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13. ABSTRACT *(Maximum 200 words)*
This constitutes an addendum to the final report for grant number DAMD17-93-J-3035. The final report already submitted covered research performed from September 22, 1993 through September 21, 1996, whereas this addendum covers research performed from September 22, 1996 through September 21, 1997. Two conditions of Schwann cell hyperplasia occur at increased incidence in NF1 patients: neurofibromas and malignant schwannomas. We continued investigations of the Kit/ stem cell factor complex in hyperplasias of these cells. In the request for no-cost extension, several experiments were listed to be completed. These included testing conditioned media provided from Dr. Nancy Rattner from wild-type and NF1 knockout mice, testing paraffin sections of malignant schwannomas provided by Dr. Viskochil, and testing various malignant neural tumors for the Kit/SCF complex. Conditioned medium from Schwann cells of NF1 knockout mice failed to convert mast cells to a connective tissue phenotype, as compared to wild-type Schwann cells, suggesting less soluble stem cell factor from the NF1 knockout Schwann cells. We also found that most neural tumors express mRNA for Kit, and also for stem cell factor.

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Thomas F. Huff   6/17/97

PI - Signature   Date

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Introduction

This constitutes an addendum to the final report for grant number DAMD17-93-J-3035. The final report already submitted covered research performed from September 22, 1993 through September 21, 1996, whereas this addendum covers research performed from September 22, 1996 through September 21, 1997. In the request for no-cost extension, several experiments were listed to be completed, in addition to continuing support for the supplement-based research. These included testing conditioned media provided from Dr. Nancy Rattner from wild-type and NF1 knockout mice, testing paraffin sections of malignant schwannomas provided by Dr. Viskochil, and testing various malignant neural tumors for the Kit/SCF complex.

Body

The body of the addendum will follow an abbreviated format (no references) as that used in the final report, and will include a listing of findings with reference to Figures in the Appendix. The findings obtained from the AASERT supplement are included a separate subsection. To emphasize the major findings conceptually, all standard procedures have been described in a Materials and General Procedures subsection at the end of this section.

Characterization of murine bone marrow-derived mast cells cultured with Schwann cell-conditioned media from wild type or NF1-deficient mice

Efforts to conduct experiments examining potential paracrine effects of mast cells and Schwann cells upon each other have been hampered by the difficulties involved in obtaining and maintaining relatively pure populations of normal human mast cells and Schwann cells. The experiments described above were done with cell lines, which represent phenotypically abnormal cells, and therefore do not serve as an adequate model system. Primary cultures of mouse mast cells are routinely established in this laboratory, and attempts to isolate and grow highly purified cultures of mouse Schwann cells have been successful in other labs, thereby offering a potential source of cells with which to study interactions in the murine system. The establishment of the *NF1* knockout mouse strains allows for comparisons between normal cells and neurofibromin-deficient cells.

We obtained conditioned media from Schwann cell cultures derived from wild-type, *NF1* +/-, and *NF1* -/- mice from the laboratory of Dr. Nancy Ratner (Department of Cell Biology, Neurobiology and Anatomy, College of Medicine, University of Cincinnati). We cultured bone marrow-derived mast cells in 10% concentrations of the various Schwann cell conditioned media and compared the cultures for differences in mast cell phenotype or rates of proliferation. Alcian blue/safranin stains were done to determine the percentage of cells containing safranin-positive (heparin-containing) granules, characteristic of the connective tissue mast cell phenotype (CTMC). Our laboratory has previously used the shifting of alcian blue/safranin staining to redder granulation as an indication of the presence of SCF in cell-conditioned medium. Cultures containing each of the conditioned media were left untreated or were supplemented with either

50 ng/ml rmSCF, 50 U/ml rmIL-3, or both. Cytospin preparations of the cells were stained with alcian blue and safranin, followed by microscopic examination. The total number of cells in five random 20X fields were counted, and the percentages of cells with safranin-positive granules were calculated (Table 1).

An increase in the percentage of safranin-positive cells in CM-containing cultures given only IL-3 (Table 1), when compared to the absence of safranin staining in cultures which contain no conditioned media, suggests that the conditioned media contains one or more factors which contribute to the phenotypic shift. The results obtained with cultures containing BALB/c 3T3 fibroblast conditioned medium are similar to those from previous work in this laboratory which led to the discovery of stem cell factor. The appearance of the cell is shown in photomicrographs in Figures 1 and 2.

Changes in the rate of proliferation of the mast cells were assessed based on incorporation of ^{3}H-thymidine during DNA synthesis. After three days of culture in the different combinations of conditioned media and additives noted above, 1 μCi of ^{3}H-thymidine was added to each well. After an additional 24 hours in culture, the amount of incorporation was determined using a scintillation counter (Figure 3).

Analysis of Kit Expression on Schwann cells of neurofibroma or malignant schwannoma tumors from paraffin sections

Neurofibromas are composed primarily of Schwann cells, but include a few other cell types, including mast cells. We wished to determine whether these Schwann cells express the Kit receptor, offering a possible explanation for their hyperplasia. Sections of formalin-fixed paraffin-embedded neurofibromas were evaluated for the expression of Kit protein by immunohistochemistry as described in Materials and Methods using the polyclonal anti-Kit antibody sold by American Research Products. Mast cells are known to express Kit protein, and therefore serve as convenient internal controls for the staining protocol. Indeed, Kit protein was easily detectable on mast cells. The surrounding Schwann cells, however, show no evidence of immunoreactive protein. By using differential interference contrast microscopy at a higher magnification we were better able to identify the Schwann cells, based on their characteristic fibrillar morphology. However, very recently, were have detected low-level Kit staining on the same tissue sections using the monoclonal anti-Kit antibody YB5.B8 under conditions where isotype-matched controls were completely negative.

The detection of Kit protein on all four of the malignant schwannoma cell lines led to our hypothesis that there may be a correlation between aberrant Kit expression by Schwann cells and a transformed phenotype. However, the results of immunohistochemical analyses using the polyclonal anti-Kit antibody sold by American Research Products revealed that Kit expression by mast cells was evident, but no Kit was detected on the Schwann cells of these tumors (Figure 4).

Malignant Schwannoma lines make MITF, the transcription factor for Kit.

MITF is a basic helix-loop-helix leucine zipper protein with structural motifs which define a family of transcription factors related to *Myc* and its partners. The *mi* or micropthalmia locus of mice encodes MITF. Mutant mice of the *mi/mi* genotype have the deletion of an arginine in the basic domain and show the abnormality in development of eyes, melanocytes, osteoclasts and mast cells. MITF binds to a hexameric motif (CACATG). Since the Kit receptor tyrosine kinase plays an important role in the development of mast cells, and since the Kit expression by cultured mast cells from *mi/mi* mice is deficient in both mRNA and protein levels, the mast cell deficiency of *mi/mi* mice is attributible at least in part to the deficient expression of Kit. Kit expression was deficient in mast cells but not in erythroid precursors, testicular germ cells, and neurons of *mi/mi* mice. This suggested that the regulation of the Kit transcription by the MITF was dependent on cell types.

Since Kit was present in malignant schwannoma lines but not normal Schwann cells, we tested these schwannoma lines for the presence of MITF by RT-PCR using primer based on the published sequence. We were particularly interested in determining whether MITF might be the transcription factor in these Schwann cells since it did not apparently control Kit expression in neurons (see above). A strong amplification product was detected from RNA derived from each of the 4 malignant schwannoma lines, with a representative ST88-14 result shown in Figure 5. The results further confirm the aberrant Kit expression and relate its regulation more to mast cells than to neurons. Interestingly, mast cell Kit shows a very high rate of Kit mutation as compared with Kit on various solid tumors such as breast carcinoma, colon cancer, or small cell lung cancer; and we have detected a Kit mutation in the schwannoma Kit.

RT-PCR analysis of RNA derived from freshly isolated surgical specimens of benign schwannomas and glioblastomas provided by Dr. Broaddus.

Dr. William Broaddus, a neurosurgeon at this institution, regularly provides us with tissue from benign schannomas such as acoustic neuromas or from malignant neural tumors such as glioblastoma. We have analyzed RNA from these tissues by RT-PCR for expression of stem cell factor, Kit, and MITF. So far, all tissues tested show varying expression of both SCF and Kit, as shown in by example using glioblastomas rc2011, 2038, 2044, and 2049 (Table II). Positive controls are HMC-1 mast cells and HT1080 human cells for Kit and SCF and 159 cells as a negative control (Table II). These same lines were tested for the presence of MITF mRNA by RT-PCR (Table III). All of the malignant gliomas, even though Kit positive, were negative for MITF mRNA, whereas the HMC-1 mast cell line and ST88-14 malignant schwannoma line were positive as expected. This result suggests that KIT expression in mast cells and in the ST88-14 malignant schwannoma may be due to the MITF transcription factor, whereas Kit expression in glial tumors is not. Furthermore, the high rate of Kit mutation in mast cells may correlate with the effects of MITF, the transcription factor for Kit in mast cells. Our PCR studies agree with the observation that neural cells do not express detectable mRNA for MITF.

Subsection on anti-Kit Ribozyme (AASERT)

The following results were obtained by research funded during the no-cost extension period from the supplement grant (Augmentation Award).

Detection of only transfected cells using Green Fluorescent Protein.

We successfully employed the tetracycline inducible anti-*c-kit* ribozyme in the murine P815 mastocytoma (see final report). However, we had not been able to establish a permanent representative of this system. We believe that this is the result of the inability of this system to support high level expression of the ribozyme. Therefore, we switched to a more transient expression system to achieve high level expression of the ribozyme. This required us to address the transfection efficiency of P815 cells. Transfection efficiency was assessed using a pCDNA3lacZ construct, using several different methods to introduce plasmid DNA into the cells. Cells were transfected with plasmid DNA at 1, 10, 20, 30 and 50 μg, allowed to recover overnight, fixed with .05% Glutaraldehyde, and incubated in an X-Gal solution. Transfected cells turn blue. Results indicate that electroporation remains our best transfection alternative with P815 cells, yielding a 10-20% transfection rate. Unfortunately, this small percentage of cells can be overwhelmed by non transfected cells within 24 hours. Therefore, this requires that we selectively remove transfected cells from non transfected cells. This problem has been solved by using a vector that encodes the green fluorescent protein (GFP) (Clontech). P815 cells were electroporated with 1 μg, 10 μg, 20 μg, and 30 μg of plasmid DNA. Transfected cells were readily visualized by fluorescent microscopy and by flow cytometry. We are now in the process of isolating these cells by Flow Sorting and by fluorescent image analysis using an optical cubes to detect GFP, Texas red, and UV on an Olympus Provis microscope with a high resolution CCD camera coupled to an image analysis program by Signal Analytics.

Successful trans cleavage using the anti-Kit ribozyme

We have previously demonstrated cis cleavage by the ribozyme to cut out excess 5' sequence from the ribozyme construct. The results were confirmed using more stringent conditions to reveal very clear cleavage by the proper orientation (Figure 6, lane 1) of the ribozyme into 56 base and 37 base fragments as predicted, whereas the incorrect orientation of the same ribozyme does not exhibit cis cleavage (Figure 6, lane 2).

Now and more importantly, we have also successfully demonstrated in vitro *trans* cleavage of the anti-*c-kit* ribozyme toward an Kit RNA in vitro. In vitro transcribed ribozyme RNA (100 pmol) was mixed with 1 pmol of target RNA; the target RNA corresponds to the ribozyme target site in P815 cells. RNAs were hybridized for 5 minutes at 70C, followed by a quick chill. Cleavage reactions were initiated by the addition of a buffer solution containing 10, 30, or 50mM $MgCl_2$. Reactions were run at 37°C or 50°C for 1 or four hours. RNA products were resolved on an 8% denaturing polyacrylamide gel and exposed to film. Cleavage products of 107 and 83 bases were observed in all instances, but not in ribozyme or target only controls (Figure 7). This indicates that our anti-*c-kit* ribozyme has the capacity to cleave its target RNA under physiological conditions.

Full-length sequencing of human Schwannoma Kit using overlapping TA clones.

We have also continued to address the nature of the Kit protein expressed in the human Schwannoma lines. Previously, we had reported that these lines expressed a truncated form of the Kit protein, which may have been a secreted form of the Kit protein. We established an ELISA protocol to detect soluble Kit in medium derived from these lines. No soluble Kit was detected in the supernatants of these lines. Therefore, we decided to identify regions of the Kit protein that might have been deleted. We sequenced overlapping TA cloned RT-PCR products of the entire *c-kit* message in ST88-14 cells. No unusual truncations or deletions were observed indicating that the message for *c-kit* was normal (Figure 8). Further characterization of the Kit protein in ST88-14 cells by Western has indicated that the truncated Kit in these lines may actually represent a non-glycosylated form of the Kit protein.

Inhibition of growth of human malignant schwannoma using a dominant negative construct of Kit

We have also begun to address the role Kit expression plays in these malignant schwannoma lines. A dominant negative (kinase deficient) *c-kit* cDNA in the pCEP4 vector was transfected into these cells. A full length *c-kit* cDNA control, an empty pCEP4 vector control, and an electroporated, no DNA control were included. Cells were cultured for two days in cDMEM; hygromycin was then added at .8 mg/ml to select for transfected cells. Preliminary results showed that hygromycin resistant cells could only be grown out of the pCEP4 only, and pCEP4Kit controls. No cells were grown out of the pCEPKit dominant negative populations. These results indicate that a Kit/SCF autocrine loop protein is required for maintaining the viability of these cells.

Subsection on Materials and General Procedure

Surgical specimens

In the no-cost extension period, prepared sections of formalin-fixed, paraffin-embedded malignant peripheral nerve sheath tumors were the kind gifts of Dr. David Viskochil (Division of Medical Genetics, University of Utah, Salt Lake City, UT). Fresh surgical specimens of vestibular schwannoma, acoustic neuroma, and meningioma tissues were kindly provided by Dr. William Broaddus (Division of Neurosurgery, Department of Medicine, Virginia Commonwealth University, Richmond, VA).

Conditioned media from Schwann cells of NF1-deficient mice

Schwann cell-conditioned media from wild type and *NF1*-deficient mice were the generous gifts of Dr. Nancy Ratner (Department of Cell Biology, Neurobiology and Anatomy, College of Medicine, University of Cincinnati). Schwann cell cultures were established from excised peripheral nerve tissue of embryonic C57BL/6 mice bearing a targeted mutation of the *NF1* gene on either one (+/-) or both (-/-) alleles. Schwann cells derived from wild-type (+/+) mice were used for comparison.

Murine mast cells grown in Schwann cell-conditioned media

Mouse bone marrow-derived mast cells, after three weeks of culture in rmSCF and rmIL-3 were seeded onto a 96-well tissue culture plates at a density of 5 x 10^5 cells/ml (10^5 cells/well) in cDMEM. The media in various wells contained either no conditioned medium (control), 10% conditioned medium from cultures of BALB/c 3T3 fibroblasts (FCM), 10% conditioned medium from cultures of Schwann cells derived from wild type C57BL/6 mice (NF1 +/+ SCCM), 10% Schwann cell-conditioned medium from mice which were heterozygous for a disruption in the *NF1* gene (NF1 +/- SCCM), or 10% Schwann cell-conditioned medium from homozygous NF1 "knockout" mice (NF1 -/- SCCM). Each of these categories included media supplemented with either 50 ng/ml rmSCF, 50 U/ml rmIL-3, both factors, or no added factors. After 4-7 days of culture under these conditions, representative plates were examined for mast cell granule phenotype, as determined by alcian blue/safranin staining, and rate of proliferation, assessed by ^{3}H-thymidine incorporation.

Alcian blue/safranin staining of murine mast cells

Partial phenotypic characterization of bone marrow-derived murine mast cells was accomplished using a two-step staining protocol. Cells were transferred to slides using a Cytospin 2 (Shandon, Inc., Pittsburgh. PA). Preparations were first covered with 0.5% alcian blue in 0.3% glacial acetic acid for 5-10 minutes, rinsed with tap water, and covered with 0.1% safranin in 0.1% glacial acetic acid for an additional 5-10 minutes. The slides were again rinsed with tap water and air dried, and coverslips were mounted using Cytoseal™ 60 (Stephens Scientific, Riverdale, NJ). Heparin, which can be found in the secretory granules of cells of the connective tissue mast cell (CTMC) phenotype, retains the safranin stain, resulting in the appearance of pink or red granules. Mucosal mast cells (MMC) do not contain heparin, and they stain alcian blue positive.

Proliferation assays for mast cells and Schwann cells

Cultures of mast cells and Schwann cells grown in 96-well plates were "pulsed" by addition of 1 µCi of ^{3}H-thymidine (ICN Pharmaceuticals, Inc., Costa Mesa, CA) to each well, followed by continued incubation for 4-24 hours. The samples were collected onto glass fiber filters using a Filtermate 196 Harvester (Packard, Meriden, CT), and the amount of ^{3}H-thymidine incorporation was determined using the TopCount scintillation counter (Packard). The level of incorporation is proportional to the rate of proliferation of the cells in the sample.

Immunohistochemistry to detect Kit in neurofibroma and malignant schwannoma tissue

Immunohistochemical staining for human Kit was performed using standard techniques. Briefly, slides containing formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylenes and rehydrated in a series of graded ethanol washes. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. A polyclonal rabbit anti-Kit antibody was used as the primary antibody at a concentration of 0.5µg/ml. This antibody was then localized using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine (Vector

Laboratories) was used as the color substrate.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The standard RT-PCR protocols used in this study were performed using the GibcoBRL Superscript Preamplification System (Life Technologies, Inc., Baltimore, MD), supplemented with AmpliTaq® DNA Polymerase (Perkin Elmer, Norwalk, CT).

The reverse transcription reaction was carried out by adding 1µg total RNA in a volume of 1µl to a reaction tube containing 10µl Milli-Q water. After addition of 1µl of a 2µM gene-specific antisense oligonucleotide primer, the mixture is incubated at 70°C for 10 minutes and then quickly chilled on ice for at least 1 minute. 7µl of a master mix containing 2µl 10X PCR buffer, 2µl 25mM $MgCl_2$, 1µl 10mM dNTP mix and 2µl 0.1M DTT are added to each tube, followed by a 5 minute incubation at 42°C. After addition of 1µl (200U) of SuperScript™ II RNase H^- reverse transcriptase, the 42°C incubation is continued for an additional 50 minutes. The reaction is terminated by incubating at 70°C for 15 minutes. After another quick chill on ice, the contents are collected by brief centrifugation, followed by the addition of 1µl (2U) *E. coli* RNase H and a 20 minute incubation at 37°C. The polymerase chain reaction was performed by first adding 2µl of each RT reaction to a fresh tube. 23µl of a master mix containing 18µl water, 2.5µl 10X PCR buffer, 1.5µl $MgCl_2$, and 1.0µl 10mM dNTP mix are added to each reaction. One paraffin bead (Ampliwax® PCR Gem 100 [Perkin Elmer] or equivalent) is added to each tube, followed by a 5 minute incubation at 80°C to melt the wax. After allowing the paraffin to reharden at room temperature, 25µl of a master mix containing 18.5µl water, 2.5µl 10X PCR buffer, 1.5µl 25mM $MgCl_2$, 1.0µl 10µM sense primer, 1.0µl 10µM antisense primer, and 0.5µl (2.5U) AmpliTaq® DNA Polymerase were added onto the top of the wax barrier. The PCR reactions were carried out in the GeneAmp® PCR System 9600 (Perkin Elmer) using programs optimized for the primers and templates being used. For mouse stem cell factor, 30 cycles were performed as follows: denature at 94°C for 15 seconds, anneal at 55°C for 15 seconds, and extend for 30 seconds at 72°C. Reaction products were resolved by agarose gel electrophoresis. For most applications, a standard 1% agarose gel was used, but for quantitative RT-PCR reactions, 4% NuSieve® 3:1 agarose (FMC BioProducts, Rockland, ME) was used to better resolve fragments with small size differences. Bands were visualized by staining with ethidium bromide and viewing on a UV light box.

Southern blotting

After agarose gel electrophoresis, gels were soaked for 40 minutes in a denaturing solution containing 1.5M NaCl and 0.5M NaOH. Gels were then soaked two times (30 minutes each) in a neutralizing solution containing 1.5M NaCl and 1M Tris pH 8.0. Nucleic acid transfer was accomplished by downward capillary action as follows: a piece of thick filter paper, cut to gel size, was soaked 20 minutes in 10X SCC and placed on a 5 inch stack of paper towels in a basin. This was followed by 2 pieces of thin filter paper (also cut and soaked), a piece of 0.2µm Nytran® (Schleicher and Schuell, Keene, NH), the gel (soaked 10 minutes in 10X SSC), a third piece of thin filter paper, and a sponge soaked in 10X SSC. The entire container was wrapped in plastic wrap and left undisturbed overnight. After transfer, the orientation of the wells were marked on the nytran with a needle, and the DNA was cross-linked to the Nytran using a UV Stratalinker™ 1800 (Stratagene, La Jolla, CA).

Oligonucleotide probes were labeled using the GibcoBRL 5' end labeling kit (Life Technologies, Inc., Baltimore, MD). 100ng of probe in 17μl water were mixed with 2μl T4 kinase (10U), 6μl 10X forward reaction buffer, and 5μl [γ-^{32}P]ATP (150μCi/μl). After incubating at 37°C for one hour, the reaction was terminated by incubating for 10 minutes at 65°C. The labeled probes were then hybridized to the blots using a Hybaid hybridization oven (Hybaid Instruments, Holbrook, NY). The Nytran filters and nylon mesh were briefly soaked in sterile water, layered upon each other, rolled into a tube and placed in a hybridization bottle. A 2X prehybridization solution was prepared by mixing 125mg yeast RNA (mixed in 1ml water and a few drops of NaOH), 2.5ml 20% SDS, 0.5g bovine serum albumin, 0.5g polyvinylpyrrolidone (PVP), 0.5g ficoll, 25ml 1M sodium phosphate pH 6.5, and 125ml 20X SSC, brought to a total volume of 250ml with water. 10ml of a 1:1 mixture of 2X prehybridization solution and water were added to the bottle containing the blots, and the bottle was incubated in the hybridization oven for 1 hour at 37°C. This solution was then poured off and replaced with fresh 1:1 prehyb mix and water to which the entire probe labeling reaction was added. The blots were incubated with the probe overnight in the oven at 37°C. After disposing of the solution into the appropriate radioactive waste container, the blots were washed twice (10 minutes each) in 30ml of 2X SSC/0.1% SDS at 37°C, followed by a 1 hour wash in 50ml of 0.2X SSC/0.1% SDS at 37°C. The excess liquid was blotted from the filters with paper towels, the blots were covered in plastic, and placed on x-ray film for 24 hours (exposure time varies with the strength of the signal).

Ribonuclease protection assay (RPA) for SCF isoforms

Total RNA was obtained by using the Ultraspec™ RNA isolation system (Biotecx Laboratories, Inc., Houston, TX). Samples were stored at -80°C at concentrations of 1-2 μg/μl until needed. The probe was generated by PCR amplification of a fragment of our cloned human stem cell factor cDNA (Ryan et al., 1994) including most of exons 5 and 6 (nucleotides 560-781 as numbered by Martin et al., 1990). The following primers were used: Primer 1: 5'-ATG**GGATCC**ATTCAAGAGCCCAGAAC-3'; primer 2: 5'-GC**TCTAGA**TGC TACTGCTGTCATTCC-3'. Nucleotides in bold type represent restriction sites for BamHI and XbaI, respectively. After amplification, the fragment was digested with the two enzymes and ligated into pBluescript II SK+ (Stratagene Cloning Systems, La Jolla, CA), which had been similarly digested. A radiolabeled transcript was obtained with the MAXIscript™ In Vitro Transcription Kit (Ambion, Inc., Austin, TX) using T3 RNA polymerase and [α-^{32}P] UTP. RNase Protection Assays (RPA) were performed with the RPA II™ kit (Ambion), following the suggested protocol. Fragments were resolved on a 5% acrylamide 8M urea sequencing gel. Protected fragments of 222 and 144 nucleotides are indicative of the exon 6-containing (soluble) and exon 6-lacking (membrane-bound) isoforms of human SCF mRNA, respectively.

Conclusions

The research described in the final report and in this addendum deals with some of the patterns of expression and biologic activities of stem cell factor and its receptor Kit. SCF is capable of acting on any cell which expresses its Kit. The Kit^{+} cells addressed in this dissertation research were mast cells and malignant schwannoma cell lines. Both Schwann cells and mast cells are found in high numbers in the neurofibroma nodules associated with NF1, and Schwann cells have been shown to make SCF. The hyperplastic Schwann cells in these lesions have an abnormally high tendency to become malignant. Many such solid tumors have been shown to involve an SCF/Kit autocrine loop. Thus, neurofibromas and malignant schwannomas appear likely to have a complex dependency on the SCF/Kit complex, whether in an autocrine or paracrine signalling pattern. This research investigates various aspects of SCF and Kit expression, with particular emphasis on which isoform of SCF might be expressed. To date, very few studies have attempted to make a distinction between different forms of SCF expression, membrane or soluble, and relate them to biologic or pathologic situations.

A major part of the research dealt with the possible etiologic role of the SCF/Kit complex in NF1 lesions. Although the manifestations of type 1 neurofibromatosis rarely lead to fatal consequences, NF1 causes significant morbidity. In addition to the medical symptoms of the pain and mechanical limitations resulting from the size and location of neurofibromas, the patient must also deal with the unfortunate social stigma associated with being deformed. Developmental dysfunctions and learning disabilities occur more frequently in children and adolescents with NF1 than in the general population, creating a tendency toward (or perhaps resulting from) a sense of low self esteem. Perhaps due to the low incidence of mortality associated with NF1, the degree of public awareness has not correlated well with the relatively high rate of occurrence of the disease. Although this may create obstacles in the way of gaining support for research efforts, it is not an indication of the relative importance of such research. The cloning of the *NF1* gene and the subsequent characterization of the gene product, neurofibromin, has greatly improved our understanding of the disease, and may possibly lead to the development of practical clinical interventions. However, even the *NF1* gene cloning studies do not provide an adequate explanation for the overproliferation of Schwann cells and mast cells in the lesions. The purpose of this study has been to obtain information about some of the very basic mechanisms at work in NF1, specifically those involving stem cell factor and Kit interactions.

We chose to focus on SCF and Kit due to a logical association of certain facts: that mast cells (which express Kit and respond to SCF) are present in the nervous system associated with normal nerves; that mast cells are increased at sites of nerve damage and repair or in nerve-associated tumors, such as neurofibromas and schwannomas; and that Schwann cells produce stem cell factor. We further sought to determine whether aberrations in the expression of either SCF (i.e. changes in isoform) or Kit (ectopic expression by Schwann cells) may be involved in the development of the tumors characteristic of NF1.

<u>Addendum Conclusions</u>: It would be informative to establish cocultures of human mast cells and Schwann cells derived from both genetically normal individuals and NF1 patients, and monitor them for changes in phenotype. These changes might relate to maturation, differentiation, proliferation, or activation. We are not aware of any studies of this sort to date, which is possibly an indication of the difficulties inherent in the derivation of pure cultures of human mast cells

and Schwann cells. In an effort to circumvent these obstacles and approximate the conditions as closely as possible, we have performed preliminary experiments in the mouse system using conditioned media from *NF1* knockout Schwann cells kindly provided by Dr. Nancy Ratner at the University of Cincinnati. We have cultured bone marrow-derived murine mast cells (BMMC) in the presence of mouse Schwann cell conditioned media (SCCM). The sources of the SCCM include Schwann cells derived from wild-type, and *NF1* heterozygous (+/-) and homozygous (-/-) knockout mice. Changes in the phenotype or growth rate of the BMMC were then measured by alcian blue/safranin staining and tritiated thymidine incorporation, respectively.

Alcian blue/safranin staining of mast cells has been used to differentiate between cells of the mucosal (MMC) or connective tissue (CTMC) phenotype. The granules of CTMC contain heparin, which binds the safranin dye, resulting in pink or red staining. Stem cell factor is produced by cells in the connective tissue microenvironment *in vivo*, and drives mast cell progenitors toward the CTMC phenotype, whereas the MMC result from the influence of IL-3 in the mucosal microenvironment. In *in vitro* studies, a shift toward red granulation in alcian blue/safranin stained mast cell cultures is an indication of the presence of SCF in the culture medium. A similar approach was used successfully by John Ryan in his dissertation research in this lab to detect biologically active SCF in other mast cell co-cultures. In our experiments with Schwann cell-conditioned media, we noted that, in the presence of IL-3 alone, SCCM from both *NF1* +/+ (wild type) and *NF1* +/- mice shifted the cultured mast cells toward the CTMC phenotype as did cultures containing fibroblast-conditioned medium (FCM). In addition, we observed that the wild type SCCM induced a greater shift than did the +/-. However, *NF1* -/- SCCM appeared to have a diminished capacity to exert this effect. It is possible that the Schwann cells of the homozygous knockout mice do not release soluble SCF into the medium (perhaps due to a change in isoform expression), and therefore do not drive the mast cells as effectively toward the CTMC phenotype. This possibility is particularly intriguing in view of the observation that the malignant schwannoma lines do not make soluble SCF. The complete loss of neurofibromin in these cells may lead to the secretion of other cytokines which, in concert with exogenous SCF and IL-3, may co-stimulate the proliferation of the cultured mast cells. Indeed, DNA synthesis was increased in mast cells grown in *NF1* -/- SCCM in the presence of both SCF and IL-3 compared to all other culture conditions. A possible explanation for why *NF1* -/- Schwann cell-conditioned meduim augments growth but does not change phenotype is that whereas other cytokines in Schwann cell conditioned medium could likely augment mast cell growth, the ability to cause a change toward the connective tissue phenotype is a biologic activity that has been much more closely linked to SCF specifically.

A possible explanation for the intermediate ability of *NF1* +/- Schwann cell-conditioned medium to cause a shift in alcian blue/safranin staining is that a mutation on a <u>single</u> *NF1* allele may cause a <u>partial</u> decrease in the production of soluble SCF by Schwann cells. If so, these cells might actually be models for Schwann cells in NF1, a genetic disease which is caused by an autosomal dominant mutation (heterozygous). On the other hand, when both alleles are non-productive, as is certainly the case for both the mouse *NF1* knockout Schwann cells and the ST88-14 malignant schwannoma, soluble SCF bioactivity, as indicated by safranin-positive granules is barely detectable (Table 1). Studies involving the ST88-14 malignant schwannoma line show that, although a single copy of the *NF1* gene appears to remain intact in these cells, *NF1* mRNA expression was found by Northern analysis to be substantially reduced or absent. Moreover, it has also been reported that ST88-14 cells express extremely low levels of neurofibromin protein. It has been suggested that the greatly reduced *NF1* expression in this tumor cell may be due to a somatic mutation acquired by the intact allele, resulting in either greatly reduced transcription or message instability. It is also possible that a homozygous mutation may lead to the transformation of the cell, as has been suggested for the ST88-14 human malignant schwannoma. Additional studies are required to determine if lack of soluble stem cell factor expression by Schwann cells might be caused by severe neurofibromin deficiency.

The findings reported here indicate a significant involvement of stem cell factor and Kit in at least some of the pathological manifestations of NF1, perhaps in part through faulty regulation of SCF isoform expression. We have found that four human malignant schwannoma cell lines all aberrantly express the Kit receptor, and that this protein may be truncated and constitutively activated. We further suggest that loss of neurofibromin function may have a direct effect on stem cell factor biologic activity, by regulating the induction toward a membrane isoform switch. There are two lines of evidence which suggest a possible role for neurofibromin in SCF isoform expression: the mouse *NF1* knockout data and the human ST88-14 malignant schwannoma data, both of which lack neurofibromin activity and appear to express the membrane isoform of SCF. In fibroblasts, there appears to be a correlation of membrane SCF with more rapid rate of growth. A better understanding of the mechanisms by which expression of the stem cell factor isoforms is regulated is necessary to define a possible relationship between SCF expression and neurofibromin function.

The conclusion fro the AASERT-associated research are that an inducible antisense hammerhead ribozyme is a powerful and promising way to inhibit Kit expression, which will likely result in apoptotic death in these cells. The ribozyme are clearly regulable by the tetracycline system, exhibit both cis and trans cleavage reactions in in vitro assays, inhibit cell-surface Kit in treated cells, and in the future may be deliverable by other vectors such a recombinant adenoviruses or retrviruses. If aberrant SCF/Kit signalling is a major etiologic factor for Schwann cell hyperplasias associated with NF1, such an approach might be an excellent therapeutic strategy.

We interpret that that KIT expression in mast cells and in the ST88-14 schwannoma may be due to the MITF transcription factor, whereas Kit expression in glial tumors is not. Furthermore, the high rate of Kit mutation in mast cells may correlate with the effects of MITF, the transcription factor for Kit in mast cells. Our PCR studies agree with the observation that neural cells do not express detectable mRNA for MITF. Thus, we anticipate that malignant gliomas may be more likely to use an autocrine growth loop rather than an activating mutation in Kit.

The malignant schwannoma cell lines and the *NF1* knockout mice likely represent good model systems in which these mechanisms can be studied. This information could lead to the development of therapeutic strategies for the treatment of the symptoms of NF1.

Appendix (Grant DAMD17-93-J-3035)

Table 1. Alcian blue/safranin staining of bone marrow-derived mast cells

Percent cells containing safranin-positive granules

	no added factors	**50 ng/ml rmSCF**	**50 U/ml rmIL-3**	**SCF + IL-3**
Cells grown in cDMEM alone	0%	86%	0%	92%

Cells grown in 10% conditioned media supplemented with 50 U/ml rmIL-3

Conditioned medium	**Safranin-positive cells**
cDMEM alone (no CM)	0%
BALB/c 3T3 fibroblast CM	17%
NF1 +/+ Schwann cell CM	30%
NF1 +/- Schwann cell CM	20%
NF1 -/- Schwann cell CM	4%

Table II. Malignant gliomas express mRNA for Kit and SCF, but not MITF.

This experiment tested six supposed glioblastoma cell lines and one normal brain tissue sample. RNA from these samples were provided by Dr. Broaddus' laboratory. The cell lines; RC 2083, RC 2085, RC 2011, RC 2038, RC 2044, RC 2049, and 164, were tested for the presence of MITF, SCF, c-kit, and actin mRNA.

The results of the gels are summarized in the following table:

Cell line	**Actin**	**c-kit**	**SCF**	**MITF**
RC 2083	negative	negative	negative	negative
RC 2085	negative	negative	negative	negative
RC 2011	positive	positive	positive	negative
RC 2038	positive	positive	positive	negative
RC 2044	positive	positive	positive	negative
RC 2049	positive	positive	positive	negative
164	negative	negative	negative	negative

It appears as if the RNA from cell lines RC 2083, RC 2085, and 164 are not intact. It could also be that the RT step using oligo dT is not allowing sufficient synthesis of cDNA. This does not seem to be the case because the oligo dT method has produced results in the past experiments. It was expected that these glioblastomas would have MITF, and other experiments did contain MITF. However, those experiments used mouse primers. A second experiment will be conducted on 7/8/97 to determine if MITF is really absent from these samples. It will use double the amount of primer in the PCR step.

Table III. Additional malignant glioma tissue lacks expression for MITF mRNA.

This RT-PCR was done on the cell lines below in order to determine which samples gave the best gel bands for MITF. A final gel including the best bands would be used to make a blot to be used at a later time.

The results of the gels are summarized in the following table:

Cell line	Results for MITF	Cell line	Results for MITF
ST88-14	positive	RC 2044	negative
HMC-1	positive	RC 2049	negative
Detroit 551	positive	5-22-2	positive
KEL-FIB	positive[1]	5-22-3	negative
RC 2083	negative	5-22-4	negative
RC 2085	negative	5-22-5	negative
164	negative	5-22-6	negative
RC 2011	negative	5-22-8	?
RC 2038	?	5-22-9	negative

Those samples marked with a ? indicate instances where a band or something resembling a band appeared in the area of the expected band, but did not appear as other positive bands did. Further analysis would be needed to determine whether or not these samples actually contain message for MITF. The final gel confirmed the results of the first two gels, and the final gel contained the samples with the best bands for MITF. The confirmation that these bands represent actual MITF message will come from a Southern blot analysis.

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[1] Many other bands were also present, but a band seemed to occur at 560 bp.

BMMC Proliferation

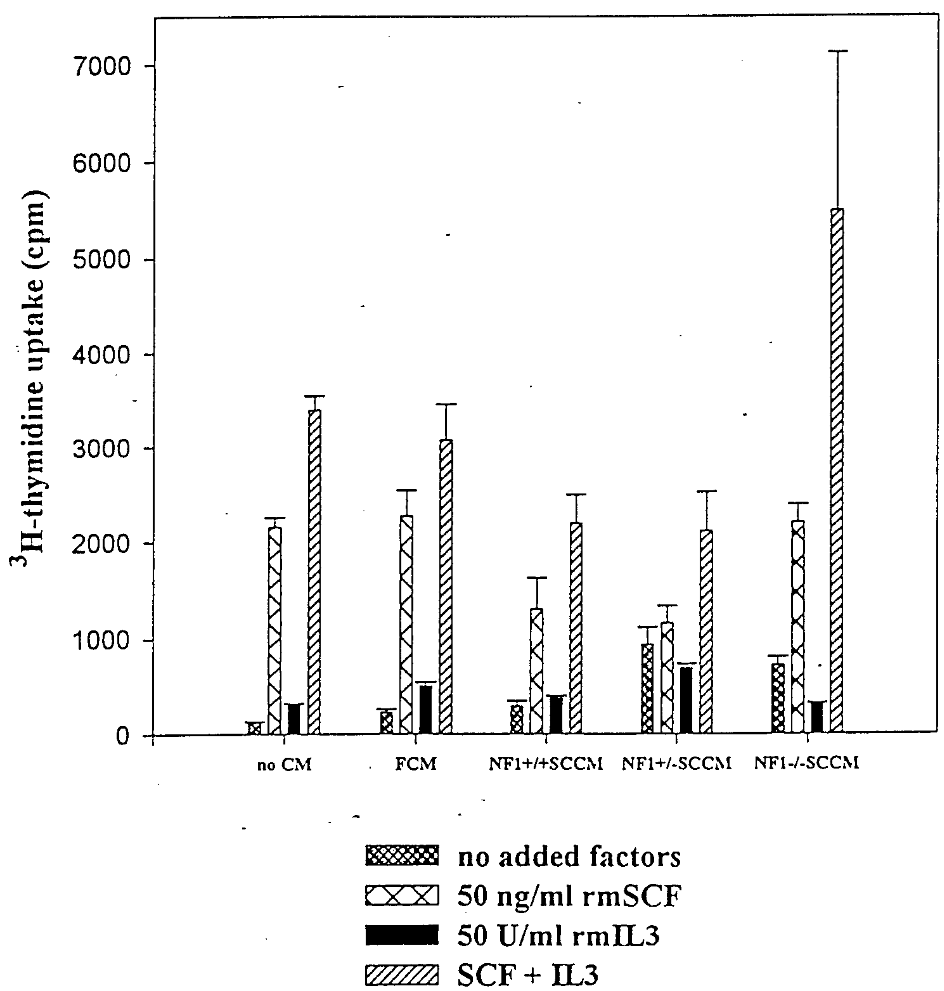


Figure 1 Proliferation of bone marrow-derived mouse mast cells grown in Schwann cell-conditioned media from wild-type or *NF1* knockout mice. Bone marrow-derived mast cells were cultured for 3 days with cytokines and 10% concentrations of the conditioned media indicated. 1 μCi of ^{3}H-thymidine was added to each well, and the plate was incubated for an additional 24 hours. Cells were harvested, and ^{3}H-thymidine incorporation was determined using a scintillation counter. Data represent the mean ± SEM of triplicate wells.

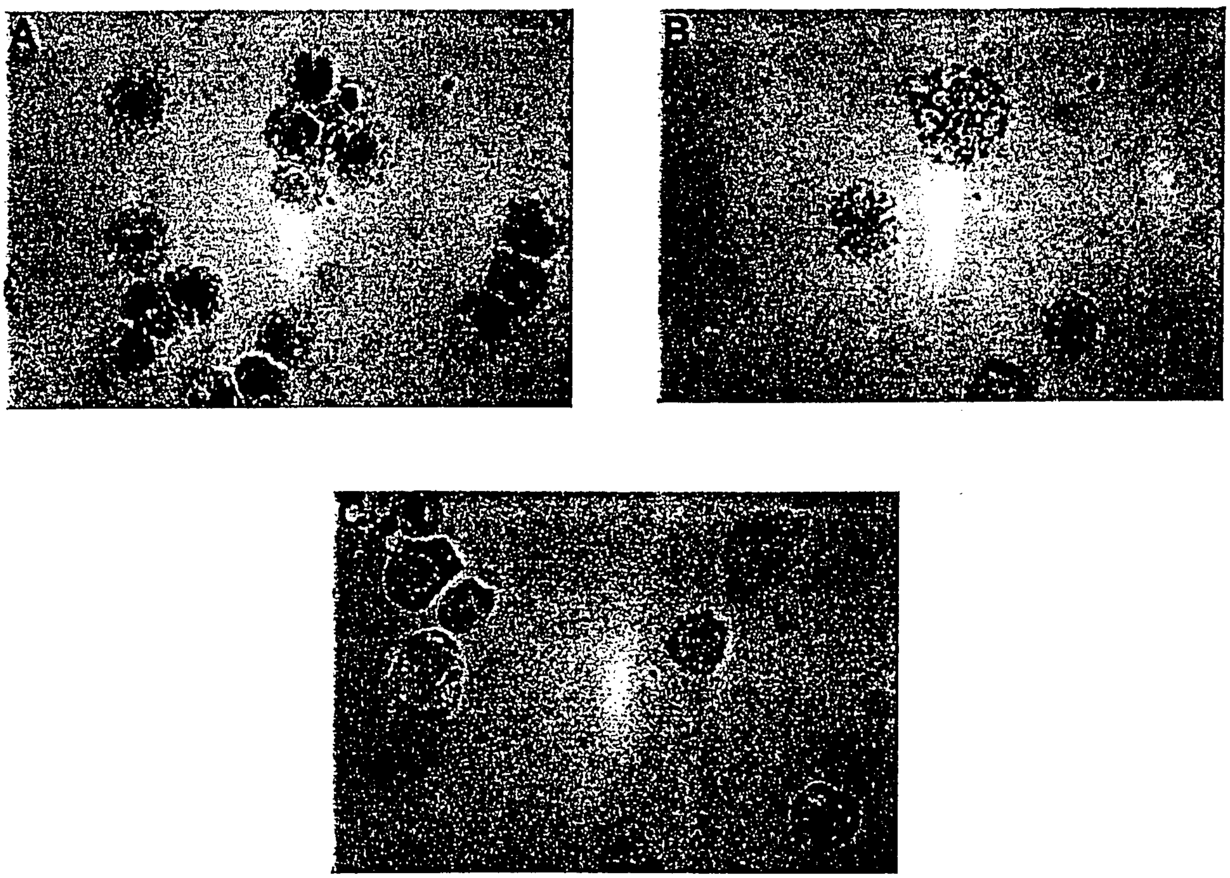


Figure 2 . Photomicrographs of alcian blue/safranin-stained bone marrow-derived mouse mast cells grown with rmSCF, rmIL-3, or both. Bone marrow-derived mouse mast cells were obtained as described in Materials and Methods. Alcian blue/safranin staining of cytospin preparations was done after 5 days of culture with (A) 50 ng/ml rmSCF, (B) 50 U/ml rmIL-3, or (C) SCF + IL-3.

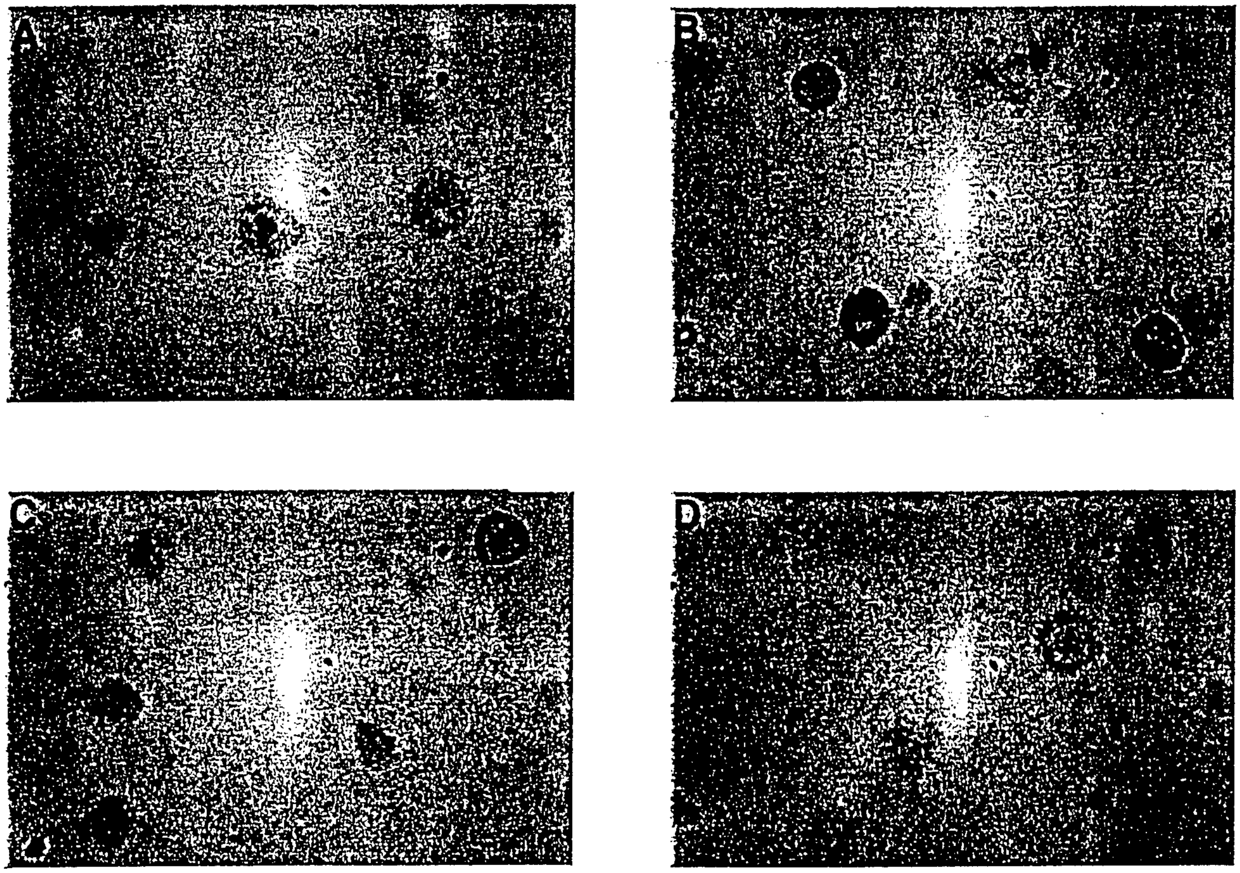


Figure 3 . Photomicrographs of alcian blue/safranin-stained bone marrow-derived mouse mast cells grown in Schwann cell-conditioned media from wild-type or *NF1* knockout mice. Alcian blue/safranin stains were done on cytospin preparations of bone marrow-derived mouse mast cells grown for 5 days with 50 U/ml rmIL-3 in the presence of various cell culture-conditioned media. Shown are representative examples of cells grown in 10% BALB/c 3T3 fibroblast conditioned medium (A), or Schwann cell-conditioned medium from wild type (B), *NF1* +/- (C), or *NF1* -/- mice (D).

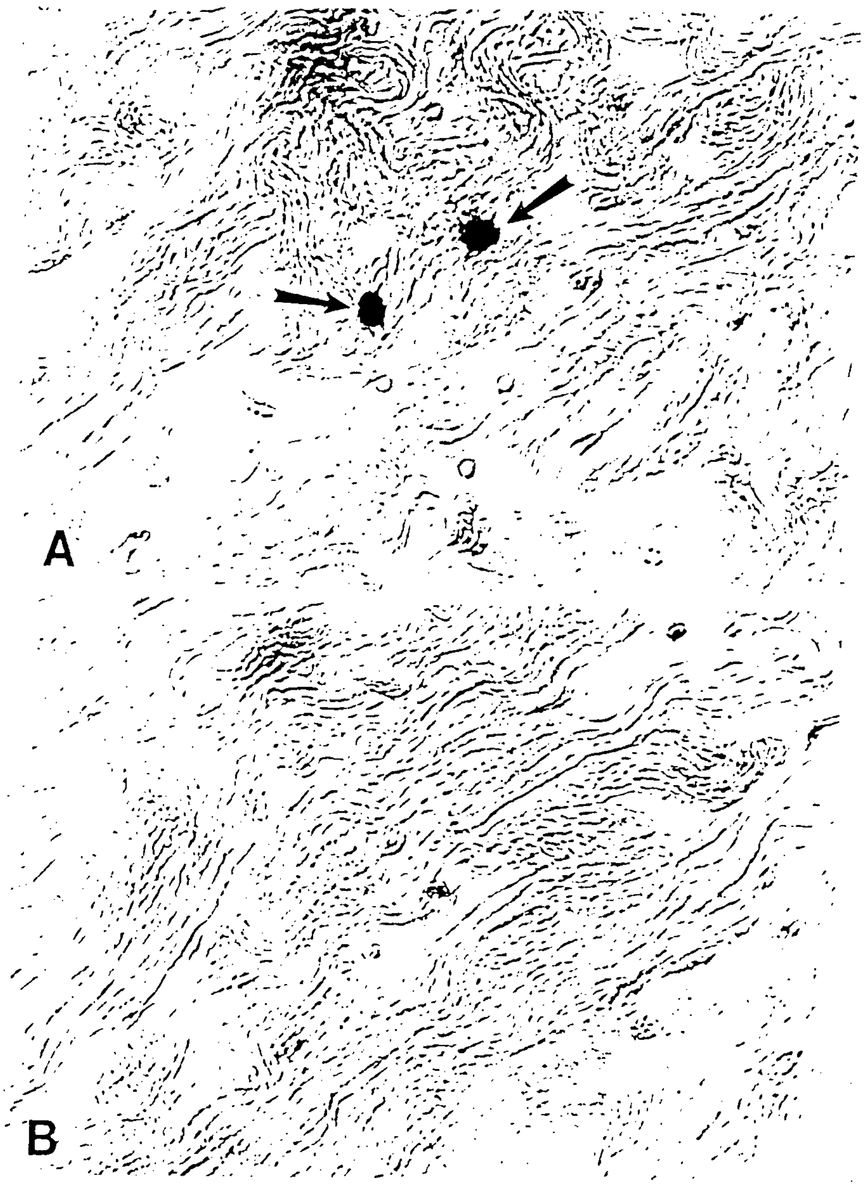
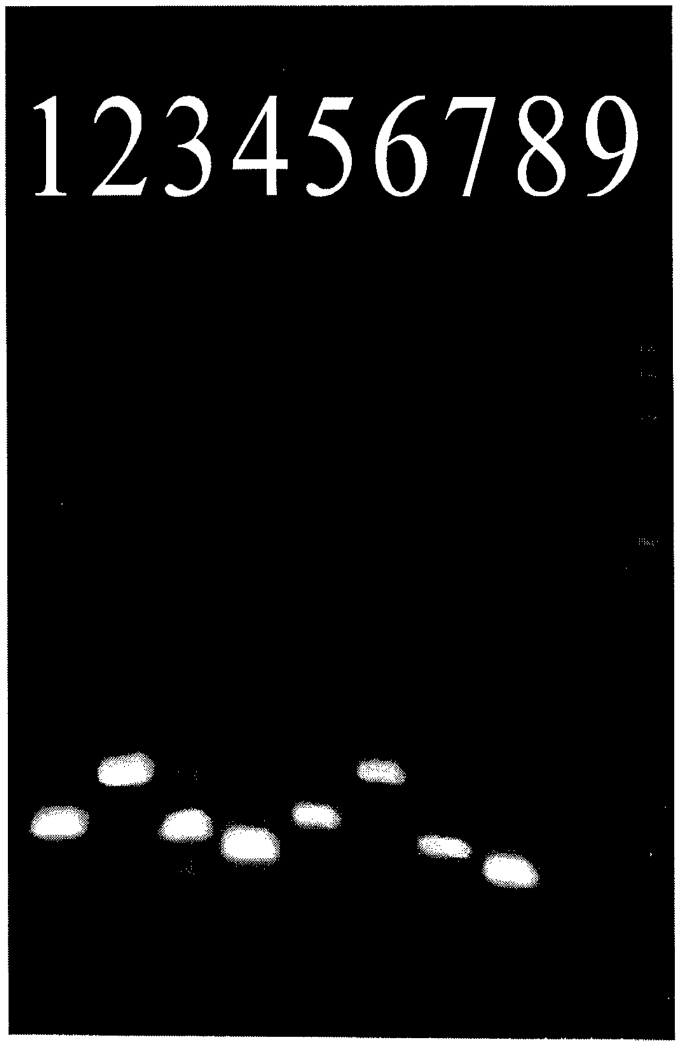


Figure 4 Photomicrographs of immunohistochemistry to detect Kit protein in malignant schwannoma tissue sections. Immunohistochemistry was performed on thin sections of human malignant schwannoma tumors as described in Materials and Methods. (A) Section stained with a polyclonal antibody against human Kit. Arrows indicate Kit$^+$ mast cells. (B) Adjacent section stained with an isotype-matched control antibody. Original magnification: 132X.



RT-PCR. Lanes 1-4 are HMC-1. Lanes 5-8 are ST88-14. Lanes 1,2,5,6 are Kit from 2 primer sets. Lanes 3 & 7 are MITF. Lane 9 is no mRNA.

Figure 5. RT-PCR for Kit and MITF mRNA in a malignant schwannoma line

Cis-In Vitro Cleavage Reaction

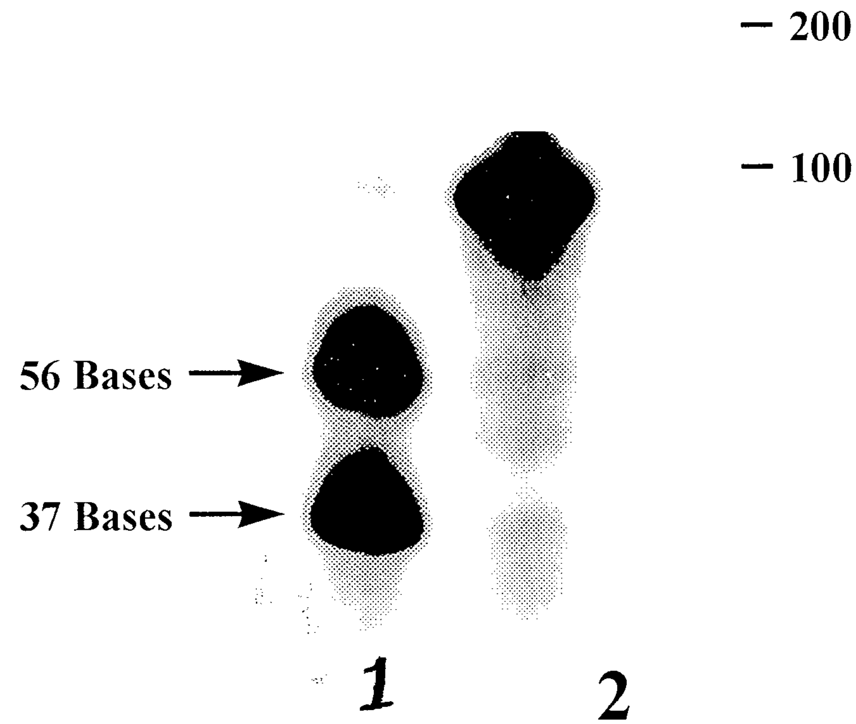


Figure 6

In Vitro *Trans* Cleavage Reaction

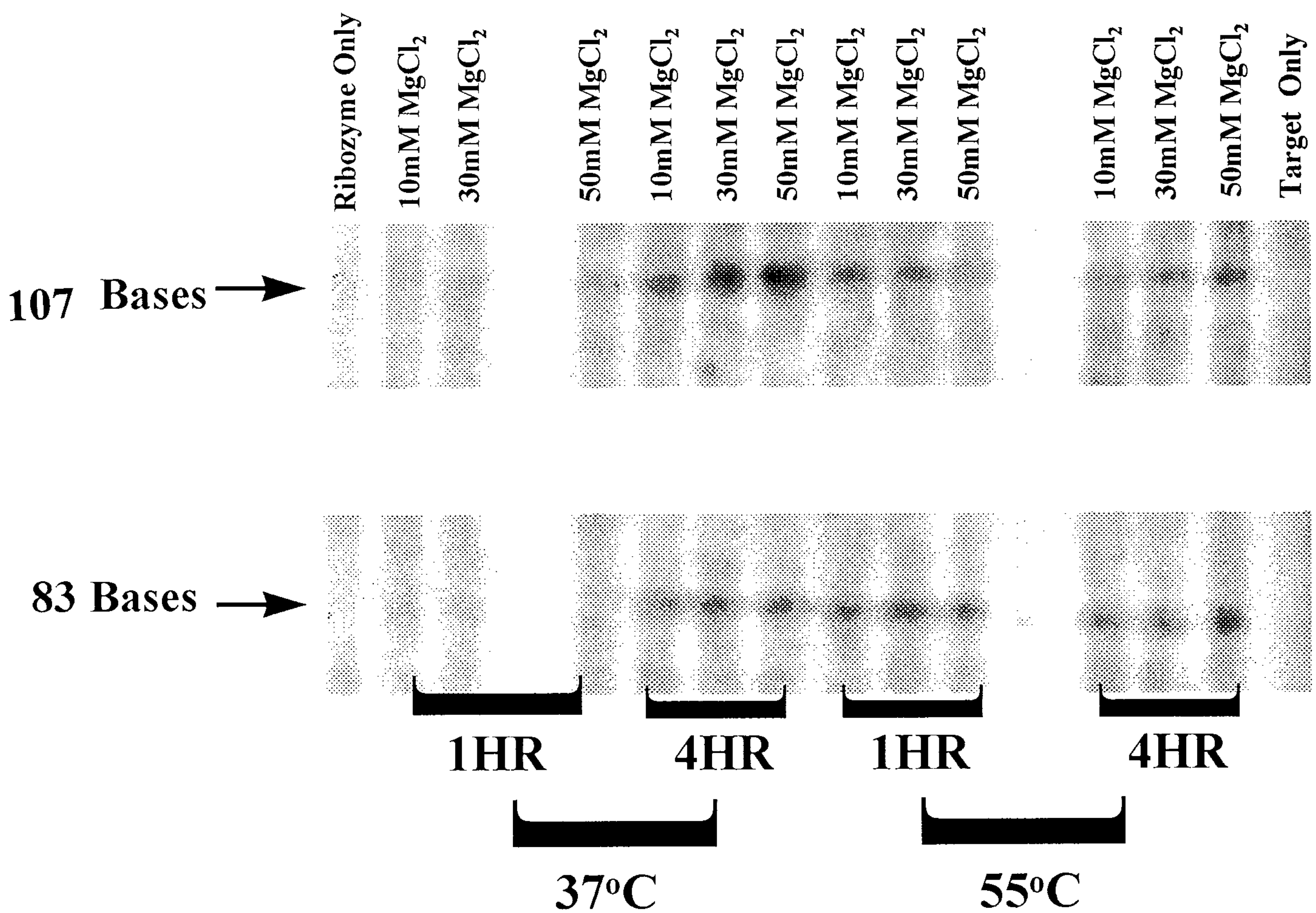


Figure 7

RT-PCR for *c-kit* mRNA in the ST8814 Human Schwannoma Line

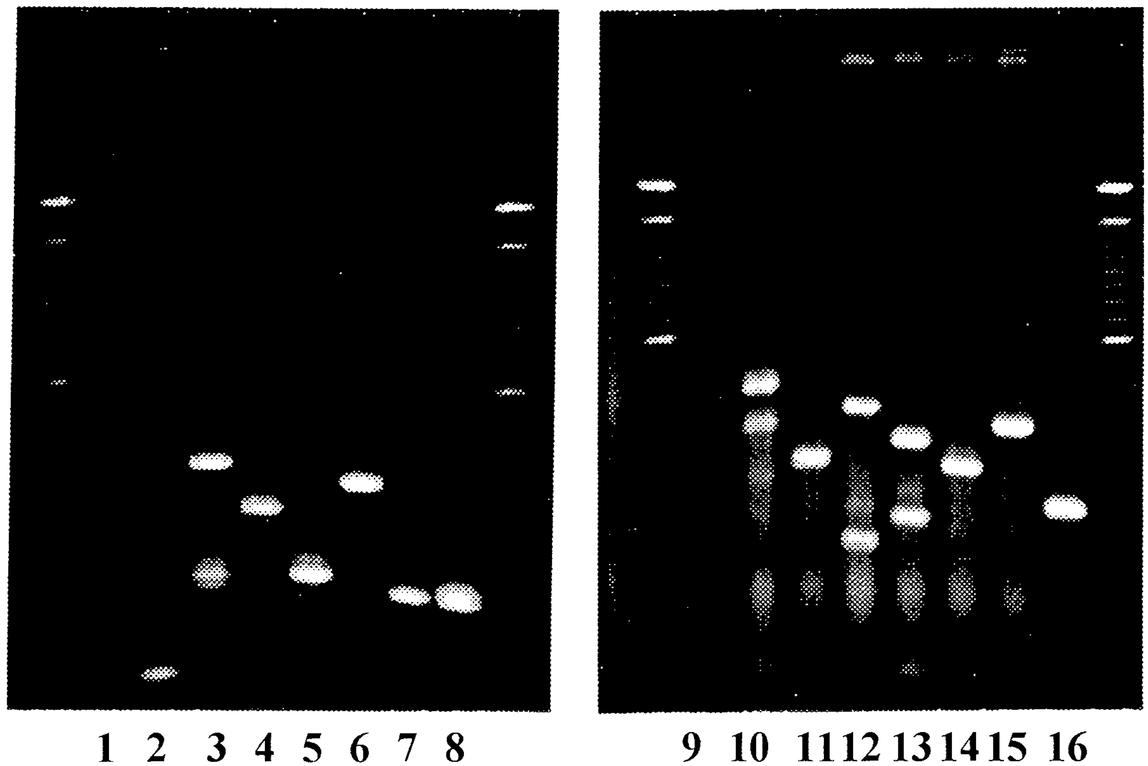


Figure 8

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Manuscript in preparation.

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