/cKit during development, we next evaluated whether or not c-Kit/SCF was playing a similar role in the NF1-derived Schwann cell lines. First we wanted to establish the degree to which these cell lines were resistant to apoptosis.

Three methods were tried for initiating cell death in the ST88 and T265 cells:

- 1) Serum deprivation produced no changes in cell survival that were evident by visual inspection over a five-day period. Note that these conditions have been shown to induce apoptosis in neonatal rat Schwann cells.
- 2) NGF treatment (10 ng/ml) in serum-free medium also produced no evidence of cell death over the same time period.
- 3) Treatment with C2 ceramide (20 μ M) for 3 hours caused noticeable changes in cell morphology (cell rounding and detachment) which were consistent with cell death. C6 ceramide at the same concentration did not have such dramatic effects, but the cells did begin to show signs of changes in morphology by 6 hours that were not evident in the cells treated with dihydro-C6 ceramide (inactive).

Experiments are underway to insure that the ceramide induced cell death is apoptosis and not necrosis. These preliminary experiments indicate that some changes in

intracellular metabolism have taken place, which allows the cells to survive the normal apoptotic process. In turn, this would contribute to tumor growth by stabilizing cells that have divided. In order to evaluate this possibility, we first asked the question as to the extent to which phosphotyrosine staining was evident indicating that the c-Kit receptor was activated in basal conditions and after stimulation with c-Kit. The results are shown in Figure 26 below.

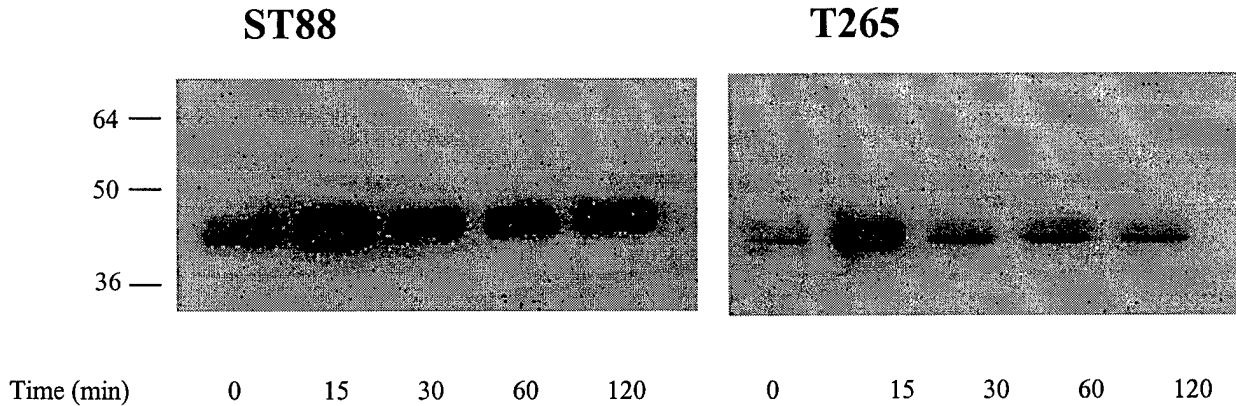


Figure 26: Anti-Phosphotyrosine Western blots of ST-88, T265

These results are consistent with activation of c-Kit by stem cell factor, exogenously applied. It is also interesting to note that at the 0 times, there was already a significant activation of a protein in the same molecular weight range as the c-Kit receptor. These results currently are being repeated by immunoprecipitation of c-Kit followed by analysis of phosphotyrosine to more specifically assign the phosphorylation to c-Kit.

Next we asked if the tumor cells could activate the antiapoptotic pathway by SCF stimulated activation of Akt.

The first thing we looked at was whether or not the key transduction molecule Akt was activated in ST88 and T265 Schwann cells by stimulation by stem cell factor. The results are shown in Figure 27.

Phospho-Akt Activation by SCF

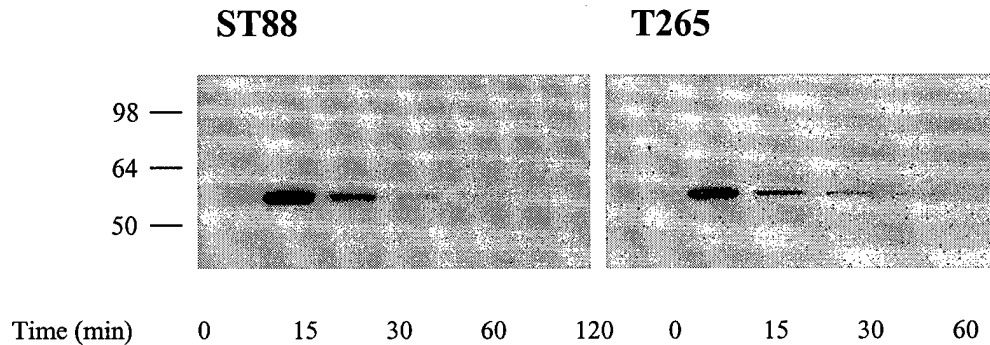


Figure 27: SCF induced Akt Activation of ST-88 and T265 Schwann Cells

ST88 and T265 cells were plated in 35 mm dishes at a density of 300,000 cells per dish. The cells were originally plated in serum containing medium, incubated in serum-free medium for four hours before SCF treatment. Stem cell factor was added after the four hour incubation period at a concentration of 20 ng/ml, and the cells were incubated with SCF for the given time points. Proteins were separated on a 4-20% gradient gel and blotted to a PVDF membrane. The membrane was probed with a Phospho-Akt antibody (1:1000).

These results are consistent with the view that there is a rapid activation of BAD Akt, which is known to be a molecule that can phosphorylate BAD and prevent it from inducing apoptosis. We were also interested to see the extent to which MAP-Kinase was activated by stem cell factor, having already shown that the proliferative response was rather modest (Badache and DeVries, 1998). Results are shown in Figure 28 below.

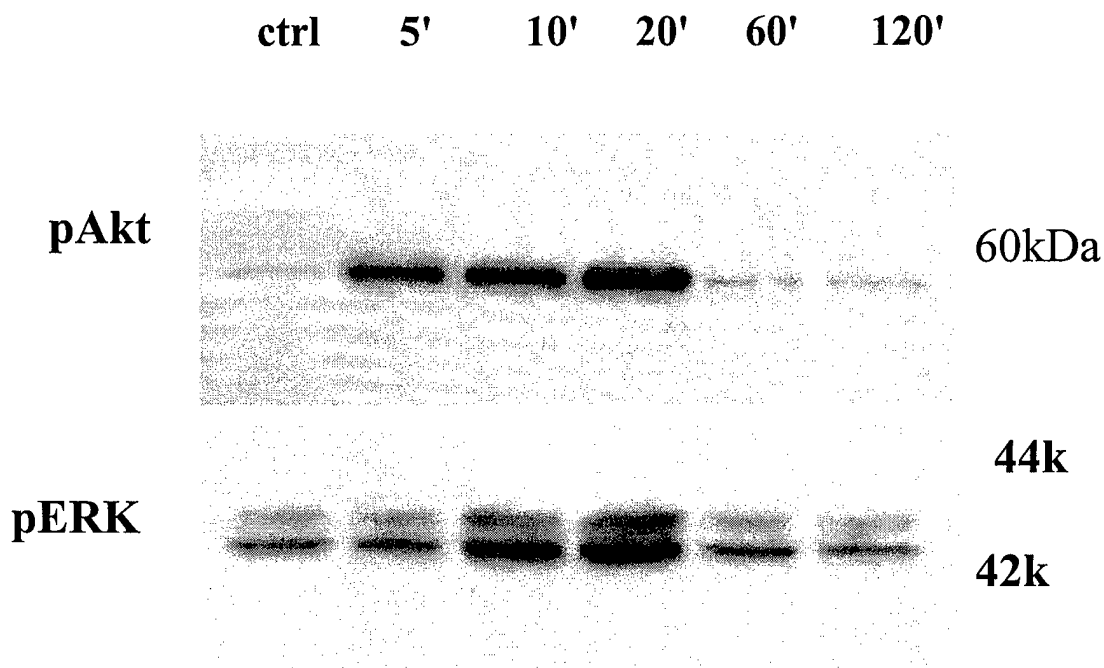


Figure 28. SCF induces phosphorylation of Akt and ERK in T265 cells. The NF derived Schwann cells were incubated with 100ng/ml of SCF for up to 120 minutes. Cells were lysed and 25ug of proteins were analyzed with a phospho specific antibody to Akt and a phospho specific antibody to ERK.

These results demonstrate an abnormality in these cells in that both MAP kinase and Akt are activated, whereas in normal rat neonatal Schwann cells as we have previously shown only Akt is activated.

In order to further evaluate the anti-apoptotic pathway in the NF1-derived Schwann cells, we looked for the next molecule on this pathways, which is downstream from Akt activation (BAD). The results are shown in Figure 29 below.



Time after SCF addition (min) 0 15 30 60 120

Figure 29: Western-blot for BAD in ST-88 cells. ST88 cells were plated in 35 mm dishes at a density of 300,000 cells per dish. The cells were originally plated in serum containing medium, but were incubated in serum free medium 4 hours before SCF treatment. Stem cell factor was added after the four hour incubation period at a concentration of 20 ng/ml, and the cells were incubated with SCF for the given time points. Proteins were separated on a 4-20% gradient gel, and blotted to a PVDF membrane. The membrane was probed with an anti-Bad antibody (1:1000). Note that this immunoreactivity probably represents phosphorylated Bad because the cells do not appear to be undergoing apoptosis, and unphosphorylated BAD is pro-apoptotic.

The only conclusion we could draw from this study is that BAD is expressed in the ST-88 cells. The activation by phosphorylation cannot be evaluated in the gels that were run since subtle differences in molecular weight due to phosphorylation can only be detected in higher percentage gels. Further studies are in progress.

In summary, it appears that c-Kit may play a role in preventing apoptosis in normal rat Schwann cells whereas in the NF1-derived Schwann cells it may function to weakly stimulate proliferation, in addition to preventing apoptosis.

Results and Progress made with Respect to Specific Aim 3: Effect of simultaneous Kit expression and neurofibromin alteration on Human Schwann Cell Proliferation.

This specific aim will be the focus of studies in the last year of this contract. The intent is to mimic the state of the Schwann cell lines that we have been studying by adding cKit expression and evaluating neurofibromin expression. We have carried out some preliminary experiments with respect to this specific aim. We do have the cDNA construct, which will allow us to express cKit and new, more stable antisense constructs that will block neurofibromin expression are being synthesized. These constructs are made with a morpholino backbone so that they are completely non-hydrolyzable. We have established a good working relationship with Dr. Pat Wood at the Miami Project to reliably obtain human nerve for preparing normal human Schwann

cells. We have established a collaboration with Dr. Wade Clap at Indiana University to obtain adenovirus constructs to allow us to effectively transfect our cells in culture. A new research associate, Dr. Lakshmi Gollapudi, who will join the lab in September, has expertise in carrying out transfection experiments. Given all these factors, we are confident that significant progress will be made this year on this specific aim.

Results and progress made with respect to all specific aims:

When we stated these investigations, we realized that the human Schwann cell lines derived from malignant peripheral nerve sheath tumor have never been completely characterized. Therefore, we carried out a careful characterization of these cells in a manuscript "Phenotypic Characterization of Human Schwann Cell Lines Derived from Malignant Peripheral Nerve Sheath Tumors." This manuscript is appended to this progress report and has been submitted to *Glia*.

E. Key Research Accomplishments

- Relationship between the absence of neurofibromin and presence of c-Kit receptor has been extended to include the NF1-derived Schwann cell lines 90.8 and 88.3.
- A developmental study has been carried out that indicates that c-Kit has a function early in Schwann cell development prior to myelination.
- The role of c-Kit in normal Schwann cells is not related to a stimulation of mitosis.
- The normal role of c-Kit in Schwann cell development is not to synergize with other mitogens such as NDF and cause increase proliferation.
- The function of c-Kit in normal rat Schwann cells is not related to stimulation of differentiation.
- Stem cell factor in neonatal rat Schwann cells activates a signal transduction pathway related to Akt. However, MAP Kinase is not activated by stem cell factor.
- Neonatal rat Schwann cells contain appropriate size message for c-Kit with the correct nucleic acid composition
- In contrast to neonatal rat Schwann cells, in a NF1-derived Schwann cell line (T265), stimulation of c-Kit with stem cell factor causes activation of both Akt and MAP Kinase.
- MAP Kinase is stimulated by addition of exogenous PDGF-BB in T265 cells.
- The PDGF-BB induced cell proliferation of T265 cells is inhibited by the MAP Kinase inhibitor, PD98059.
- PI-3-Kinase is involved in the proliferation of T265 cells, since inhibition of PI-3-Kinase with either wortamin or LY294002 causes a dose dependent decrease in proliferation.
- The type of adenylyl cyclases in normal human Schwann cells and in the T265 cells have been characterized. Normal human Schwann cells contain only type 3 adenylyl cyclase while the T265 contain types 2, 3, and possibly 5.
- PGE2 secretion is markedly elevated in all Schwann cell lines although it reaches its highest level of secretion in the Schwann cell lines that are lacking neurofibromin.
- The type of prostaglandin receptors found in NF cells have been characterized as EP2 and EP4 in contrast to the normal adult human Schwann cell, which contains only EP4 and the neurofibromin containing STS26T cell line, which contains EP2.

- The intracellular levels of cAMP in the NF1 and non-NF1-derived Schwann cells are markedly increased by application of exogenous PGE2.
- The intracellular levels of cAMP are markedly elevated by extracellular application of PDGF-BB.
- The proliferation induced by PDGF-BB is dependent on continued synthesis of prostaglandin since inhibitors of prostaglandin synthesis, such as indomethacin attenuate the proliferation.
- The proliferation induced by PDGF-BB is also markedly dependent on intact protein Kinase A. Since inhibition of this key transduction molecule by the drug H89 greatly attenuates the PDGF-BB stimulated proliferative response of T265 cells.
- Akt is activated by stem cell factor in both the ST88 and T265 cell lines as is MAP Kinase.
- The key transduction molecule involved in apoptosis (BAD) is present in ST88 cells.
- The NF1-derived Schwann cell lines are completely resistant to conditions that normally favor apoptosis in neonatal rat Schwann cell.

F. Reportable outcomes

Manuscripts:

Three manuscripts are currently in preparation pending replication of experiments. In addition, a manuscript that characterizes the NF-1 derived Schwann cell lines is included with this progress report.

Patents and Licenses Applied for and/or issued:

N/A

Degrees obtained that are supported by this award

It is anticipated that two graduate students who have been working on this project, Ian Dang and Naser Muja, will be receiving their Ph.D. in Neuroscience toward the end of this year.

Development of Cell lines, tissue, or serum repositories

The NF-1 derived Schwann cell line, T265, has been set up in our laboratory and is completely characterized. Several investigators are currently using this cell line in their own neurofibromatosis investigations, including Dr. Lynne Fieber at the University of Miami and Dr. Jeff DeClue at the National Institute of Health.

Information such as animal bases, and animal models, etc:

N/A

Funding applied for based on work supported by this award

A new grant to the US Army Division of Materiel Command will be submitted September 6th, based on results supported by this award.

Employment or Research Opportunities Applied for and/or Received on Experiences/Training supported by this Award

N/A

G. Conclusions:

The information gathered under the auspices of this research proposal can be summarized as follows:

First, we have demonstrated that, like so many other molecules that are important in cancer etiology, c-Kit appears to have a function in Schwann cell biology early in development. That function is not related to proliferation or differentiation but most closely related to the prevention of apoptosis. This view is supported by the fact that the transduction pathway (Akt activation) is consistent with the role of c-Kit/stem cell factor in prevention of apoptosis. Adding to the list of receptor abnormalities in NF1-derived Schwann cells, we have discovered that activation of the cKit receptor in NF1-derived Schwann cell lines, such as the T265 cell line, activates not only Akt but MAP Kinase as well, indicating a partial role for this molecule in stimulating the abnormal proliferation that is characteristic of these cells.

Additionally, we have extended our observations to the PDGF-BB receptor and found that the relationship of lack of neurofibromin and the presence of this receptor is also consistent with the expression of the PDGF-BB receptor as well. Activation of this receptor via exogenous PDGF-BB results in further increases in transduction molecules previously shown to be specifically related to NF-derived cells: cAMP and prostaglandins.

In addition, for the first time we have characterized the type of adenylyl cyclases that are present in normal human Schwann cells and transformed cells and found increased expression of several unique isoforms of this enzyme in the transformed cells relative to the single type of enzyme that is present in the normal human cells.

We also have described the type of prostaglandin receptors that are present in the transformed cells relative to normal human Schwann cells and found a similar situation; increased expression of these receptors in the transformed cells, which could lead to an augmentation of a response to exogenous PDGF.

Studies are underway to characterize the anti-apoptotic pathway in the NF-derived Schwann cells, which includes activation of PI3-Kinase, Akt, and the phosphorylation of

BAD. Taken together, these findings have given us new understanding as to the molecular dynamics of the proliferation, which is characteristic of Schwann cells derived from NF-1 tumors.

H. References:

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Appendices:

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Phenotypic Characterization of Human Schwann Cell Lines Derived from Malignant Peripheral Nerve Sheath Tumors

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Abstract

The phenotypic characteristics were characterized for two cell lines derived from NF1 patients (ST88-14 and NF1T cell lines), a new cell line (T265-2C) derived from an NF1 patient, as well as a Schwann cell line derived from a malignant Schwannoma from a non-NF patient (STS26T). Morphologically, all the cells from each cell line are more pleiomorphic than the primary Schwann cells. The NF1T cell line has the greatest resemblance to primary Schwann cells since these cells are spindle-shaped, bipolar cells with long processes. The antigenic profile of each cell line was evaluated using the markers CNPase, GFAP, myelin basic protein, galactocerebroside, and sulfatide and nerve growth factor receptor. All cells in each cell line was positive for all these markers with the exception of nerve growth factor receptor, for which immunoreactivity was detected only in the NF1T cell line. This immunoantigenic profile is consistent with a Schwann cell origin for each cell line.

The doubling times of each cell line was determined by analysis of growth curves. The T265 cells divided the most rapidly with the doubling time of 40.7 hours; the ST88-14 had a doubling time of 48.6 hours; the STS26T had a doubling time of 66.3 hours while the NF1T were the slowest dividing cells with a doubling time of 81 hours.

Fluorescent activated cell sorter analysis of cell cycle kinetics was carried out utilizing BrdU incorporation in conjunction with a specific antibody to be BrdU to detect cells in the S phase of the cell cycle or utilizing propidium iodine staining and evaluation of cells in the S phase. The results were consistent with the previously determined doubling times.

INTRODUCTION

A number of approaches have been used to culture and study primary human Schwann cells *in vitro*. Murray and Stout were the first to obtain cultured human Schwann cells derived from either peripheral nerve tumors (Murray and Stout, 1940) or normal adult and fetal spinal nerves (Murray and Stout, 1942). Initial techniques for culturing human Schwann cells utilized successive reexplantation of adult nerves in culture. In this technique, the first cells to emerge from the explant were characterized as fibroblasts, followed at a later time by the migration from the explant of typical spindle shaped Schwann cells. Although cultures obtained in this way were highly enriched in Schwann cells, without mitotic stimulation of Schwann cells, the cultures quickly were overrun with the more mitotically active fibroblasts. Although the initial workers did not attempt to establish long term cultures, they were able to perform initial microscopic and histochemical analysis of cultured human Schwann cells. Forty years later, Askanas et al (1980), utilized this technique with adult human sural nerve biopsy material to obtain primary SC cultures which were reduced in fibroblast contamination. Moretto et al (1984) isolated human adult Schwann cells from autopsy material by enzymatic digestion and maintained them in culture for up to five months using crude extracts of glial growth factor which stimulates Schwann cell proliferation. Pleasure and colleagues (1986) described a procedure for the culture of human Schwann cells isolated from human dermal neurofibromas via enzymatic digestion using a modification of the procedure used by Brockes et al (1979). Scarpini et al (1987) using the explantation technique of Askanas (1980) to culture human SC from adult and fetal nerves were the first to demonstrate a number of SC antigenic markers by immunocytochemistry. Following from this, Scarpini et al (1988b) describe the isolation of Schwann cells from human fetal sciatic nerves by chemical and mechanical disassociation. Kim et al (1989) utilized fetal dorsal root ganglia to isolate SC in culture and to assess their antigenic properties and proliferative capacity. A variation of the sequential explantation technique was utilized by Armati and Bonner (1990) in conjunction with a media which would selectively inhibit the growth of fibroblasts. Rutkowski et. al.(1992) modified this technique to include the Schwann cell mitogen cholera toxin in the original explant to expand Schwann cell numbers. These workers also found that a combination of cholera toxin, forskolin and glial growth factor would inhibit fibroblast proliferation while stimulating at least half of the Schwann cells to divide. Morrissey et. al further refined this methodology by utilizing heregulin, a cloned growth factor related to glial growth factor to selectively stimulate Schwann cell proliferation and suppress fibroblast proliferation when combined with agents which raise the intracellular concentration of cyclic AMP such as cholera toxin and forskolin (Morrissey et al, 1991).

In summary human Schwann cells *in vitro* have not been extensively studied for several reasons. In the first place, primary cultures of relatively pure human Schwann cells are both difficult to establish. In addition it is difficult to reliably obtain a continuing source of starting human nerve from which to derive human Schwann cell cultures. Although the final cell culture are enriched in Schwann cells, in time fibroblasts will become the predominant cell in the culture. Therefore it would be useful to have stable and characterized human Schwann cell lines available for long term studies of these cells.

The establishment of transformed Schwann cell-derived lines from tumors of nerve sheath origin has been accomplished for both rodent and human cells. Several Schwannoma derived cell lines have been reported and well studied with respect to their Schwann cell characteristics. These cell lines are commonly produced by ethylnitrosourea induction of tumors in vivo during gestation (Fields et al, 1975; Pfeiffer and Wechsler, 1972; Bansal and Pfeiffer et al, 1987). The establishment of human Schwannoma derived cell lines have also been reported although they are less common, more difficult to establish in culture and not very well characterized with respect to their Schwann cell phenotype (Fletcher et al, 1991; Weichselbaum et al, 1985).

In this report we characterize the antigenic phenotype, doubling time and morphological characteristics of a Schwann cell line derived from a human malignant Schwannoma (STS26T), two human Schwann cell lines derived from NF-1 patients (ST88 and NF1T), which have already been established, in addition to a new human Schwann cell line that we established from a malignant peripheral nerve sheath tumor in a patient diagnosed with neurofibromatosis type I.

MATERIALS AND METHODS

Cell Lines Culture Conditions

All cell lines, both tumor and primary, were maintained according to standard cell culture protocols (Freshney, 1987). They were grown in high glucose Dulbecco's Modified Eagles Medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, (Gibco), 0.5% penicillin-streptomycin (50 U/ml penicillin/ 50 µg/ml streptomycin) (Gibco), 1 mM sodium pyruvate (Sigma Chemical Co., St. Louis, MO), and buffered with 3.7 g/l sodium bicarbonate (Gibco) to pH 7.2. Media reconstituted from powder was prepared using Millipore (Bedford, MA) ionpure charcoal filtered deionized water and sterilized using a 0.02 µm bottle filtration unit (Costar, Cambridge, MA).

Primary human tissue or tumor specimens were initially collected in either Hanks Balanced Salt Solution (HBSS) (Gibco) or RPMI 1640 media (Gibco). All subsequent enzymatic digestion of primary tissue was done in RPMI 1640 media as the base medium. RPMI 1640 media was routinely supplemented with 2 mM L-glutamine (Gibco), 0.5% penicillin-streptomycin (50 U/ml penicillin/ 50 µg/ml streptomycin) (Gibco), and buffered with 10 mM HEPES (N-2-hydroxyethylpiperazine N'-2-ethane sulfonic acid) (Sigma), pH 7.2- 7.4.

The cell culture media was also supplemented with 5-20% fetal bovine serum (FBS) (Gibco, Lot # 46K7319) depending on the protocol. All cell cultures, both primary and tumor cells, were grown on tissue culture plasticware (Costar, Cambridge, MA) at 37° C in a humidified 5% CO₂/95% air atmosphere in a NuairTM autoflow CO₂ water-jacketed incubator (Nuair, Plymouth, MN). All cell cultures were maintained in this manner unless otherwise specified. Tissue culture reagents were obtained from Gibco Laboratories, Grand Island, NY unless otherwise stated. All other chemicals were obtained from Sigma Chemical Co., St Louis, MO unless otherwise specified.

Trypsinization and Freezing of Cell Cultures

Cell cultures were passaged periodically as they became confluent. The confluent culture flasks were rinsed once with sterile, calcium (Ca⁺²) and magnesium (Mg⁺²) free-Dulbecco's phosphate buffered saline (DPBS) (Gibco). Followed by incubation with a sterile solution of DPBS (minus Ca⁺² and Mg⁺²), 0.025% trypsin (Difco, Detroit, MI) and 0.02% ethylenediamine-tetraacetic acid-NH₄ (EDTA-Na₄) (Sigma) for 10 minutes at 37° C to detach the cells which were subsequently collected by centrifugation at 1000 RPM for 10 minutes. The cell number was determined with a Neubauer hemocytometer and cell viability was determined with the addition of trypan blue (Sigma) to a final concentration of 5% of the total volume.

Cell culture suspensions were cryopreserved by suspending 0.5-1 x 10⁷ cells suspended in 0.9 ml DMEM with 10% FBS were mixed with an equal volume (0.9 ml) of an ice chilled solution containing one part DMEM with 10% FBS, two parts FBS and one part anhydrous DMSO (dimethyl sulfoxide). Aliquots of this mixture were quickly transferred into a 1.8 ml cryovial (Costar) and placed on ice. The cryovials were then

transferred serially from the ice bath to a -20° C non-frost free freezer overnight, then to a -70° C freezer the next night and finally into liquid nitrogen.

Cells were reestablished in tissue culture by rapid thawing of cryovials in a 37° C water bath. The contents of the cryovial were then removed sterilely and added to 10 ml of media previously warmed to 37° C. The cell pellet was collected by centrifugation at 1000 RPM for 10 minutes and gently resuspended into 1 ml fresh DMEM containing 10% FBS and plated into a 75 cm² tissue culture flask at a density of 1 x 10⁶ cells per 10 ml. The cultures were incubated overnight to allow the cells to attach followed by a change of media the next day to remove non-viable cells.

Isolation and Establishment of Cell Lines

All human tumor cell lines were established directly from freshly excised solid tumor specimens received from patients undergoing tumor resection. Three lines were established from patients diagnosed with Neurofibromatosis Type 1 (NF1) and one line was from a non-NF1 patient. All of the tumor lines were studied between passage 3-45.

The ST88-14 tumor cell line

The ST88-14 peripheral nerve sheath tumor (PNST) cell line was established in the laboratory of Dr. Cynthia Morton by Dr. Jonathan Fletcher in the Department of Pathology at Brigham and Women's Hospital, Boston, MA (Fletcher et al, 1991). This cell line was derived from a patient diagnosed with NF1 and has been partially described elsewhere (DeClue et al, 1992 and Reynolds et al, 1992). In brief, a solid tumor specimen was enzymatically disaggregated in RPMI 1640 media using 200 U/ml collagenase (Gibco) plus 0.005% deoxyribonuclease I (DNase I) (Sigma Chemical Co.) for several hours. A single cell suspension was collected and centrifuged to remove the enzymes. The pellet was resuspended and plated onto tissue culture plasticware coated with 25 µg/ml fibronectin (Collaborative Research, Cambridge, MA). For the earliest passages, the cultures were maintained in RPMI Medium 1640 (Gibco) supplemented with 20% FBS and 3 mg/ml bovine pituitary extract (Collaborative Research).

All studies in our laboratory utilizing this line were performed no later than passage 40. At passages greater than this the cell culture would begin to slow its doubling time therefore an earlier passage was reestablished from a frozen stock for use in our experiments.

The STS-26T tumor cell line

The STS-26T and NF-1T soft tissue sarcoma cell lines were established in the laboratory of Dr. John Little by William Dalhberg at the Harvard School of Public Health, Boston, MA (Dalhberg et al, 1993). Briefly, an isolated grade III malignant Schwannoma in an individual without Neurofibromatosis Type 1 (NF1) was minced and enzymatically digested with 0.2% collagenase for 30-90 minutes to achieve a single cell suspension. The cells were then resuspended and washed twice in normal saline prior to a final resuspension into Eagle's minimum essential medium (Gibco) supplemented with 10-20% FBS (Integren), penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were seeded into culture dishes and maintained at 37° C in a humidified incubator with 5% CO₂/95% air.

This cell line was initially determined to be of Schwann cell origin based on electron micrograph (EM) studies. It has also been shown to contain clonal chromosomal

aberrations and to form tumors in nude mice following transplantation (Dalhberg, personal communication).

The NF-1T tumor cell line

The NF-1T cell line was derived from a malignant soft tissue sarcoma in a patient diagnosed with NF1. This cell line was established in culture as described for the STS-26T cell line. Previous studies on this line have determined it to be highly radioresistant to treatment with ionizing radiation during exponential growth (Weichselbaum et al, 1985). This increased level of radioresistance may reflect the ability of these cells to adequately repair potentially lethal x-ray damage and thus be more incurable in vivo with radiation therapy.

The T265-2c tumor cell line

The T265-2c cell line was established in the laboratory of Dr. George DeVries by Karen Klein at the Medical College of Virginia, Richmond, VA from a malignant Schwannoma derived from a patient with NF1. Tumor pieces were dissected free of visible necrotic tissue, normal connecting tissue and blood vessels under sterile conditions. All tumor specimens were then cut into smaller pieces approximately 5 mm in diameter and placed in an enzymatic digestion cocktail containing RPMI 1640 (Gibco, Grand Island, NY), 5% FBS (Gibco), 0.1% collagenase P (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.1% hyaluronidase (BMB), 0.01% dispase, grade II (0.5 U/ml) (BMB) and 0.002% DNase I (Type IV) (Sigma). Tissue digestion proceeded for 3 hours at 37° C in a humidified 5% CO₂/95% air incubator with gentle agitation. A turbid single cell suspension was centrifuged to collect the cells as a pellet which was resuspended in RPMI 1640 without FBS and counted using a hemocytometer. Approximately 1 x 10⁷ cells were obtained from the original total tumor sample (slightly less than 5 grams total). A final single cell suspension was made at 0.5 x 10⁷ cells per 5 ml of high glucose DMEM, with 5% FBS, plated onto 0.01% poly-d-lysine (Sigma) coated 100 mm tissue culture plasticware and allowed to adhere overnight. Dishes were incubated at 37° C in a humidified 5% CO₂/95% air incubator overnight. The following day, the cell cultures were rinsed gently with fresh media to remove the cells which did not attach to the substratum and then refed with DMEM without FBS to discourage the growth of contaminating normal fibroblasts which are extremely serum dependent. This media, however, was supplemented with a 10% volume of sterile filtered, conditioned media from the ST88-14 cell line which was used in an attempt to encourage the growth of the Schwann cell-derived elements in the culture. This supplementation was maintained throughout multiple passages until a morphologically homogeneous subculture was established at approximately the 15th passage. Subcultures derived from multiple passages of the initial culture dishes were characterized with respect to their Schwann cell phenotypes as determined by immunocytochemistry. Multiple aliquots of all subcultures were frozen at various passages and all subcultures were maintained up to the 15th passage. All experiments performed on this cell line were done between passages 15 to 35.

Antigenic profile of Human Schwann Cell Lines

Using standard procedures as described by Harlow and Lane (1988) the antigenic profile of all human Schwann cell lines was determined. All immunocytochemistry was performed in situ on subconfluent cell cultures plated onto 12 mm glass cover slips (Fisher

Scientific, Pittsburgh, PA) or Lab-Tek Chamber Slides (Nunc, Inc., Naperville, IL). All cover slips or chamber slides were pre-treated overnight at 4° C with a solution of 0.01% poly-d-lysine in deionized water (dH₂O). This was rinsed three times in sterile deionized water and allowed to dry prior to plating the cells. Cultures were then replated at the appropriate density and grown to sub-confluence in DMEM with 10% FBS. At sub-confluence, the growth media was changed to DMEM without FBS for 4-24 hours prior to fixation. The cell cultures were washed 2 x 5 minutes in Dulbecco's phosphate buffered saline containing Ca⁺² and Mg⁺² (DPBS) prior to fixation. Cells were fixed for 5 minutes in a 10% sodium acetate buffered Formalin solution (Fisher Scientific) which yielded a final concentration of 4% paraformaldehyde. Following fixation, the cultures were washed three times in DPBS prior to an overnight incubation at 4° C with the primary antibodies. All primary antibodies were prepared at their proper dilution as determined by previous titration (see Table 1). The antibodies were diluted in DPBS with 1% goat serum to block non-specific binding and 0.1% Triton X-100 to permeabilize the cell membranes. The following day, the primary antibodies were removed and the cells were rinsed 3 x 15 minutes in DPBS prior to addition of the secondary antibodies.

Primary polyclonal antibodies used in the experiments included antisera to; glial fibrillary acidic protein (GFAP), 2',3'-cyclic nucleotide phosphohydrolase (CNPase), the major PNS myelin glycoprotein O (PO), laminin, S100 and myelin basic protein (MBP). Rabbit anti-bovine GFAP IgG was purchased from Dako Corp. (Carpinteria, CA). Rabbit anti-bovine CNPase IgG1 was a gift from Dr. Terry Joe Sprinkle, Department of Neurology, Medical College of Georgia. Rabbit anti-bovine PO IgG was a gift of Dr. Bruce Trapp, Department of Neurology, Johns Hopkins University, Baltimore, MD. Rabbit anti-mouse laminin IgG was purchased from Gibco Laboratories. Rabbit anti-bovine S100 IgG was purchased from Dako Corp. Rabbit anti-bovine MBP was purchased from Advanced Immunochemical Services (Long Beach, CA). Chicken anti-mouse GM2 ganglioside was obtained from Dr. Robert Yu, Medical College of Virginia, Richmond VA.

Primary monoclonal antibodies derived from hybridomas included antibodies to; nerve growth factor receptor (NGFr), galactocerebroside (GalC), sulfatide and bromodioxuridine (BrdU). Mouse anti-human NGFr (ATCC 8737, Rockville, MD) IgG was obtained from the American Type Tissue Collection (ATCC, Rockville, MD). Mouse anti-bovine GalC IgM (O1) was a gift from Dr. Richard Quarles (National Institute of Neurological Disorders and Stroke, National Institute of Health) The mouse anti-bovine sulfatide IgM (O4) was a gift from Steven Pfeiffer (University of Connecticut Medical School, Farmington, CT). Mouse anti-BrdU IgG1 was purchased from Dako Corp. Secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG or IgM) were conjugated with either TRITC-Rhodamine, FITC-fluorescein, or with the enzyme, horseradish peroxidase. Stock solutions were diluted in DPBS to the appropriate concentrations; 1/100 for fluorochrome conjugated antibodies and 1/500 for peroxidase conjugated antibodies. Cell cultures were incubated with the appropriate secondary antibody for 1-2 hours at room temperature and rinsed 3 x 15 minutes in DPBS prior to visualization.

Most of the secondary antibodies used for these experiments were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) unless otherwise stated. These included; fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG, IgM, (H+L) F(ab')₂, rhodamine isothiocyanate (Rho) conjugated goat anti-rabbit IgG (H+L) and rhodamine isothiocyanate rabbit anti-goat IgG. Other secondary antibodies used in these experiments included; peroxidase conjugated goat anti-mouse IgG, IgM and peroxidase

conjugated goat anti-rabbit IgG and were obtained from either Boehringer Mannheim Biochemicals (Indianapolis, IN) or Jackson ImmunoResearch Laboratories, Inc. Affinity purified goat anti-chicken IgG (H+L) was obtained from Vector Laboratories, Inc. (Burlingame, CA). Also purchased from Jackson ImmunoResearch Laboratories were affinity column purified rabbit IgG, normal rabbit serum, affinity purified mouse IgG and IgM, normal mouse serum and normal goat serum.

Immunofluorescent Antibody Staining

Visualization of antibody immunoreactivity depended upon the type of label on the secondary antibody. Cell cultures where the secondary antibody was conjugated with a fluorochrome label were mounted under a glass coverslip using a solution of 0.1 M N-propyl gallate in glycerol and PBS (1:9) to prevent fluorochrome bleaching. Slide preparations were immediately ready for microscopic analysis and were stored indefinitely in the dark at 4° C.

Immunoreactivity of the fluorochrome conjugated secondary antibodies was visualized using a Nikon UFX-IIA inverted microscope equipped with an epifluorescent light source and appropriate light filtration. Black and white print photographs were taken through either a 10X, 20X or 40X objective using a Nikon FX-35WA camera and Ilford HP-5 film at ASA 400. Film was developed according to specification and printed onto Kodak paper in order to provide the best possible image.

Peroxidase Conjugated Antibody Staining

Where the secondary antibody was peroxidase conjugated then the antibody-antigen complex was reacted with 3,3- diaminobenzidine (DAB) (Sigma) in the presence of hydrogen peroxide (H₂O₂). Following removal of the secondary antibody solution, the cells were rinsed once in 50 mM Tris (pH 7.4) and the enzyme reaction was initiated by incubating the fixed cells in a solution of 0.025% DAB and 0.015% H₂O₂ in 50 mM Tris. Positive antibody immunoreactivity is visualized in a specific time period (5 minutes) by the production of a brown precipitant. The enzyme reaction was stopped by diluting, washing and storing in the Tris buffer. Cells were prepared for permanent storage by rinsing once quickly in deionized water and mounting cell side up under glass coverslips using a warmed glycerine jelly solution (10 gm gelatin/60 ml dH₂O/70 ml glycerine) which solidifies at room temperature. Positive immunoreactivity is permanent and the slide can be stored indefinitely.

Immunoreactivity was visualized using a Nikon diaphot-TMD inverted microscope equipped with a tungstun light source for brightfield microscopy. Photographs were taken with Pan-X black and white print film at ASA 50 and developed following standard procedures. Film was printed onto Kodak paper to optimize the sharpest image.

Cell Counts to Determine Doubling Times

Cell line doubling times were determined from the slopes of the growth curves derived from hemocytometer counts as described by Macieira-Coelho et al (1966). Cell lines were plated into 6-well culture dishes (Costar) in DMEM plus 10% FBS and allowed to attach for 24 hours. Once the cells had attached, the first set of triplicate wells was counted and averaged with their standard errors and the first time point was established. At each 72 hour time point thereafter cells, in triplicate wells, were counted and averaged following trypsinization. All the remaining wells were refed with fresh media at each 72

hour interval up to the last time point. Cell numbers were determined in a 1 ml volume using a Neubauer hemacytometer. Cell viability was determined using a 5% trypan blue exclusion assay.

BrdU Incorporation and Flow Cytometry Analysis

Cell cycle profiles and the percentage of total cells dividing, as measured by bromodeoxyuridine (BrdU) incorporation during DNA replication, were examined by Fluorescent Activated Cell Sorter (FACS) analysis. Human tumor cell lines and normal fibroblast cells were maintained for 72 hours in DMEM with 10% FBS prior to flow cytometry analysis. The media was pulse labeled by addition of 2 μ M BrdU (Sigma) for the last 24 hours prior to harvesting the cells. The treated cell cultures were harvested and prepared for flow cytometric analysis following the method of Beisker et al (1987). Briefly, approximately 2×10^6 cells per 75 cm² flask were removed from the flask with a trypsin solution, washed in DPBS and centrifuged to obtain a pellet. The pellet was resuspended in 1 ml cold DPBS and fixed for 10 minutes with an equal volume of cold absolute ethanol to obtain a final concentration of 50% ethanol. After fixation the cells were centrifuged 10 minutes at 2000 RPM in a Sorvall GLC-1 tabletop centrifuge at room temperature. This cell pellet was resuspended in 2 ml DPBS containing 1 mg/ml RNase (Type III, Sigma) and incubated for 20 minutes at 37° C to eliminate contaminating RNA. Cells were then centrifuged and resuspended in 0.1 N HCl with 0.7% Triton X-100 for 10 minutes at room temperature to permeabilize the cell membranes. The cells were centrifuged, resuspended into 1 ml distilled deionized water, heated to 100° C for 10 minutes to denature the DNA and then rapidly chilled on ice prior to the addition of 5 ml of cold DPBS plus 0.5% Tween-20 (PBT).

The cell nuclei were then stained using a monoclonal antibody to BrdU (Dako Corp, Carpinteria, CA) as described by Collins and Chu (1990). The cells were centrifuged and the pellet was resuspended into 500 μ l PBT containing 0.5% BSA and the anti-BrdU monoclonal antibody at a final concentration of 2 μ g/ml. The cell suspension was incubated for one hour at room temperature to bind the primary antibody. After this time, the cells were washed twice in PBT and resuspended into 500 μ l PBT containing the secondary FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc) at a 1/100 dilution. This suspension was incubated 30 minutes at room temperature. Following this incubation the cells were washed in DPBS and resuspended into 1 ml DPBS containing 10 μ g/ml propidium iodide (PI) which was used as a ubiquitous fluorescent DNA counterstain to assess total cell number. The cell solution was filtered through a 35 μ m Nitex filter mesh (Tetko Inc., Elmsford, NY) prior to flow cytometry analysis.

Cell cycle phases were calculated for each of the cell lines as previously described (Vick et al, 1992). The fluorescent intensity of the FITC conjugated goat anti-BrdU antibody complex was used to determine the percent of cells which incorporated BrdU in S phase of the cell cycle as compared to the total population of cells analyzed. Simultaneous measurements of green fluorescence (FITC-labeled anti-BrdU antibody) and red fluorescence (PI-DNA binding) were performed with an EPICS 753 Fluorescent Activated Cell Sorter (FACS) (Coulter Electronics, Hialeah, FL)

RESULTS

Isolation and establishment of the T265-2c cell line derived from a malignant tumor of the peripheral nervous system

The human cell line T265-2c was derived from a malignant Schwannoma excised from a patient diagnosed with Neurofibromatosis type 1 (NF1). A single cell suspension was isolated by enzymatic digestion of the original tumor specimen. Four subcultures (designated A-D) were maintained for their initial passages in media without FBS to decrease fibroblast growth and encourage serum-independent transformed cell growth. Initially, all subcultures contained a morphologically heterogeneous population of cells which grew well, but exhibited a substantial amount of detached and floating cells with each passage. Over time the subcultures began to change their culture characteristics with respect to morphology and growth rates. Some subcultures were not continued beyond passage 15 due to culture senescence whereas clonal foci formation was evident in two of the four subcultures. In these subcultures, the highly proliferative cells derived from the foci eventually outgrew the quiescent cells and became stable morphologically homogeneous culture. Subculture 2C was chosen for propagation and further study based on its initial Schwann cell like morphology and antigenic properties including strong immunoreactivity for S100 and CNPase.

The T265-2c cell line retains the greatest degree of contact inhibition compared to the three other tumor-derived cell lines. They grow as a true monolayer and do not pack very densely (see Fig. 1D). As the culture dish approaches confluency, their growth rate decreases (Klein, unpublished observations). This cell line generates the lowest number of cell per culture flask.

Cell morphology and culture characteristics of the four Schwannoma cell lines

The culture characteristics of the four Schwannoma-derived human cell lines varied considerably. Each cell line was distinctly unique with respect to cell morphology, plating efficiency, doubling times, degree of contact inhibition and culture confluency.

The ST88-14 cell line was morphologically heterogeneous at low confluency containing both bipolar spindle shaped cells and more pleiomorphic flattened cells (Fig. 1A). At high confluency, as the cells would pack closer they would look less bipolar and display no inhibition for multilayering. These cells could grow to high densities in culture with no apparent decrease in proliferative capacity. Eventually, a palasaiding pattern of tight cellular packing would appear which resembled what is observed in histological sections of Schwannoma tumor specimens.

The STS-26T cell line is more homogeneous in culture with a high mitotic index and population doubling time. At low confluency they do not resemble primary Schwann cells as these cells are flat and pleiomorphic. At high confluency the cells retract away from the substratum and form dense islands of cells which resembles a statburst pattern (see figure 1B). There is no apparent contact inhibition similar to what is seen in foci formation. In

addition, they are reported to form tumors in nude mice (personal communication with W. Dalhberg).

The NF1T cell line has the greatest resemblance to primary Schwann cells in culture. They are spindle shaped bipolar cells with long processes. They retain their spindle shape even at high confluency, form intricate lacy networks of cells and never completely obscure the substratum (see figure 1C). In contrast to the other cell lines, the NF1T cell line has the slowest population doubling time (see below) and appear to retain many characteristics of fully differentiated Schwann cells (see below).

Immunocytochemical staining of the Schwann cell tumor lines for Schwann cell specific markers

All of the tumor-derived Schwann cell lines were characterized with respect to their immunoreactivity to antibodies specific for glial cell antigenic markers. These included; 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), the major peripheral myelin glycoprotein (P0), laminin, glial fibrillary acidic acid (GFAP), S100, myelin basic protein (MBP), galactocerebroside (O1), sulfatide (O4) and nerve growth factor receptor (NGFr). Typical example of staining are shown in Figure 2 (ST88-14) and Figure 3 (T265). Positive immunoreactivity was demonstrated for cultured cells in vitro using either a fluorochrome-conjugated or peroxidase conjugated secondary antibody to visualize antibody binding to the Schwann cell antigenic marker. All cell cultures were paraformaldehyde fixed and permeabilized prior to staining.

All cell lines were found to be positive to varying degree for many of the Schwann cell specific markers. The only major exception was the NGF receptor. In this case, all cell lines demonstrated negative immunoreactivity except the NF1T cell line which was positive. Immunocytochemical analysis of the cell lines derived from the malignant tumors revealed that the pattern of expression for Schwann cell specific antigenic markers is consistent with a Schwann cell origin for each of the cell lines. On the basis of their immunoreactivity, the cultured cells were determined to be of Schwann cell origin. A summary of the immunocytochemical characterization is shown in Table 1.

Theoretical doubling times and proliferative capacity of the tumor cell lines

The tumor derived Schwann cell lines were plated in 6-well culture dishes and counted in triplicate at 72 hour intervals for a two week period. Fresh media with 10% FBS was added to the remaining cultures at every time point. The theoretical cell cycle doubling times could be determined from the curves generated by the cell count data. Asynchronous cell population doubling times were calculated using the equation of the curve which is generated at the point on the curve where the cells are in a log phase of their cell cycle with adequate nutrients in the media and space to grow. The theoretical cell population doubling times as calculated from the growth curves are as follows: ST88 = 48.66 hours, STS-26T = 66.32 hours, NF1T = 80.99 hours and T265-2C = 40.66 hours.

Fluorescent activated cell sorter (FACS) analysis of cell cycle kinetics

Cell cycle kinetics of the four tumor-derived cell lines were analyzed using a fluorescent activated cell sorter (FACS). The cells were pulsed for 24 hours with 2 $\mu\text{M}/\text{ml}$ bromodeoxyuridine (BrdU) followed by 10 $\mu\text{M}/\text{ml}$ propidium iodide (PI) prior to analysis. Cells which incorporated BrdU during DNA synthesis were identified using FITC conjugated anti-BrdU antibodies. Total cell number was determined by counterstaining DNA with propidium iodide (PI). Cell cycle phases were determined using relative PI intensity to calculate the percent of cells in each phase of the cell cycle relative to total DNA content. Propidium iodide was used as a DNA counterstain to determine the total number of cells analyzed per cell line. Cell cycle profiles were calculated from a total of 10,000 cells sorted.

Table 2 shows both the percent of cells in each phase of the cell cycle as measured by FACS analysis of propidium iodide staining only. As well as the percent of cells which incorporated BrdU during S phase of the cell cycle and therefore underwent DNA synthesis within a 24 hour period. Note that by the criterion of either cell cycle analysis or BrdU labeling, the fastest dividing cell line is the T265-2C line while the NF1T cells are the most slow dividing cell lines. There is a good agreement with respect to the mutation rate of all cell lines when the cell cycle analysis and BrdU analysis are compared.

DISCUSSION

Our current knowledge of normal Schwann cell (SC) proliferation and differentiation has been obtained predominately through the study of rodent SC both *in vivo* and *in vitro*. Our understanding of normal human SC biology is less advanced due to their decreased availability and the difficulties in obtaining sufficient numbers of cells to study *in vitro*.

Four cell lines established previously from malignant SC tumors were characterized. Three cell lines (ST88-14, NF1T and T265-2C) were from individuals diagnosed with Neurofibromatosis type 1 (NF1). The fourth cell line (STS-26T) was from a sporadic malignant SC tumor in a non-NF1 patient. All cell lines were characterized with respect to their SC phenotype and degree of differentiation. Immunocytochemical analysis of SC antigenic markers was performed on fixed cultures *in situ*.

One of the hallmarks of a transformed cell is the loss of a differentiated phenotype and reversion to a more primitive embryological state of differentiation. To assess the level of differentiation of the four SC lines, they were assayed for their expression of SC proteins which may have been retained following malignant transformation. The antigenic staining profiles of the four cell line demonstrated positive immunoreactivity for many SC specific markers of differentiation (Table 2). Although the levels of immunoreactivity varied depending on the cell line, the expression indicated a general retention of some aspects of a differentiated SC phenotype. The presence of S100, laminin and CNPase was consistent with a SC origin for each of the cell lines. In addition, the presence of the myelin specific markers MBP and P0 (although decreased) indicate that these cell lines exhibited the potential for a myelin-forming SC phenotype. The decreased expression of GalC and sulfatide as demonstrated by both immunocytochemistry and subsequent lipid analysis is consistent with a loss of axonal contact as would be expected for normal SC isolated *in vitro* (Jessen and Mirsky, 1991).

Myelin forming SC which lose axonal contact begin to decrease expression of the myelin proteins and increase the expression of a different set of proteins which includes GFAP and NGFr (Jessen and Mirsky, 1991). While all cell lines demonstrated positive immunoreactivity for the polyclonal anti-GFAP antiserum, there is the possibility that this reagent cross-reacts with other filamentous proteins such as vimentin and is not specific for GFAP. A better indicator of the SC response to loss of axonal contact is the expression of NGFr. All cell lines, except the NF1T cell line, demonstrated initial negative immunoreactivity for anti-NGFr monoclonal antibodies. The ability of the NF1T cell line to express NGFr constitutively may indicate that these cells retain the capacity to respond appropriately to the loss of axonal contact even following transformation. This was the first indication that the NF1T cell line had a more differentiated, less transformed phenotype than the other SC lines.

The NF1T cell line was also the only line which demonstrated positive immunoreactivity for the low affinity nerve growth factor receptor (NGFr) *in situ*. This observation provides further evidence that the NF1T cell line is the most differentiated SC tumor line. It may also indicate that they retain their ability to respond appropriately to axonal controls in a fashion similar to that of normal primary SC. In other experiments

utilizing tumor tissue, Baron and Kreider (1991) were able to co-culture human Schwann-like cells (SLC) isolated from benign neurofibromas with rat DRG neurites. They demonstrated that these SLC were able to weakly associate with the axons and after several weeks in culture, changed their antigenic phenotype from NGFr+, MAG-, GalC- to NGFr-, MAG+, GalC+ as demonstrated by immunocytochemical analysis. In their experiments, the isolated SLC were initially immunoreactive for the low affinity NGFr+ as is our NF1T cell line. This observation is relevant since NGFr is one of the markers which is regulated by axonal contact both *in vivo* and *in vitro* (Yan et al, 1987; DiStefano et al, 1988; Johnson et al, 1988; Lemke et al, 1988; Jessen and Mirsky, 1991).

It is interesting to note the unique properties of the NF1T cells, which divided most slowly of all the cell lines. These cells also retained the spindle shaped morphology of Schwann cells and they were much less pleomorphic than any of the other cell lines. The extent to which the cells are dividing may be related to the degree of malignant transformation. Recent investigations have revealed that all of these cell lines do not express neurofibromin protein (Badache and DeVries, 1998). Given this fact, it is interesting to speculate as to why the doubling times are so disparate. It is possible that the *in vitro* doubling times have changed with increasing passing so that they no longer resemble the *in vivo* counterparts of these cells. All of the cells were derived from peripheral nerve malignant sheath tumors, and on this basis would be expected to have the same degree of malignant transformation. However, with increased time in culture, there may be further loss of control of growth characteristics. The changes in intracellular transduction pathways of these cell lines are currently under investigation.

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TABLE 1**Immunoreactivity of Human Schwann Cell Lines**

| | ST88-14 | NF1T | STS-26T | T265-2c |
|----------------|---------|------|---------|---------|
| CNPase | ++ | ++ | ++ | ++ |
| P0 | + | + | + | + |
| MBP | + | + | + | + |
| GFAP | + | + | + | + |
| Laminin | ++ | ++ | ++ | ++ |
| S100 | ++ | + | + | + |
| NGF receptor | - | + | - | - |
| Gal C (O1) | +/- | +/- | +/- | +/- |
| Sulfatide (O4) | +/- | +/- | +/- | +/- |

(++) strongly positive immunoreactivity

(+) positive immunoreactivity

(+/-) faintly positive immunoreactivity

(-) negative immunoreactivity

Table 2
Cell Cycle Phase Analysis of Human Schwann Cell Lines

| | RU486 |
|--------------------------------|--------------|
| ST88-14 Cells | |
| G ₀ /G ₁ | 53.9 +/- 4.0 |
| S | 29.8 +/- 2.0 |
| G ₂ /M | 16.6 +/- 3.0 |
| STS-26T Cells | |
| G ₀ /G ₁ | 49.2 +/- 4.0 |
| S | 30.9 +/- 2.0 |
| G ₂ /M | 19.8 +/- 3.0 |
| NF1T Cells | |
| G ₀ /G ₁ | 36.8 +/- 6.0 |
| S | 39.7 +/- 4.0 |
| G ₂ /M | 23.5 +/- 4.0 |
| T265-2C Cells | |
| G ₀ /G ₁ | 65.7 +/- 4.0 |
| S | 17.5 +/- 2.0 |
| G ₂ /M | 16.8 +/- 3.0 |

(*) Total population of cells analyzed equals 100 percent and represents 10,000 cells

Figure Legends

Figure 1 - Phase contrast photomicrographs of four Schwannoma-derived human cell lines (panels A-D) showing the morphology of the cells in culture. Panel A) ST88-14 cells, B) STS-26T cells, C) NF-1T cells, D) T265-2c cells. See text for a detailed description of the cell lines.

Figure 2 - Fluorescent and phase contrast photomicrographs showing the immunocytochemical antigenic staining characteristics of the ST88-14 cell line. Note the presence of both bipolar spindle shape cells as well as more pleiomorphic shaped cells in panel A. Panel B-F demonstrate positive immunoreactivity for Schwann cell antigens; panel B) S100, C) laminin, D) CNPase, E) P0 and F) MBP. Timed matched controls were negative for antibody reactivity.

Figure 3 - Photomicrograph of the T265-2c cell line subclone following establishment of the tumor cells in culture. Panel A is a phase contrast photograph of the cells in culture. Panel B, C, E and F demonstrate positive immunoreactivity for Schwann cell antigenic markers; B) MBP, C) laminin, E) S100 and F) GFAP. NGF receptor (panel D) was negative for immunoreactivity by immunocytochemical analysis.

Figure 1

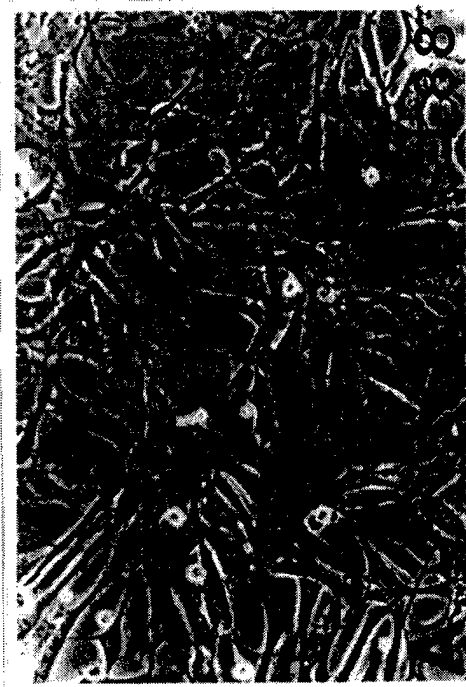
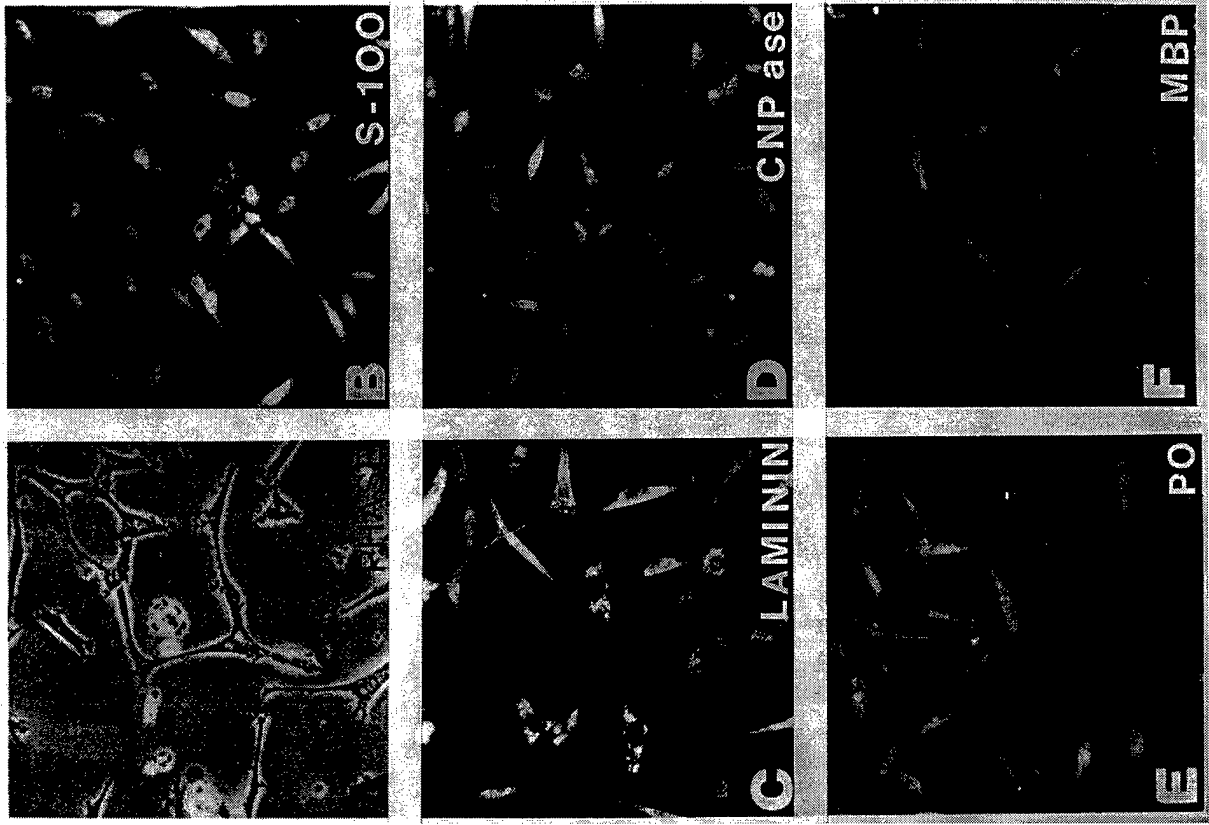


Figure 2



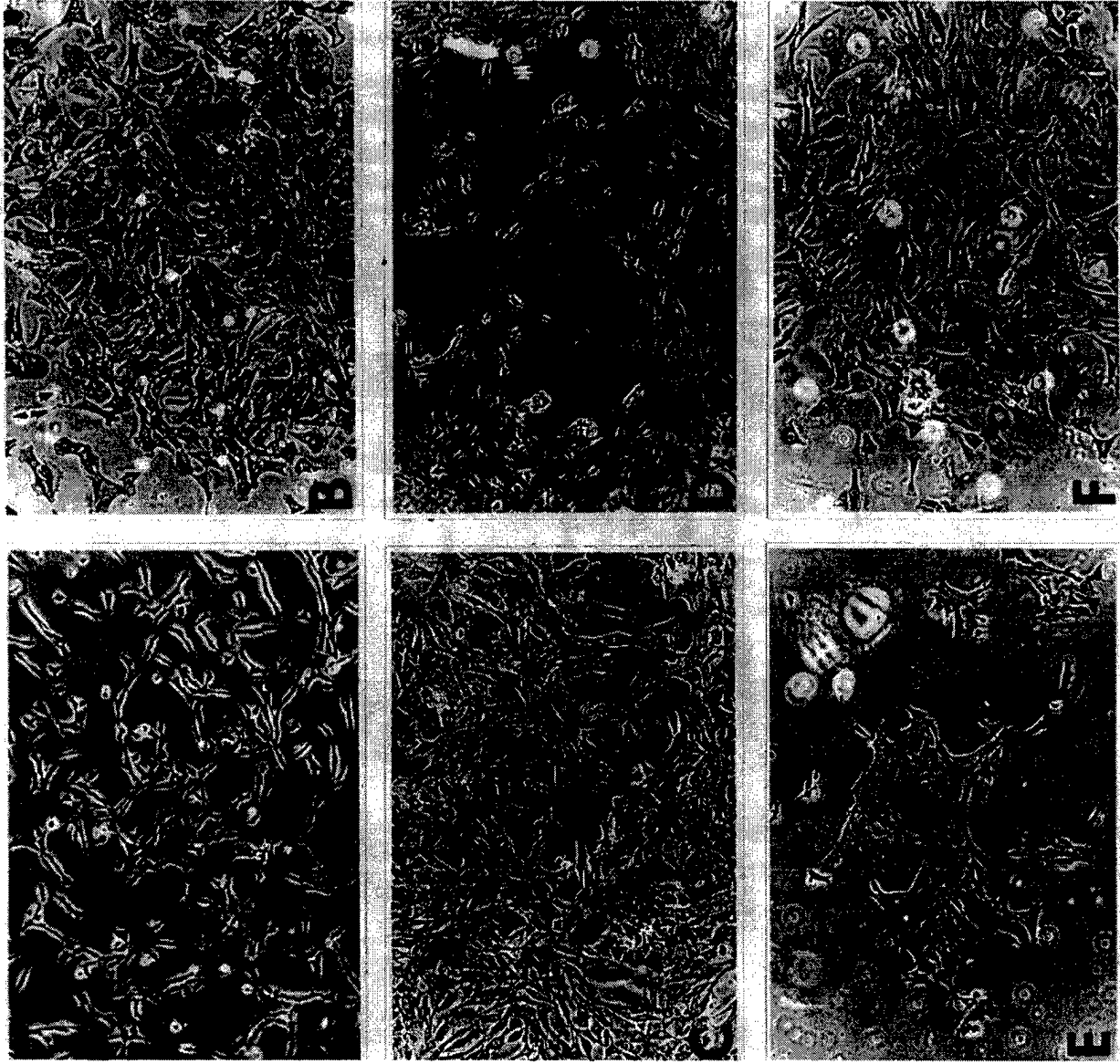


Figure 3