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Stem Cell Factor and Kit Expression in Type I Neurofibromatosis

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Stem Cell Factor and Kit Expression in Type I Neurofibromatosis

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Dedication

This work is dedicated to the memory of my cousin, Elaine Rebecca Roth, who died of a malignant schwannoma June 24, 1972.

Acknowledgments

I wish to express my sincere appreciation to Dr. Thomas F. Huff, my mentor, my counselor, and my friend. His gentle guidance and patience has been invaluable for the completion of this work. Dr. Huff has been an excellent role model for me as I have pursued my education in the classroom and laboratory, and remains so as I continue my education in the "School of Life".

I also thank the members of my advisory committee, Dr. Daniel Conrad, Dr. Steven Grant, Dr. John Tew, and Dr. Eric Westin. Their suggestions and encouragement along the way have been greatly appreciated.

Finally I want to thank my family, although I'm not sure I know how to effectively do so. My wife Terri has made tremendous sacrifices to allow me to achieve this goal. It is impossible for me to express my love and appreciation for her with words on a page. My sons Benjamin, Joseph and Daniel are not even aware of the sacrifices they've made during my graduate school experience, but I wish to assure them that I have remained very aware. My solemn promise to each of the members of my family is that I will make every effort to become more than just a part-time father and husband. To achieve this goal will be more satisfying and will mean more to me than any degree or title I may possess.

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List of Abbreviations

ARIA	Acetylcholine Receptor Inducing Activity
BMMC	Bone Marrow-Derived Mast Cells
cAMP	Cyclic Adenosine Monophosphate
cDMEM	Complete Dulbecco's Modified Eagle's Medium
CIAP	Calf Intestinal Alkaline Phosphatase
CM	Conditioned Medium
CTMC	Connective Tissue Mast Cell
DNF	Damselfish Neurofibromatosis
DRG	Dorsal Root Ganglion
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ENU	N-Nitroso-N-Ethylurea
$Fc_{\epsilon}RI$	High Affinity Receptor for the Fc Portion of Immunoglobulin E
FCM	Fibroblast Conditioned Medium
FITC	Fluorescein Isothiocyanate
GAP	Guanosine Triphosphatase Activating Protein
GDP	Guanosine Diphosphate
GGF	Glial Growth Factor

GRD	GAP-Related Domain
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HRG	Heregulins
IL-3	Interleukin-3
IPTG	Isopropylthiogalactoside
Kb	Kilobases
KL	Kit Ligand
LB	Luria Broth
LIF	Leukemia Inhibitory Factor
MCSF	Macrophage Colony Stimulating Factor
MGF	Mast Cell Growth Factor
MMC	Mucosal Mast Cell
Nb	*Nippostrongylus brasiliensis*
N-CAM	Neural Cell Adhesion Molecule
NDF	*neu* Differentiation Factor
NF1	Type 1 Neurofibromatosis
NGF	Nerve Growth Factor
NGF-RII	Nerve Growth Factor Receptor II
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphatidylinositol 3'-Kinase

PLCγ1	Phospholipase C gamma 1
PMA	Phorbol-12-Myristate-13-Acetate
PNS	Peripheral Nervous System
PSI	Pounds per Square Inch
PVP	Polyvinylpyrrolidone
rmIL-3	Recombinant Mouse Interleukin-3
rmSCF	Recombinant Mouse Stem Cell Factor
RNA	Ribonucleic Acid
RNase	Ribonuclease
RPA	Ribonuclease Protection Assay
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCCM	Schwann Cell-Conditioned Medium
SCF	Stem Cell Factor
SCF mRNA	Stem Cell Factor Messenger RNA
SCLC	Small Cell Lung Carcinoma
SDGF	Schwannoma-Derived Growth Factor
SDS	Sodium Dodecyl Sulfate
SF	Steel Factor
Sl	Murine *Steel* Locus
SLF	Steel Factor
SSC	Salt and Sodium Citrate

TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
W	Murine *White Spotting* Locus

Abstract

STEM CELL FACTOR AND KIT EXPRESSION IN TYPE I NEUROFIBROMATOSIS

By Kenneth Eugene Roth, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Neurofibromatosis type I (NF1) is an inherited disease characterized by the appearance of multiple neurofibromas and an increased incidence of malignant schwannomas, both of which contain hyperproliferative Schwann cells. Our laboratory previously reported that Schwann cells produce stem cell factor (SCF), a multi potential growth factor known to be involved in mast cell migration and growth. Given the fact that mast cell numbers are increased in both neurofibromas and malignant schwannomas, we set out to evaluate a potential role for SCF in the development of NF1 lesions. First we studied the effects of high doses of recombinant SCF on mast cell numbers *in vivo*, and found that dermal and peritoneal mast cell numbers were decreased. Next, we examined the expression of stem cell factor and its receptor, Kit, in neurofibromas and malignant schwannoma tumors and cell lines.

Using an RNase protection assay, we find that each of four human malignant schwannoma cell lines express only the membrane-bound isoform of SCF messenger RNA.

In contrast, neurofibroma, vestibular schwannoma, and acoustic neuroma tissues, as well as the majority of human fibroblast sources, all express the soluble form. Low level expression of Kit protein was detected on all four malignant schwannoma cell lines. However, Kit expression by Schwann cells was not indicated by immunohistochemical analyses of neurofibroma and malignant schwannoma sections, although Kit was readily detected on the mast cells within these lesions. We report that bone marrow-derived mouse mast cells appear to be driven toward a connective tissue phenotype when cultured in the presence of conditioned media from Schwann cells of wild type and *NF1* +/- knockout mice. This effect was not observed in cultures containing conditioned medium from Schwann cells of *NF1* -/- knockouts. In addition, the latter appeared to augment the proliferation of mast cells in response to exogenous cytokines.

Together, these results suggest a significant role for stem cell factor and Kit in the lesions of NF1. In addition, functional neurofibromin, the product of the *NF1* gene, may be required for proper regulation of SCF isoform expression. The mechanisms by which this expression is regulated remain to be clearly defined.

Introduction

Type 1 neurofibromatosis (NF1) is among the most common inherited human diseases, affecting one out of 3500 individuals worldwide (Stumpf et al., 1987). The responsible gene has been cloned and is located on the long arm of chromosome 17 (Barker et al., 1987). The *NF1* cDNA reveals that the gene encodes a large protein, called neurofibromin, which contains a region with homology to mammalian Ras GTPase-activating protein (GAP) and the yeast GAP-like proteins IRA1 and IRA2 (Xu et al., 1990). Each of these proteins has been shown to inactivate Ras p21, the product of the *ras* protooncogene (Tanaka et al., 1990). Ras p21 is activated when it is bound to guanosine triphosphate (GTP), and becomes inactive when GTP is converted to guanosine diphosphate (GDP) by its intrinsic GTPase activity. GAP and GAP-related proteins induce this activity, thereby serving as negative regulators of Ras. It has been reported that the GAP-related domain of neurofibromin indeed has GAP-like activity against Ras p21, supporting the postulation that neurofibromin functions as a tumor suppressor (Weinberg, 1991).

The clinical manifestations of NF1 are generally associated with the peripheral nervous system. Two phenomena which occur at an increased rate of incidence in NF1 patients involve hyperproliferation of Schwann cells, which are intimately associated with peripheral nerves and are normally responsible for the formation of the myelin sheath around them. These conditions are termed neurofibromas and malignant schwannomas. The appearance of

neurofibromas is a hallmark characteristic of the disease. These are benign peripheral nerve tumors consisting primarily of Schwann cells, which account for 60-85% of the cellular composition. Fibroblasts make up an additional 10-20%, with pericytes, perineurial cells, mast cells, endothelial cells and smooth muscle cells also present (Peltonen et al., 1988). It has long been known that neurofibroma tissues contain increased numbers of mast cells (Isaacson, 1976; Johnson et al., 1989), but the mechanism by which this occurs has not been well understood. Mast cells can normally be found in perineurial and epineurial spaces of peripheral nerves (Bienenstock et al., 1991), and are often seen in close proximity to Schwann cells (Isaacson, 1976). In addition, dramatic increases in mast cell numbers are observed in situations involving peripheral nerve injury and repair (Isaacson, 1976). Neither the means by which this occurs, nor a potential role for mast cells in these processes has yet been clearly defined.

The second NF1-related condition involving altered Schwann cell growth is the malignant schwannoma, which is also known by other names, including neurofibrosarcoma and malignant peripheral nerve sheath tumor. These occur much less frequently than neurofibromas, with an incidence of up to 2% in NF1 patients (Ponder, 1990; Nimura, 1992). This is, however, a significantly higher rate of occurrence than that seen in genetically normal individuals (<<1% (Cutler and Gross, 1936)). Given the known involvement of Ras p21 in the regulation of cellular proliferation, and the evidence that neurofibromin is able to functionally regulate the activity of Ras p21, it seems plausible that the NF1 gene defect alone could be responsible for these Schwann cell disorders. This, however, does not appear to be the case. All patients with NF1 appear to have mutations within the same region of

chromosome 17 (Barker et al., 1987; Collins et al., 1988; Seizinger et al., 1987), but not all have neurofibromas (Ponder, 1990; Riccardi, 1982) and even fewer develop malignant schwannomas, suggesting that additional mutations or other influences are required (Ratner et al., 1990).

Our laboratory described a soluble factor produced by normal but not *Steel* (*Sl*) murine fibroblasts which could promote the growth of mast cells in vitro (Jarboe et al., 1989). Since then the *Sl* product, now known as stem cell factor (SCF), has been more fully characterized in both rodent and human, and is known to exist as either a soluble or membrane-bound protein, dependent upon whether the portion of the primary gene transcript encoded by exon 6 is included in the final messenger RNA (Flanagan et al., 1991). The exon 6-containing mRNA gives rise to a protein that contains a protease-sensitive site in an extracellular membrane-proximal location. This isoform of SCF, after being initially expressed as a membrane-inserted protein, is efficiently cleaved by an unidentified protease, releasing a biologically active growth factor. Soluble SCF has been described as a potent chemotactic agent for cells that express its receptor Kit, the product of the protooncogene c-*kit* (Blume-Jensen et al., 1991; Meininger et al., 1992). We recently reported that normal rodent and human Schwann cells, and the human malignant schwannoma cell line ST88-14, all produce SCF (Ryan et al., 1994). Interestingly, Hirota and colleagues noted higher levels of stem cell factor mRNA in neurofibroma tissue compared with normal skin and suggested that SCF may be involved in the increased numbers of mast cells in these lesions (Hirota et al., 1993) Since mast cells express the Kit receptor, one might speculate that the mast cells in a neurofibroma may be recruited to the site by the chemotactic activity of soluble SCF. We have also

reported that, although normal rodent and human Schwann cells do not express Kit, the ST88-14 malignant schwannoma line aberrantly expresses Kit mRNA and protein (Ryan et al., 1994). These findings have led us to consider whether the abundant growth of Schwann cells found in neurofibromas and malignant schwannoma tumors might be due to the simultaneous expression of SCF and its receptor by Schwann cells, resulting in an autocrine growth loop. In this study, we evaluate Kit expression in three additional human malignant schwannoma cell lines and in excised neurofibroma and schwannoma tumors. In addition, we determine which isoform of SCF mRNA is produced by these and other sources.

Literature Review

Neurofibromatosis type I (NF1) and neurofibromin

The first full characterization of NF1 is generally credited to Friedrich von Recklinghausen, who described the disease in 1882 (von Recklinghausen, 1882). It is for this reason that NF1 has become known as von Recklinghausen neurofibromatosis, or more simply, von Recklinghausen's disease. There were, however, some less detailed medical descriptions recorded prior to von Recklinghausen's account. Two independent reports had previously described the disease in multiple family members (Virchow, 1857; Hitchcock, 1862), while the first known descriptions date back to the eighteenth century (Akenside, 1785; Tilesius, 1793). It was not until early in the twentieth century that neurofibromatosis was determined to be a genetic disorder (Thomson, 1900; Adrian, 1901; Prieser and Davenport, 1918). With a worldwide rate of incidence of about 1 in 3500 (Stumpf et al., 1987), NF1 is actually quite a common disease, yet it has generally received very little public attention. Perhaps the most significant influence promoting public awareness of neurofibromatosis has been the incorrect assumption that Joseph Merrick, a grotesquely disfigured man of Victorian England whose life was chronicled in the literary work, *The Elephant Man* (Treves, 1923), suffered from NF1 and some of its most serious complications (Montague, 1971; Sparks, 1980). However, after extensive examination of Merrick's remains and medical records, it has been suggested that he actually did not have NF1, but likely

suffered from a disease known as the Proteus syndrome (Cohen, 1988). Nonetheless, Merrick's life and social struggles have been portrayed in theater, cinema and television, bringing widespread attention to neurofibromatosis, albeit through an error in diagnosis. Even today some still refer to NF1 as, "the Elephant Man's disease." Interestingly, it has also been suggested that Quasimodo, the hunchback in *Notre Dame de Paris*, was created by playwright Victor Hugo based upon his acquaintance with someone who had NF1 (Hecht, 1989; Solomon, 1968).

Neurofibromatosis type 1 is caused by a mutation in a gene (*NF1*) which has been mapped to the long arm of human chromosome 17 (Barker et al., 1987), and has since been cloned (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990). It is inherited in an autosomal dominant fashion with a penetrance of the mutated gene considered to be very nearly 100 percent (Riccardi, 1992). The disease is characterized by the formation of multiple cutaneous and subcutaneous neurofibromas, which are benign tumors of the nerve sheath. Although these lesions are rarely fatal, they are disfiguring and can be quite painful. There is remarkable variability in the number, location and size of the tumors, all of which can contribute to the degree of disfigurement and pain. Additional symptoms of NF1 include localized hyperpigmentation of skin, termed café-au-lait spots, axillary freckling, iris hamartomas (Lisch nodules), and sometimes more serious tumors, such as optic gliomas and neurofibromas of spinal nerve roots. NF1 patients appear to have an increased incidence of certain cancers (Riccardi, 1992; Huson and Hughes, 1994), particularly malignant schwannomas (neurofibrosarcomas), which are generally fatal (Bernards et al., 1992). The gene is very large, spanning approximately 350 kilobases (kb) of genomic DNA, encoding an

mRNA of 11 to 13 kb, and containing at least 56 exons (Heim et al., 1994). Sequence analysis reveals an open reading frame of 2818 amino acids, although alternative splicing may encode different protein isoforms (Marchuk et al., 1991). This large protein, called neurofibromin, contains a region of 360 amino acids which shows significant similarity to the catalytic domains of mammalian GTPase activating protein (GAP), as well as to the *IRA1* and *IRA2* gene products in yeast, which are inhibitors of the *ras* protooncogene product (Xu et al., 1990). Interestingly, during the course of efforts to characterize the *NF1* gene, three active genes were found embedded within an intron. These genes, *OMGP, EVI2B,* and *EVI2A*, are transcribed in the opposite orientation from the *NF1* gene, and encode oligodendrocyte-myelin glycoprotein and ecotropic viral integration sites 2B and 2A, respectively (Viskochil et al., 1991).

Ras p21 (hereafter referred to simply as Ras) is the product of the *ras* protooncogene, and can act as a source for either mitogenic or differentiation-inducing signals, depending on the cell type (Bar-Sagi and Feramisco, 1985; Hagg et al., 1986; Noda et al., 1985). The protein is physiologically quiescent when bound to guanosine diphosphate (GDP), and becomes activated when the GDP is replaced with guanosine triphosphate (GTP). It returns to the inactive state when the GTP is hydrolyzed to GDP by a GTPase activity intrinsic to Ras. GAP and other GAP-like proteins inhibit Ras function by stimulating this hydrolysis. The GAP-related domain (GRD) of neurofibromin has been shown to bind Ras proteins avidly and to stimulate their GTPase activities (Martin et al.,1990c), suggesting that neurofibromin functions as a tumor suppressor (Weinberg, 1991).

Perhaps due in part to its extensive size, the *NF1* gene seems to have a predisposition

to mutate, with 50% of NF1 cases thought to be due to new mutations. Although the GRD of neurofibromin has been studied rather extensively, computer searches have revealed little similarity between the remainder of sequence and any known proteins in current databases. Therefore, the full function of neurofibromin, and any pathological changes which may occur as a consequence of a mutation at the *NF1* locus, are not yet well defined. There is evidence that the function of neurofibromin is not limited to its ability to regulate Ras activity. Johnson and coworkers showed by a series of transfection studies that neurofibromin, when overexpressed in NIH 3T3 cells, can inhibit cell growth independently of its GAP-like activity. They also found that melanoma and neuroblastoma cell lines which lack neurofibromin do not have increased levels of active Ras (Johnson et al., 1994). These findings are consistent with later work done by Griesser, et al. which showed that melanocytes cultured from NF1 patients (from both café-au-lait spots and normally pigmented skin) had reduced levels of neurofibromin, but still showed similar amounts of GTP-bound Ras when compared to melanocytes from healthy donors (Griesser et al., 1995). In a study conducted to determine if neurofibromin is involved in melanogenesis, it was found that coexpression of neurofibromin in a melanoma cell line caused an increase in expression of a reporter gene under the control of the tyrosinase gene promoter (Suzuki et al., 1994). Tyrosinase is a rate-limiting enzyme in melanin biosynthesis and is expressed only in melanin-producing cells. These investigators further showed that the GRD of neurofibromin was mainly responsible for this induction (Suzuki et al., 1994).

Two alternatively spliced isoforms of *NF1* messenger RNA have been identified and termed type 1 and type 2 (Anderson et al., 1993; Nishi et al., 1991). The two differ by a 63

base pair insertion in the GAP-related domain of the type 2 isoform. Bernards and coworkers showed by S1 nuclease protection experiments that the two *NF1* mRNAs are widely expressed in human cell lines and tissues, but that the ratio of the two forms differed substantially between cell lines (Bernards et al., 1992). Gutmann and colleagues have reported that type 1 appears to be associated with cellular proliferation, and that the isoform expression switches to predominantly type 2 when the cells undergo differentiation (Gutmann et al., 1993).

Until recently, NF1 research has been somewhat hampered by the lack of a good animal model system. In the 1980s a naturally occuring disease in the bicolor damselfish (*Pomacentrus partitus*), termed damselfish neurofibromatosis (DNF), was introduced as a possible model for human NF1 (Schmale et al., 1983; Schmale et al., 1986). These fish exhibit multiple neurofibromas and malignant schwannomas, many of which are hyperpigmented, indicating similarities to some of the manifestations of human NF1. However, while NF1 in humans is inherited as an autosomal dominant mutation, DNF is a transmissible disease (Schmale and Hensley, 1988). Nakamura and coworkers induced multiple neurofibromas, melanomas, Wilms' tumors, and pheochromocytomas in Syrian golden hamsters by transplacental administration of *N*-Nitroso-*N*-ethylurea (ENU) and considered this as an animal model for human NF1 (Nakamura et al., 1989). Perhaps the most promising model to date is a strain of mouse recently developed which contains a targeted mutation in the *NF1* gene (Jacks et al., 1994). Although animals which are heterozygous for this mutation (homozygotes die in utero) do not exhibit the classical symptoms of human NF1, they do have a high disposition to develop pheochromocytomas and myeloid leukemia,

both of which occur with increased frequency in human NF1 patients. Surely these models will allow for a better understanding of NF1 and the symptoms associated with the disease.

Schwann cells

Axons in the nervous system are supported, protected, and in some cases myelinated by various cell types collectively known as glial cells (from the Greek word for "glue"). In the peripheral nervous system (PNS) the glial cell providing these functions is the Schwann cell. Schwann cells are derived from the neural crest cells which form during neural tube development in embryogenesis (Dupin et al., 1990; Zimmer and Le Douarin, 1993). As the embryo develops, neural crest cells migrate and eventually differentiate into highly specialized cell types of neuronal, glial, melanocytic and mesectodermal phenotypes (Le Douarin and Dupin, 1993). Fully differentiated neural crest derivatives include Schwann cells, ganglion satellite cells, most of the neurons of the PNS, melanocytes, and part of the cranial mesenchyme (Dupin et al., 1990). During early nervous system development, Schwann cell precursors migrate with the developing axons and multiply rapidly to accommodate axonal growth (Asbury, 1967; Lemke, 1990). Jessen and Mirsky have characterized Schwann cell development in the embryonic rat peripheral nervous system according to changes in antigenic properties (Jessen and Mirsky, 1991). They report that Schwann cell precursors, identifiable by embryonic day 15, give rise to two morphologically and antigenically distinct mature Schwann cell types. These are the myelin-forming cells associated with axons of larger diameter, and the non-myelin-forming cells which associate with smaller axons.

Schwann cell development is dependent upon a close association between Schwann

cells and axons. *In vivo* studies have demonstrated that neurites supply Schwann cells with mitogenic signals (Asbury, 1967; Aguayo et al., 1976; Raine, 1977). These findings were supported by *in vitro* studies which showed that contact with the neuronal surface caused Schwann cells to replicate (Salzer and Bunge, 1980; Ratner et al., 1987). The activities responsible for this stimulation have been localized to the neuronal cell surface (Salzer and Bunge, 1980; Ratner et al., 1988; Mason et al., 1989; Wood and Bunge, 1975; Salzer et al., 1980; Sobue et al., 1983), but no specific mitogenic proteins had been identified until the recent characterization of a family of proteins arising from alternative splicing of a single gene. These proteins include glial growth factor (GGF, Marchionni et al., 1993), heregulins (HRG, Holmes et al., 1992), *neu* differentiation factor (NDF, Wen et al., 1992), and acetylcholine receptor inducing activity (ARIA, Falls et al., 1993). GGF has been shown to be a potent mitogen for rat Schwann cells (Lemke and Brockes, 1984). Morrissey and others have determined that heregulin is involved in the axon-induced mitogenesis of human Schwann cells and that the receptor tyrosine kinase $p185^{erbB2}$ appears to be the cognate protein on the Schwann cell (Morrissey et al., 1995; Levi et al., 1995).

The mitogenic effect of axons can be mimicked *in vitro* by agents which elevate cyclic adenosine monophosphate (cAMP), suggesting that axon-associated signals mediate their effects by elevating cAMP. In further *in vitro* studies it was found that the cAMP-induced progression from a premyelination state to a myelination state required that the cells withdraw from the cell cycle. This raises the possibility that, in order for a mature Schwann cell to begin myelination *in vivo*, it may require signals which suppress proliferation (Jessen and Mirsky, 1991). Direct cell-cell contact is not an absolute requirement for neurons to regulate

all Schwann cell gene expression, however. Using an *in vitro* co-culture system in which primary neurons and adult Schwann cells are separated by a microporous membrane, investigators showed that diffusible molecules produced by neurons can repress expression of the nerve growth factor receptor $p75^{NGFR}$ in Schwann cells, while inducing expression of the myelin protein P_0 (Bolin and Shooter, 1993).

Under normal circumstances in the fully developed organism, the Schwann cell no longer undergoes cell division (Peters and Muir, 1959; Asbury, 1967). The intimate axon-Schwann cell association is retained, and the Schwann cell remains essentially quiescent throughout the life of the animal (Asbury, 1967) unless disrupted by injury or disease, resulting in alteration of the normal metabolism of either of the cell types. Under pathological conditions such as peripheral nerve injury, demyelination, and tumorigenesis, the Schwann cell can be reactivated to enter the cell cycle (DeVries, 1993). The signals required for this reactivation are not yet known, although changes in Schwann cell gene expression resulting from loss of axonal contact have been described. While neonatal rat Schwann cells grown alone in culture express membrane-bound stem cell factor (SCF), those grown in contact with dorsal root ganglion (DRG) neurites do not (Ryan et al., 1994). This raises the possibility that loss of axonal contact induces expression of SCF by Schwann cells. Expression of other proteins is induced following loss of axonal contact as a result of nerve damage. These include vimentin, neural cell adhesion molecule (N-CAM), glial maturation factor β, and nerve growth factor receptor II (NGF-R II). Expression of each of these proteins decreases with subsequent nerve regeneration (Jessen et al., 1987a; Lemke and Chao, 1988; Taniuchi et al., 1988; Bosch et al., 1989; Neuberger and Cornbrooks, 1989; Mitchell et al., 1990). In

contrast, loss of axonal contact due to nerve damage causes a decrease in expression of myelin-associated glycoprotein, the myelin basic proteins, the Schwann cell myelin-associated proteins P_2 and P_0, and galactocerebroside. Expression of these proteins returns to normal levels following nerve regeneration (Jessen et al., 1987b; Lemke and Chao, 1988; Gupta et al., 1990; LeBlanc and Poduslo, 1990; Mitchell et al., 1990).

These examples of Schwann cell gene regulation by axons are likely involved in the process by which damaged nerves are repaired. A number of studies suggest that the presence of Schwann cells is very important in nerve repair. Following crush injury to the optic nerves of rats, regions containing resident Schwann cells were regenerated, whereas no regeneration occurred in those areas in which Schwann cells were absent (Berry et al., 1992a). In addition, when sections of optic nerve were engrafted between the stumps of freshly transected sciatic nerves, regeneration only occurred through grafts containing Schwann cells (Hall et al., 1992). One possible explanation for this preferential regrowth is that the Schwann cells may be able to overcome the inhibitory effects of putative reactive elements within the glial microenvironment. Oligodendrocytes and central nervous system (CNS) myelin have been implicated in the failure of CNS axons to regenerate after injury (Berry, 1982; Schwab and Thoenen, 1985; Caroni and Schwab, 1988), perhaps because of molecules associated with both elements which inhibit axonal regrowth (Schwab and Caroni, 1988). It is also suggested that Schwann cells contribute to axonal regeneration because they secrete trophic molecules (Varon and Williams, 1986), and sequester growing axons within basal lamina tubes (Berry et al., 1988; Hall and Berry, 1989), possibly isolating growth cones from inhibitory influences (Berry et al., 1991, 1992b).

Mitogenic activity can be extracted from neurofibromas (Ratner et al., 1990), but it is not clear whether this is important in tumor formation, or whether similar mitogens are present in normal peripheral nerves. However, Schwann cells, but not fibroblasts, isolated from NF1 tumors show angiogenic and invasive properties in the chick chorioallantoic membrane (Sheela et al., 1990). These effects were not seen when cells from normal human peripheral nerves were used, suggesting that neurofibroma Schwann cells secrete one or more growth factors that normal Schwann cells do not. It is unclear whether Schwann cells isolated from normal regions of peripheral nerves of NF1 patients display these abnormal angiogenic and invasive characteristics. This information would be helpful in determining why tumors occur only in limited locations and only in some patients, even though the mutation is ubiquitously expressed (Xu et al., 1990; Wallace et al., 1990). Either the *NF1* mutation causes all Schwann cells to secrete angiogenic activity, or additional mutations or other epigenetic changes lead to secretion of this activity only by the tumor cells (Eccleston, 1992). The latter possibilities would explain why neurofibromas are restricted to discrete areas.

Mast cells in neurofibromas

Mast cell numbers increase in response to nerve damage and repair. This phenomenon has been observed in amputation neuromas (Olsson, 1971), peripheral nerve injury and pathological conditions involving Schwann cells, such as the lesions of NF1 (Isaacson, 1976). A greater degree of mast cell hyperplasia is seen in NF1 tumors than in other forms of nerve damage (Johnson et al., 1989). The involvement of mast cells in these lesions continues to be a focus of investigation in this laboratory. We have suggested (Roth et al., submitted for

publication) that mast cells or their precursors are perhaps recruited to the site by the chemotactic activity of soluble stem cell factor (possibly produced by Schwann cells). Alternatively, they may be trapped there by the binding of Kit on their surfaces to membrane-associated SCF expressed by the already hyperplastic Schwann cells in the growing lesion. Although a clearer understanding of the means by which mast cells accumulate at the site of a neurofibroma is important, perhaps more so is the potential contribution of mast cells to the pathogenesis of the tumor itself, or of NF1 in general. Riccardi has hypothesized that mast cells accumulate following local trauma (or the elaboration or sequestering of a tropic factor) and secrete various substances as usual. Due to an *NF1* mutation, the response is abnormal, resulting in a proliferation of Schwann cells, fibroblasts, perineurial cells, etc., the manifestation of which is a growing neurofibroma (Riccardi, 1990). Although this is speculation at this point, it is not without merit. Mast cells secrete a variety of substances with potential mitogenic effects, including heparin and histamine, as well as the well known mitogen transforming growth factor-β (Pennington et al., 1991). Giorno and others have demonstrated by transmission electron microscopy that neurofibromas contain, in addition to fully granulated mast cells, both partially and fully degranulated cells, indicative of activation and mediator release (Giorno et al., 1989). These findings suggest that mast cells are active components of neurofibromas rather than mere bystanders.

Additional evidence of active involvement of mast cells in the symptoms of NF1 come from clinical observations. Rapidly growing neurofibromas are often accompanied by inflammatory reactions such as pruritus and facial flushing (Giorno et al., 1989; Rothe et al., 1990). The pruritus is not controlled with standard H1 antihistamines such as

diphenhydramine hydrochloride (Riccardi, 1987), but the mast cell stabilizing drugs ketotifen and disodium cromoglycate are effective (Riccardi, 1981; Riccardi, 1987). In some cases, treatment with ketotifen even resulted in a decrease in neurofibroma growth (Riccardi, 1987).

The Schwann cells of NF1 tumors may also produce substances which exhibit mitogenic activities through both autocrine and paracrine mechanisms. Autocrine and paracrine growth factors in the supernatant of NF1 neurofibroma cultures have been reported which could increase Schwann cell growth *in vitro* (Riccardi, 1986). Nerve growth factor (NGF) is secreted by Schwann cells, especially in the presence of gangliosides (Ohi et al., 1990), and has been shown to induce mast cell proliferation (Matsuda et al., 1991). TGFβ messenger RNA has been detected in rat Schwann cells, suggesting that TGFβ may be produced as an autocrine growth factor by Schwann cell tumors (Ridley et al., 1989). In addition, a schwannoma-derived growth factor (SDGF) has been isolated which has homology to epidermal growth factor (EGF) and glial growth factor (GGF), and which can stimulate proliferation of Schwann cells, fibroblasts and astrocytes (Kimura et al., 1990). In light of the current evidence, it seems possible that Schwann cell-mast cell interactions are important in the maintenance of both populations within a neurofibroma, and may make significant contributions to tumorigenesis.

Mast cell overview

It is interesting that the first detailed description of NF1 was reported by Friedrich von Recklinghausen, as mentioned above (von Recklinghausen, 1882), because he also may well have been the first to describe mast cells when he recorded his observations of granular cells

in unstained preparations of frog mesentery (von Recklinghausen, 1863). The actual credit for establishing that mast cells were a distinct lineage, however, goes to Paul Ehrlich. He found that granules within cells obtained from frog mesentery exhibited metachromatic staining with aniline dyes (Ehrlich, 1878). The name he gave these cells, "mästzellen," is derived from the German word "mästen," which means "feed or fatten," because Ehrlich believed that the intracellular granules were the result of ingestion of particles, or over-feeding. Ehrlich also noted that mast cells had a tendency to localize around blood vessels and nerves, and in areas of inflammation or neoplasia.

Over the years, theories concerning the origin and lineage relationships of mast cells have evolved along with advances in scientific investigation. At first some thought that mast cells arose from lymphocytes, fibroblasts or macrophages (Valent et al., 1989; Burnet, 1977; Zucker-Franklin et al., 1981; Czarnetzki et al., 1982). Others believed they were derived from mesenchymal cells, plasma cells, histiocytes, endothelial cells, or degenerate cells (reviewed in Michels, 1963). The current theory is that mast cells arise from hematopoietic stem cells in the bone marrow. Kitamura and colleagues demonstrated this using adoptive transfer experiments involving the mast cell-deficient *W/W^v and Sl/Sl^d* mice. They found that they could cure the mast cell deficiency in *W/W^v* mice by injecting them with bone marrow from congenic normal littermates or from *Sl/Sl^d* mice (Kitamura et al., 1978). Others observed that mast cells could be grown *in vitro* from bone marrow when cultured in the presence of a variety of cell culture-conditioned media now known to contain interleukin 3 (IL-3), a growth factor for murine mast cells (Hasthorpe, 1980; Nabel et al., 1981; Razin et al., 1981; Schrader et al., 1981; Tertian et al., 1981; Yung et al., 1981). Both *in vitro* (Kirshenbaum et al., 1991;

McCarthy et al., 1980) and *in vivo* (Födinger et al., 1994) studies have suggested that human mast cells also originate in the bone marrow. Mast cells in the human are now known to arise from $CD34^+$ hematopoietic progenitors, but not from $CD34^-$ bone marrow cells (Kirshenbaum et al., 1991).

Although it is generally accepted that mast cells originate in the bone marrow, their relationship to other hematopoietic lineages is still unclear. Mast cells were long thought to have arisen from the same progenitor as the basophil, because both have granules which exhibit metachromatic staining, both contain histamine, and both express the high affinity receptor for IgE ($Fc_\epsilon RI$). Similarities between the two cell types have been observed at the ultrastructural level as well (Zucker-Franklin, 1980). This common progenitor theory has become less popular, however, as the current body of knowledge has grown. Mast cells and basophils differ in nuclear morphology, granule content, location in the organism, and response to external stimuli (reviewed in Galli and Lichtenstein, 1988; Valent and Bettelheim, 1990). Also, mast cells leave the bone marrow as immature progenitors, completing their differentiation in the peripheral tissues, while basophils are not released until they are fully mature, terminally differentiated cells (McNeil, 1996). The fact that mast cells express the transcription factors GATA-1, GATA-2 and GATA-3 (Martin et al., 1990a; Zon et al., 1991) is another point which distinguishes them from the basophil lineage. Rather, these findings suggest that mast cells may share a common progenitor with erythrocytes and megakaryocytes, which also express the GATA binding proteins.

A possible relationship between mast cells and the monocyte/macrophage lineage has also been suggested based upon the observation that human mast cells are recognized by some

monoclonal antibodies specific for differentiated macrophages (Valent et al., 1989). Also, both cell types differentiate in the periphery, and both activated macrophages and activated mast cells produce cytokines (reviewed in Costa et al., 1993; Galli et al., 1991; Galli et al., 1993; Gordon et al., 1990; Paul et al., 1993). However, Agis and others have found that human mast cells are not derived from monocytes cultured in the presence of stem cell factor, and suggest that the mast cell does not arise from a monocyte/macrophage precursor (Agis et al., 1993). This group went on to show that mast cells developed from a peripheral blood progenitor which was $CD34^+$, Kit^+, Ly^-, $CD14^-$, and $CD17^-$. These data indicate that mast cells do not appear to originate from circulating monocytes ($CD14^+$), basophils ($CD17^+$), or lymphocytes (Ly^+), but demonstrate that mast cells are replenished directly from early hematopoietic progenitors and therefore represent a unique hematopoietic cell lineage (Agis et al., 1993). In spite of the advances being made in the field of mast cell biology, a specific relationship of the mast cell to other hematopoietic lineages continues to be debated.

There is considerable structural and functional heterogeneity within the mast cell lineage in both the human and rodent. Mast cells found in human lung and intestinal mucosa were shown by electron microscopy to contain granules with many scroll-like crystal structures, (Craig et al., 1988; Weidner and Austen, 1990). These granules contain a subclass of tryptic serine proteases (tryptases), leading to the classification of these cells as MC_T, for "mast cell/tryptase" (Irani et al., 1986). A second subset of mast cells is found in the connective tissue stroma of skin, intestinal submucosa, breast parenchyma and lymph nodes, and interacts with microvascular and neural networks. Electron miscroscopy of these cells reveal very few scroll-like structures (Craig et al., 1988; Weidner and Austen, 1990), and they

have been named MC_{TC}, because their granules contain both tryptases and chymases, as well as an exopeptidase, carboxypeptidase A (Irani et al., 1986). The two subsets of human mast cells also exhibit functional differences. For instance, MC_{TC} are responsive to the complement component C5a, morphine derivatives, and various neuropeptides, but MC_T are not sensitive to these stimuli (Cohan et al., 1989).

Two subsets of mast cells in rats and mice have been characterized based upon tissue localization, T cell-derived growth factor dependence, and protease expression. The connective tissue mast cell (CTMC) and the mucosal mast cell (MMC) in rodents are in many ways comparable to the MC_{TC} and the MC_T of the human, respectively. The CTMC and MMC in the mouse can be distinguished by their differential expression of a large family of serine proteases (McNeil and Austen, 1994). Jarboe and colleagues have identified two types of murine mast cell progenitors: one that is dependent upon interleukin-3 (IL-3), and another that is not, but instead responds to a fibroblast-derived factor (Jarboe and Huff, 1989; Jarboe et al., 1989). These two progenitors may give rise to MMC and CTMC, respectively (Kobayashi et al., 1986). There is evidence, however, that murine mast cells can change their phenotypic characteristics in response to alterations in their microenvironment (McNeil and Austen, 1994; Ghildyal et al., 1992; Gurish et al., 1992). Mast cells which appear to be morphologically differentiated are long-lived, can proliferate extensively, and are able to change their phenotype (Takagi et al., 1992). It may be that this ability to change phenotype in response to environmental stimuli enables murine mast cells to adapt their functional capabilities in response to diseases or immunologic responses. In the human system, however, the ability of mast cells to switch from one phenotypic subset to the other has not been

demonstrated. Some investigators have suggested that human mast cells may commit to either the MC_T or the MC_{TC} subset early and proceed upon distinct pathways (Irani et al., 1992; Craig et al., 1989).

It seems that the bulk of our understanding would implicate the mast cell as an "evil-doer" which presents itself as a mere nuisance, as in those who suffer milder forms of allergy, or as a serious threat, as in the case of victims of anaphylaxis. The mast cell's involvement in type I hypersensitivity, allergic asthma and numerous inflammatory processes is widely accepted (reviewed in McNeil, 1996; Galli and Costa, 1995). Moreover, there is significant evidence that mast cells are involved in sudden infant death syndrome (Platt et al., 1994). Although logic dictates that the mast cell's evolutionary persistence implies an essential function in normal physiology, our inability to assign functions to the mast cell that are beneficial to the organism underlies the "riddle of the mast cell" (McNeil, 1996; Galli, 1990).

Kit, the product of the c-kit protooncogene

In the course of maintaining inbred strains of mice for use in research, scientists are sometimes given the opportunity to study the functions of unidentified gene products which have been altered in some way due to spontaneous mutations in these strains. These "experiments of nature" have been extremely important in our understanding of the functions of stem cell factor (SCF) and its receptor, Kit. Much of what we now know about the involvement of SCF and Kit in hematopoiesis and developmental programs involving melanocytes, germ cells and mast cells was predicted based on studies with mutant mice long before the gene products were identified.

For a number of years it has been known that mutations at either the dominant white spotting (*W*) locus on chromosome 5 or the steel (*Sl*) locus on chromosome 10 resulted in similar phenotypic changes. These include changes in coat pigmentation, severe macrocytic anemia, sterility, and a dramatic reduction in the number of mast cells (Kitamura et al., 1978; Kitamura and Go, 1979; Russell, 1979; Silvers, 1979). Following transplantation and embryo fusion studies using *W* and *Sl* mutant mice and *in vitro* studies using cells and tissues from them, it was suggested that deficits in the *W* mutant mice were expressed by the cells in the affected lineages, and those in the *Sl* mutants were expressed by cells in the microenvironment necessary for the development of the affected lineages (Russell, 1979; Silvers, 1979). The similarities of phenotypic changes resulting from mutations at either locus suggested that the *W* locus might encode a receptor expressed by the cells of the affected lineages, while the *Sl* locus might encode the cognate ligand (Russell, 1979). More recent work has confirmed that the *W* locus is allelic with the c-*kit* gene, which encodes a receptor tyrosine kinase (Chabot et al., 1988; Geissler et al., 1988a, b), and that *Sl* encodes the corresponding ligand, which has been given several names, including stem cell factor (SCF, Zsebo et al., 1990a, b; Martin et al., 1990b), Kit ligand (KL, Huang et al., 1990), mast cell growth factor (MGF, Williams et al., 1990; Copeland et al., 1990; Anderson et al., 1990), and steel factor (SLF or SF, Witte, 1990; Williams et al., 1992a).

The *kit* gene was first characterized as the viral oncogene v-*kit* of a feline sarcoma virus (Besmer et al., 1986). It was later determined that v-*kit* arose from transduction and truncation of the cellular protooncogene c-*kit* (Yarden et al., 1987; Qiu et al., 1988). The c-*kit* gene was first cloned in the mouse from a brain cDNA library (Qiu et al., 1988). The

predicted amino acid sequence was found to display significant homology to members of a family of transmembrane receptors now known as type III receptor tyrosine kinases (RTKs), which includes the receptor for macrophage colony stimulating factor (MCSF, encoded by c-*fms*), the platelet-derived growth factor (PDGF) receptors alpha and beta (Besmer et al., 1986; Yarden et al., 1987; Qiu et al., 1988), and the more recently described receptor Flk2/Flt3 (Lyman et al., 1993).

The Kit protein contains an extracellular ligand-binding domain with five immunoglobulin-like regions. These regions contain several intramolecular disulfide bonds and potential N-glycosylation sites (Yarden et al., 1987; Qiu et al., 1988). The intracellular portion of the protein contains a kinase region which is split into two parts by a noncatalytic stretch of 77 amino acids known as the kinase insert (Yarden et al., 1987; Qiu et al., 1988). It has been demonstrated in the murine system that a specific tyrosine residue within the kinase insert (Y719) is critical for signal transduction through physical association with phosphatidylinositol 3'-kinase (PI3K), and that Y719 must be phosphorylated before this association can occur (Serve et al., 1994).

Upon binding to stem cell factor, Kit is induced to dimerize, and this dimerization correlates with autophosphorylation and activation of its kinase (Blume-Jensen et al., 1991; Philo et al., 1996). After activation, Kit rapidly associates with specific cytoplasmic signaling proteins involved in cell proliferation and survival, including PI3K and phospholipase C-γ1 (PLCγ1) (Rottapel et al., 1991). In contrast to the loss of Kit function observed in the various *W* mutant mice, some activating mutations have been reported in both mouse and human. The factor-independent tumorigenic murine mastocytoma cell line P815 was found

to express a constitutively tyrosine-phosphorylated Kit receptor, in the absence of endogenous or exogenous SCF (Rottapel et al., 1991). Furitsu and coworkers discovered that the Kit protein in the human mast cell leukemia cell line HMC-1 was also constitutively phosphorylated in the absence of SCF. In addition, they found two point mutations in the region encoding the cytoplasmic domain of the receptor (Furitsu et al., 1993). These findings are suggestive that some activating c-*kit* mutations may contribute to transformation of some cells. Nagata et al. have identified a point mutation in the catalytic domain of Kit in patients who have mastocytosis with an associated hematologic disorder. Identical or similar amino acid substitutions in mast cell lines resulted in ligand-independent autophosphorylation of the receptor (Nagata et al., 1995).

In addition to activating mutations of the receptor, aberrant signal transduction through Kit may result from autocrine stimulation arising either from ectopic expression of Kit in tissues that normally express SCF, or from ectopic expression of SCF in tissues that normally express Kit. Indeed, coexpression of the receptor and ligand has been reported in gynecological tumors (Inoue et al., 1994), colon tumor cell lines (Toyota et al., 1993), in the majority of surgical breast tumor specimens and tumor cell lines (Hines et al., 1995), and in at least 70% of small cell lung carcinoma (SCLC) tumors and tumor-derived cell lines (Hibi et al., 1991). Moreover, Krystal et al. showed that transfection of a c-*kit* expression vector into an SCLC line that expressed SCF but not Kit resulted in constitutive tyrosine phosphorylation of immunoprecipitated Kit protein and more vigorous cell growth in serum-free medium compared to control-transfected cells. This group also reported that transfection of an SCLC line which normally coexpresses SCF and Kit with a dominant negative, kinase-

defective c-*kit* gene resulted in a significant loss of growth factor independence (Krystal et al., 1996).

The phenotype of the *W* and *Sl* mutant mice suggest that SCF and Kit are likely involved in development of hematopoietic, melanoblast, and germ cell lineages in the developing mouse embryo. Studies examining the patterns of expression of the two proteins have confirmed this involvement in terms of the migration and spatial distribution, as well as the proliferation and differentiation of the cells at their definitive sites in the developing embryo (Matsui et al., 1990; Orr-Urtregger et al., 1990; Keshet et al., 1991). However, expression was also detected in tissues other than those apparently affected in the *W* and *Sl* mutants. These findings have indicated a possible role for Kit signaling in the development of cells in the placenta, the nervous system, the septa of the heart, the lung, the facial chondrogenic nuclei, and the midgestational kidney (Galli et al., 1994). Clearly, SCF and Kit are of vital importance in the early development of the animal. In fact, loss of function mutations in both alleles of either locus leads to an inability to properly develop, resulting in the death of the fetus in utero (Nocka et al., 1989).

Stem cell factor

After it had been established that the *W* locus of the mouse was allelic with c-*kit*, it was reasonable to imagine that the ligand might be encoded at *Sl*, in accord with Russell's earlier proposal (Russell, 1979). Experiments using combinations of mast cells or their precursors and fibroblasts derived from *W* or *Sl* mutant mice provided strong biological evidence that fibroblasts can produce membrane-bound (Kitamura et al., 1989; Fujita et al.,

1989; Kitamura and Fujita, 1989) and soluble (Jarboe and Huff, 1989) forms of the Kit ligand. Soon after these reports, Nocka et al. purified and characterized a mouse fibroblast-derived factor which could stimulate growth of normal mast cells, but not mast cells derived from *W* mutant mice (Nocka et al., 1990). Later that year three groups simultaneously reported the cloning and characterization of the growth factor (designated SCF in this review) which represented the product of the *Sl* locus, and a ligand for the receptor encoded at c-*kit* (Williams et al., 1990; Copeland et al., 1990; Anderson et al., 1990; Zsebo et al., 1990a, b; Martin et al., 1990b; Huang et al., 1990).

Physical characterization of the SCF protein indicates that it is heavily glycosylated, and exists as noncovalently associated dimers (Arakawa et al., 1991; Lu et al., 1991; Langley et al., 1992). In the human, alternative splicing of the mRNA gives rise to two forms of SCF protein: soluble or membrane-bound (Anderson et al., 1991). The full-length mRNA encodes a transmembrane protein of 248 amino acids, which includes a 25 amino acid cleaved signal peptide, a 189 amino acid extracellular domain, which includes a membrane-proximal proteolytic cleavage site encoded by exon 6, a hydrophobic membrane-spanning region of 21-23 amino acids, and a short intracellular domain of 36-37 amino acids. This form is efficiently cleaved by an unidentified protease and released as soluble SCF. Exon 6 is not included in the alternative SCF mRNA, which encodes a transmembrane protein that lacks 28 amino acids, including those which comprise the protease recognition site (Anderson et al., 1991). This form is much more resistant to proteolysis and generally remains associated with the cell membrane. Three isoforms of SCF have been reported in the mouse, including exon 6-containing and exon 6-lacking forms similar to those found in the human, and a third form

which contains a smaller 16 amino acid deletion of the exon 6 sequence. This deletion also eliminates the protease recognition site, giving rise to a predominantly membrane-bound protein (Anderson et al., 1990; Flanagan et al., 1991). The transcriptional and post-translational events necessary for production of either soluble or membrane-bound SCF are obvious points at which a given cell may regulate SCF isoform expression. The biological effects of membrane-bound vs. soluble SCF may be significantly different (see later discussion), as has been reported with respect to bone marrow progenitor cells (Toksoz et al., 1992).

Although most functional studies of SCF have focused on the biological activities of the extracellular portions of the proteins (soluble or membrane-bound), there is limited evidence that the short cytoplasmic tail may have biological importance which has not yet been defined. This is suggested by the phenotype of mice bearing a mutation designated Sl^{17H}, which produces a splicing defect resulting in a cytoplasmic tail of 28 amino acids, rather than 36. The phenotype of Sl^{17H}/Sl^{17H} mice includes sterility in males but not in females, indicating that some biological activity resides in the cytoplasmic tail of SCF (Brannan et al., 1992).

SCF expression appears to be tightly regulated during mouse embryogenesis. Examination of the genital ridges of 10-day embryos revealed a gradient of SCF expression along the route of migration of primordial germ cells between the dorsal mesentery and the gonad anlage. Interestingly, an inverse gradient of c-*kit* expression was observed, with SCF expression highest in the genital ridge and c-*kit* expression highest in the dorsal mesentery. By day 11.5, when colonization of the gonad is completed and germ cell migration is no longer necessary, SCF expression along the migratory route ceases, but remains high in the

gonad throughout sexual differentiation (Matsui et al., 1990; Orr-Urtregger et al., 1990; Keshet et al., 1991).

In the ovary of the adult mouse, c-*kit* is expressed only by the oocytes (Arceci et al., 1992; Orr-Urtregger et al., 1990; Keshet et al., 1991) and SCF is expressed at highest levels in the surrounding granulosa cells (Keshet et al., 1991). The observed pattern of SCF expression during sexual development supports the hypotheses of several groups that cells lining the migratory route may guide the primordial germ cells by way of haptotaxis through expression of membrane-bound SCF, whereas expression of SCF in the gonad anlage may indicate a chemotactic role for soluble SCF in directing homing of germ cell precursors (Flanagan and Leder, 1990; Williams et al., 1990; Matsui et al., 1990; Flanagan et al., 1991; Keshet et al., 1991). Similar haptotactic/chemotactic roles for SCF in the development of pigmentation have been suggested by studies of SCF and c-*kit* expression during melanocyte migration (Keshet et al., 1991). SCF expression can be detected in the subdermal mesenchymal cells of the limb buds before melanocytes begin to invade, and persists during and after melanocyte colonization. c-*kit* is detected in the melanocyte precursors and remains throughout their proliferation and differentiation (Keshet et al., 1991).

SCF and c-*kit* also appear to be important in the early development of the brain. Between gestational days 12 and 15, SCF is expressed in the floor plate of the neural tube, followed by expression in the ventrolateral regions associated with motor neuron differentiation (Matsui et al., 1990; Orr-Urtregger et al., 1990). c-*kit* expression has been detected in migrating neural crest cells and in the differentiating neurons in the dorsal neural tube and dorsal ganglia, suggesting that SCF may function as a chemoattractant for neurons

and/or axonic processes (Keshet et al., 1991).

Expression of c-*kit* is high in the embryonic liver at gestational day 11.5, a time at which the liver is heavily involved in hematopoiesis (Orr-Urtregger et al., 1990; Keshet et al., 1991), consistent with the widely accepted view that SCF is an important hematopoietic growth factor. In the adult mouse hematopoietic system, c-*kit* mRNA expression is found predominantly in those lineages affected by *W* mutations, including mast cells and early erythroid and myeloid cell lines (André et al., 1989; Nocka et al., 1989). Using ^{125}I-labeled SCF in binding studies, Metcalf and Nicola found that Kit is widely distributed among cells in adult murine bone marrow. They reported expression on blasts, promyelocytes/myelocytes, promonocytes, monocytes, eosinophilic myelocytes, eosinophils, and some lymphocytes (Metcalf and Nicola, 1991). In general, analysis of the distribution of c-*kit* mRNA and Kit protein on hematopoietic cells indicates that levels of Kit are highest during early stages of development and progressively diminish in parallel with maturation of the various hematopoietic lineages (Papayannopoulou et al., 1991). The mast cell represents an exception, in that mature mast cells continue to express the receptor and exhibit responsiveness to SCF.

The biological consequences of SCF/Kit interactions include adhesion, chemotaxis or haptotaxis, survival, proliferation, differentiation, and activation or modulation of secretion (reviewed in Galli et al., 1994). The various functions would require that the expression of the appropriate isoform of SCF be specifically regulated, although the mechanism of this regulation is presently unknown. The membrane-bound form would understandably be involved in adhesion to Kit-bearing cells. Studies using cells expressing the *Sl*^{d} allele, which

makes only soluble SCF, have demonstrated that a membrane-associated form of SCF is necessary for adhesion to mast cells (Flanagan et al., 1991; Kaneko et al., 1991; Adachi et al., 1992). Conversely, Adachi and coworkers have shown that mast cells derived from *W/W* mice, which express no Kit on their surface, were greatly impaired in their ability to bind to fibroblasts (Adachi et al., 1992). The same group demonstrated that Kit tyrosine kinase activity was not necessary for adhesion to fibroblasts by using mast cells derived from W^v/W^v or W/W^{42} mutants, which bear Kit receptors with greatly diminished or completely absent kinase activity, respectively (Adachi et al., 1992). As mentioned above, it has been suggested that membrane-bound SCF may promote attachment of mouse primordial germ cells during embryonic development (Matsui et al., 1991). It has also been shown that neutralizing antibodies to Kit can inhibit the binding of human megakaryocytes to fibroblasts (Avraham et al., 1992). These studies offer strong evidence that SCF and Kit can function as adhesion molecules.

The defects in coat pigmentation, mast cell deficiency, and sterility characteristic of *W* and *Sl* mutant mice are known to reflect an impairment of the migration of cells of the melanocyte, mast cell, or germ cell lineage during embryonic development (Russell, 1979; Silvers, 1979; Kitamura, 1989). Upon identification of the proteins encoded at these loci, it was reasonable to assume that this migration may be due to a chemotactic response of the Kit-bearing cells to SCF. It has been shown that subcutaneous injections of recombinant SCF into the skin of Sl/Sl^d mice, which ordinarily lacks mast cell precursors and mature mast cells (Kitamura and Go, 1979; Hayashi et al., 1985), resulted in the appearance of large numbers of mast cells at the site of injection (Zsebo et al., 1990b; Tsai et al., 1991). *In vitro* studies

have demonstrated that porcine aortic endothelial cells transfected with wild-type human c-*kit* cDNA exhibited chemotactic responses to concentrations of recombinant human SCF as low as 50 pg/ml (Blume-Jensen et al., 1991). It was further shown that Kit tyrosine kinase activity was necessary for chemotactic responsiveness to SCF (Meininger et al., 1992).

Given the fact that certain Kit-expressing cells, such as oocytes, melanocytes, and mast cells are able to reside in the tissues for long periods of time without undergoing proliferation, one might imagine that SCF is involved in promoting their survival. There are several lines of evidence that support this theory, and that suggest that the membrane-associated form of SCF is responsible. When normal mast cells were injected into the dermis of *Sl/Sl*^{d} mice, which lack membrane-bound SCF, they did not survive (Gordon and Galli, 1990). However, the mast cells did survive when injected into the skin of *W*^{v}/*W*^{v} mice, suggesting that membrane-bound SCF is necessary for, or at the least, more effective than soluble SCF for promoting the survival of mast cells (Gordon and Galli, 1990). Toksoz et al. demonstrated that the membrane-bound form of SCF maintained hematopoiesis for longer periods than the soluble form (Toksoz et al., 1992). Although membrane-bound SCF is better at maintaining the survival of mouse primordial germ cells *in vitro* (Dolci et al., 1991; Matsui et al., 1991), it has been shown that soluble SCF can also maintain the survival of mouse primordial germ cells (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991), hematopoietic stem cells (Bernstein et al., 1991; McNiece et al., 1991; Metcalf and Nicola, 1991; Tsuji et al., 1991; Cicuttini et al., 1992; Migliaccio et al., 1992; Williams et al., 1992b), human melanocytes (Funasaka et al., 1992), human mast cells (Irani et al., 1992; Valent et al., 1992; Mitsui et al., 1993), or human NK cell precursors (Uittenbogaart et al., 1992) *in vitro*,

and can promote the survival of mouse mast cells *in vitro* or *in vivo* (Zsebo et al., 1990b; Tsai et al., 1991). In addition, Iemura and others have provided evidence that SCF promotes the survival of mast cells by suppressing apoptosis (Iemura et al., 1994).

An *in vitro* mast cell proliferation assay has been the standard bioassay for characterizing SCF bioactivity since the cloning of the gene (Williams et al., 1990; Copeland et al., 1990; Anderson et al., 1990; Zsebo et al., 1990a, b; Martin et al., 1990b; Huang et al., 1990). There can be little argument about the ability of SCF to induce proliferation in mast cells. However, the effects of SCF on the proliferation of other Kit-bearing lineages is most dramatic in the context of synergism with other stimuli (reviewed in Galli et al., 1994). SCF is much more effective in inducing proliferation of cultured mouse primordial germ cells when leukemia inhibitory factor (LIF) is added as a cofactor (Matsui et al., 1991). Human melanocytes proliferate *in vitro* when SCF is provided with PMA, but SCF alone has little effect (Funasaka et al., 1992). Similarly, SCF by itself has only modest effects on the development of colonies of hematopoietic cells *in vitro*, but acts in synergy with many other growth factors to promote the production *in vitro* of early and intermediate precursors of erythroid, myeloid, and lymphoid lineages (Galli et al., 1994).

Finally, SCF can function as an agent to stimulate mediator release from mast cells through Kit signaling. SCF-induced mediator release has been demonstrated in purified human skin mast cells (Columbo et al., 1992) and from purified mouse peritoneal mast cells (Coleman et al., 1993) *in vitro*. In addition, *in vitro* studies with human lung (Bischoff and Dahinden, 1992) or skin (Columbo et al., 1992) mast cells or with purified mouse peritoneal mast cells (Coleman et al., 1993) also showed that soluble SCF can enhance the level of mast

cell secretion and mediator release observed in cells stimulated through the high affinity IgE receptor (FcεRI). This effect was seen at SCF concentrations substantially lower than those required to induce mediator release directly. Taken together, these data indicate that SCF is involved in mast cell development and function. It is not clear to what extent SCF may also influence the functional activity of other lineages that express Kit.

Materials and Methods

Animals

Female BALB/c mice, 8-10 weeks of age, were purchased from the National Cancer Institute, Frederick, MD. They were maintained in laminar flow housing and provided with food and water ad libitum.

Antibodies

An affinity-purified rabbit IgG polyclonal anti-human c-*kit* antibody raised against a synthetic peptide (American Research Products, Inc., Belmont, MA) was the primary antibody used for immunohistochemistry. Control experiments were performed using rabbit IgG purified from serum of non-immunized animals (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Immunofluorescent staining was done with a monoclonal anti-human c-*kit* antibody (clone 1.D9.3D6, Boehringer Mannheim Biochemica, Indianapolis, IN). Non-specific interactions were blocked with a solution containing purified human IgG (Jackson). A mouse IgG_1 (Biosource International, Camarillo, CA) was used as a negative control. In the immunofluorescence studies, anti-c-*kit* antibodies were localized using a biotin-conjugated $F(ab')_2$ goat anti-mouse IgG (Jackson), followed by fluorescein isothiocyanate (FITC)-labeled streptavidin (Pierce, Rockford, IL).

Cells and cell lines

The murine fibroblast cell line BALB/c 3T3, the human fibroblast lines CCD18, HS68 and MRC-9, and the human fibrosarcoma cell line HT1080 were all obtained from American Type Culture Collection, Rockville, MD. The four human malignant schwannoma cell lines were generous gifts of Dr. George DeVries, Department of Cell Biology, Neurobiology and Anatomy, Loyola University, Chicago, IL. The ST88-14 line was derived from a peripheral nerve sheath tumor of a patient diagnosed with NF1 (Fletcher et al., 1991), and partially described elsewhere (DeClue et al., 1992; Reynolds et al., 1992). Due to contamination of this line, a separate clone was obtained directly from Dr. Jonathan Fletcher, Department of Pathology, Brigham and Women's Hospital, Boston, MA. Other malignant schwannoma cell lines include the STS-26T line, which was derived from an isolated grade III malignant schwannoma in a patient who did not have NF1 (Dahlberg et al., 1993), the NF-1T cell line, derived from a malignant soft tissue sarcoma in an NF1 patient (Dahlberg et al., 1993), and the T265-2c cell line, which was established in the laboratory of Dr. George DeVries from a malignant schwannoma in a patient with NF1. The HMC-1 cell line was derived from a patient with mast cell leukemia, and was the kind gift of Dr. Joseph H. Butterfield, M.D., Allergic Diseases and Internal Medicine, Mayo Clinic, Rochester, MN. All cells except HMC-1 were maintained in Dulbecco's modified Eagle's medium supplemented with 2mM L-glutamine, 50μg/ml gentamicin, 100U/ml penicillin, 100μg/ml streptomycin, and 10% fetal calf serum (cDMEM) in a humidified chamber at 37°C and 5% CO_2. HMC-1 cells were grown in Iscove's modified Dulbecco's medium with 10% defined, iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, Utah), 1.2mM monothioglycerol (Sigma Chemical

Company, St. Louis, MO), 2mM L-glutamine, 50μg/ml gentamicin, 100U/ml penicillin, and 100μg/ml streptomycin at 37°C and 5% CO_2.

Total RNA was extracted from surgical tissue and cultured cells in this laboratory using the Ultraspec™-II RNA isolation system (Biotecx Laboratories, Inc, Houston, TX), which involves extraction with acid guanidinium thiocyanate and phenol-chloroform (Chomczynski and Sacchi, 1987).

Primary human fibroblast cultures designated 8N, Edwards, 16BN, and 30K were established and maintained in the laboratory of Dr. Dorne Yager (Division of Surgery, Department of Medicine, Virginia Commonwealth University, Richmond, Virginia), who kindly provided total RNA samples extracted from the cells. RNA specimens from the small cell lung cancer cell lines H146, H209, H249, and WBA, and from the breast cancer line MCF7, were the generous gifts of Dr. Geoffrey Krystal (Division of Hematology/Oncology, Department of Medicine and Department of Microbiology and Immunology, Virginia Commonwealth University and McGuire VA Medical Center, Richmond, VA).

Cytokines and growth factors

Recombinant mouse interleukin-3 (rmIL-3) and recombinant mouse stem cell factor (rmSCF) were purchased from BioSource International, Camarillo, CA. Recombinant murine stem cell factor used in the *in vivo* experiments involving intraperitoneal injections ($rmSCF^{169}$) was cloned and expressed in this laboratory (see below).

Cloning, expression, and purification of rmSCF^{169}

The production of recombinant mouse SCF^{169} was accomplished by Dr. Julie Leftwich, a postdoctoral fellow in this laboratory, and has not been reported elsewhere. Therefore, the protocol used is briefly stated here. A clone of mouse SCF was obtained by screening an NIH 3T3 cDNA library in a Lambda Zap II vector (Stratagene, La Jolla, CA) with a 5' end-labeled 30 base oligonucleotide probe (nucleotides 251-281) derived from the published sequence (Zsebo et al., 1990b). Nitrocellulose filters lifted from the library were incubated for 18 hours at 37°C with 10^6 cpm/ml of probe. The filters were washed 4X at 37°C for 10 minutes with 2X SSC, 0.2% sodium dodecyl sulfate (SDS), followed by one wash at 50°C for 30 minutes with 0.5X SSC, 0.2% SDS. 1 of 3 positive clones was chosen from 10^6 original plaques, and *in vitro* excision using the R408 helper virus was performed. The resulting pBluescript phagemids containing the cDNA were transfected into XL-1 Blue host cells. The clone was sequenced using the Sequenase Version 2 kit (United States Biochemical, Cleveland, OH) and the coding gene region was found to have identical predicted peptide sequences as those published (Zsebo et al., 1990b).

To express recombinant SCF proteins, BglII-BSU36I fragments encoding the secreted form of SCF were subcloned into PET-3d utilizing a 5' linker adding an NcoI site and a start codon and a 3' linker adding a stop codon and a BamHI site. These manipulations placed the coding region under the control of a T7 promoter which is inducible by the addition of isopropylthiogalactoside (IPTG). The expressed mRNA encodes a protein 169 amino acids long, and has been designated rmSCF^{169}.

To purify rmSCF^{169} protein, expression vector-containing bacteria were grown at

28°C in LB/Mg/glucose/amp to an OD_{600} of 0.6-0.8 at which time IPTG was added to a final concentration of 0.8 mM. The cells were cultured an additional 2 hours, pelleted, and resuspended in lysing buffer. The cells were then subjected to French Press (PSI = 20,000) and the supernatant was clarified and partially purified using sizing chromatography. The resulting preparations of recombinant SCF proteins were approximately 80-90% pure.

Surgical specimens

Formalin-fixed, paraffin-embedded neurofibroma specimens were obtained from Dr. Audrey Steck (Division of Pathology, Department of Medicine, Virginia Commonwealth University, Richmond, VA). Additional neurofibroma tissue was generously provided by Dr. Fu-Tong Liu (Division of Allergy, La Jolla Institute for Allergy and Immunology, San Diego, CA). 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).

Bone marrow-derived murine mast cells

Mouse bone marrow cells were obtained by flushing femurs with a 22 gauge needle into 10 ml of cDMEM supplemented with 50 ng/ml recombinant mouse stem cell factor (rmSCF, BioSource International, Camarillo, CA) and 50 U/ml recombinant mouse

interleukin-3 (rmIL-3, BioSource). The cells were initially maintained in a 25 cm^2 tissue culture flask in a humidified chamber at 37°C and 5% CO_2. After overnight incubation, the non-adherent cells were transferred to a fresh 25 cm^2 flask and placed back into the incubator, while the adherent cells were discarded. After the second night of incubation, the cell suspension was removed and centrifuged. The medium was discarded and the cell pellet was resuspended in a 75 cm^2 tissue culture flask in 30 ml of cDMEM supplemented with rmSCF and rmIL-3 at the above concentrations. The culture was maintained for a total of 4 weeks, with non-adherent cells being removed, centrifuged, and resuspended in fresh medium every 3-7 days.

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 (see above) were seeded onto a 96-well tissue culture plates at a density of 5×10^5 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) (Jacks et al., 1994), 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 (see below), and rate of proliferation, assessed by ^{3}H-thymidine incorporation (see below).

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 (Enerback, 1966). 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.

Immunofluorescence for the detection of Kit on malignant schwannoma cell lines

Each of four human malignant schwannoma cell lines, and the KEL-FIB human fibroblast line (negative control) were cultured at low density on 2-well chamber slides (Nunc Inc., Naperville, IL) in cDMEM until firmly attached. The non-adherent HMC-1 cell line (positive control) were grown in a tissue culture flask and then transferred to slides using a Cytospin 2 (Shandon, Inc., Pittsburgh, PA). All specimens were fixed by immersion in acetone for 15 minutes at -20°C, drained, rinsed in deionized water and dried. 500μl of blocking solution (1% BSA, 100mM Tris pH 7.4, 150mM NaCl) containing 10μg/ml human IgG were added to each chamber, and slides were incubated at 4°C for 30 minutes. The blocking solution was removed and replaced with blocking solution containing either 20μg/ml anti-human c-*kit* or IgG_1 isotype control antibodies, followed by incubation at 4°C for 20 hours. Slides were washed 3 times in wash solution (100mM Tris pH 7.4, 150mM NaCl) for 10 minutes each. 0.5ml of a 1:500 dilution of a biotinylated $F(ab')_2$ goat anti-mouse IgG in blocking solution was added to each slide for 1 hour at 4°C. Slides were again washed three

times in washing solution, and then incubated one hour in 0.5ml of blocking solution containing a 1:1000 dilution of streptavidin-FITC at 4°C in the dark. Coverslips were placed on the slides using 5% N-propyl gallate (Sigma Chemical Company, St. Louis, MO) to prevent quenching. Slides were analyzed on the Meridian Ultima confocal interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI).

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.

Quantitative reverse transcription polymerase chain reaction of mouse SCF

A quantitative RT-PCR protocol was developed which requires the use of a specially designed "cRNA control fragment" in competitive reactions with RNA isolated from the cells under study. This cRNA controls for variations during reverse transcription, and serves as an internal standard for the polymerase chain reaction. A murine stem cell factor control fragment was constructed by inserting a 102 base pair piece of double-stranded DNA into our mSCF clone. The insert was obtained by digesting the plasmid pBluescript II SK+

(Stratagene Cloning Systems, La Jolla, CA) with the restriction enzymes KpnI and SstI. The products were separated by agarose gel electrophoresis, and the band corresponding to the 102 bp fragment was cut from the gel and minced with a razor blade. The DNA was extracted from the agarose by adding phenol, vortexing for 30 seconds, and incubating in a dry ice and ethanol bath for 5 minutes. After centrifugation, the fragment was precipitated from the aqueous phase by addition of 3M sodium acetate and 95% ethanol, followed by overnight incubation at minus 20°C.

Our mouse SCF clone was digested with NsiI, which cuts at a single site within the SCF sequence (nucleotide 492 by the numbering of Anderson, et al., 1990), but does not cut the vector (pBluescript II SK+). This plasmid and the insert fragment were blunt-ended with T4 DNA polymerase. Both samples were then incubated with calf intestinal alkaline phosphatase (CIAP) to remove 5' phosphate groups, followed by phenol-chloroform extraction and ethanol precipitation. A blunt end ligation was performed using T4 DNA ligase at a plasmid:insert ratio of 1:100.

E. coli XL1 blue bacteria were made competent for transformation by incubating in cold 100 mM $CaCl_2$ for 10 minutes on ice. Cells were transformed by treatment with DMSO and addition of the products of the ligation reaction, followed by 30 minutes incubation on ice. Bacteria were heat-shocked by incubating in a 42°C water bath for 90 seconds, and then chilled on ice. Bacteria were grown in Luria broth (LB) containing no antibiotics for an hour in a 37°C shaker. Samples were then streaked onto LB agar plates containing ampicillin and incubated at 37°C overnight. Colonies were plucked and subcultured in liquid LB containing ampicillin and incubated overnight at 37°C with shaking.

Plasmid DNA was extracted from the bacterial cultures using the Insta-Prep Kit (5' to 3', Inc., Boulder, CO). Samples were digested with the restriction enzyme XhoI to determine if an insert had been ligated into the plasmid. The vector, pBluescript II SK+, contains a single XhoI site. The 102 base pair insert also contains this site. If the insert is missing, the enzyme cuts at only one site. If it is present, two sites are cut, releasing a fragment of 594 base pairs. A clone containing the insert was chosen, and submitted to the MCV Molecular Biology Core Facility for large-scale plasmid preparation. The cRNA was generated by *in vitro* transcription using T7 RNA polymerase. The control fragment contains the same primer binding sites as natural SCF mRNA, but will generate an RT-PCR product which is 102 base pairs larger.

For competitive RT-PCR, decreasing amounts of control fragment RNA of known concentrations are added to a series of tubes containing a constant amount of total cellular RNA. Standard reverse transcription and polymerase chain reactions are carried out, resulting in competition between the two templates for primers and other reaction components. Following amplification, the products are resolved by agarose gel electrophoresis and subjected to Southern blot analysis (see below). The intensities of the bands on the resulting autoradiograph are compared by densitometry. The quantity of mRNA in the original unknown sample is ascertained by determining the amount of control fragment needed to generate a band of equal intensity.

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.

ELISA for human SCF

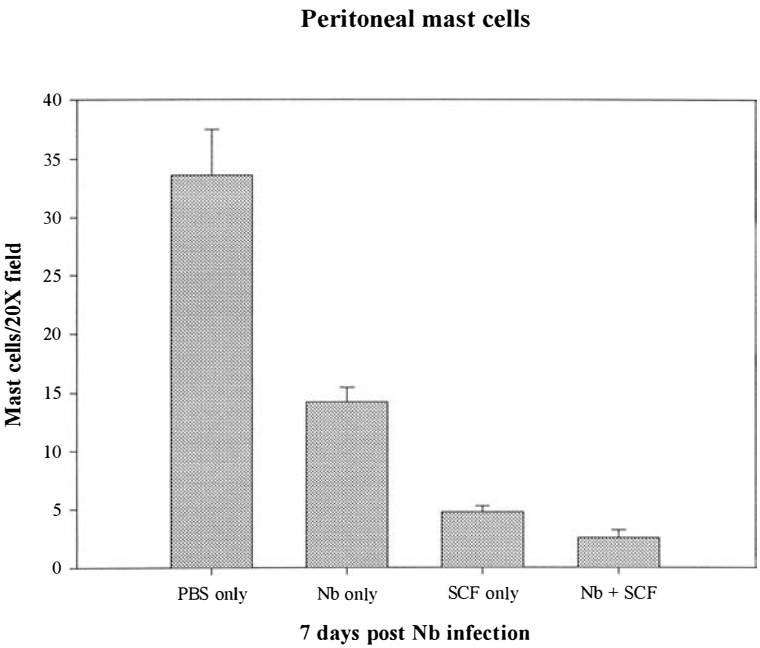
Conditioned media were assayed for immunoreactive stem cell factor using a Quantikine™ human SCF kit (R&D Systems, Minneapolis, MN). The kit contains all the necessary antibodies and standards, and the manufacturer's suggested protocol was followed. The plates were read at 450 nm on a V-max Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA). The range of the assay was from 31.25 to 1000 pg/ml.

Results

In vivo effects of intraperitoneal injections of rmSCF^{169}

To determine if intraperitoneal SCF injections might lead to the migration of mast cell precursors into the peritoneal cavity and subsequent maturation and proliferation, mast cells in the peritoneum were quantitated. Mice were given daily intraperitoneal injections of 3 µg of rmSCF^{169} in 100 µl sterile phosphate-buffered saline (PBS). A control group was injected daily with PBS alone. After 16 days of injections, a group of SCF-treated mice and a group of control mice were infected with the hookworm *Nippostrongylus brasiliensis* (Nb) by subcutaneous injection of 600 stage 3 larvae/mouse in 200 µl PBS. Mice were sacrificed after 22 days of SCF (or PBS) injections. In Nb-infected animals, this corresponded to day 7 post infection. Five ml of a 0.22 M sucrose solution were injected into the peritoneal cavity, followed by gentle massage to dislodge cells. After the muscle membrane lining the cavity was surgically exposed, the fluid was removed with a Pasteur pipette through a small hole in the membrane. The cells in these samples were transferred to slides with a Cytospin 2 cytocentrifuge, and stained with acid toluidine blue to detect mast cells. Although peritoneal wash-out cells such as these are a rich source of mature mast cells in untreated mice, the numbers were markedly decreased in response to SCF treatment (Figure 1). As previously reported, peritoneal mast cell numbers were also greatly diminished in response to Nb infection alone (Huff et al., 1995).

Figure 1. Effects of rmSCF^{169} injections on peritoneal mast cell numbers. Mice were given daily intraperitoneal injections of PBS alone or 3 µg of rmSCF^{169} in PBS for 22 days, at which time the mice were sacrificed. Nb infection was established by subcutaneous injection of 600 stage 3 larvae of the hookworm *Nippostrongylus brasiliensis* seven days prior to sacrifice. Cytospin preparations of peritoneal washouts were stained with 0.2% acid toluidine blue, and mast cells were enumerated. Data represent the mean ± SEM of mast cell counts in five random 20X microscopic fields from three mice.


Peritoneal mast cells
Mast cells/20X field
40
35
30
25
20
15
10
5
0
PBS only
Nb only
SCF only
Nb + SCF
7 days post Nb infection

Next we wished to determine if mast cell numbers might be systemically affected. Subcutaneous injections of SCF have been shown to result in a local mast cell hyperplasia at the site of injection (Zsebo et al., 1990b; Tsai et al., 1991). We tested to determine if prolonged intraperitoneal injections of high doses of rmSCF^{169} would lead to a systemic increase in mast cell numbers. Mice were sacrificed and five ml of air were injected subcutaneously into the back skin of mice just over the spine. The back skin was then everted to reveal a transparent bubble of dermal tissue. This tissue was dissected away and dermal spreads were prepared on slides. After air drying, the slides were stained with 0.2% acid toluidine blue and the mast cells were counted. No significant difference in the number of mast cells was observed between control and SCF-treated subjects after 22 days of injections (and 7 days of Nb infection, where applicable), suggesting that intraperitoneal administration of high doses of SCF does not appear to cause a systemic mast cell hyperplasia (Figure 2).

Having demonstrated that mast cell numbers were locally but not systemically decreased, we wished to determine if intraperitoneal SCF treatment might affect Nb egg production and worm expulsion. The degree of Nb infection in control and SCF-treated mice was monitored by performing daily fecal egg counts on days 4-11 following larval injections. To do this, we collected feces by housing the mice in wire bottomed cages. Fecal droppings were collected on moist paper towels, retrieved, and eggs were counted using the Ovassay™ fecal diagnostic system (Pitman-Moore, Inc., Mundelein, IL). The results of this experiment are shown in Figure 3. No significant differences were noted in the number of Nb eggs expelled by either PBS control or SCF-injected animals. As an alternative means to determine the severity of infection, adult Nb worms in the small intestines of mice sacrificed on days 7

Figure 2. Effects of rmSCF^{169} injections on dermal mast cell numbers. rmSCF^{169} injections and Nb infections were performed as in Figure 1 legend. Dermal tissue was obtained by injecting a 5 ml air bolus under the skin just over the spine. Skin was surgically everted, and the membranous dermal tissue was excised and placed on slides. After air drying, the slides were stained with acid toluidine blue. Data represent the mean ± SEM of mast cell counts in 5 random 20X microscopic fields from 3 mice.

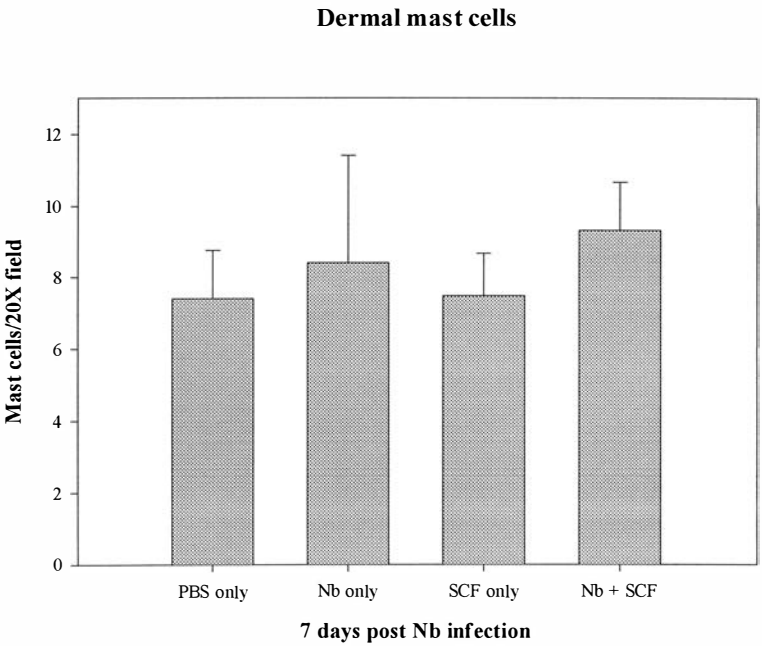
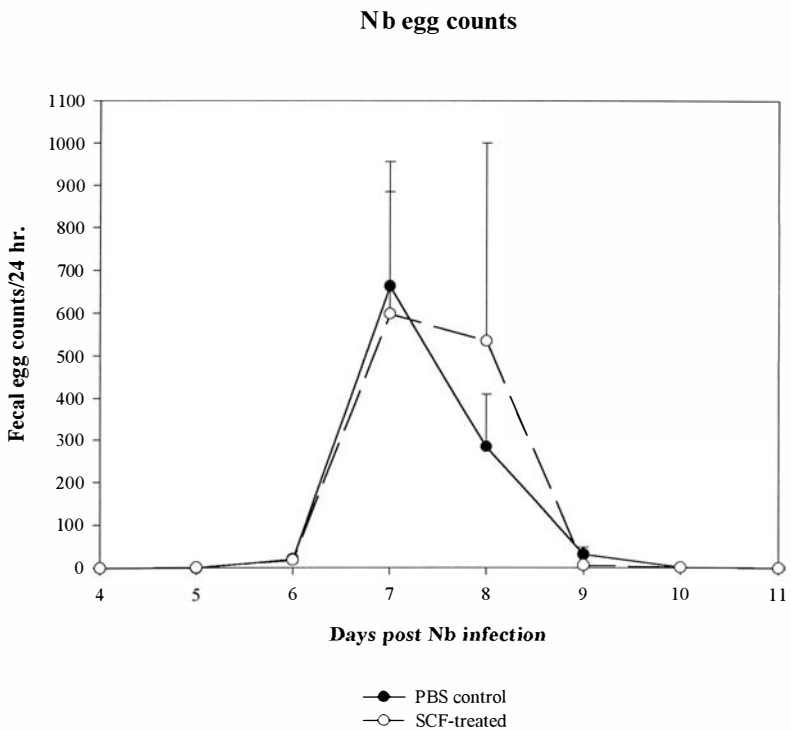

Dermal mast cells
Mast cells/20X field
12
10
8
6
4
2
0
PBS only
Nb only
SCF only
Nb + SCF
7 days post Nb infection

Figure 3. Effects of rmSCF^{169} injections on severity of *Nippostrongylus brasiliensis* infection: fecal egg counts. Nb-infected mice were housed in wire-bottomed cages, and fecal droppings were collected every 24 hours for the indicated days. Nb eggs were counted using a commercial fecal diagnostic kit. Data represent the mean ± SEM of counts obtained from triplicate test subjects. Injections and Nb infections were performed as described in Figure 1 legend.


Nb egg counts
Fecal egg counts/24 hr.
1100
1000
900
800
700
600
500
400
300
200
100
0
4
5
6
7
8
9
10
11
Days post Nb infection
PBS control
SCF-treated

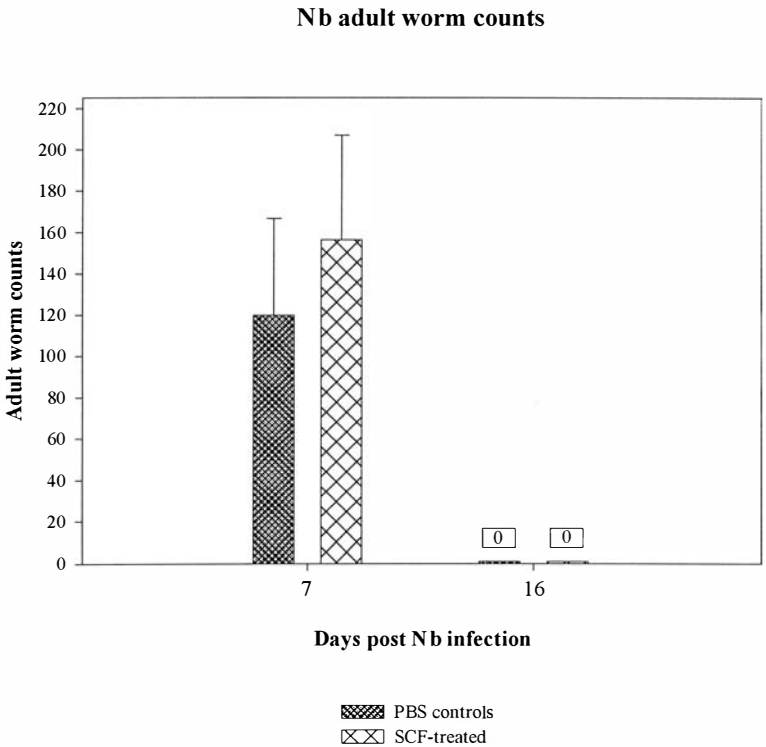
and 16 post infection were counted. The small intestine (15 cm section) of each mouse was removed and opened using surgical scissors. The worms were counted using a dissecting stereomicroscope either directly in the gut section, or as a suspension in PBS. As seen in Figure 4, a large number of worms were observed in the gut of both control and SCF-treated mice after 7 days of Nb infection, and the worms were completely cleared in both groups by day 16. This time course is consistent with previous results in our laboratory using this model system. Stem cell factor injections did not appear to have any effect on the number of worms in the small intestine, or on the rate at which they were cleared.

Quantitation of stem cell factor isoforms by competitive RT-PCR

As a result of differential splicing and postranslational events, human stem cell factor can be found as a soluble or membrane-bound protein (Anderson et al., 1990; Anderson et al., 1991; Flanagan et al., 1991). The biological activities of SCF/Kit interactions include adhesion, migration (by way of chemotaxis or haptotaxis), survival, proliferation and differentiation, and in some cases, activation (reviewed in Galli et al., 1994), and significantly different activities have been associated with each isoform (Toksoz et al., 1992). We therefore felt that it was reasonable to hypothesize that altered regulation of SCF isoform expression may have pathological consequences.

To evaluate the levels of expression of one isoform compared to the other, we set out to develop a means to quantitate the relative amounts of each mRNA produced by a given population of cells. This laboratory had previously designed a competitive reverse transcription polymerase chain reaction (RT-PCR) protocol with which to quantitate levels

Figure 4. Effects of rmSCF^{169} injections on severity of *Nippostrongylus brasiliensis* infection: adult worm counts. Daily injections and Nb infections were performed as in Figure 1 legend. Mice sacrificed at day 16 post infection received continued daily injections of rmSCF^{169} or PBS (31 days total). 15 cm sections of small intestines were removed, and adult Nb worms were counted. Data represent the mean ± SEM of worm counts in four mice. No worms were seen in any of the mice at day 16.

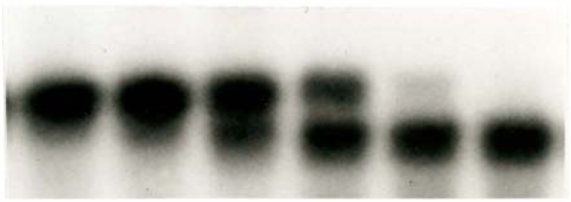

Nb adult worm counts
Adult worm counts
220
200
180
160
140
120
100
80
60
40
20
0
0
0
7
16
Days post Nb infection
PBS controls
SCF-treated

of mRNA expression for the gamma chain of the murine high-affinity IgE receptor ($Fc_{\epsilon}RI\gamma$). We used an adaptation of this technique in an attempt to determine the quantities of mRNAs for both isoforms of stem cell factor.

As described in Materials and Methods, this protocol involves the use of a panel of known quantities of a cRNA control fragment which is co-transcribed and co-amplified in the RT-PCR reactions with cellular RNA, resulting in a competitive advantage for the species of higher molar concentration. The products are distinguished from each other by agarose gel electrophoresis, based on their size differences, and the relative quantities are determined by comparing the densities of the resulting bands. The original amount of target mRNA can be calculated based on the amount of cRNA control fragment needed to generate a similar amount of product. We initially developed a protocol to evaluate mouse SCF expression. By using an antisense PCR primer which targets a sequence within exon 6, the quantity of mRNA for the soluble isoform can be determined. In a separate set of reactions using an antisense primer which anneals upstream of exon 6, the combined quantities of both isoforms is obtained. The molar concentration of the membrane-bound isoform is calculated by subtracting the value obtained for the soluble isoform of mRNA from that obtained for the combined forms.

The results of a representative experiment are shown in Figure 5. The DNA from this gel was subjected to Southern blot analysis and visualized by hybridization with a radiolabeled oligonucleotide probe, followed by autoradiography (see Materials and Methods). In this experiment, the amount of SCF mRNA in 1 μg of total cellular RNA (the amount added to the original reaction mixture), is determined to be between 0.2 and 2.0 *picograms*, *based* on

Figure 5. Competitive RT-PCR for quantitation of mouse SCF mRNA. A constant amount (1 μg) of total RNA from BALB/c 3T3 fibroblasts was added to decreasing amounts of mSCF cRNA control fragment, as indicated. Samples were then reverse transcribed to cDNA and amplified by polymerase chain reaction. Shown is an autoradiograph of the Southern blot of the amplified products.


CF
BALB/c 3T3
200 20 2 0.2 0.02 0.002
picograms of CF

the relative intensities of the bands. Alternatively, the bands on an autoradiograph can be assigned numerical values by densitometry, allowing for more accurate calculations.

We developed a similar protocol for the evaluation of human SCF. However, although the technique is highly sensitive and can be performed on very small quantities of RNA, we concluded that it is overly labor intensive in requiring a dose response curve for each experimental sample. Therefore we sought to determine if our samples could be assayed using a ribonuclease protection assay (RPA), which is ten times more sensitive that Northern analysis, and could be used in side-by-side comparisons between samples.

Two isoforms of human stem cell factor messenger RNA can be detected by a ribonuclease protection assay

Schwann cells are a source of SCF (Ryan et al., 1994) and mast cells, which express Kit and respond to SCF, are hyperplastic in neurofibromas (Isaacson, 1976). In light of the above mentioned activities associated with the two isoforms of SCF (and their possible contributions to pathological conditions), we set out to examine the expression of stem cell factor isoforms in tissues from NF1 and non-NF1 patients.

In an effort to acquire a means by which the two isoforms of human stem cell factor messenger RNA (SCF mRNA) could be discretely detected, a ribonuclease (RNase) protection assay (RPA) was developed. RPA involves hybridization of a radiolabeled RNA probe to a target mRNA by complementary base-pairing, and subsequent ribonuclease digestion of all unhybridized RNA. This study utilizes a probe which is complementary to the sequence encoding most of exons 5 and 6 of the SCF mRNA. A protected fragment 222

nucleotides in length results from hybridization of the probe with an mRNA which includes sequence encoded by exon 6 (the soluble isoform). When exon 6 is absent, as in the case of the membrane-bound isoform, the protected fragment is 144 nucleotides long (see Figure 6).

To assess the ability of the RPA to detect the mRNA isoforms, the procedure was performed on total RNA extracted from human fibroblast-derived cell lines, because fibroblasts are known to express SCF (Kitamura et al., 1989; Fujita et al., 1989; Kitamura and Fujita, 1989; Jarboe and Huff, 1989). Autoradiographic analysis of the gel reveals that bands representing protected fragments of apparent lengths of 222 and 144 nucleotides result, indicating that SCF mRNAs have been detected for the soluble and membrane-bound isoforms, respectively (Figure 7).

In addition to the fibroblast-derived cell lines, we performed the procedure on RNA extracted from primary human fibroblast cultures which had been derived by explantation of surgical skin tissue. In 7 out of 7 samples tested, a band corresponding to a protected fragment of 222 nucleotides was observed, suggesting expression of the soluble isoform of SCF mRNA (Figure 8). As part of a collaborative effort led by Dr. Dorne Yager (Division of Surgery, Department of Medicine, Virginia Commonwealth University, Richmond, Virginia), we included primary fibroblasts derived from keloid tissue in this study. Keloids are characterized as progressively enlarging scars resulting from excessive collagen deposition during connective tissue repair, and Dr. Yager's work includes investigation of the mechanisms by which keloids are formed. We sought to evaluate whether a change in SCF isoform expression by the resident fibroblasts played a part in this mechanism. Our preliminary conclusions are that there appears to be no change in SCF isoform expression by

Figure 6. Ribonuclease protection assay for human SCF mRNA. Schematic representation of the hybridization specificity of the probe used for RPA.

Ribonuclease Protection Assay

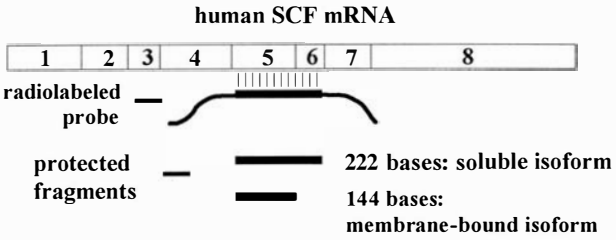


Figure 7. Autoradiograph of RPA on human fibroblast-derived cell lines. RPA was performed on 10 μg of total RNA as described in Materials and Methods. The human fibroblast cell lines used are: (lane 1) CCD18; (lane 2) Detroit 551; (lane 3) HS68; (lane 4) KEL-FIB; (lane 5) MRC-9. Size markers (M) are shown to the left. Expected fragment sizes of 222 and 144 nucleotides indicating the soluble and membrane-bound isoforms of SCF mRNA, respectively, are indicated.

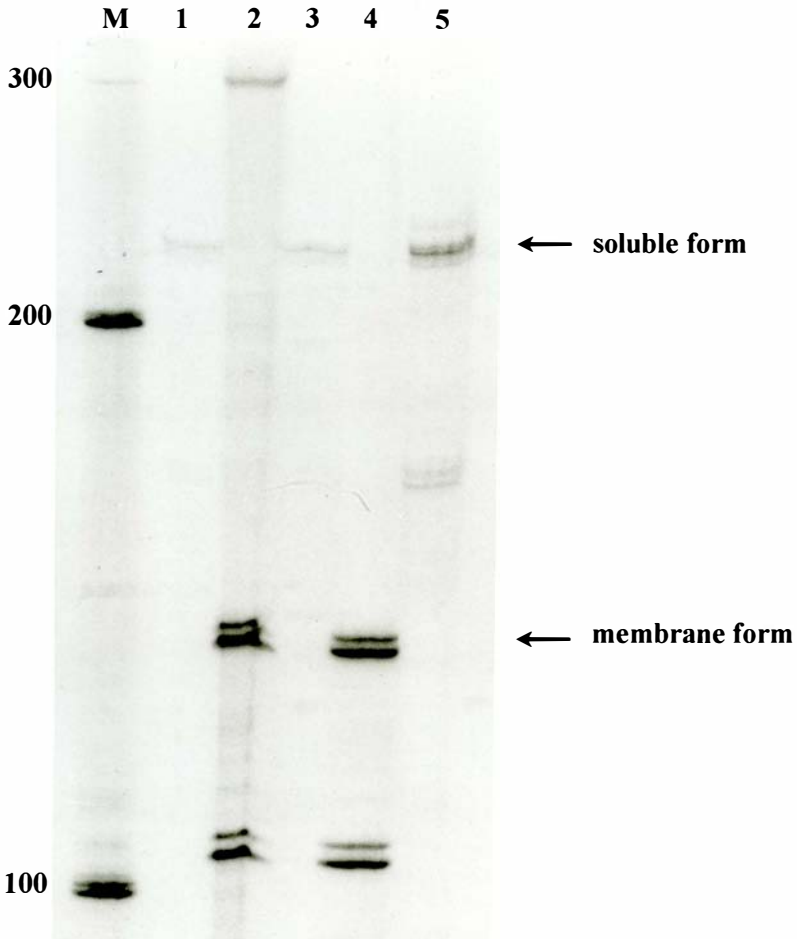
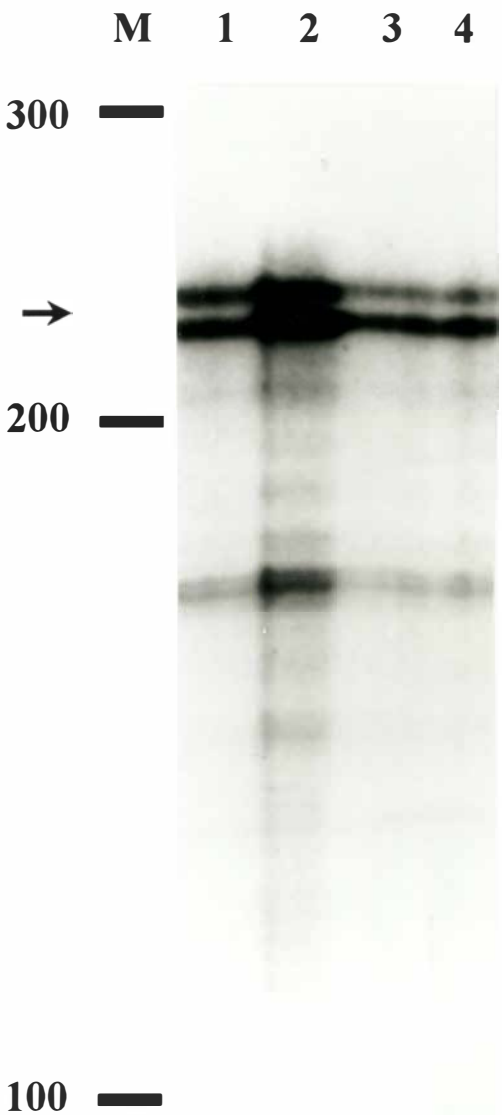

M
1
2
3
4
5
300
200
100
soluble form
membrane form

Figure 8. Autoradiograph of RPA on primary human skin fibroblasts. RPA was performed on 10 μg of total RNA extracted from cultures of primary fibroblasts established by explantation of human skin tissue. Data are representative of seven subjects tested. Samples are: (lane 1) 8N-normal skin; (lane 2) Edwards-keloid tissue; (lane 3) 16BN-normal skin from an African-American donor; (lane 4) 30K-keloid tissue. Size markers (M) are represented at the left. Arrow indicates expected 222 nucleotide fragment size for the soluble isoform of SCF mRNA.


M
1
2
3
4
300
200
100

keloid-derived fibroblasts, compared to those derived from normal skin.

Human malignant schwannoma cell lines express only the membrane-bound isoform of stem cell factor messenger RNA

The malignant schwannoma is a rare form of cancer which seems to have an increased rate of incidence in NF1 patients (Bernards et al., 1992). We obtained cell lines derived from four human malignant schwannomas and extracted RNA for analysis by RPA. In each of the four human malignant schwannoma cell lines, a protected fragment of an apparent length of 144 nucleotides is seen (Figure 9, arrow), whereas no 222 nucleotide fragment is evident. These data indicate that the cell lines exclusively express mRNA for the membrane-bound isoform of SCF.

Other human malignant cell lines do not exhibit exclusive expression of the membrane-bound isoform of stem cell factor messenger RNA

The preferential expression of membrane-bound SCF mRNA by the malignant schwannoma cell lines caused us to ponder whether this pattern of expression was in any way related to the transformed phenotype of the cells. In order to determine if other human malignancies also expressed the membrane-bound form, we obtained total RNA samples extracted from human small cell lung carcinoma cell lines and a human breast cancer cell line, and examined them for similar patterns of expression. Each of the samples tested appears to express the soluble isoform of SCF mRNA (Figure 10).

Figure 9. Autoradiograph of RPA on human malignant schwannoma cell lines. RPA was performed on 10 μg of total RNA extracted from malignant schwannoma cell lines as described in Materials and Methods. Samples are: (lane 1) NF-1T; (lane 2) ST88-14; (lane 3) STS-26T; (lane 4) T265. Size markers (M) are shown. Arrow indicates the expected 144 nucleotide fragment size for the membrane-bound isoform of SCF mRNA.

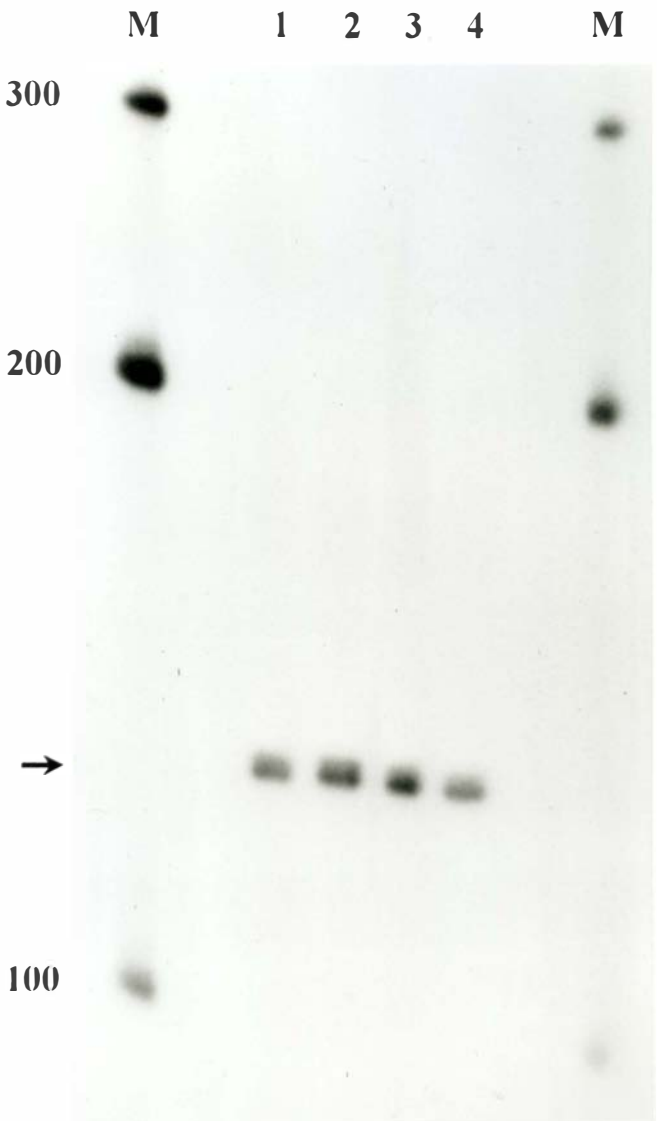
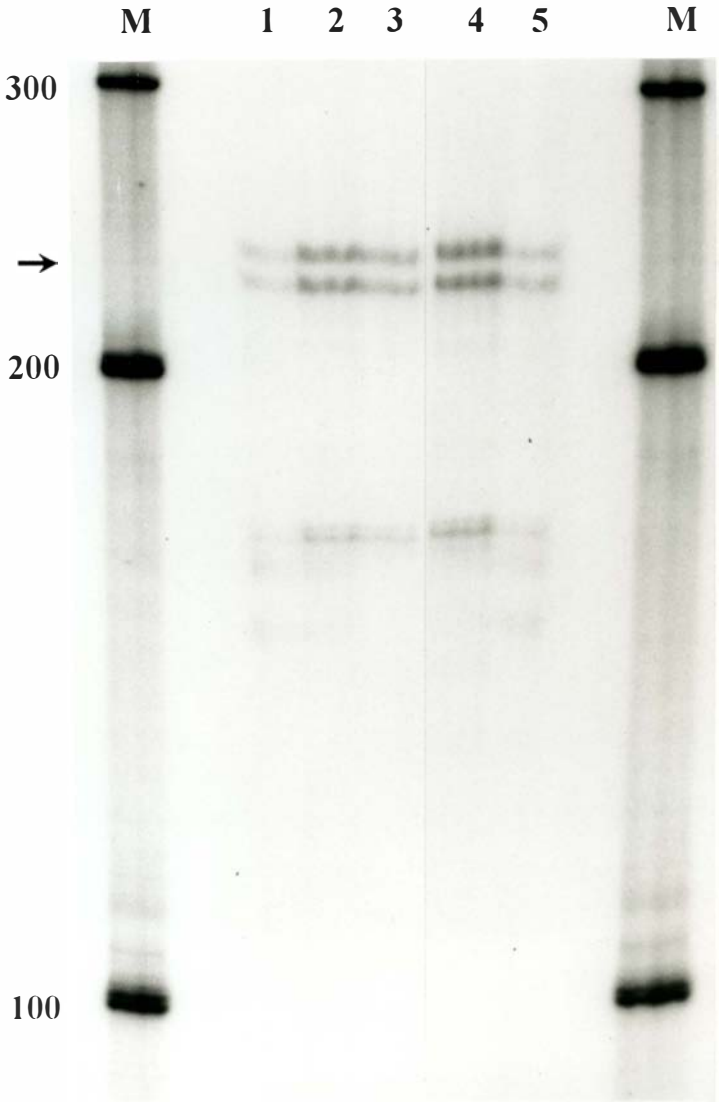

M
1
2
3
4
M
300
200
100

Figure 10. Autoradiograph of RPA on human small cell lung carcinoma cell lines and a human breast cancer-derived cell line. RPA was performed on 10 μg of total RNA extracted from human small cell lung carcinoma (SCLC) cell lines or a breast cancer line as described in Materials and Methods. Samples are: (lane 1) H146-SCLC; (lane 2) H209-SCLC; (lane 3) H249-SCLC; (lane 4) WBA-SCLC; (lane 5) MCF7-breast cancer line. Size markers (M) are shown. Arrow indicates the expected 222 nucleotide fragment size for the soluble isoform of SCF mRNA.


M
1
2
3
4
5
M
300
200
100

Expression of the exon 6-containing mRNA correlates with release of soluble SCF protein

Production of soluble stem cell factor involves at least two steps: a splicing event which includes the exon 6-encoded sequence in the mRNA, and proteolytic cleavage of the protein, which is initially expressed as a membrane-inserted protein (Anderson et al., 1991). It is possible that SCF expression may be regulated, at least in part, at the proteolysis step (for example, no cleavage of an exon 6-containing protein, resulting in membrane-bound SCF). In an effort to ascertain whether detection of exon 6-containing mRNA transcripts by ribonuclease protection assay correlated with release of soluble stem cell factor by the same population of cells, we performed a sensitive enzyme-linked immunosorbent assay (ELISA) on the culture supernatants of the cell lines used. SCF was detected in the supernatants of cell lines which were found by RPA to express the soluble isoform of SCF mRNA (Figure 11). However, the culture supernatants of the four malignant schwannoma cell lines, which expressed only mRNA for the membrane-bound isoform as determined by RPA, contained no detectable SCF (Figure 11).

Neurofibroma tissue and other Schwann cell-containing tumors express soluble SCF mRNA

Total RNA was extracted from surgically excised neurofibroma, and subjected to RPA analysis. Figure 12 indicates that this tumor expresses the soluble (arrow), but not the membrane-bound form of SCF mRNA. Other Schwann cell-containing tumors, including an additional neurofibroma, a vestibular schwannoma, and an acoustic neuroma were evaluated in a similar fashion. For comparison, a meningioma specimen was also included in this experiment. No 144 nucleotide protected fragments were observed in any of the samples,

Figure 11. Enzyme-linked immunosorbent assay to detect soluble human SCF in cell culture-conditioned media. ELISAs were performed on conditioned media from the indicated cell lines using a commercial kit. Results were obtained using an automated plate reader. CCD18, HS68, and MRC-9 are human fibroblast cell lines. HT1080 is a human fibrosarcoma cell line which was shown by RPA to express the soluble isoform of SCF mRNA (data not shown). NF-1T, ST88-14, STS-26T, and T265 are human malignant schwannoma cell lines. Sensitivity of the assay ranged from 31.25 to 2000 pg/ml of human SCF. Data represent the mean of duplicate wells. Similar results were obtained in three separate experiments.

Human SCF

ELISA

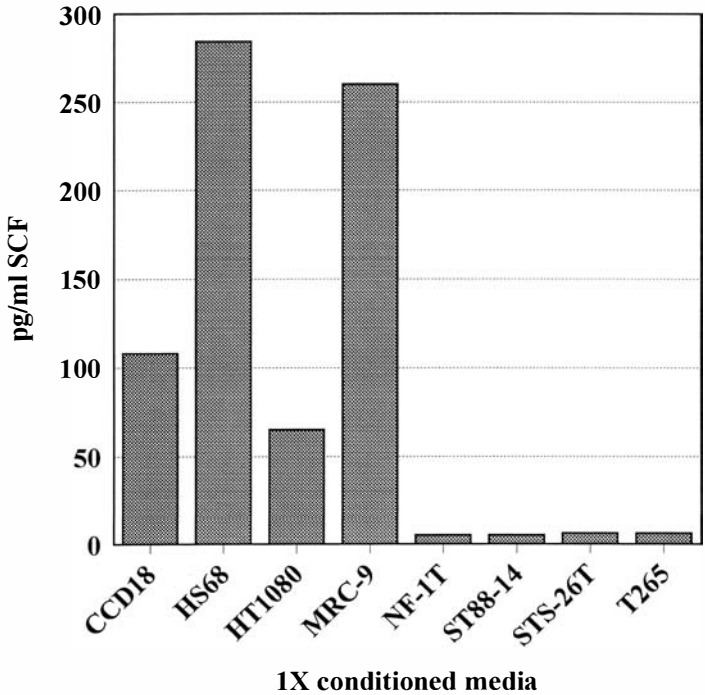
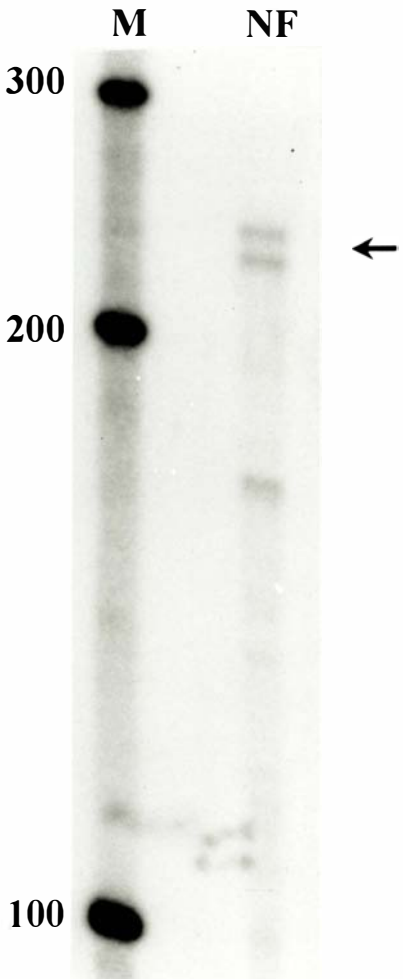


Figure 12. Autoradiograph of RPA performed on neurofibroma tissue. 20 μg of total RNA extracted from neurofibroma tissue was subjected to RPA as described in Materials and Methods. Size markers (M) are shown. Arrow indicates the 222 nucleotide expected fragment size for the soluble isoform of SCF mRNA. NF: neurofibroma.


M
NF
300
200
100

while the majority of the tumors revealed a fragment of 222 nucleotides, indicating expression of the soluble isoform of SCF mRNA (Figure 13). No SCF mRNA was detected in the meningioma tissue.

Human malignant schwannoma cell lines exhibit low level expression of Kit

Normal rat and human Schwann cells have been shown to express stem cell factor, but not its receptor, Kit (Ryan et al., 1994). In that study, however, it was found that the ST88-14 human malignant schwannoma cell line simultaneously expressed both proteins, suggesting that these cells may proliferate in response to autocrine stimulation (Ryan et al., 1994). Having obtained three additional human malignant schwannoma cell lines, we sought to determine if they also expressed Kit. In the course of pursuing a related project in this laboratory, Christopher Shelburne analyzed the four cell lines by indirect immunofluorescence and confocal laser cytometry. Figure 14 indicates that each of the four lines exhibits fluorescence indicative of a low level expression of Kit (panels A-D). Corresponding samples incubated with a non-specific mouse IgG_1 showed no fluorescence (not shown). Further analyses suggest that the Kit protein in each of these lines is truncated in some way, and bears a novel point mutation which may cause the receptors to be constitutively activated (manuscript in progress). HMC-1 cells, which express Kit abundantly, are shown as a positive control (panel E).

Kit is not detected on Schwann cells of neurofibroma or malignant schwannoma tumors

Neurofibromas are composed primarily of Schwann cells, but include a few other cell

Figure 13. Autoradiograph of RPA performed on human Schwann cell-containing tumors. RPA was performed on 10 μg of total RNA extracted from human tumors as described in Materials and Methods. Tumors include: (lane 1) neurofibroma; (lane 2) acoustic neuroma; (lane 3) meningioma; (lane 4) vestibular schwannoma. Size markers (M) are shown. Arrow indicates the expected 222 nucleotide fragment for the soluble isoform of SCF mRNA.

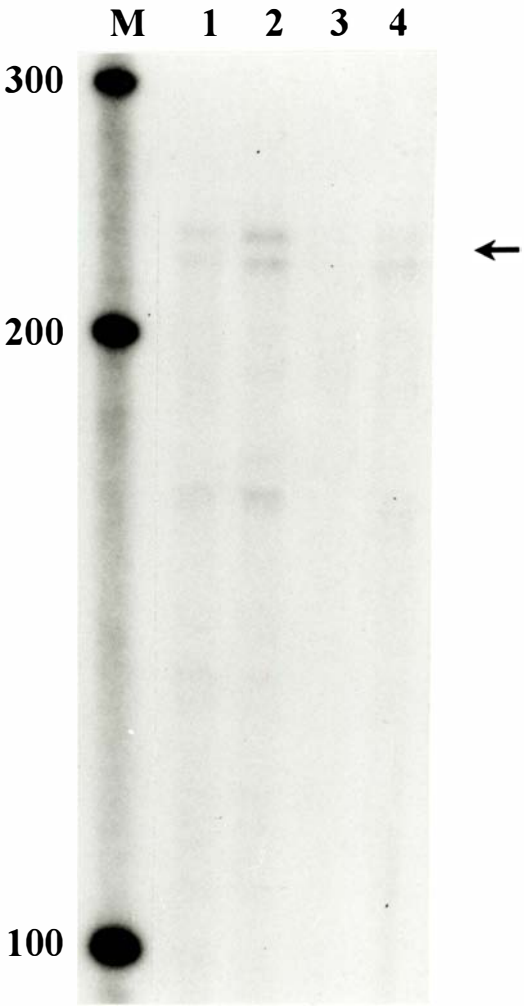
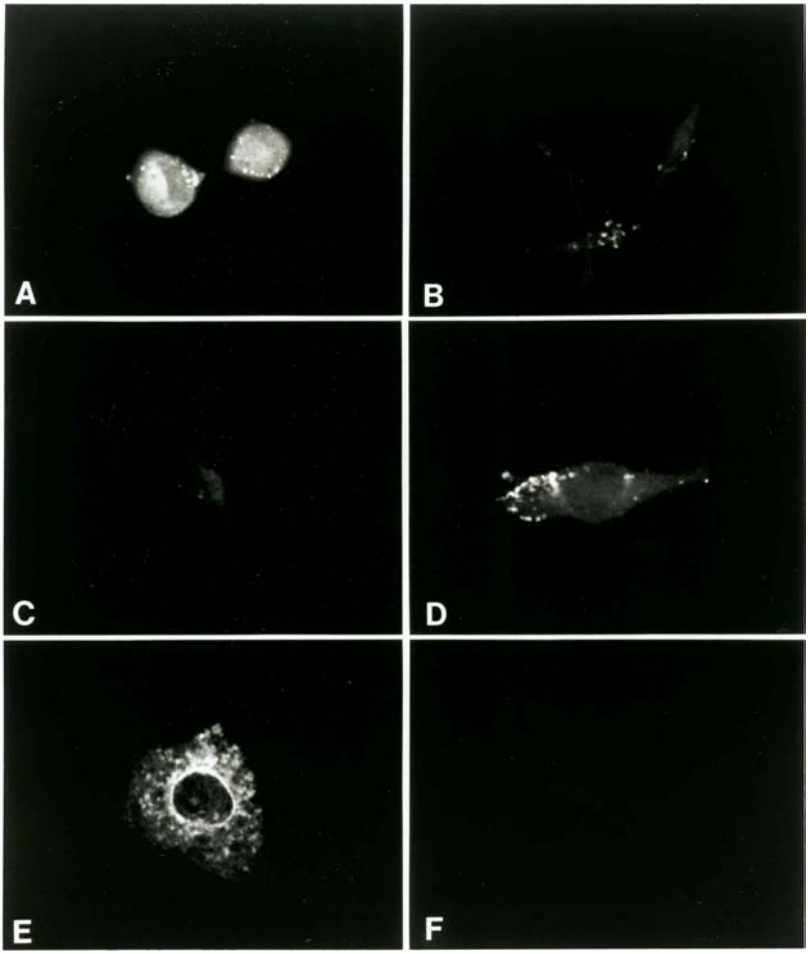

M
1
2
3
4
300
200
100

Figure 14. Photomicrographs of indirect immunofluorescence to detect Kit protein expression by human malignant schwannoma cell lines. Cells were incubated with a monoclonal antibody against human Kit. Bound antibodies were visualized using a fluorochrome-labeled secondary antibody, followed by confocal laser cytometry. Samples are: (A) NF-1T; (B) ST88-14; (C) STS-26T; (D) T265. The human mast cell line HMC-1 (E) were used as a positive control. The negative control was the KEL-FIB human fibroblast cell line (F).


A
B
C
D
E
F

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. 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 (Figure 15, arrows). The surrounding Schwann cells, however, show no evidence of immunoreactive protein. By using differential interference contrast microscopy at a higher magnification (Figure 16) we were better able to identify the Schwann cells, based on their characteristic fibrillar morphology.

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 of malignant schwannoma sections did not support this hypothesis. Once again, Kit expression by mast cells was evident, but no Kit was detected on the Schwann cells of these tumors (Figure 17).

Growth of the HMC-1 cell line is not supported by coculture with or conditioned media from human malignant schwannoma cell lines

In light of the fact that both Schwann cells and mast cells are found in increased numbers in neurofibromas (Isaacson, 1976; Peltonen et al., 1988; Johnson et al., 1989), and that Schwann cells make stem cell factor (Ryan et al., 1994), we wanted to investigate the possibility that either cell type may be inducing the proliferation of the other. We obtained

Figure 15. Photomicrographs of immunohistochemistry to detect Kit protein in neurofibroma tissue sections. (A) Immunohistochemistry was performed on neurofibroma sections using a polyclonal antibody agains human Kit as described in Materials and Methods. Arrows indicate Kit^+ mast cells. (B) Adjacent section stained with an isotype-matched control antibody. Original magnification: 66X.

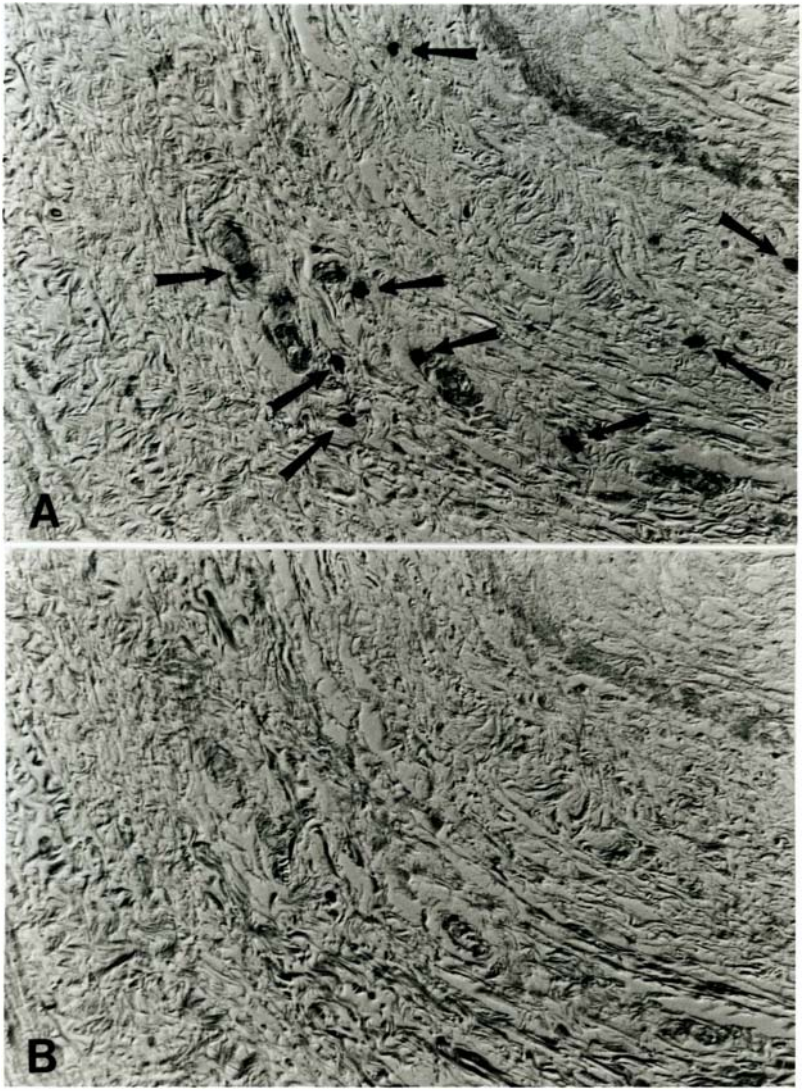

A
B

Figure 16. High magnification photomicrographs of immunohistochemistry to detect Kit protein in neurofibroma sections. Sections of neurofibroma depicted in Figure 15 viewed at a higher magnification. (A) Kit^+ mast cell. (B) Adjacent section stained with an isotype-matched control antibody. Original magnification: 330X.

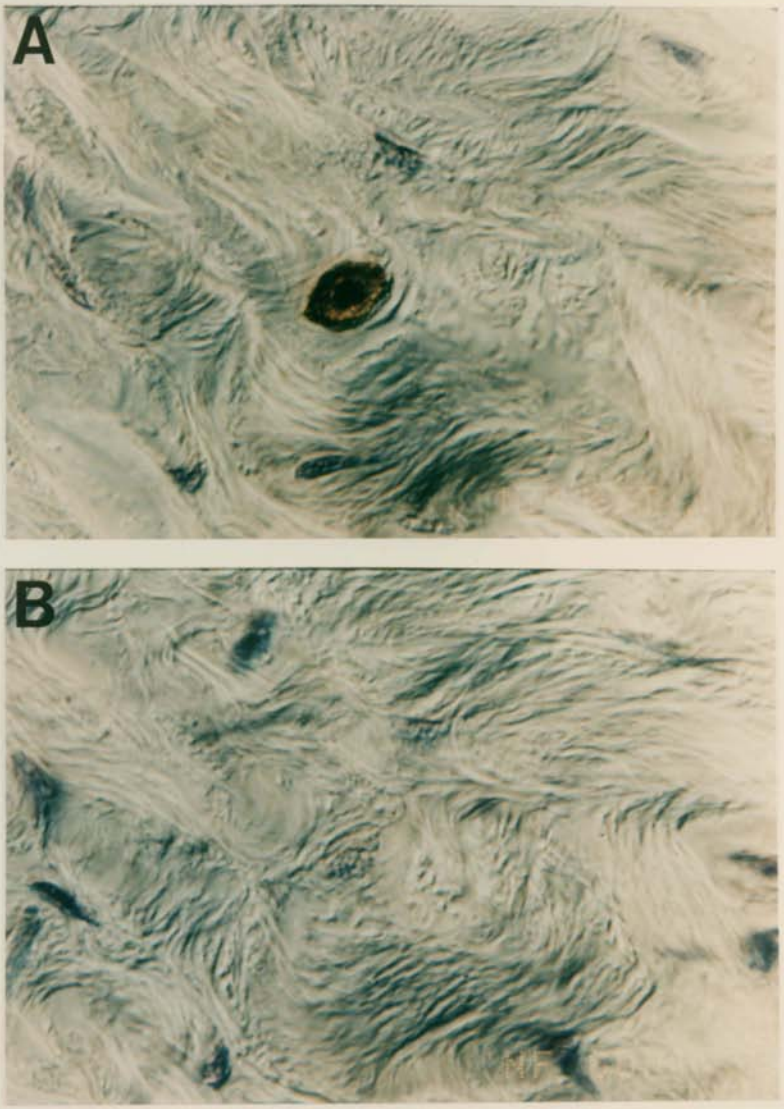
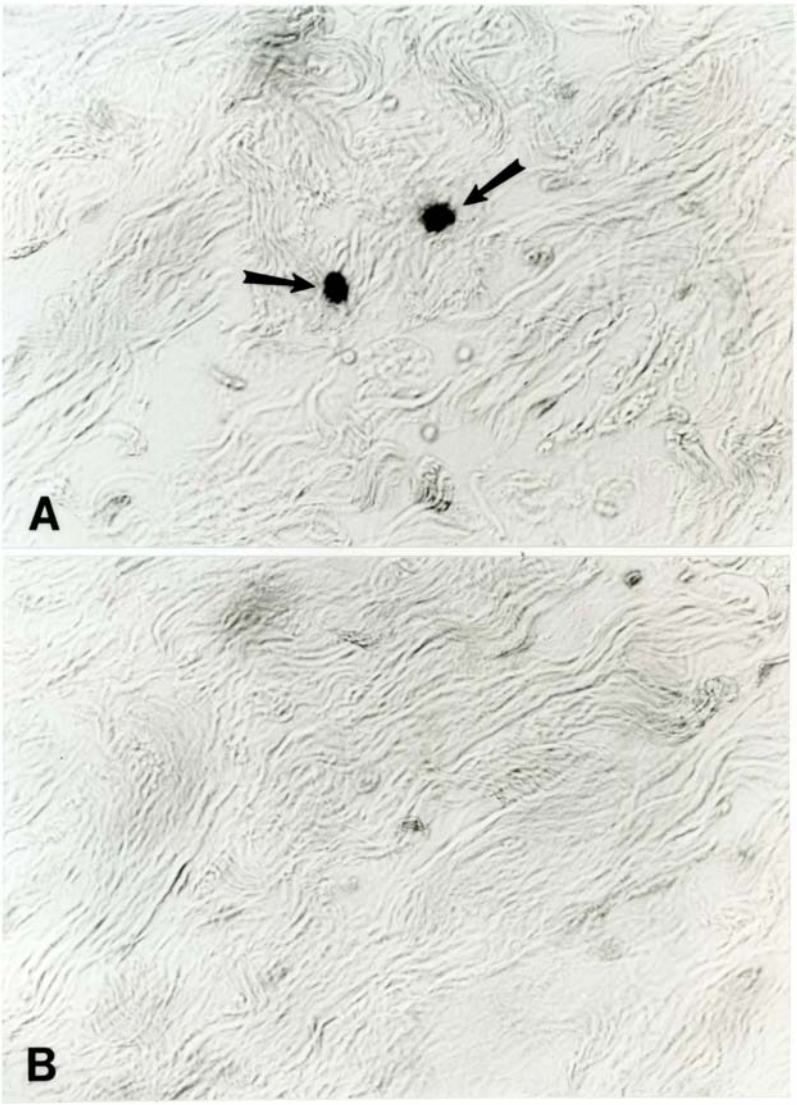

A
B

Figure 17. 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.


A
B

the HMC-1 cell line, which was derived from a patient with mast cell leukemia, and is thought to represent a human mast cell line (Butterfield et al., 1988), to grow in coculture with the malignant human schwannoma cell lines.

Twelve separate experiments involving HMC-1 cells in coculture with malignant schwannoma cell lines, or HMC-1 cells grown in the presence of conditioned media (CM) derived from the schwannoma lines were performed. The proliferation of HMC-1 cells, determined either by ^{3}H-thymidine uptake assays or by increases in cell number, was not up-regulated in any of the experiments. Typical results are represented in Figure 18. Oddly, in early cultures of HMC-1 cells in the conditioned medium of the ST88-14 schwannoma line, we noted a rapid demise of the HMC-1 cells (data not shown). The cell death was not occurring through an apoptotic pathway, according to the results of a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Upon receipt of a different clone of the ST88-14 line from Dr. Jonathan Fletcher (Department of Pathology, Brigham and Women's Hospital, Boston, MA), the experiments were repeated, and this phenomenon was no longer observed (Figure 19), suggesting that the original clone likely contained a toxic contaminant.

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.

Figure 18. Proliferation of HMC-1 cells cocultured with irradiated human fibroblast or malignant schwannoma cell lines. Malignant schwannoma and KEL-FIB cells were irradiated with ^{127}Cs (4000 rads) and seeded onto a 96 well plate. HMC-1 cells were cocultured with the irradiated cells for 2 days. Each well was incubated for 4 hours after the addition of 1 μCi of ^{3}H-thymidine, and the cells were harvested with a PHD cell harvester. Incorporation of ^{3}H-thymidine was determined using a scintillation counter. Data represent the mean ± SEM of triplicate wells.

HMC-1 Proliferation

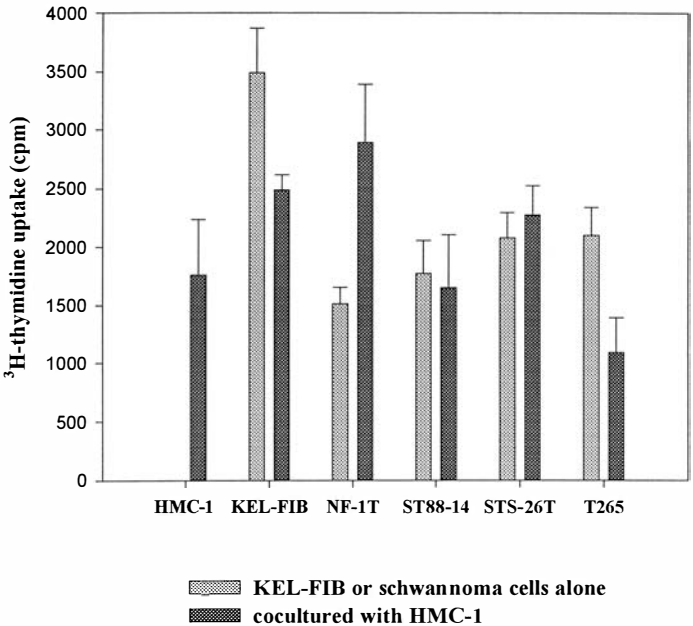
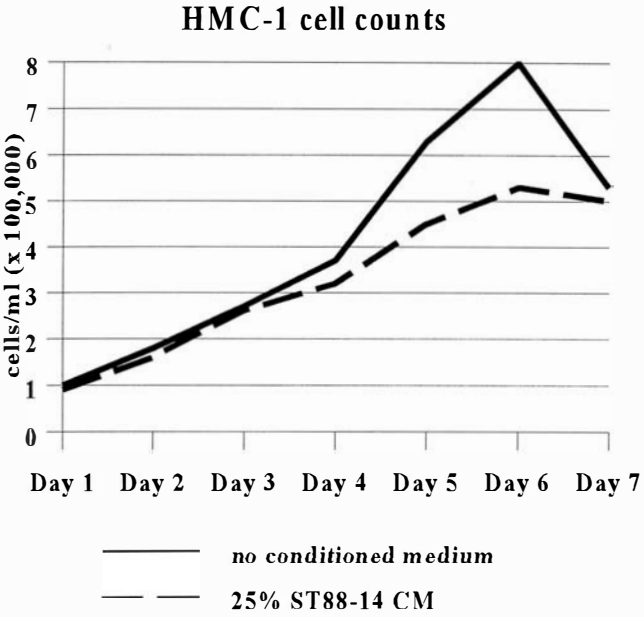


Figure 19. Daily cell counts of HMC-1 cells grown in 25% ST88-14 CM. HMC-1 cells were grown in a 25 cm^2 flask in a 25% concentration of conditioned medium from cultured ST88-14 human malignant schwannoma cells. The number of viable HMC-1 cells was determined daily by trypan blue exclusion.


HMC-1 cell counts
cells/ml (x 100,000)
8
7
6
5
4
3
2
1
0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
no conditioned medium
25% ST88-14 CM

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 (Jacks et al., 1994) 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 (Ryan, 1992). 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). Figure 20 depicts the appearance of cells grown in the absence of any conditioned media, and is

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

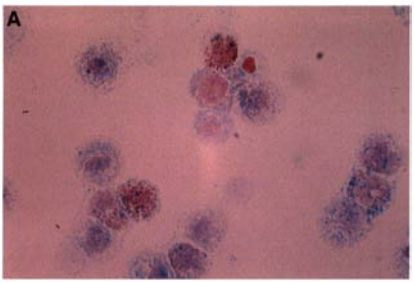
Percent cells containing safranin-positive granules

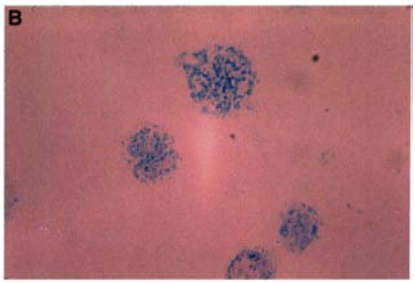
	no added factors	**50 ng/ml rmSCF**	**50 U/ml rmIL-3**	**SCF + IL-3**
Cells grown in cDMEM alone	0%	86%	0%	92%

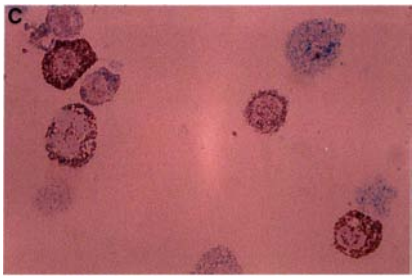
Cells grown in 10% conditioned media supplemented with 50 U/ml rmIL-3

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%

Figure 20. 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.


A


B


C

consistent with the results of previous studies in this laboratory.

Cells grown in the presence of various conditioned media are represented in Figure 21. 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 (Jarboe et al., 1989) which led to the discovery of stem cell factor.

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 22).

Figure 21. 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).

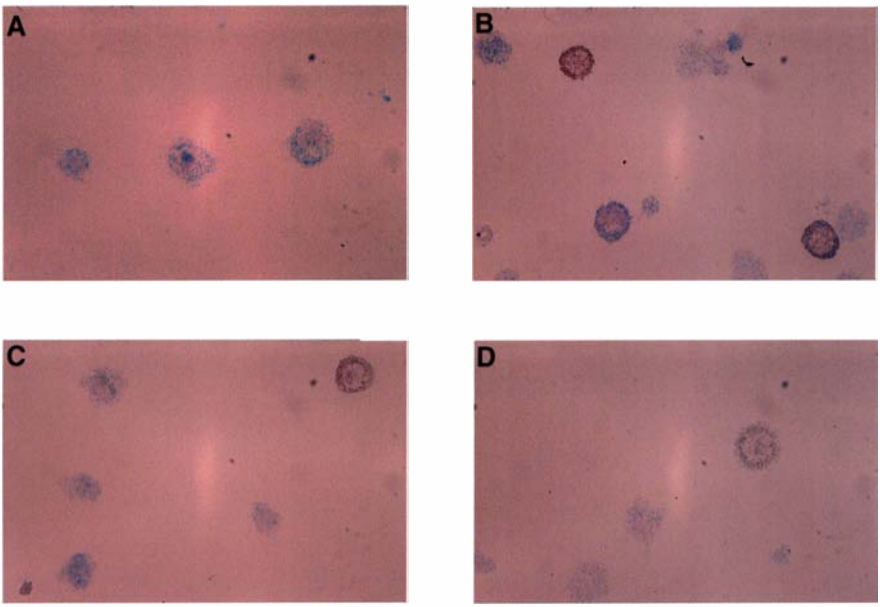
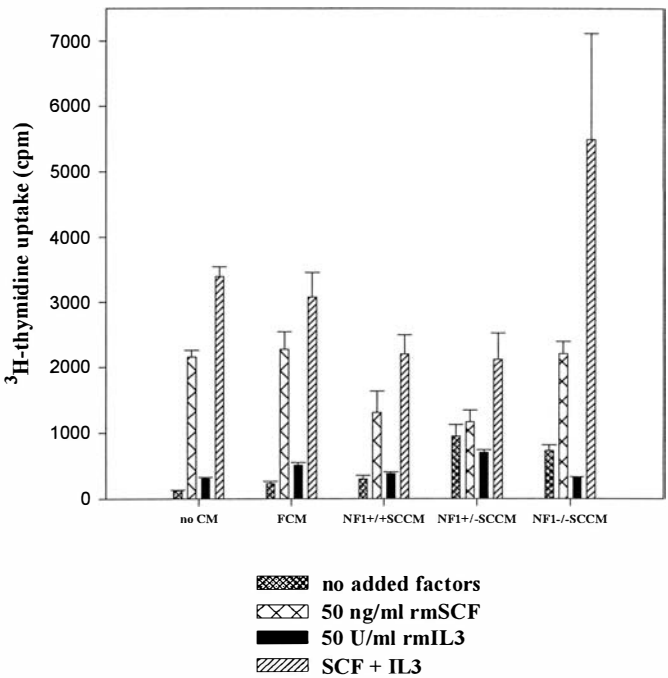

A
B
C
D

Figure 22. 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.

BMMC Proliferation



Discussion

The research described in this dissertation deals with some of the patterns of expression and biologic activities of stem cell factor. SCF is capable of acting on any cell which expresses its receptor, 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 dissertation 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.

Membrane-bound and soluble SCF clearly have different biologic activities. Membrane-bound SCF is more effective to induce proliferation and differentiation (Miyazawa et al., 1995), whereas soluble SCF is more likely to be involved in chemotaxis of Kit^+ cells (Blume-Jensen et al., 1991) and augmentation of mediator release from mast cells (Columbo

et al., 1992; Wershil et al., 1992). Based on the previous work of John Ryan in this laboratory, we suspected that Schwann cell-derived SCF might play a role in the mast cell hyperplasia associated with NF1 neurofibromas. However, it was not clear whether the SCF might be more likely to recruit mast cells, or cause mast cells to proliferate *in situ*, or both. Furthermore, we did not know what SCF isoform might be made by the Schwann cells.

Before investigating soluble or membrane SCF effects on cells we suspected of expressing Kit only weakly, we first tested the effect of high concentrations of soluble SCF (recombinant) on strongly Kit^+ cells, peritoneal mast cells. SCF and Kit have been implicated in biologically relevant interactions involving adhesion, migration, survival, proliferation, differentiation, and activation (reviewed in Galli et al., 1994). We suspected that changes in expression of SCF and/or Kit resulting from a mutation of the *NF1* gene leads to the hyperproliferation of Schwann cells in a neurofibroma, perhaps partly due to recruitment of mast cells. Therefore, we first wanted to demonstrate, in a more straight forward model system, stem cell factor-induced recruitment or proliferation of mast cells *in vivo*, so we injected high doses of $rmSCF^{169}$ into the peritoneal cavities of mice, and later examined them for site-specific mast cell hyperplasias. In addition, we compared the responses of otherwise healthy mice to those which had been infected with the intestinal helminth *Nippostrongylus brasiliensis* (Nb). It is known that Nb infection results in a mucosal mastocytosis (Befus and Bienenstock, 1984), and has been used by this and other labs as a model system to study mast cell development. We expected that administration of $rmSCF^{169}$ would result in increased numbers of peritoneal mast cells, and further thought that the greater numbers of mast cells in the infected mice would lead to a more rapid clearance of the adult worms in the gut,

thereby suggesting that SCF might be acting as a therapeutic agent.

Surprisingly, SCF treatment resulted in decreases in the number of mast cells found in the peritoneal cavities of mice treated with rmSCF^{169}, compared to control mice injected with phosphate-buffered saline (PBS) alone (Figure 1). The drop in peritoneal mast cell number in response to SCF treatment is similar to that previously observed with Nb infection (Huff et al., 1995). Kasugai and colleagues have also noted a loss of mast cell precursors in the blood of mice infected with Nb (Kasugai et al., 1995). Our results are, however, in contrast to previous studies in which subcutaneous injections of recombinant SCF in mice led to the appearance of large numbers of mast cells at the dermal site of injection (Zsebo et al., 1990b; Tsai et al., 1991). There was no significant difference in the numbers of mast cells in back dermis preparations of control or SCF-treated mice (Figure 2), indicating that intraperitoneal injections of rmSCF^{169} do not seem to confer systemic effects. In addition, no significant differences in the degree of Nb infection were noted between PBS-treated controls and SCF-treated mice, in terms of the number of adult worms in the gut (Figure 4), or the daily expulsion of eggs in the feces (Figure 3).

To explain why the intraperitoneal administration of high doses of rmSCF^{169} does not lead to proliferation of mast cells, but rather reduction, we suggest that the mast cells could be activated and induced to degranulate, thereby rendering them undetectable by our methods. SCF has been shown to induce mediator release from purified human skin mast cells (Columbo et al., 1992) and from purified mouse peritoneal mast cells (Coleman et al., 1993) *in vitro*. This hypothesis is supported by our observations that SCF treatment combined with 7 day Nb infection, which contributes both IgE plus antigen, resulted in lower numbers of

peritoneal mast cells than with either treatment alone, suggesting augmented degranulation. Alternatively, the lower numbers of mast cells detected in SCF-treated mice may reflect a change in the pattern of mast cell homing, as suggested by Kasugai and colleagues to be the mechanism of loss of mast cell progenitors from blood during Nb infection (Kasugai et al., 1995). Further studies would be needed in this model system to test these hypotheses. We conclude from these experiments that the effects of high concentrations of soluble SCF are less predictable than previously thought.

The major part of the dissertation 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 (Dilts, 1997), 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 (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990) 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 (Bienenstock et al., 1991); that mast cells are increased at sites of nerve damage and repair (Isaacson, 1976; Olsson, 1971) or in nerve-associated tumors, such as neurofibromas and schwannomas (Isaacson, 1976); and that Schwann cells produce stem cell factor (Ryan et al., 1994). 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.

Human stem cell factor exists as either a soluble or membrane-bound protein, resulting from alternative splicing and specific proteolysis (Anderson et al., 1991). Each isoform is associated with distinctly significant biological activities (see discussion in Literature Review), which led us to the notion that a failure in SCF regulation resulting in aberrant expression of either form may contribute to pathology. Since we wanted to evaluate the relative expression of the two forms of SCF mRNA, we developed techniques with which we could discriminate and quantitate them. Although a competitive RT-PCR protocol was effective at quantitating mRNA levels (Figure 5), we found that a ribonuclease protection assay (RPA) was better suited for sample-to-sample comparisons. Therefore, RPA was used throughout this study to detect SCF mRNA isoforms.

In early experiments using RNA extracted from human fibroblast-derived cell lines, we determined that we were able to detect either isoform of SCF mRNA by this method (Figure 7). However, in addition to the bands representing the protected fragments of 144 nucleotides (membrane) and 222 nucleotides (soluble), we also noted additional bands indicating protected fragments of approximately 105, 175, and 300 nucleotides. Since the radiolabeled transcript used as a probe in this protocol is approximately 300 nucleotides long, the band of that size likely represents excess probe which remained unhybridized and undigested. By reducing the concentration of ribonucleases, and lowering the temperature of the digestion reaction, the 105 nucleotide fragment was eliminated, leaving the expected fragments, appearing as doublets, and the unexpected doublet around 175 nucleotides. Doublets such as these may result from transient strand separation at AU-rich regions of the RNA, allowing for RNase cleavage at that site, or they may represent non-specific cleavage due to secondary RNA structure. Similar unexpected fragments have been reported elsewhere (Huang et al., 1992; Kauma et al., 1996).

Having developed an RNase protection assay capable of detecting both SCF isoforms, we examined SCF isoform expression from malignant schwannoma cell lines and from neurofibroma tissues. The malignant schwannoma is a rare cancer of Schwann cell origin affecting much less than 1% of the general population (Cutler and Gross, 1936). This rate of incidence is increased to up to 2% in NF1 patients (Ponder, 1990; Nimura, 1992), which supports the notion that neurofibromin may function as a tumor suppressor (Weinberg, 1991). Because of the infrequency of occurrence of malignant schwannomas, cells and tissue samples from such tumors are in short supply, and are very difficult to obtain for research purposes.

We have, however, obtained four cell lines which have been derived from human malignant schwannomas, and have used them throughout this study in place of actual tumors or primary cultures of schwannoma-derived cells. We note here that cell lines are not completely accurate representations of the phenotypic characteristics of their *in vivo* cell counterparts, and interpretations of the data must be made with that in mind. We examined the stem cell factor mRNA expression of the malignant schwannoma cell lines by RPA. In every case, only the membrane-bound isoform was detected (Figure 9). These findings were in contrast to results obtained with RNA from primary cultures of normal and keloid-derived human fibroblasts (Figure 8). The same pattern was observed in multiple subsequent experiments using the same or different RNA extractions. In view of the fact that, of the five human fibroblast cell lines used in the early RPA experiments (Figure 7), the two lines which expressed mRNA for the membrane-bound isoform of SCF grew much more rapidly than the other three which expressed the soluble isoform mRNA (Table 2), we wondered if the expression of primarily membrane-bound SCF correlated with the transformed phenotype of these cell lines. Therefore, we sought to determine if other SCF-expressing malignant cells exhibited a similar pattern of isoform expression.

A panel of small cell lung carcinoma (SCLC) cell lines have been characterized with respect to stem cell factor expression (Krystal et al., 1996). We obtained RNA extracted from these, as well as from a breast cancer cell line, to address the question of a potential link between membrane-bound SCF expression and malignancy. The detection of only the soluble isoform of SCF mRNA in each of these samples (Figure 10) leads us to the conclusion that expression of the membrane-bound form is not required for a malignant phenotype in stem

Table 2. Patterns of stem cell factor and Kit expression and growth rates of cultured cells

Cell lines	SCF isoform	Kit expression	Rate of growth
CCD18	soluble	ND*	slow
Detroit 551	membrane	ND	rapid
HS68	soluble	ND	slow
HT1080	soluble	ND	slow
KEL-FIB	membrane	negative	rapid
MRC-9	soluble	ND	slow
Primary fibroblasts	soluble	negative	ND
NF-1T	membrane	positive	rapid
ST88-14	membrane	positive	rapid
STS-26T	membrane	positive	rapid
T265	membrane	positive	rapid

*ND = not determined

cell factor-expressing cells. However, these data do not rule out the possibility that any correlation between preferential expression of the membrane-bound isoform and malignancy (or perhaps simply hyperplasia) may be dependent upon the cell type. In other words, it may be true for Schwann cells, but not small cell lung carcinoma or breast cancer cells.

The fact that these malignant schwannoma lines exclusively express the membrane isoform of SCF is striking in itself. A search of the literature does not reveal any other primary cell preparation or cell lines which exhibit such tight regulation toward the membrane SCF isoform. Thus, these malignant schwannoma lines may prove to be a good model system for studying how the alternative splicing of exon 6 of SCF is regulated. Majumdar and colleagues have studied SCF isoform regulation, but only in SCF transfectants, not in naturally occurring circumstances (Majumdar et al., 1996).

To explore the possibility that all Schwann cell hyperplasias, not just Schwann cell malignancy, might be characterized by expression of the membrane-bound isoform of stem cell factor, we extracted RNA from tissues obtained by surgical excision of neurofibroma, vestibular schwannoma, acoustic neuroma, and meningioma tumors. Except for meningiomas, these all contain increased numbers of Schwann cells, but the Schwann cells are not transformed. Meningiomas were included as an example of a neural-associated tumor not involving Schwann cells. Ribonuclease protection assays performed on these samples indicate that the soluble isoform of stem cell factor, but not the membrane-bound form, is being expressed in these tissues (Figures 12 and 13). As expected, the meningioma showed little SCF expression of either isoform. It should not be assumed that the source of the SCF mRNA being detected in the other tumors is exclusively Schwann cells, because the tumors

contain other SCF-producing cell types, including fibroblasts. The methods used here do not allow for the determination of the source of the mRNA from a mixed population of cells. Nevertheless, Schwann cells do represent a major cellular constituent of each of these lesions, and if there had been exclusive expression of membrane SCF, it would likely have been revealed by the RNase protection assay.

In an earlier study in this laboratory, it was reported that the ST88-14 human malignant schwannoma cell line expressed Kit, while normal human and neonatal rat Schwann cells did not (Ryan et al., 1994). These findings, along with the detection of stem cell factor production by the same cells, were suggestive that the ST88-14 cells might be responding to an autocrine growth loop. Coexpression of SCF and Kit has been reported in a variety of malignancies (Hibi et al., 1991; Toyota et al., 1993; Inoue et al., 1994; Hines et al., 1995), and transfection studies have provided strong evidence of a correlation between coexpression and growth factor independence in small cell lung carcinoma lines (Krystal et al., 1996). With the acquisition of three additional human malignant schwannoma cell lines, through related studies performed by Christopher Shelburne in this laboratory, we sought to determine if these also expressed Kit. Indirect immunofluorescence staining, using a monoclonal antibody directed against human Kit, was performed on the three cell lines, as well as the ST88-14 line. The preparations were analyzed using a confocal laser cytometer, which revealed expression of Kit protein at low levels on all four malignant schwannoma lines (Figure 14). Additional characterizations, including immunoprecipitation of Kit protein from the cell lines, suggest that the protein may be truncated. Moreover, RT-PCR amplification and subsequent sequencing of fragments of the c-*kit* mRNA indicate a novel point mutation in the kinase

region of Kit which may lead to constitutive receptor activation (Shelburne et al., manuscript in preparation). Taken together, these findings offer two possible explanations for the transformed phenotype of the malignant schwannoma cell lines: the cells are constantly induced to proliferate due to simultaneous expression of SCF and Kit, or they bear a mutation in Kit which causes it to be constitutively activated, leading to unregulated growth.

As mentioned above, although the use of cell lines as a model system offers the advantages of abundance and availability, their phenotype may diverge from that of the corresponding cells *in vivo*. To further characterize the Schwann cells of malignant schwannomas as well as those of neurofibromas *in situ*, we obtained formalin-fixed, paraffin-embedded sections of each tumor type and examined them for Kit expression. No primary cell cultures were available from these sources to perform experiments similar to those performed on the malignant schwannoma lines. As we have reported in the Results section, by performing immunohistochemical analyses of the sections, using a polyclonal antibody specific for Kit protein, we were able to detect abundant Kit expression by the resident mast cells (Figures 15-17). However, no Kit protein was detected on the Schwann cells by this method. We can not rule out the possibility that Kit may be expressed at levels too low to be detected by this method. Indeed, mature mast cells such as these are likely to express much higher numbers of Kit molecules than other cells (reviewed in Galli et al., 1994). We are currently developing techniques whereby we can adapt the immunohistochemistry protocol such that it can be analyzed with the highly sensitive confocal laser cytometer, thereby offering the same degree of sensitivity as was used with the malignant schwannoma cell lines.

Production of substances with potential mitogenic effects has been observed in mast

cells (Pennington et al., 1991) and Schwann cells (Ridley et al., 1989; Ohi et al., 1990). In addition, Schwann cells derived from a neurofibroma of an NF1 patient appear to secrete growth factor activity which is not observed in Schwann cells from genetically normal individuals (Sheela et al., 1990). We have hypothesized that NF1 Schwann cells produce factors (SCF and perhaps others) which are chemoattractive and mitogenic for mast cells. Thus, the mast cells might be drawn to the site where they, in turn, secrete growth factors to which the Schwann cells are responsive, resulting in the formation of a neurofibroma. Others have suggested similar theories concerning the involvement of mast cells in neurofibroma growth. Riccardi has suggested that mast cells may accumulate following local trauma, or in response to tropic factors, and that their secretions may induce abnormal proliferation in other cells due to the *NF1* mutation, resulting in neurofibroma growth (Riccardi, 1990).

To test whether human Schwann cells might upregulate the growth of human mast cells (such as might occur in NF1 neurofibromas), we used malignant schwannoma lines and the HMC-1 mast-like line, using both co-culture experiments and conditioned medium experiments. No upregulation of HMC-1 growth or differentiation was observed. The slight decreases in HMC-1 growth were likely due to slight nutritional deprivation due to dilution with spent (conditioned) medium. Also, since HMC-1 cells have an activating Kit mutation (Furitsu et al., 1993), any upregulation due to SCF might be hard to detect. In addition, the malignant schwannoma lines might not well represent Schwann cells from NF1 neurofibromas.

It would likely 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 (Figure 21), 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 (Figure 11). 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 (Figure 22), 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 single *NF1* allele may cause a partial 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 (Reynolds et al., 1992), 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 (Reynolds et al., 1992). Moreover, it has also been reported that ST88-14 cells express extremely low levels of neurofibromin protein (DeClue et al., 1992). 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 (Reynolds et al., 1992). 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 (Reynolds et al., 1992). 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 observation that only the membrane-bound form of SCF mRNA is expressed by the four schwannoma cell lines implies that there may be a correlation between SCF isoform expression and control of Schwann cell proliferation. It is interesting that faster rates of proliferation were observed in cultures of fibroblast cell lines which expressed the membrane-bound isoform, compared to soluble SCF-producing cells (Table 2). Although no malignant schwannoma solid tumors were available for analysis by RPA, neurofibroma, vestibular schwannoma, and acoustic neuroma tumors, each of which is characteristically slow-growing and non-invasive, all expressed the soluble SCF isoform (Figures 12 and 13, Table 3). In the future, it is hoped that, through collaborations with other laboratories, the experimental design

Table 3. Patterns of SCF and Kit expression by solid tumors

Tumor	SCF isoform	Kit expression
neurofibroma	soluble	negative
malignant schwannoma	ND*	negative
vestibular schwannoma	soluble	ND
meningioma	none detected	ND
acoustic neuroma	soluble	ND

*ND = not determined

can be expanded to include cultures of Schwann cells in mast cell-conditioned media, as well as cocultures of the two cell types from normal and knockout mice.

In summary, 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 first determined that high doses of recombinant stem cell factor may cause degranulation or rerouting of peritoneal mast cells, because their numbers are decreased in mice after repeated daily intraperitoneal injections of rmSCF[169]. In conjunction with Christopher Shelburne, 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 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.

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