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Stem Cell Factor in Mast Cell and Schwann Cell Proliferation and Hyperplasia

John Joseph Ryan

School of Basic Health Sciences
Virginia Commonwealth University

This is to certify that the dissertation prepared by John Joseph Ryan entitled Stem Cell Factor in Mast Cell and Schwann Cell Proliferation and Hyperplasia has been approved by his committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

[redacted]

Thomas F. Huff, Ph.D., Director of Thesis

[redacted]

Daniel H. Conrad, Ph.D., School of Basic Health Sciences

[redacted]

Eric H. Westin, M.D., School of Medicine

[redacted]

George H. DeVries, Ph.D., School of Basic Health Sciences

[redacted]

Darrell L. Peterson, Ph.D., School of Basic Health Sciences

[redacted]

Francis L. Macrina, Ph.D., Department Chairman

[redacted]

S. Gaylen Bradley, Dean, School of Basic Health Sciences
Chairman, MCV Graduate Committee

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

By

John Joseph Ryan

Director: Thomas F. Huff, Ph.D.

Associate Professor, Microbiology and Immunology

Medical College of Virginia

Virginia Commonwealth University

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Abbreviations

^{3}H-TdR	Tritiated Thymidine Deoxyribonucleotide
ARSC	Adult Rat Schwann Cells
BFU	Blast Forming Unit
cDMEM	Complete Dulbecco's Modified Eagle's Medium
CFU	Colony Forming Unit
CM	Conditioned Medium
CTMC	Connective Tissue Mast cell
DEPC	Diethylpyrocarbonate
FACS	Fluorescence-Activated Cell Sorter
FCM	Fibroblast-Conditioned Medium
FITC	Fluorescein Isothiocyanate
HSC	Adult Human Schwann Cells
IL	Interleukin
LT	Leukotriene
MCCP	Mast Cell-Committed Progenitor
MMC	Mucosal Mast Cell
Nb-MLN	Mesenteric Lymph Nodes from Mice Infected 12-15 Days with *Nippostrongylus brasiliensis*
NF	Neurofibromatosis
NGF-R	Nerve Growth Factor Receptor

NGS	Normal Goat Serum
NRSC	Neonatal Rat Schwann Cells
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
PG	Prostaglandin
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PWM-SCCM	Pokeweed Mitogen-Stimulated Spleen Cell-Conditioned Medium
rmSCF	Recombinant Mouse Stem Cell Factor
RPMC	Rat Peritoneal Mast Cell
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Sl	Murine *Steel* Locus
TRSC	Transfected Rat Schwann Cells
W	Murine *White Spotting* Locus

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Abstract

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

John Joseph Ryan

Virginia Commonwealth University

Advisor: Thomas F. Huff,Ph.D.

Stem cell factor (SCF) is a recently characterized hematopoietic growth factor capable of stimulating the proliferation and differentiation of many hematopoietic cells, including the mast cell. We have cloned the gene encoding SCF from a cDNA library prepared from NIH 3T3 fibroblasts, and have characterized the ability of recombinant SCF to induce the development of mast cell-committed progenitors (MCCP), found in the mesenteric lymph nodes of mice infected with *Nippostrongylus brasiliensis* (*Nb*-MLN), but not naive mice. We have also examined Schwann cells, mast cells, and reproductive tissues for their ability to produce SCF.

We have found that recombinant SCF alone can promote the formation of mast cell colonies from MCCP in methylcellulose. SCF affected not only MCCP proliferation, but could also induce their differentiation to connective tissue

phenotype mast cells. SCF was also capable of generating mast cell colonies from precursors found in the peritoneal cavity of naive mice. Unexpectedly, infection of mice with *Nippostrongylus* caused a loss of the precursors from the peritoneal cavity by 14 days after infection.

Mast cells are found near Schwann cells, and increases in mast cells have been noted concomitant with Schwann cell neoplasia, and nerve damage and repair. Schwann cells from several sources were shown to produce mRNA for SCF, and the protein was also detected on the cell surface by immunofluorescent staining, or as a secretable product in cell-conditioned supernatant. Schwann cells were also analyzed for the ability to produce the receptor for SCF, c-*kit*. Immunofluorescent staining and polymerase chain reaction (PCR) analysis has shown that a human malignant schwannoma expresses c-*kit*, while normal human Schwann cells as well as SV-40 transfected, primary neonatal and primary adult rat Schwann cells do not. Thus normal production of SCF could form an autocrine growth loop in some Schwann cell neoplasias which aberrantly express c-*kit*.

Lastly, rat and human reproductive tissues were examined for expression of the SCF gene, using PCR and northern analysis. We found that SCF is expressed in a differential manner by various maternal and fetal tissues during pregnancy and the menstrual cycle. While rat tissues contained easily detectable SCF mRNA, human tissues appeared to express this gene in a lower quantity.

Introduction

Greater than 1 in 5 United States citizens suffers from some form of allergic disease (Bernstein and Lawrence, 1990), and many of these clinical phenomena have an associated mast cell hyperplasia. These include active inflammation, scleroderma, rheumatoid arthritis, keloid formation, neurofibromata, dense fibrosis, peripheral neuropathies, and posttraumatic nerve damage. Mast cell degranulation is also known to play an important role in asthma, allergic rhinitis, and atopic dermatitis. Furthermore, the proposed role of heparin in bone resorption implies a role for mast cells in rheumatoid arthritis, renal osteodystrophy, and postmenopausal osteoporosis. Treatment for these diseases often involves the use of drugs which affect mast cell function, such as antihistamines, corticosteroids, ß-adrenoreceptor agonists, and mast cell stabilizing drugs. Leukotriene antagonists are now being tested.

The mast cell is an excellent system for the study of cell differentiation, since the signals required for development are relatively few, and the changes that occur are both unique and large scale. Mast cells develop prominent cytoplasmic granules containing histamine, polysulfated proteoglycans, and unique neutral proteases. Jarboe and Huff have described a late stage, non-granulated mast cell-committed progenitor (MCCP) present in the mesenteric lymph node of mice infected with *Nippostrongylus brasiliensis* (*Nb*-MLN), but not naive MLN (Jarboe et al., 1989). This

progenitor could be stimulated by fibroblast-conditioned medium to develop mast cell colonies in methylcellulose in the absence of IL-3, and the factor present in this conditioned medium was deemed to be novel.

A recently cloned hematopoietic growth factor, stem cell factor (SCF), has been shown to induce mast cell development, function, and migration (Williams et al., 1990; Zsebo et al., 1990; Jozaki et al., 1991; Bischoff and Dahinden, 1992; Nakajima et al., 1992; Wershil et al., 1992). SCF is the ligand for the c-*kit* proto-oncogene product, a receptor tyrosine kinase, and was shown to be produced by Buffalo rat liver cells, a human fibrosarcoma cell line, and NIH 3T3 fibroblasts in either a membrane-bound or secretable form (Flanagan and Leder, 1990; Williams et al., 1990; Huang et al., 1990; Zsebo et al., 1990). However, its activities are not limited to mast cells, as SCF can induce the proliferation and/or development of murine CFU-S, erythrocytes, lymphocytes, megakaryocytes, melanocytes, and germ cells (reviewed in (Williams et al., 1992), and is expressed in the nervous system (Keshet et al., 1991). This wide range of activities described for SCF has made it a focal point in the study of hematopoiesis and the development of melanocytes, germ cells, and the nervous system. Since its identification in October, 1990, over 200 publications have shown SCF to play an important role in many forms of cell development.

We were interested in the ability of SCF to induce mast cell proliferation and differentiation from MCCP, and have sought to determine if SCF could substitute for fibroblast-conditioned medium. The availability of a recombinant molecule for studies of mast cell development using the MCCP would allow direct insight into the regulation of gene transcription during differentiation.

Mast cells have also been shown to reside in perineurial and epineurial spaces of peripheral nerves (Bienenstock et al., 1991), often in close proximity to the Schwann cells which myelinate these nerves (Isaacson, 1976). Moreover, mast cells increase dramatically in peripheral nerve injury and repair (Isaacson, 1976), and in Schwann cell neoplasias such as the lesions of neurofibromatosis (Isaacson, 1976; Riccardi, 1981). The origin and role of mast cells in these phenomena is poorly characterized. The localization of SCF transcripts in the nervous system during murine embryogenesis (Keshet et al., 1991), and the known role of SCF in mast cell development implied that this growth factor may be produced by neuron-associated cells, such as Schwann cells. We have examined normal, transformed, and malignant Schwann cells from rat and human sources for their ability to produce SCF and its receptor, c-*kit*. Since SCF and c-*kit* have also been shown to influence germ cell migration and development (Keshet et al., 1991; Horie et al., 1991; Manova et al., 1990; Sorrentino et al., 1991), we have also examined maternal and fetal tissues during embryogenesis in the rat and human.

Literature Review

I) The Mast Cell

The mast cell was first described in 1878 by Paul Ehrlich (Ehrlich, 1878), who named these granular, metachromatic cells from the German word *mastung*, meaning "to chew or masticate". Ehrlich thought the cells, which he obtained from frog mesentery, were overfed. Unna noted the increased presence of mast cells in urticaria pigmentosa skin lesions (reviewed in (Michels, 1938), and later these cells were shown to contain large amounts of histamine and heparin in their cytoplasmic granules. Wilander demonstrated the discharge of mast cell granules in 1939 (Reviewed in(Bernstein and Lawrence, 1990), and Riley and West showed a correlation between mast cells numbers in tissue and histamine content (Riley and West, 1953). The metachromatic staining of these cells was also determined to be due to the presence of heparin in their cytoplasmic granules.

However, research in mast cells made even greater strides in the 1960's. Mast cells were shown to degranulate with specific antigen after sensitization (Mota,I, et al., 1960), and the Ishizakas identified and characterized the sensitizing antibody as IgE (Ishizaka, K, et al., 1966). The Ishizakas also demonstrated the presence of high affinity receptors for IgE (FcϵRI) on the surface of mast cells and basophils, and its role in antigen-induced degranulation (Ishizaka, 1972)). In the same decade, Enerbach showed that two phenotypes of mast cells, connective tissue and mucosal,

existed (Enerback, 1966).

Mast cells are ovoid or irregular shaped, with a diameter of 9-12 μm and a large oval nucleus. Mast cell granules are easily apparent in the cytoplasm, with a diameter of 0.2-0.4 μm. Unlike other hematopoietic cells, mature mast cells are only found in tissues, often at sites of internal-external boundaries such as the nasal and bowel mucosa, alveolar wall, conjunctiva, dermis and around blood vessels (Schwartz and Huff, 1992). Although the mast cell was originally thought to be related to the basophil, which was also described by Ehrlich (Ehrlich, 1879), we now know that there are important differences between these two cells and that they have separate lineages (Schwartz and Huff, 1992). Both basophils and mast cells express FcϵRI, are metachromatic and contain histamine. However, while basophils are terminally differentiated, polymorphonuclear and can invade tissues or circulate in the blood, mast cells are capable of proliferation, are mononuclear, and are found exclusively in tissues. Furthermore, mast cells express neutral proteases and the cis-acting DNA binding proteins GATA-1,-2, and -3, which are not found in basophils (Martin and Orkin, 1990; Zon et al., 1991). Thus it is apparent that mast cells share some functional and staining characteristics with basophils, but the two cells are in fact quite different in their location and granule contents, and appear to be derived from separate progenitors.

Mast cell degranulation releases potent inflammatory mediators in a complex process that can be induced by many stimuli. The process can occur through cross linking of FcϵRI molecules on the cell surface, which activates phospholipase C, leading to the generation of inositol triphosphate and diacylglycerol. The combined effort of these compounds results in the mobilization of cytosolic and extracellular

calcium, and the activation of protein kinase C (PKC). The action of PKC ultimately results in the formation of the fusogens lysophosphatidic acid and lysophophatidylcholine, which allow granules to be released from the cell (Schwartz and Huff, 1991). Also, the initial activation of phospholipase C results in protein kinase A activation, which is important in histamine release. The breakdown of diacylglycerol leads to formation of arachidonic acid metabolites, which cause smooth muscle contraction, neutrophil chemotaxis, and mucus secretion (Bernstein and Lawrence, 1990; Katz et al., 1991). This cross linking of FcεRI molecules induces mast cell proliferation as well (Ashman et al., 1991; Plaut et al., 1989). Alternatively, mast cell degranulation can be induced through the action of many agents other than IgE immune complexes. These include IgG immune complexes, the complement components C3a and C5a, calcium ionophores, polylysine, polyarginine, compound 48/80, morphine sulfate, neuropeptides such as substance P, f-met peptides, and mellitin (Schwartz and Huff, 1991).

Minutes after degranulation, pruritis and urticaria occur, and a well-documented "wheal and flare" reaction appears. A late phase of these reactions also takes place 3 to 12 hours after the immediate phase. This phase is characterized by less edema but more pain than occurs in the immediate reaction and an influx of activated eosinophils (Plaut et al., 1989).

Mast cell mediators can be characterized into three classes: preformed, granule-derived mediators, membrane lipid-derived mediators, and cytokines (Katz et al., 1991). The preformed mediators found in mast cell granules are the biogenic amines histamine and serotonin, serine proteases such as chymase, tryptase, and carboxypeptidase A, acid hydrolases (Katz et al., 1991), and the proteoglycans

heparin and chondroitin sulfate. Histamine and serotonin induce vasodilation and spasmogenic reactions of smooth muscle, and can result swelling, pruritis, or difficulty breathing. The function of the neutral proteases is less well understood. Chymase is known to induce serous cell secretion, while tryptase enhances histamine-induced smooth muscle contraction. Carboxypeptidase A and acid hydrolases are degradative enzymes that can induce connective tissue damage. Heparin slows coagulation, and it and other proteoglycans can act as immunomodulatory agents.

Mast cell membrane lipid-derived mediators are also important in the inflammatory process. Leukotriene (LT) B_4 induces neutrophil chemotaxis, while LTC_4 causes smooth muscle contraction, as does prostaglandin (PG) D_2 (Katz et al., 1991). Platelet activating factor causes platelet and neutrophil activation.

Cytokine production by mast cells is a recent discovery (Plaut et al., 1989; Gordon et al., 1990), and has served to further the known role of mast cells in inflammation. IgE cross linking was shown to induce the production of the interleukins (IL) IL-1, IL-3, IL-4, IL-5, and IL-6, as well as granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN) gamma, transforming growth factor (TGF) ß, and tumor necrosis factor (TNF) α (Katz et al., 1991). This set of lymphokines resembles those produced by the T_H2 subset of T lymphocytes (Plaut et al., 1989). Furthermore, these lymphokines are well suited for a role in allergic responses: IL-1 induces lymphocyte activation; IL-3 is important in stem cell and mast cell development; IL-4 induces IgE production and mast cell maturation; IL-5 enhances eosinophil differentiation; and IL-6 plays an important role in B cell development (Katz et al., 1991). Furthermore, GM-CSF induces myeloid cell development, and TNFα induces neutrophil chemotaxis (Katz et al., 1991). Taken

together, these cytokines enhance the production and differentiation of cells important in the allergic response, while inducing production of IgE antibody, and induce the chemotaxis of cells with a known role in inflammation.

Mast cells were shown to develop from bone marrow stem cells in a complex series of experiments using the mast cell deficient mice $WBB6F_1$-W/W^v. These mice were shown to be repopulated with mast cells after transplantation with normal syngeneic or semi-syngeneic bone marrow (Galli, 1990). It is now understood that mast cells leave the bone marrow in an immature form and migrate through the blood to mucosal and connective tissues, where they complete their differentiation. The signals that instruct mast cell development are not fully understood, but are known to involve IL-3, IL-4, and the recently described product of the mouse *Steel* (*Sl*) locus, stem cell factor (SCF) ((Schwartz and Huff, 1992). While mouse mast cells can be derived from cultures of bone marrow grown with IL-3 alone, human mast cells do not express IL-3 receptors, and may rely solely on SCF for their development (Schwartz and Huff, 1992). The lineage that gives rise to mast cells has not been fully characterized, but will be discussed below.

Ginsburg suggested that there were two subsets of mast cells, T cell dependent and T cell independent (Kishimoto et al., 1979). These are now known by a variety of names: IL-3 dependent and IL-3 independent, as called mucosal (MMC) and connective tissue (CTMC) mast cells in the mouse, and MC^T and MC^{TC} in the human (Schwartz and Huff, 1992). Although there are some interspecies differences, the murine MMC and CTMC share many features with the human MC^T and MC^{TC}, respectively (Schwartz and Huff, 1991).

The two phenotypes of mast cells differ in their size, life span, staining

characteristics, and granule contents. MMC stain with alcian blue but not safranin, are smaller, have fewer granules and less histamine than CTMC, produce chondroitin sulfate E proteoglycan, and LTC_4, and have a life span of about six weeks that is dependent upon IL-3 stimulation. MMC mast cells also express the mouse mast cell proteases (MMCP) 1, 2, and 4 (Gurish et al., 1992; Reynolds et al., 1990). By comparison, CTMC stain with alcian blue and safranin, are larger, have more granules and more histamine, produce heparin proteoglycan and PGD_2, and have a life span of at least six months in the absence of IL-3 stimulation. CTMC express MMCP-3, 4, 5, and 6 (Gurish et al., 1992; Reynolds et al., 1990). Mouse CTMC are also sensitive to compound 48/80 and other non-IgE stimuli to which MMC do not respond. While MMC are found in the lamina propria, crypts, and between columnar epithelial cells, CTMC are located in dermis, in fibrous capsules of many organs, and in peritoneal fluid. Human mast cells are less well understood. The most noted difference in human mast cell subsets is the expression of the serine protease chymase (Schwartz and Huff, 1991). While all human mast cells appear to express tryptase, chymase is expressed by mast cells found in greatest numbers in skin and intestinal submucosa, while those that express only tryptase are located primarily in the lung and intestinal mucosa (Irani et al., 1987). While there do not appear to large differences in histamine content, heparin synthesis, or arachidonic acid metabolites, MC^{TC} cells respond to compound 48/80, while MC^{T} cells cannot, mimicking the responses of CTMC and MMC, respectively. Also, immunodeficient patients who lack IL-3 have been shown to lack MC^{T}, and have normal MC^{TC} (Irani et al., 1987). Thus while there are differences between species, it is thought that MC^{T} are similar to MMC, while MC^{TC} resemble CTMC. In the murine system it is

apparent that both MMC and CTMC are derived from a common bone marrow progenitor, since normal bone marrow injected into W/W^v mice gives rise to both phenotypes, and culture-derived mast cells can also give rise to MMC or CTMC (Kanakura et al., 1988). Some data suggest that Phenotypic changes can occur bidirectionally multiple times, and appear to depend upon the microenvironment in which the mast cells are placed (Kanakura et al., 1988).

II) *Nippostrongylus brasiliensis* Infection and the Generation of Mast Cell-Committed Progenitors

It has long been known that infection of mice with the rodent hookworm *Nippostrongylus brasiliensis* (*Nb*) results in a mucosal mastocytosis and concomitant increase in IgE production (Befus and Bienenstock, 1984). Therefore this infection has been used to study the roles and development of mast cells. Subcutaneous injection of the third larval form of *Nb* into mice or rats is followed by migration of the larvae to the lungs within 15 hours, where they molt to the fourth stage and migrate to the small intestines via the trachea and esophagus by day 3 (Ogilvie and Jones, 1971). Here the larvae molt once more to the fifth, or adult stage. The worms reach a length of 2-6mm, and begin to produce eggs by day 6 (Ogilvie and Jones, 1971). Only about 55% of the worms injected reach maturity, but each adult female worm can produce up to 1000 eggs a day for a period of about six days, at which time the worms are usually expelled from the intestines (Ogilvie and Jones, 1971). The worms remain in the jejunum until expulsion, and although anemia is common during infection, they do not suck blood from their host (Ogilvie and Jones, 1971). The immune response to *Nb* infection results in an influx of leukocytes to the mesenteric lymph node. These include plasma cells, lymphocytes, mononuclear cells,

macrophages, eosinophils, and a notable mastocytosis (Ogilvie and Jones, 1971).

Bone marrow progenitors are known to traffic to the mesenteric lymph node (MLN) during infection with *Nb* (Ashman et al., 1991), and the mast cell hyperplasia coincident with infection is thought to be the result of both progenitor trafficking and local proliferation (Keller et al., 1974). However, while mast cells increase from their initially low numbers in the lamina propria and small intestine muscularis and submucosa during infection (Ogilvie and Jones, 1971; Arizono and Nakao, 1988), the peak in mast cell numbers seems to follow expulsion of *Nb* (Woodbury et al., 1984), with a small increase at day 5 and a large peak at day 15-17 (Keller et al., 1974; MacDonald et al., 1980). However, it has been noted that blood levels of mast cell-specific rat mast cell protease II (RMCP II) peak at day 10, coinciding with a decrease in fecal egg counts and the expulsion of *Nb* (Woodbury et al., 1984). Rats are resistent to reinfection, and mast cell numbers rapidly peak at day 5-6 (Ogilvie and Jones, 1971). Thus *Nb* infection and the associated mastocytosis is a reliable model system in which to study mast cell development and function.

In addition to the increase in mast cells in the mesenteric lymph node, findings from the laboratory of William Paul have indicated that *Nb* infection may result in an increase in mouse mast cell and basophil precursors in the bone marrow and spleen. These progenitors, characterized by a lack of B and T cell markers, consist of 1-5% FcERI positive cells, and could produce IL-3 and IL-4, after activation with plate-bound IgG and IgE (Ben Sasson, et al., 1990). FACS analysis of this population demonstrated that the IL-4 producing capabilities could be segregated with the FcERI positive cells (Seder, et al., 1991a). Histochemical and elctron microscopic analysis showed these cells to have basophilic cytoplasm,

histamine-containing granules, and the majority appeared basophil or basophil-myelocyte morphology (Seder, et al., 1991b). This population also contained 3-4% mast cell precursors, and methylcelluolose culture of these cells with IL-3 gave a mixture of mast cell and basophil colonies (Seder. et al., 1991b). Thus *Nb* infection and the associated mastocytosis appears to be a reliable system in which to study mast cell development and function.

Several groups have demonstrated the ability of mast cells to be maintained on fibroblast monolayers, and these initial results indicated that mast cell proliferation on these monolayers was contingent upon direct contact with the fibroblasts (Jarboe et al., 1989). Jarboe, et al have described a cell termed the mast cell committed progenitor which can be obtained from the MLN of mice infected for 14 days with *Nb* (*Nb*-MLN), but not naive mice (Jarboe et al., 1989). This committed progenitor was shown to proliferate and differentiate on either embryonic skin or 3T3 fibroblast monolayers, which do not produce IL-3 or IL-4, and could develop into mature CTMC (Jarboe et al., 1989). The cell was characterized as a nongranulated progenitor with a density of 1.060 to 1.070 g/cc (Jarboe et al., 1989), and is thought to be more mature than the bone marrow mast cell progenitor (Ashman et al., 1991). Commitment of this cell to the mast cell lineage was determined by its inability to yield other colony types in response to known growth factors, and the fact that culture of this cell with concentrated fibroblast-conditioned medium (FCM) or a source of IL-3 yielded almost 100% mast cells (Jarboe et al., 1989). The activity of FCM in this system was in direct contrast to the results of other workers, and was the first report of mast cell progenitor development in culture in the absence of IL-3 or direct contact with fibroblasts (Jarboe et al., 1989). Also,

since normal bone marrow was able to generate mast cell colonies in response a source of IL-3 but not in response to fibroblast-CM, the committed progenitor was judged to be more mature than bone marrow mast cell progenitors (Jarboe et al., 1989).

Since naive MLN cells were not active in the methylcellulose mast cell progenitor assay using concentrated fibroblast-CM, the committed progenitor was thought to arise due to the unique microenvironment present in the *Nb*-MLN (Jarboe et al., 1989). Further work by Ashman, et al. characterized this microenvironment and recreated *in vitro* the stimuli sufficient to yield committed progenitors from normal bone marrow by determining the ability of *Nb*-MLN cells to produce cytokines involved in mast cell development during *Nb* infection. This was accomplished by stimulating *Nb*-MLN cells with *Nb* exosecretory antigen, and assaying culture supernatants (Ashman et al., 1991). It was shown that IL-3 and IgE peaked on day 11 of infection, three days before the peak in committed progenitor development (Ashman et al., 1991). IL-4 production by *Nb*-MLN cells peaked at day 8 of infection (Ashman et al., 1991). These stimuli are known for their ability to enhance mast cell development and proliferation. IFN-γ, which inhibits mast cell proliferation, peaked at day 14, and may play a role in suppressing progenitor development (Ashman et al., 1991). Most importantly, normal bone marrow was cultured in conditions mimicking the *Nb*-MLN, and it was shown that stimulation with IL-3 and IgE immune complexes yielded cells which could now respond fibroblast-CM alone (Ashman et al., 1991). IgE immune complexes alone could even generate committed progenitor cells from normal bone marrow in the absence of IL-3, possibly by inducing autocrine IL-3 production by these cells (Ashman et al., 1991).

According to this data, the mast cell lineage contains a committed progenitor which is more mature than the bone marrow mast cell progenitor but is still nongranulated. This cell can be generated from normal bone marrow through IgE receptors.

No known growth factor was able to mimic the activity of fibroblast-CM (Jarboe et al., 1989). In an effort to characterize this factor and the committed progenitor, mast cell deficient mice were examined for their activity in the committed progenitor assay. In this manner it was found that fibroblast-CM from W/W^v, but not Sl/Sl^d mice was active in the assay. Conversely, *Nb*-MLN cells from Sl/Sl^d but not W/W^v mice were able to respond to fibroblast-CM, while both populations could respond to sources of IL-3 (Jarboe and Huff, 1989). It was therefore apparent that mice of the W/W^v strain lacked the ability to form committed progenitors, but could produce uncommitted progenitors as well as the fibroblast factor, while mice of the Sl/Sl^d genotype had the reverse mutation. Because the W locus has been demonstrated to encode the receptor tyrosine kinase c-*kit* (Chabot et al., 1988; Geissler et al., 1988), which is expressed on *Nb*-MLN cells or developing committed progenitors cultured in FCM express c-*kit*, but not naive MLN (Leftwich et al., 1992), c-*kit* expression is likely to play an important role in committed progenitor development. This theory was given further credence when Leftwich, et al. showed that anti-sense oligonucleotides to c-*kit* were shown to block committed progenitor development in response to fibroblast-CM, but not PWM-SCCM (Leftwich et al., 1992).

III) The *W* Allele and c-*kit* Receptor Tyrosine Kinase

Mutations in the murine White Spotting (*W*) allele on chromosome 5 are

known to cause a phenotype that includes defects affecting coat color, hematopoiesis, and fertility (Geissler et al., 1988). This same set of developmental defects is seen in mice with mutations at the Steel (*Sl*) locus (Bernstein et al., 1990), indicating a possible relationship between these two genes. These cell populations all require extensive migration during their development, and as such the *W* and *Sl* loci have been postulated to be function in cell-cell contact and migration.

An intricate series of experiments by Kitamura demonstrated the relationship between *W* and *Sl* (reviewed in (Bernstein et al., 1991)). It was shown that while normal bone marrow could be transferred to W/W^v mice and cure their hematopoietic defect, W/W^v bone marrow could not rescue irradiated normal animals. Conversely, Sl/Sl^d bone marrow could cure W/W^v mice or rescue normal irradiated mice, but adoptive transfer of normal bone marrow to Sl/Sl^d mice did not correct their defect (Bernstein et al., 1991). From these experiments it was inferred hat the *W* mutation affected a progenitor cell, while *Sl* affected the tissue microenvironment.

The receptor tyrosine kinase (RTK) *c-kit* was isolated due to its homology to the retroviral *v-kit* sequence, which induces feline sarcomas (Qiu et al., 1988). The characterization of this gene lead to its being linked with the *W* locus. Linkage to *W* was inferred from research showing *kit* expression in populations affected by *W* mutations, a decrease in c-*kit* message and protein in *W* mutants, and rearrangements of the c-*kit* gene in *W* mutants (Bernstein et al., 1990). Interspecific backcross analysis and somatic cell hybrid analysis showed that the *c-kit* proto-oncogene is encoded by the *W* locus (Chabot et al., 1988; Geissler et al., 1988), and as will be

described in detail below, the ligand for this proto-oncogene is the product of the *Sl* allele.

Mouse c-*kit* is known to encode a 975 amino acid glycoprotein with a 21 amino acid hydrophobic signal sequence, 518 amino acid extracellular domain that comprises 5 immunoglobulin-like repeats, a 24 amino acid transmembrane domain, and a large cytoplasmic tail that encodes 461 amino acids, making up a tyrosine kinase domain ((Qiu et al., 1988). The RNA encoding this structure is approximately 5.5 kb in the mouse (Qiu et al., 1988). Although the predicted molecular weight of the protein is 109 kDa, there are nine potential sites for N-glycosylation (Majumder et al., 1988). Antibodies raised against the *v-kit* protein have been used to detect *kit* expression in various tissues, demonstrating the size of the *kit* protein to range from 145-160 kDa (Majumder et al., 1988). c-*kit* has been mapped to the long arm of human chromosome 4 (4q31-34), a region that encodes genes similar to the murine *W* locus, including phosphoglucomutase-2, alpha-feto protein, and albumin (Qiu et al., 1988; Chabot et al., 1988).

The *v-kit* protein is 95% homologous to its cellular counterpart at the amino acid level (Qiu et al., 1988). However, this acute transforming retrovirus encoded by the Harvey-Zuckerman 4-feline sarcoma virus (HZ4-FeSV) produces an 80 kDa *gag-kit* fusion protein that lacks extracellular and transmembrane domains, and has tyrosine kinase activity (Bernstein et al., 1990; Majumder et al., 1988). The structure of HZ4-FeSV is known to be: 5' *gag-kit-pol-env* 3' (Qiu et al., 1988).

One of the most interesting findings about c-*kit* structure has been its homology to the M-CSF and PDGF receptors, both of which are RTKs (Majumder

et al., 1988; Qiu et al., 1988). All three RTK's have 5 Ig-like repeats (Bernstein et al., 1990), a transmembrane domain, and a cytoplasmic tyrosine kinase that is split into a tripartite structure, with the ATP binding and tyrosine kinase domains separated by a 70-100 amino acid interkinase domain which is thought to function in substrate recognition (Majumder et al., 1988; Qiu et al., 1988). The overall structures of the three proteins are similar on the extracellular domain as well; in each, four of the five Ig-like domains have paired cysteine residues (Majumder et al., 1988). However, the amino acid homology is 20-26% between the three, and the interkinase domains have little homology, probably owing to their recognition of unique substrates (Qiu et al., 1988).

RTK-mediated signalling is generally comprised of four events: receptor-ligand binding, receptor clustering, internalization of the receptor-ligand complex via clathrin-coated pits, and activation of the tyrosine kinase (Qiu et al., 1988; Majumder et al., 1988). The internalization of the receptor results in its degradation and subsequent down-regulation (Majumder et al., 1988). c-*kit* has been shown to undergo autophosphorylation in an *in vitro* kinase assay (Majumder et al., 1988), and the level of kinase activity is related to the severity of *W* mutations in the mouse (Majumder et al., 1988). Mutations which completely abolish kinase activity are lethal in their homozygous form (Majumder et al., 1988), however in heterozygous form, these mutations tend to be less severe than mutations which do not completely abrogate *kit* function (Bernstein et al., 1991). This initially perplexing finding was understood only after the revelation that *kit* receptors must dimerize to transduce a signal (Lev et al., 1991; Williams et al., 1992). Dimerization would be hindered by the presence of mutant, nonfunctional *kit* molecules; however a mutation which

abolishes *kit*, while lethal in homozygous form, would not produce defective molecules to interfere with dimerization in the heterozygous state. The finding that Kit functions as a dimer has lead some to postulate that what has been noted as autophosphorylation of receptors is actually trans-phosphorylation (Williams et al., 1992).

Both the PDGF and MCSF receptors, which share *kit* structure, have been shown to associate with phosphoinositol 3' kinase (PI3K) via their kinase insert domains following ligand binding (Lev et al., 1992). PI3K is comprised of 85 kDa and 110 kDa fragments (Lev et al., 1992). The 85 kDa portion contains two *src*-homology domains, which are thought to play a role in association of tyrosine kinases and their substrates (Lev et al., 1992). Studies have shown an association of the 85 kDa PI3K fragment with *kit* after SCF stimulation (Lev et al., 1991; Lev et al., 1992), which is coupled with *kit* autophosphorylation (Lev et al., 1992). Furthermore, site-directed mutagenesis of the kinase insert domain decreases it association with PI3K, as well as PI3K activity and receptor autophosphorylation (Lev et al., 1992). However, although PI3K is responsible for generating inositol phosphates, EKR stimulation did not result in detectable changes in inositol phosphate metabolism (Lev et al., 1991). Use of the EKR chimeric protein showed that phoshpolipase C (PLC) gamma, another mediator of inositol lipid metabolism, and the serine/threonine kinase *Raf*1 are both tyrosine phosphorylated following receptor-ligand interaction (Lev et al., 1991). Thus it appears that c-*kit* can associate with several second messenger molecules, but the effects on these molecules has not been determined.

Although murine *W* mutations affect the hematopoietic, germ cell and melanocytic lineages (Russell, 1979), studies of *kit* expression have linked it to an even more diverse group of tissues. Initial studies using a monoclonal antibody raised against the *v-kit* P80 protein demonstrated *kit* expression not only in the expected organs of spleen and testis, but a complex pattern of labelling was also seen in the brain (Majumder et al., 1988). Kit protein has also been detected in human bone marrow, peripheral blood, and fetal liver (Papayannopoulou et al., 1991), as well as mast cells and bone marrow mononuclear cells (Lerner et al., 1991). Almost all *in vitro* derived clonogenic progenitors from bone marrow and CFU-S express Kit (Williams et al., 1992). Slot blot analysis of feline RNA demonstrates *kit* expression in bone marrow, thymus, spleen, liver, lymph node, kidney, lung, and brain (Qiu et al., 1988). Still other studies have shown *kit* expression in melanocytes and placenta (Bernstein et al., 1990) and the erythroleukemic cell lines HEL and LAMA (Ikeda et al., 1991). However, *kit* is not expressed in the lymphoid cell lines HL60, KG1, and U937, or non-erythroid myeloid cell lines (Ikeda et al., 1991; Bernstein et al., 1990). *In situ* hybridization studies in the developing mouse embryo have shown a lack of *kit* expression prior to day 9 of gestation, followed by expression in brain, neural tube, and liver on day 11.5; melanocyte precursors demonstrate *kit* expression by day 13.5 (Keshet et al., 1991). Although *W* mutant mice are almost completely devoid of mast cells, levels of *kit* message do not appear to differ between W/W^{v} and normal mice (Bernstein et al., 1991); however this may only indicate that transcription of the mutant allele is not affected, while the protein is nonfunctional.

The expression of c-*kit* in primordial germ cells could explain the infertility

associated with many *W* mutations. Accordingly, recent findings have shown that expression of c-*kit* on spermatogonia correlates with germ cell proliferation in the mouse (Manova et al., 1990; Sorrentino et al., 1991). Kit has also been demonstrated on murine oocytes, with a decrease in protein levels concomitant with ovulation (Horie et al., 1991; Manova et al., 1990). The present findings indicate a role for the early development and migration of both male and female germ cells. This is in agreement with the lack of migration of these progenitors seen in many *W* mutants.

Since *W* mice have obvious coat color defects, it was logical to examine melanocytes for Kit expression; in fact human melanocytes have been shown to express Kit, and proliferate in response to SCF stimulation (Funasaka et al., 1992). Studies using EKR also showed this chimera to be tyrosine phosphorylated in melanocytes following ligand binding (Funasaka et al., 1992). Although Kit appeared to be upregulated in some melanoma cell lines examined, the Kit receptor was not autophosphorylated, as would be expected if it were constituitively activated (Funasaka et al., 1992). Also, SCF had no activity on primary nodular or metastatic melanomas that proliferate in the absence of exogenous factors (Funasaka et al., 1992). From these results it appears that Kit functions in normal melanocyte development, but may not play a role in melanoma formation.

While *W* mutants have not shown any obvious neurological deficits (Russell, 1979), *kit* and its ligand are visible at the earliest stages of neural differentiation, and there is a complex expression of both receptor and ligand in developing mouse brain, as detected by *in situ* hybridization (Keshet et al., 1991). Also, the outer boundary of the developing neural tube is surrounded by *kit*-expressing cells, which appear to

be migratory neural crest cells (Keshet et al., 1991). This has lead some to propose a role for the SCF-Kit complex in axon guidance during fetal development.

Some data suggest that c-*kit* may play a role in some leukemias and solid tumors. An increase in tyrosine kinase levels, or the expression of retroviral tyrosine kinases have previously been linked to neoplastic phenomena: *c-erbB2* is increased in mammary carcinomas and NIH 3T3 cells, while *v-erbB* induces avian erythroblastosis (Majumder et al., 1988). Since *W* mice are known to have decreased CFU-spleen, BFU-erythrocyte, and CFU-erythrocyte (Geissler et al., 1988), initial investigations have examined the expression of c-*kit* in hematopoietic cells. Monoclonal antibody YB5.B8 was raised against the blast cells of an acute myelogenous leukemia (AML), and this antibody has been demonstrated to recognize Kit on the surface of these cells (Lerner et al., 1991). Another anti-c-*kit* monoclonal antibody, SR-1, recognizes Kit expressed on the erythroleukemia cell line OCIM1 (Broudy et al., 1992). Kit has also been detected on the blasts cells of acute nonlymphoblastic leukemia (Ikeda et al., 1991). The strongest link between Kit expression and neoplasia appears to be AML, since *kit* transcripts have been noted in 20 of 25 AML patients examined (Ikeda et al., 1991). Cells extracted from AML patients showed 7 of 12 populations had autophosphorylation of Kit in the absence of stimulation, and 6 of 11 populations could proliferate in the presence of rmSCF. However, Kit expression did not appear to correlate to French-American-British classifications or clinical symptoms (Ikeda et al., 1991). In a non-hematopoietic neoplasia, *kit* expression was detected in 81% of small cell lung cancer cell lines and 12 of 13 tumors tested (Sekido et al., 1991).

A human equivalent to the *W*-encoded mutations in melanocyte development has apparently been found in the autosomal dominant disorder piebaldism. This disease results in the complete absence of melanocytes, and a subsequent lack of pigmentation, in patches of hair and skin, particularly on the forehead, chest, abdomen, and extremities (reviewed in (Spritz et al., 1992)). This trait has mutations associated with it at chromosome 4q12; human c-*kit* is located on chromosome 4q11-12 (Spritz et al., 1992). Recently, missense and frameshift mutations in the *kit* tyrosine kinase domain resembling the W^{57} allele in the mouse were found in 3 patients with piebaldism. The other defects in hematopoiesis and fertility associated with the *W* allele have not been noted in piebald individuals (Spritz et al., 1992). These findings add more credence to the proposed role for Kit in melanocyte migration and development.

IV) Stem Cell Factor and the *Sl* Allele

A dramatic series of papers in the October, 1990 issue of *Cell* reported the cloning of the *Sl* locus. These showed that the *Sl* gene encodes a glycoprotein growth factor which is now referred to as Stem Cell Factor (SCF) (Huang et al., 1990), Mast Cell Growth Factor (Williams et al., 1990), Steel Factor (Williams et al., 1992), or Kit Ligand (Huang et al., 1990). These publications also demonstrated that SCF was in fact the ligand for the c-*kit* proto-oncogene product (Flanagan and Leder, 1990; Zsebo et al., 1990; Williams et al., 1990; Huang et al., 1990), proving a hypothesis put forth by several investigators. Murine SCF maps to the distal region of chromosome 10, and is deleted in some *Sl* mutants (Copeland et al., 1990), while human SCF maps to chromosome 12q14.3-12qter (Geissler et al., 1991). The message encoding

mouse SCF is about 6kb (Zsebo et al., 1990). Although the active material isolated from BRL-3A cells migrated at 28-35 kDa, it could form noncovalent dimers of 70-90 kDa (45), which correlates to the data above showing the necessity of dimer formation for Kit function. SCF is a glycoprotein composed of 248 amino acids arranged into extracellular, transmembrane and cytoplasmic domains (Williams et al., 1992). The external domain is comprised of 164 amino acids, and is preceded by a 25 amino acid signal peptide, which is cleaved during processing. The extracellular domain includes a 12-28 amino acid membrane spacer region and a membrane tether portion. A proteolytic cleavage site within the membrane spacer encodes a protease recognition site, which allows cleavage of the SCF molecule to yield a secreted form. The transmembrane domain is followed by a short cytoplasmic tail, which possesses no known enzymatic activities (Williams et al., 1992). The glycoprotein contains 4 sites for N-glycosylation, 3 for O-glycosylation, and is, in fact, heavily glycosylated, as $rmSCF^{164}$ has a predicted molecular weight of 18 kDa but migrates at about 30 kDa (Lu et al., 1991). The Kit receptor binds SCF with an affinity of $K_D = 3 \times 10^{-8}M$ (Flanagan and Leder, 1990).

The overall domain architecture, dimerization, and proteolytic cleavage of SCF is similar to M-CSF (Bazan, 1991). The two growth factors have 16% amino acid identity, with 32% conserved amino acids, and a homologous three dimensional structure that includes the membrane spacer region and two disulfide bonds in the extracellular domain (Bazan, 1991). Other growth factors are known to be produced in membrane-bound or secreted forms, including TGFα, EGF, and TNFα (Flanagan et al., 1991).

The observation that SCF can be produced as either a membrane-bound or

secreted growth factor was not completely unexpected. Jarboe, et al. has reported the ability of Balb/c 3T3 fibroblast-conditioned medium to support the development of mast cell progenitors, in the same manner that others had shown fibroblast monolayers to induce mast cell differentiation (Jarboe et al., 1989). It is now known that alternative splicing of exon 6 determines the ability to produce either membrane or secreted SCF (Williams et al., 1992). This exon encodes a 28 amino acid segment of the protein that includes the protease recognition site; in its absence, only membrane SCF can be made. The expression of the protease recognition site results in about a 1:1 ratio of secreted and membrane SCF (Flanagan et al., 1991). The full length SCF transcript has been termed KL-1, and the truncated, membrane-only form KL-2 (Flanagan et al., 1991). Either form is effective in mediating cell-cell adhesion as a membrane protein (Flanagan et al., 1991). Truncation of SCF can be easily detected by the size of the protein, since secreted SCF has a molecular weight of 25 kDa, and membrane SCF a molecular weight of 38 kDa (Flanagan et al., 1991). The ratio of KL-1 to KL-2 has been shown to vary greatly in tissues, from 5:1 to 0.6:1 (Flanagan et al., 1991).

The different forms of SCF can explain the results of Jarboe, et al., since Balb/c 3T3 fibroblasts have been shown to produce large amounts of KL-1. Thus conditioned medium from these cells would contain much secreted SCF (Flanagan et al., 1991). However, it is apparent that membrane SCF may have a more important *in vivo* role than secreted SCF. Transfer of normal spleens to Sl/Sl^d mice resulted in a local curing of their hematopoietic deficit only; no systemic effects were seen. Also, creation of cell lines which could produce either membrane bound or secreted SCF transcripts only showed that membrane bound alone was capable of

supporting longterm hematopoiesis. Furthermore, *in vitro* expansion of primordial germ cells required the presence of membrane bound, and not secreted SCF. Finally, soluble rmSCF could only support *in vivo* hematopoiesis in large doses (Williams et al., 1992). Thus it appears that membrane-bound SCF may be more relevant to *in vivo* cell development.

The most dramatic evidence of the important role membrane bound SCF plays in development and migration may be yielded from studies of the *Sl/Sl*^{d} mice, as reviewed by (Williams et al., 1992). These mice have a diluted coat color, macrocytic anemia, are sterile, and like other *Steel* mutants, they cannot be cured by bone marrow transplantation (Russell, 1979; Williams et al., 1992). However, initial studies showed these mice to contain message for SCF. Southern blot analysis showed *Sl*^{d} message to contain a 4kb deletion, resulting in a net loss of 175bp of translated sequence. Importantly, this deleted region encodes the transmembrane and cytoplasmic domains of SCF. Therefore, these mice can produce only the secreted form of the growth factor. Furthermore, this form has been shown to be active (Flanagan et al., 1991; Brannan et al., 1991). The fact that these mice can produce secreted SCF, yet still have the pleiotropic abnormalities associated with *Sl* mutations implies that membrane-bound SCF is critical for progenitor development.

The designation of the *Sl* gene product as a "stem cell factor" has been drawn from its ability to induce the proliferation of many hematopoietic lineages, often acting at early stages in development. SCF has been shown to induce the proliferation of murine CFU-S alone, and can synergize with IL-1 and IL-3 (de Vries et al., 1991). As stated earlier, almost all *in vitro*-derived hematopoietic progenitors

have been shown to express Kit, with the exception of IL-7-responsive cells (Ogawa et al., 1991). Also, while SCF has been shown to synergize with many known growth factors in the delta assay used by Moore and Warren, there is no evidence that SCF can support the self renewal of hematopoietic stem cells (reviewed in (Williams et al., 1992)). Therefore the actual ability of the *Sl* product to act as a "stem cell factor" may be somewhat overstated. However, the ability of SCF to act upon such a wide range of progenitor cells makes the possible clinical uses of SCF both diverse and important.

Since both *W* and *Sl* mutants have deficiencies in erythropoiesis, the effect of SCF on erythrocyte development has been examined in depth. SCF has been shown to synergize with erythropoietin (Epo), enhancing colony number and size from BFU-E and CFU-E, and may in fact be required for BFU-E differentiation to CFU-E (Williams et al., 1992). Also, SCF can correct the anemia of Sl^d mice and induce baboon erythropoiesis (Andrews et al., 1991). In erythrocytic diseases, SCF has been shown to enhance erythroid colony formation from Diamond-Blackfan anemic bone marrow, and can increase fetal hemoglobin in BFU-E from sickle cell anemia patients (Williams et al., 1992).

SCF also has an important role in mast cell development and function. Both culture-derived and peritoneal mouse mast cells have been shown to proliferate in response to SCF from 3T3 fibroblasts (Jozaki et al., 1991), and human mast cells can be derived by culturing human cord blood in SCF (Williams et al., 1992). SCF has been shown to induce mast cell chemotaxis (Meininger et al., 1992). Histamine release from rat or human mast cells can be stimulated by SCF as well, and some studies suggest the ability of SCF to induce mast cell-dependent acute inflammation

is 10 fold greater than substance P on a molar basis (Bischoff and Dahinden, 1992; Nakajima et al., 1992; Wershil et al., 1992). Injections of SCF have also proven to induce mast cell degranulation and associated respiratory distress in mice (Williams et al., 1992). From these studies it is apparent that SCF is important in mast cell development, maintenance, and function.

Although *Steel* mice have no obvious T or B lymphocyte defects (Russell, 1979), SCF has been shown to influence the development of lymphocyte precursors. SCF can promote the proliferation of both murine $B220^+$ and $B220^-$ cells; however as these cells mature, they lose SCF-responsiveness, and SCF has no effect on mature B lymphocytes (Williams et al., 1992). In the T cell system, SCF transcripts have been detected in several thymic stromal cell lines, and SCF can enhance the proliferation of thymic lobe cells, especially in the presence of IL-7 (Williams et al., 1992). Synergism of SCF with IL-2 or IL-7 to increase the growth of $CD4^-8^-$ cells in semisolid culture has also been noted, but as with B cells, SCF has no effect on mature peripheral T lymphocytes (Williams et al., 1992).

Steel mutant mice are also not known to have mutations in megakaryocytopoiesis, however there is data to support a role for SCF in development of this lineage. SCF has been demonstrated to synergize with a number of cytokines to increase colony formation from BFU-MK and CFU-MK (Briddell et al., 1991). Although SCF can act at many stages of megakaryocyte maturation, no consist difference in platelet levels has been shown in *Sl* mice (Williams et al., 1992). Thus the role of SCF in megakaryocyte development is not fully understood.

One of the most obvious mutations in *Steel* mice is their pigmentation abnormalities. Since melanocytes are migrating neural crest cells, studies have

examined the effect of SCF on the migration and proliferation of these cells and their precursors (Williams et al., 1992). Kehet, et al. published data showing a probable chemotactic role for SCF in the migration of melanoblasts during mouse development. These cells appeared to migrate along a SCF gradient, and melanoblasts were shown to express c-*kit* (Keshet et al., 1991). However, expression of SCF and its receptor appeared to decrease with melanocyte development (Keshet et al., 1991), and SCF can induce melanocyte proliferation only in the presence of phorbol esters; alone it supports their survival, and does not have any effect on melanin production (Williams et al., 1992). However, injections of anti-Kit antibodies into pregnant and non-pregnant mice showed that this antibody could modulate coat color in not only the developing fetus, but adult mice as well. This fits well with the data discussed above concerning the expression of c-*kit* and human piebaldism. The absence of SCF could prevent the proper migration of melanocyte progenitors, leaving the affected areas devoid of pigment. Thus it is obvious that SCF plays an important part in melanoblast migration and development, but its role in mature melanocyte function needs further study.

The sterility of *Steel* mice also indicates an importance of SCF in germ cell development. In fact, *in situ* hybridization studies in the developing mouse have shown that primordial germ cells, which express Kit, may migrate along the genital ridge using a SCF gradient, much as the data presented for melanoblast migration has demonstrated (Keshet et al., 1991). SCF was shown to be expressed in the genital ridges in correlation with germ cell colonization, and expression ceases after colonization. In the adult animal, SCF is expressed in the granulosa cells surrounding the oocytes, and the oocytes are known to express Kit (Keshet et al.,

1991). This pattern of SCF expression could explain the observed sterility of *Steel* mice.

Lastly, as stated above for c-*kit* expression, *Steel* mice are not known to have any obvious neurological abnormalities (Russell, 1979). But as has been found for its receptor, SCF is expressed in and around the developing nervous system, and may function in neuron organization. *In situ* hybridization studies show SCF expression in neural floor plate early in gestation (Day 9.5), and later is expanded to include the ventrolateral regions, which are associated with motor neuron differentiation (Keshet et al., 1991). Recent experiments have indicated a role for the neural floor plate in organization of motor neuron tracts (Reviewed in (Keshet et al., 1991)). The *in situ* study also demonstrates *kit* expression in differentiating neural tubes and around its outer boundary, leading the authors to speculate that SCF may function as a chemoattractant for neurons or axons. By day 14.5, the forming motor neuron columns demonstrate SCF expression on neurons and cells in the ventral marginal zone that appear to be migrating toward the developing motor columns (Keshet et al., 1991). However, the expression of SCF in developing nervous tissue differs from that seen in the development of the germ cell pathway. While germ cell development appears to follow a SCF gradient, SCF is expressed in a localized manner in the nervous tissue, postulating the use of secreted SCF in neuronal migration and development (Keshet et al., 1991). SCF expression in the brain is equally complex to the aforementioned results concerning *kit* expression. Here again, SCF is postulated to play a trophic role in development. The embryonic thalamus shows high levels of SCF message (Keshet et al., 1991). This area functions to

mediate the organization of afferent inputs from the hypothalamus, olfactory, optic and spinal tracts, and the trigeminal sensory nuclei (Keshet et al., 1991), all of which requires extensive migratory regulation. SCF is also expressed in other areas of the developing brain, including the floor plate through the hindbrain and the length of the neural tube (Keshet et al., 1991). These findings implicate a potentially important role for this growth factor-receptor complex in the developing nervous system.

V) Mast Cells and the Nervous System

Mast cells are known to associate with peripheral nerves (Bienenstock et al., 1991), usually located in the nerve sheath and endoneurium (Olsson, 1971). Some studies have shown close membrane association of mast cells and neurons (Bernstein and Lawrence, 1990). There is also literature to support mast cell/neuron "cross talk": electrical stimulation of nerves can induce mast cell degranulation, while mast cell degranulation has been shown to induce chloride ion flux in neurons (Bienenstock et al., 1991). Furthermore, sensory neurons participate in inflammatory responses in many tissues, including the joints, skin, eyes, and respiratory tract (Bienenstock et al., 1991). There are many inflammatory diseases with no known role for IgE, including late onset and exercise-induced asthma, perennial rhinitis, angioneurotic edema, chronic idiopathic pruritis, and cholinergic urticaria (Church et al., 1989). Many of these are thought to involve nerve function, and all respond to standard H1 antihistamines (Church et al., 1989). Mast cells are known to respond to neuropeptides such as substance P and somatostatin, which can induce a weal and flare reaction upon injection into dermis (Church et al., 1989). Substance P has also been shown to elicit histamine release from human MC^{TC} (Church et al.,

1989), and substance P-induced inflammation can be blocked by antihistamines (Bienenstock et al., 1991).

Mast cell numbers are increased in response to nerve damage and repair, as seen in amputation neuromas (Olsson, 1971) and peripheral nerve injury (Isaacson, 1976); however, dramatic increases in mast cell numbers have also been noted to occur in pathological conditions involving Schwann cells (Isaacson, 1976). Schwann cells ensheathe and myelinate the peripheral nerves, and form the basal lamina upon which neurons reside (Ridley et al., 1989). The proliferation of these cells is tightly regulated, occurring only during development, after nerve damage, or in neoplastic phenomena such as neurofibromatosis (NF) (Davis and Stroobant, 1990 and Neuberger and DeVries, 1992).

NF is one of the most common autosomal dominant disorders found in man, affecting 1 in 3500 people of all ethnic groups (Xu et al., 1990). This disorder has several characteristics associated with it, including multiple neurofibromas, cafe-au-lait spots, axillary freckling, Lisch nodules, bony lesions, optic gliomas, and schwannomas (Gutmann et al., 1991). Half of all cases are spontaneous, and there is a wide range in severity of the disease (Goldberg and Collins, 1991). Two forms of NF are known to exist: NF1, or von Recklinghausen NF, and NF2, which usually manifests itself as bilateral acoustic NF (BANF). Neurofibromas, the hallmark of NF, are complex benign nerve sheath tumors composed of Schwann cells, perineurial cells, endothelial cells, fibroblasts, mast cells and other mononuclear leukocytes, and extracellular matrix material (Giorno et al., 1989; Riccardi, 1987). The NF-associated cafe-au-lait spots are the result of hyperpigmentation caused by melanocytes (Riccardi, 1981). Malignant neurofibrosarcomas and schwannomas can

occur in NF, but are rare (Ponder, 1990). Thus while this disease is rarely fatal, it can be extremely disfiguring.

The gene for NF1 has been cloned and localized to pericentromeric chromosome 17 (Marchuk et al., 1991; Goldberg and Collins, 1991) It is a very large gene, encompassing 240 kilobases that encode a message of 13,000 bases, with a resulting 2818 amino acid product. *Nf1* is expressed in many tissues, and is conserved among species (Goldberg and Collins, 1991). The *Nf1* gene encodes a glycoprotein with a molecular weight of 250 kDa, and appears to be part of a 400-500 kDa complex (Gutmann et al., 1991; DeClue et al., 1991). NF1 has homology to the GTPase activating protein (GAP) and the yeast protein IRA1, both of which function to inhibit *ras* protooncogene signalling (Xu et al., 1990; Goldberg and Collins, 1991). Furthermore, NF1 can also interact with ras, indicating a possibly similar role to GAP and IRA1 (Goldberg and Collins, 1991). The NF1 gene is highly mutable (1 in 10^4), offering an explanation for the high incidence of spontaneous NF cases (Ponder, 1990). But although *nf1* is apparently ubiquitously expressed, NF seems to affect cells of neural crest origin alone (Gutmann et al., 1991).

The Schwann cell-containing lesions of NF contain large increases in mast cells. Mast cells are common in NF tumors and the associated schwannomas (Isaacson, 1976), and are present in greater numbers in these neoplasia than those seen most forms of nerve damage (Johnson, et al.,1989). There are apparent interactions between Schwann cells and mast cells that may regulate the proliferation and activation of both cell types. Nerve growth factor, a Schwann cell product, has been shown to induce mast cell proliferation (Matsuda et al., 1991), while mast cells are postulated to increase the *in vitro* growth of Schwann cells (George H. DeVries,

unpublished observations). Schwann cells are thought to be dependent upon glycosaminoglycans in the ground substance for their state of differentiation (Kessler et al., 1976; Poole and Zetter, 1983), and the action of mast cell proteases and glycosaminoglycan secretion may be able to affect this. Inflammatory reactions such as pruritis and facial flushing are common in NF, often accompanying rapidly growing neurofibromas (Giorno et al., 1989; Rothe et al., 1990). Standard H1 antihistamines such as diphenhydramine hydrochloride do not decrease this pruritis (Riccardi, 1987). However the mast cell stabilizing drugs ketotifen and disodium cromoglyate are effective (Riccardi, 1987; Riccardi, 1981), and use of ketotifen also showed some decrease in neurofibroma growth (Riccardi, 1987). Thus mast cell-Schwann cell interactions may be important in the growth and maintenance of both populations.

Schwann cells can be induced to proliferate by glial growth factor (GGF), transforming growth factor (TGF) ß1, TGFß2, and agents which increase cyclic AMP (cAMP) levels, such as dibutyryl cAMP or forskolin (Davis and Stroobant, 1990). In the presence of suboptimal doses adenylate cyclase - activating agents, platelet-derived growth factor (PDGF), and acidic and basic fibroblast growth factors (FGF) can also function as Schwann cell mitogens (Davis and Stroobant, 1990). PDGF, FGFs, and TGFs-ß are involved in tissue repair after injury, and are released by platelets, endothelial cells, mesenchymal cells, macrophages, and in the case of TGFß, mast cells (Davis and Stroobant, 1990; Katz et al., 1991). Heparin, another mast cell product, is also known to potentiate the activities of FGFs (Rothe et al., 1990). Therefore, Schwann cell proliferation appears to be linked to tissue repair, and may be influenced in part by mast cells.

Schwann cells are not known to produce many growth factors, although they

do secrete NGF, especially in the presence of gangliosides (Ohi et al., 1990). Riccardi has reported the presence of autocrine and paracrine growth factors present in the supernatant of NF1 neurofibroma cultures, which could increase Schwann cell growth *in vitro* (Riccardi, 1986). Ridley, et al. have detected TGFß mRNA in rat Schwann cells, and suggest that TGFß may be produced as an autocrine growth factor by Schwann cell tumors (Ridley et al., 1989). Finally, a schwannoma-derived growth factor (SDGF) has been isolated that has homology to EGF and glial growth factor, and can stimulate proliferation of Schwann cells, fibroblasts, and astrocytes (Kimura et al., 1990).

Materials and Methods

Animals

Female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). All mice were used at eight to ten weeks of age unless otherwise stated. Female Sprague Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) at 200-250g weight, and were used within 3 months of delivery. Neonatal rat were also purchased from Harlan on day 1 after birth, and were used at two days of age. New Zealand White rabbits were purchased from Blue and Grey Rabbitry (Aylett, VA) at 2.5kg weight.

Infection with *Nippostrongylus brasiliensis (Nb)*

Nb were maintained as described (Huff and Justus, 1988). To obtain the mast cell-committed progenitor (MCCP), mice were injected subcutaneously in the nape of the neck with 750 to 1000 L3 larvae, and were sacrificed at day 12-15 of infection.

Cells and Cell Lines

The contact-inhibited murine fibroblast cell line BALB/c 3T3 clone A31, the human fibrosarcoma cell line HT-1080, the murine mastocytoma P815, and the murine myelocytic cell line Wehi-3 were obtained from American Type Culture Collection (Rockville, MD). A rat Schwann cell line transfected with SV40 large T antigen (TRSC) has been described previously (Tennekoon et al., 1987) and was obtained

from George DeVries, Virginia Commonwealth University. The human malignant peripheral nerve sheath tumor line clone ST88-14 was generously given by Dr. Cynthia Morton, Brigham and Women's Hospital, Harvard University. Neonatal rat Schwann cells were prepared as described by Brockes, et al. (Brockes et al., 1979). Adult rat and human Schwann cells were prepared from sciatic or sural nerves, respectively, by a modification of Morrissey, et al. (Morrissey et al., 1991), using a digestion buffer of 0.05% collagenase, 1.25 u/ml dispase, 1.0 mg/ml hyaluronidase, 0.02 mg/ml DNase, 15% fetal calf serum, and 25 mM HEPES in DMEM. The murine mastocytoma cell line MMC-34 was the gift of Dr. Reuben Siraganian, National Institute of Dental Research, NIH, and has been described previously (Barsumian et al., 1985). The murine mast cell line PT18 was the kind gift of Dr. Daniel Conrad, Department of Microbiology and Immunology, Virginia Commonwealth University, and was originally produced by Dr. Dov Pluznik, Division of Cytokine Biology, Food and Drug Administration. The human small cell lung cancer lines H146 and H510 were generously given by Dr. Geoff Krystal, Department of Hematology and Oncology, Medical College of Virginia. All cells were maintained in Dulbecco's medium which was supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 1mM sodium pyruvate, 1mM oxaloacetic acid, 4nM 2-mercaptoethanol, and 10% FCS (cDMEM). The human SCF-responsive cell line MO7E was the kind gift of Dr. James Griffin, Dana Farber Cancer Institute, Harvard University. These cells were maintained in cDMEM supplemented with 10 ng/ml rhGM-CSF and 50 ng/ml rhSCF.

Preparation of Cell-Conditioned Medium (CM)

Cells were grown in a confluent state for 7 to 14 days in cDMEM, until medium was light orange in color. Conditioned supernatant was removed, centrifuged at 600 x g, and concentrated by ultrafiltration on a YM-10 filter (Amicon, Danvers, MA). Samples used in the MO7E proliferation assay were first filtered through an XM-50 ultrafiltration unit in 1.5M NaCl, and dialyzed against three changes of phosphate buffered saline. Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCCM) was prepared as described previously (Jarboe and Huff, 1989).

Recombinant Human Stem Cell Factor

rmSCF[164] was purchased from Genzyme Corporation (Boston, MA), and stored at -70°C.

Mast Cell-Committed Progenitor Assay

MCCP respond to 3T3 fibroblast-conditioned medium or IL-3 by formation of mature mast cell colonies in methylcellulose (Jarboe et al., 1989). A 2 ml mixture containing 6 x 10^5 *Nb*-MLN cells/ml, 0.8 ml methylcellulose medium [11.5% (v/v) 2x MEM alpha medium (Gibco, Grand Island, NY), 70% (v/v) fetal calf serum, 2.5% deionized bovine serum albumin, 2.5% *l*-glutamine, 2.5% streptomycin solution, 50 ug/ml gentamicin, 5 x 10^{-5} 2-ME], and 1.0 ml 3% 4,000 centipoise methylcellulose (Fisher Scientific, Fair Lawn, NJ) was supplemented with 0.2 ml growth factor, unless otherwise indicated. This mixture was cultured in 24 well plates at 37°C in 5% CO_2 for 13 days. Individual colonies were then enumerated with an inverted microscope at 100X magnification, and cytospin slides prepared for differential staining. The number of specific colony types in culture was determined by multiplying the number of total colonies counted by the percentage of cells present, as determined by cell staining.

Isolation of Ribonucleic Acid

RNA was isolated from cultured cells by the RNAzol extraction procedure (Biotecx Laboratories Inc, Houston, TX), which uses a solution of guanidine isothiocyanate, phenol, and 2-mercaptoethanol to obtain a two phase separation of RNA from DNA and proteins. Concentration of RNA was determined by absorbance at 260nm, where 1 OD unit equals 40 μg/ml.

cDNA Library Screening and Sequence Analysis

A mouse NIH 3T3 fibroblast cDNA library packaged in Lambda Zap II vector (Stratagene, La Jolla, CA) was screened with a 5' end-labelled 30-mer oligonucleotide probe specific for mouse SCF, which was derived from the published sequence (Zsebo et al., 1990). Nitrocellulose filters lifted from the library were incubated overnight at 37°C with 10^6 cpm/ml of probe. Filters were then washed 4 by 10 minutes at 37°C with 2X SSC, 0.2% SDS followed by one 30 minute wash at 50°C with 0.5X SSC, o.2% SDS. From approximately 10^6 original plaques, 3 positive clones were obtained and In Vitro Excession using a R408 helper virus was performed as advised by Stratagene. The resulting pBluescript phagemid containing the cDNA was transfected into XL-1 Blue host *E. coli*. One clone was chosen for further study and was sequenced using US Biological Sequenase Version 2.

Expression Vector

A BglII-BSU36I fragment encoding the secreted form of SCF was subcloned into PET-3d utilizing a 5' linker, which added an NCOI site and a start codon, and a 3' linker, adding the additional amino acids ASSLR, a stop codon, and a BAMHI site to the 3' end. These manipulations result in the expression of a message encoding a protein of 169 amino acids; therefore we have designated our recombinant Stem Cell

Factor protein rmSCF^{169}.

Protein Purification

BL-21 *E. coli* containing the expression vector were grown at 28° C in LB (1% tryptone, 0.75% yeast extract, 0.34M NaCl) supplemented with 0.01 Mg, 22mM glucose, and 25μg/ml ampicillin to an OD_{600} of 0.6-0.8, at which time IPTG was added to a final concentration of 1 mM. The cells were then cultured for 2 hours, pelleted and resuspended in 10mM tris, 0.5 mM EDTA, pH 7.8. Cells were then subjected to French Press (PSI = 20,000), and the supernatant was clarified and resuspended in 6M urea, 50 mM tris pH 7.8, 1% triton X-100, 5% glycerol, 0.1 mM phenylmethylsulfonylfluoride, and 0.1 mM dithiothreitol, incubated overnight at 4°C with rocking, clarified by centrifugation, and partially purified using sizing chromatography. The resulting preparations of rmSCF^{169} were approximately 60-65% pure, as determined by densitometric analysis of polyacrylamide gels. To evaluate the activity of SCF^{169}, preparations were tested in parallel with rmSCF purchased from Genzyme for their ability to stimulate proliferation of the mouse mast cell line MC9.

Polymerase Chain Reaction and Nucleic Acid Transfer

RNA (2μg) samples were subjected to reverse transcription using 200U mouse mammary leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD) and oligonucleotide primers (see below) at 37°C for 60 minutes. cDNA was amplified using the Geneamp system of DNA amplification (Perkin Elmer Cetus, Norwalk, CT). Samples were incubated in a thermocycler (Perkin Elmer Cetus, Norwalk, CT) with oligonucleotide primers and Taq polymerase. Denaturing was carried out at 95°C for 2 minutes, followed by primer annealing at 50°C for 2 minutes, and primer extension at 72°C for 2 minutes. After 30 cycles were completed, samples were

incubated at 72°C for 7 minutes, then stored at 4°C. The amplified product was subjected to agarose gel electrophoresis and blotted to 0.2 μm nytran (Schleicher and Schuell, Keene, NH). Nytran filters were crosslinked using an ultraviolet Stratalinker 2400 (Stratagene, La Jolla, CA). Filters were probed overnight with a ^{32}P-end labelled oligonucleotide at 37°C in a solution of 50% formamide, 0.1% SDS, .05M sodium phosphate pH 6.5, 5x SSC, 5x Denhardt's solution, and 0.25 mg/ml yeast RNA, followed by washing with a solution of 0.2% SDS, 2X SSC at 37°C for 4 x 10 minutes, then 30 minutes at 60°C in a solution of 0.2% SDS, 0.5X SSC. Filters were exposed to Kodak X-OMAT AR film (Eastman Kodak Co, Rochester, NY) at -70°C for 1 to 5 days.

Oligonucleotide Primers and Probes

5' and 3' oligonucleotide primers were designed with Primer Detective (CLONTECH Laboratories, Inc., Palo Alto, CA) from published sequences of the human and rat stem cell factor genes (Martin et al., 1990), the rodent IL-3 genes (Cohen et al., 1986), the human c-*kit* sequence (Vandenbark et al., 1992), the rat c-*kit* sequence (Tsujimora, et al.,1991), the human actin sequence (Gunning et al., 1983), the mouse actin sequence (Leader et al., 1986), the human IL-3 sequence (Phillips et al., 1989), and the human GM-CSF sequence (Kaushansky et al., 1986). Oligonucleotide probes of 30 nucleotides in length were designed in the same manner. Oligonucleotide primers and probes were confirmed to lack significant homology to other rodent and human sequences in the Genbank and EMBL databases by the FastA algorithm of the GCG sequence analysis program. These primers and probes were synthesized in the Nucleic Acid Core Facility at Virginia Commonwealth University using an Applied Biosystems 380A DNA synthesizer. The sequences of each PCR primer and

probe is in Appendix A.

MO7E Proliferation Assay

1 x 10^4 MO7E cells were placed into culture with cDMEM supplemented with the indicated concentration of growth factor or cell-conditioned medium. After 48 hours of incubation at 37°c and 5% CO_2 in a humidified incubator, 1.0 μCi ^{3}H-TdR was added to the cultures for 16 hours. Cells were then harvested onto absorbent glass paper and radioactivity associated with the cells was determined by liquid scintillation analysis (Packard 2200, Packard Instrument Co., Downers Grove, IL).

Antibodies

The following were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA): FITC-goat anti-rabbit IgG (H+L) F(ab')2; normal rabbit serum; rabbit IgG, whole antibody; PE-goat anti-mouse IgG (H+L) F(ab')2; normal mouse serum; mouse IgG, whole antibody. Mouse anti-human c-*kit* mAb was purchased from Boehringer-Mannheim Biochemical, (Indianapolis, IN). Rat anti-mouse IL-3 was purchased from Genzyme Immunobiologicals (Boston, MA). Mouse anti-human c-*kit* antibody YB5.B8 was the gift of Dr. Leonie Ashman, Department of Microbiology and Immunology, The University of Adelaide, Adelaide, South Australia. Rabbit anti-human SCF^{169} or anti-mouse SCF^{169} were produced in our laboratory by immunizing New Zealand White rabbits with 50μg purified $rhSCF^{169}$ or $rmSCF^{169}$ in complete Freund's adjuvant, followed by boosting with 50μg $rhSCF^{169}$ or $rmSCF^{169}$ in incomplete Freund's adjuvant. Animals were bled, serum harvested by centrifugation, and IgG fractions were collected using a protein A column (Pierce Chemical Co., Rockford, IL). Mouse anti-human nerve growth factor was originally obtained from ATCC, and was the kind gift of Dr. George DeVries, Virginia

Commonwealth University, and was used as a neat solution.

Hitochemical Staining of Cells

Toluidine blue stain was prepared by mixing 0.2% toluidine blue powder and 0.1M citric acid in 50% ethanol. The final pH of this solution was 1.0. Alcian blue stain was prepared at a final concentration of 0.5% in 0.3% glacial acetic acid. Safranin stain was made to a final concentration of 0.1% in 0.1% acetic acid. Cytospin slides were stained at room temperature with acidic toluidine blue for 45 minutes to detect the presence of metachromatic cells (mast cells). Alternatively, slides were stained with alcian blue for 10 minutes, followed by safranin for 10 minutes. A cell was denoted as "positive" for safranin staining if >20% of the granules in the cell were stained with safranin.

Immunofluorescent Staining of Cells

Cells were harvested by centrifugation and washed once in PBS supplemented with 10% NGS (PBS/NGS). 1 X 10^6 cells were incubated in a volume of 100 μl with primary or control antibody at the concentration denoted for 45 minutes at 4°C. Cells were then washed twice with PBS/NGS, and resuspended in a 100μl volume, to which 5 μl secondary antibody was added. Cells were incubated at 4°C for 30 minutes, washed twice with PBS/NGS, and analyzed by flow cytometry.

Flow Cytometry

Fluorescently labelled cells were analyzed on an Epics 753 Flow cytometer (Coulter Corp., Hialeah, FL) or a Becton Dickinson Facscan Flow Cytometer (Becton Dickinson Immunocytometry Systems, Braintree, MA). FITC and PE emissions were observed using standard filters. Propidium iodide staining was used to exclude non-viable cells, after which 10,000 cells were analyzed.

Stimulation with PMA

Nonadherent cells were incubated in cDMEM containing 50 ng/ml PMA for 60 minutes at 37°C with rocking. After incubation, cells were washed with cDMEM to remove PMA, and used as described.

Isolation of Peritoneal Cells

Murine or rat peritoneal cells were harvested through peritoneal lavage by injecting PBS (5 ml/mouse; 35 ml/rat) into the peritoneal cavity of freshly sacrificed animals. PBS was extracted with a pasteur pipette, and cells were obtained by centrifugation.

Percoll Density Separation

Percoll was purchased from Pharmacia Fine Chemicals (Piscataway, NJ) at a density of 1.31 g/ml. This stock solution was diluted with 10X PBS and dH_2O to yield the desired final density. Cells were layered on percoll gradients and centrifuged at 1200 x g in an IEC tabletop centrifuge model HN-SII (IEC, Needham Heights, MA) for 25 minutes at room temperature. Cells were removed from gradient interfaces with a pasteur pipette and washed with cDMEM by centrifugation. Peritoneal mast cells (>98%) were obtained by centrifuging $2x10^7$ peritoneal cells on a gradient of percoll at density 1.082 g/ml as described above, and harvesting the pellet

Placental Tissue RNA

In collaboration with Dr. Scott Kauma, Department of Obstetrics and Gynecology, Medical College of Virginia, placental tissues were obtained from donors after informed consent, tissues were homogenized with a Tekmar Tissuemizer (Tekmar Co., Cincinatti, OH), and RNA was extracted as described.

Northern Analysis

RNA was separated by electrophoresis on a 1% formaldehyde agarose gel at 25v for

16 hours, then capillary blotted to 0.2μm nytran filters (Schleicher and Schuell, Keene, NH) and probed with a ^{32}P-labelled 539 nt human SCF cDNA probe, which was random prime labelled using an oligolabelling kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) at the manufacturer's specifications. cDNA probe was placed at a final concentration of $1x10^6$ cpm/ml. Filters were pre-washed at 42°C in a solution of 20 mM tris, pH 8.0, 1M NaCl, 1mM EDTA, and 0.1% SDS for 1 hour with rocking. Filters were then pre-hybridized with a solution of 50% formamide, 5x SSC, 5x Denharts, 0.1% SDS, 10mM sodium phosphate, pH 6.5, and 100 μg/ml salmon sperm DNA for 1 hour at 42°C with rocking, then probed for 16 hours under the same conditions. Filters were washed in a solution of 20 mM sodium phosphate, and 0.1% SDS with 4x SSC, followed by 2x SSC, then 0.1x SSC. Each wash was carried out at 56°C. Filters were exposed to Kodak X-OMAT AR film (Eastman Kodak Co, Rochester, NY) at -70°c for 1 to 5 days.

Isolation of Polyadenylated RNA

Columns were prepared with oligo-dT sepharose beads (Pharmacia LKB Biotechnology, Uppsala, Sweden) and washed with 5 column volumes of binding buffer (0.01M tris, pH 7.5, 0.5M NaCl, 1mM EDTA). RNA (1-2 mg) was resuspended at 5 mg/ml in elution buffer (.01M tris, pH 7.5, 1mM EDTA) and heated to 65°C for 5 minutes, then kept on ice. RNA was diluted 1:1 with 2x binding buffer and applied to the column. Effluent was collected and re-applied twice, after which time the column was rinsed with 5 volumes binding buffer, followed by 5 volumes wash buffer (0.01 M tris, pH 7.5, 0.1M NaCl, 1mM EDTA). Eluate was then collected by washing the column with 2 volumes elution buffer. RNA in this fraction was precipitated by the addition of 0.1 volume 3M sodium

acetate and 2.5 volumes 100% ethanol for 16 hours at -80°C. Precipitate was collected by centrifugation at 15,000 rpm in a microcentrifuge (Costar, Cambridge, MA), and resuspended in DEPC-dH_2O. Concentration of RNA was determined by absorbance at 260 nm.

Results

I) Known Growth Factors Cannot Mimic 3T3-CM Activity

This laboratory has previously described a mast cell colony stimulating activity present in the supernatant of 3T3 fibroblast cultures. Initial experiments demonstrated that the factor was a protein with Mr 5-50 kDa (Jarboe et al., 1989). This factor was able to give rise to mast cell colonies from *Nb*-MLN cells after a two week culture period in methylcellulose. To determine the identity of the colony stimulating factor in 3T3-CM, various known growth factors were added to methylcellulose cultures to mimic the activity found in the fibroblast-conditioned medium. In addition to the list of known growth factors previously shown to be unable to support the development of mast cell colonies from *Nb*-MLN cells, bFGF (5 ng/ml,25 ng/ml,50 ng/ml), TBFß (1 ng/ml,5 ng/ml,10 ng/ml), and IL-9 (5,10,100 u/ml) also had no activity. These and other previously tested growth factors are shown in Table VII (See Appendix B).

Of the factors tested, only IL-3 was found to generate mast cell colonies from *Nb*-MLN cells. However, 3T3 fibroblasts have been shown not to produce IL-3 (Jarboe et al., 1989), and RNA extracted from BALB/c 3T3 fibroblasts was demonstrated by RT-PCR to lack message for IL-3 (Figure 32, Appendix B). Therefore we could not attribute the activity in 3T3-CM to this growth factor. This

lead us to the conclusion that an uncharacterized mast cell growth factor was produced and secreted by 3T3 fibroblasts.

II) Cloning of Mouse SCF[169]

After having concluded that the fibroblast-produced growth factor was likely to be a new one, our laboratory contacted AMGEN Biologicals to initiate a collaborative cloning effort. This project began immediately as a six month unfunded collaboration, during which time our *Nb*-MLN culture system was shown to be able to read blinded samples (Table IX, Appendix B). However, this collaboration was ended when AMGEN set new stipulations not part of the original agreement. As a result of the delay, we were unable to clone the growth factor prior to the 1990 *Cell* publications by three groups describing the cloning and isolation of a mast cell growth factor encoded by the *Sl* locus (Copeland et al., 1990; Flanagan and Leder, 1990; Anderson et al., 1990; Williams et al., 1990; Huang et al., 1990; Zsebo et al., 1990). However, we did use the published information to derive clones of mouse and human SCF in order to more quickly accomplish our goals of elucidating the signals for mast cell growth and differentiation.

In a joint effort lead by Dr. Julie Leftwich of this laboratory, a clone of mouse SCF was obtained by screening a cDNA library from NIH 3T3 fibroblasts. Using a labelled oligonucleotide probe, a clone of 826 nucleotides that encodes the full length mSCF protein and included 289 nucleotides of untranslated 5' sequence was isolated as described in Materials and Methods. A prediction of the three dimensional structure of this protein, as determined by the Chou-Fasman algorithm is shown in

Figure 1. A portion of the clone encoding 169 amino acids at the amino terminus of the SCF protein was inserted into the expression vector PET-3d (Figure 1), as described in detail in Materials and Methods. This fragment includes the extracellular region and extends to, but does not include the so-called "membrane tether" region of the protein, which is known to include a proteolytic cleavage site (Williams et al., 1992). As shown in Figure 2, the amino acid sequence of our SCF^{169} clone is nearly identical to that obtained by AMGEN, with the exception of five amino acids found at the carboxyl terminus of our clone. Thus, our clone will be referred to as SCF^{169}, and AMGEN's as SCF^{164}.

III) Recombinant Stem Cell factor Promotes Maximal Mast Cell Colony Formation from Committed Progenitors

Because no known factor could mimic the effect of 3T3-CM in the committed progenitor assay, and 3T3 fibroblasts were shown to produce SCF (Zsebo et al., 1990), we determined if $rmSCF^{169}$ or $rmSCF^{164}$ could replace FCM in methylcellulose cultures.

Nb-MLN cells ($6x10^5$/ml) were cultured in the presence of $rmSCF^{169}$ or $rmSCF^{164}$ at 5, 50, 100, or 200 ng/ml for 13 days, and the number of mast cell colonies was determined as described in Materials and Methods. As shown in Figure 3, both forms of SCF were able to generate mast cell colonies in these cultures. $rmSCF^{164}$ has greater activity in this assay due to its greater purity. While $rmSCF^{169}$ is 99% pure, $rmSCF^{169}$ is approximately 65% pure, as determined by densitometric analysis of polyacrylamide gels, and has hence been denoted as "crude" $rmSCF^{169}$ in Figure 3. This dose response curve was used to calculate the half-maximal

Figure 1

Chou-Fasman algorithm prediction of rmSCF three dimensional structure. rmSCF[169] is contained between the two vertical bars. Diamonds represent area of hydrophilicity; ellipses indicate areas of hydrophobicity. Arrowhead shows "membrane tether" region.

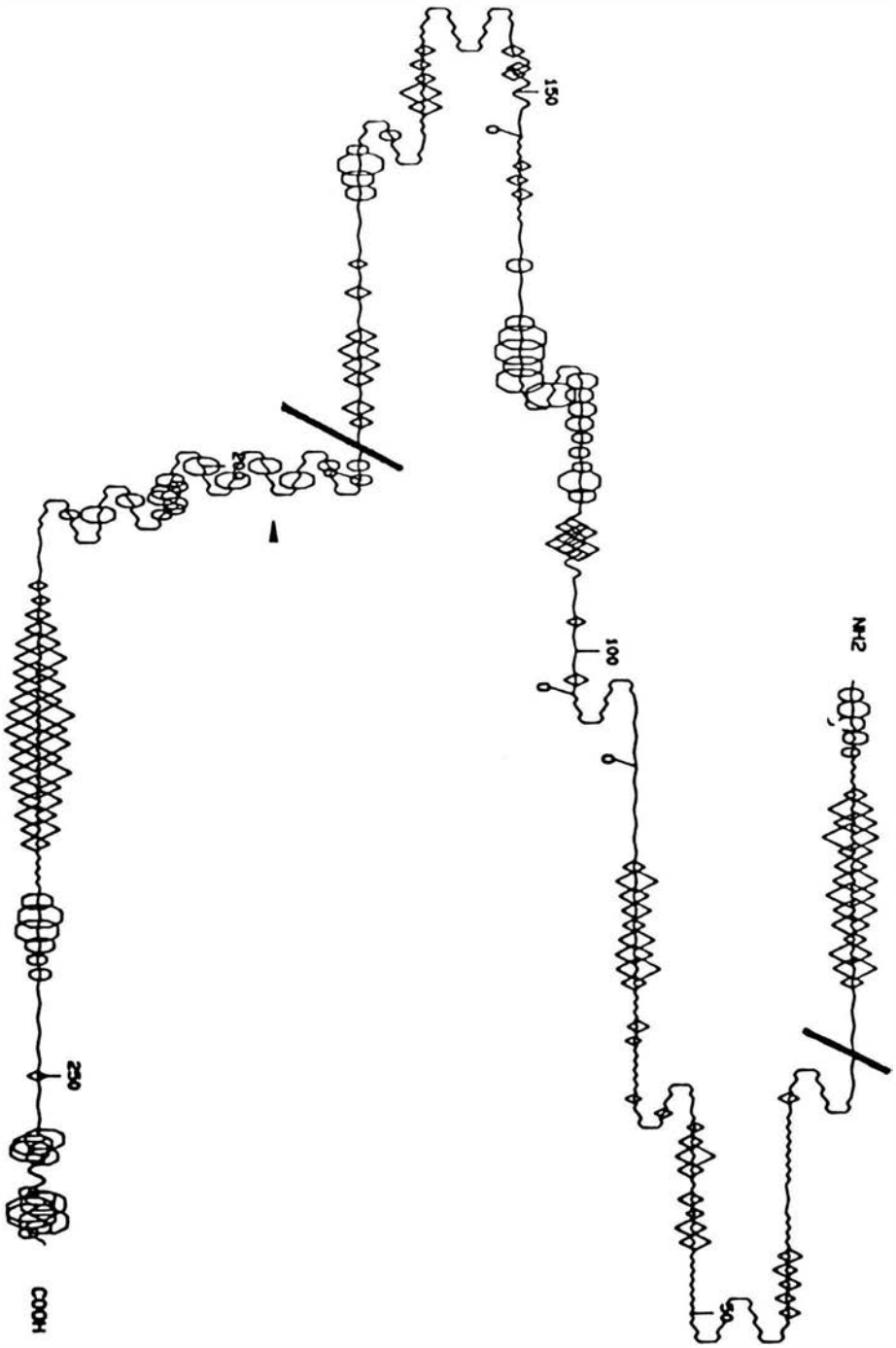
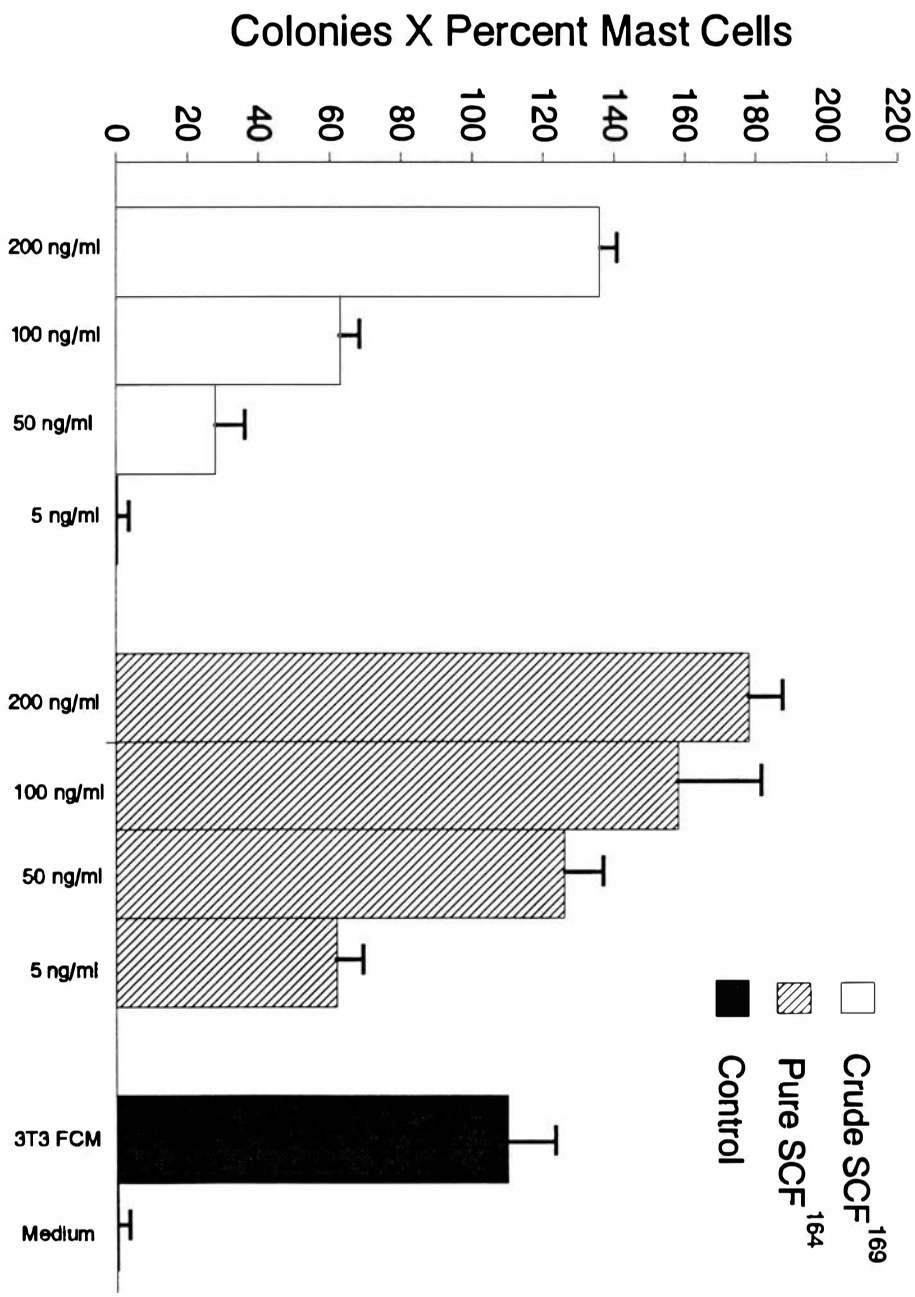

NH2
50
100
150
200
250
COOH

Figure 2
Amino acid sequences of rmSCF^{164} (Top) and rmSCF^{169} (Bottom).

```
          .         .         .         .         .
  1 MEICGNPVTDNVKDITKLVANLPNDYMITLNYVAGMDVLPSHCWLRDMVI 50
    .||||||||||||||||||||||||||||||||||||||||||||||||||
  2 KEICGNPVTDNVKDITKLVANLPNDYMITLNYVAGMDVLPSHCWLRDMVI 51
          .         .         .         .         .
 51 QLSLSLTTLLDKFSNISEGLSNYSIIDKLGKIVDDLVLCMEENAPKNIKE 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
 52 QLSLSLTTLLDKFSNISEGLSNYSIIDKLGKIVDDLVLCMEENAPKNIKE 101
          .         .         .         .         .
101 SPKRPETRSFTPEEFFSIFNRSIDAFKDFMVASDTSDCVLSSTLGPEKDS 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
102 SPKRPETRSFTPEEFFSIFNRSIDAFKDFMVASDTSDCVLSSTLGPEKDS 151
          .
151 RVSVTKPFMLPPVAASSLR 169
    |||||||||||||||
152 RVSVTKPFMLPPVA
```

Figure 3
Stimulation of committed progenitor development by recombinant SCF. *Nb*-MLN cells were cultured in the presence of the indicated concentrations of rmSCF^{169}, rmSCF^{164}, 15X 3T3-CM, PWM-SCCM, or medium alone for 13 days, after which time colonies were counted and cytospin preparations made. Percent mast cells was determined by acidic toluidine blue staining of cytospin preparations. Shown are the means and standard error measurements of duplicate wells in a 24 well plate from one of 5 representative experiments.


Colonies X Percent Mast Cells
0
20
40
60
80
100
120
140
160
180
200
220
200 ng/ml
100 ng/ml
50 ng/ml
5 ng/ml
200 ng/ml
100 ng/ml
50 ng/ml
5 ng/ml
3T3 FCM
Medium
Crude SCF169
Pure SCF164
Control

concentration of rmSCF^{164} as 12 ng/ml. SCF stimulation of *Nb*-MLN cells proved to be greater than 3T3-CM both in terms of the number and size of colonies generated in most experiments (Figure 3 and Figure 4). While 3T3 FCM gave rise to 110 mast cell colonies, rmSCF^{169} and rmSCF^{164} at 200 ng/ml yielded 136 and 178, respectively. Moreover, the number of cells in colonies derived from 3T3 FCM stimulation was about 100, whereas either source of SCF lead to the formation of colonies containing 500 cells or greater at dosages of 100-200 ng/ml. This difference in colony size could be due to a greater amount of SCF present in rmSCF than is found in 3T3 FCM, or the stimulation of distinct mast cell progenitor subsets by 3T3 FCM and SCF. Three subsets of mast cell progenitors have been described in the peritoneal cavity, based upon the colony size and cell phenotype they generate (Kanakura et al., 1988). By colony size criteria, 3T3 FCM could be postulated to act upon Medium (M)-CFU-Mast, which yield colonies containing 32-500 cells, while rmSCF could stimulate Large (L)-CFU-Mast, which form colonies with greater than 500 cells each (Kanakura et al., 1988).

Mast cells have two distinct phenotypes: connective tissue and mucosal (See Literature Review). Although all mature mast cells will stain metachromatically with toluidine blue, connective tissue phenotype mast cells stain preferentially with berberine sulfate or safranin, both of which bind to the heparin proteoglycan possessed by these, but not mucosal mast cells (reviewed in and Schwartz and Huff,1992). While M-CFU-Mast colonies stain with safranin or berberine sulfate, L-CFU-Mast do not (Kanakura et al., 1988). The state of differentiation of the cells derived from culture with SCF was determined by staining cytospins prepared from methylcellulose cultures with toluidine blue, berberine sulfate or alcian blue/safranin.

The phenotype of these cells is shown in Figure 5. Toluidine blue staining resulted in the appearance of metachromatic granules in 99% of cells stimulated with 100 ng/ml of rmSCF^{169} or rmSCF^{164}, respectively, and 90% of cells cultured with 3T3 FCM (Average of five separate experiments). Thus these cells appear to be mature mast cells.

Stimulation with 100 ng/ml rmSCF^{164} yielded cultures where 54% of the cells stained with berberine sulfate, while 67% of cells grown with 3T3 FCM were berberine sulfate positive. Safranin staining gave similar results, with 82% of cells cultured with SCF staining positive, versus 89% of 3T3 FCM-stimulated cells (Average of five separate experiments). These results indicate that culture of *Nb*-MLN cells with recombinant SCF resulted in the formation of colonies with heparin-containing mast cells, indicative of the connective tissue phenotype. Thus rmSCF alone can substitute for 3T3 FCM both in the proliferation and differentiation of mast cell-committed progenitors.

Previous results from this laboratory have shown that 3T3 FCM is not active on naive MLN cells (Jarboe et al., 1989). After demonstrating the activity of rmSCF on *Nb*-MLN cells, we cultured naive MLN cells under similar conditions, but were not able to obtain mast cell colonies (Data not shown). Similarly rhSCF, which has been shown to be 800 fold less active on rodent progenitors than on human progenitors (Zsebo et al., 1990), also lacked activity in this assay (Data not shown).

IV) Schwann Cell Neoplasia and Mastocytosis: The SCF Connection

Schwann cells myelinate the peripheral nervous system, and are the major cell type found in neurofibromas and schwannomas. Mast cells have been shown to associate with Schwann cells (Isaacson, 1976), and a prominent mast cell hyperplasia

Figure 4
Photomicrograph of developing (Day 13) colonies in methylcellulose after stimulation of *Nb*-MLN cells with (A) 15X 3T3 FCM, or (B) 50 ng/ml rmSCF[169]. Magnification 200x.

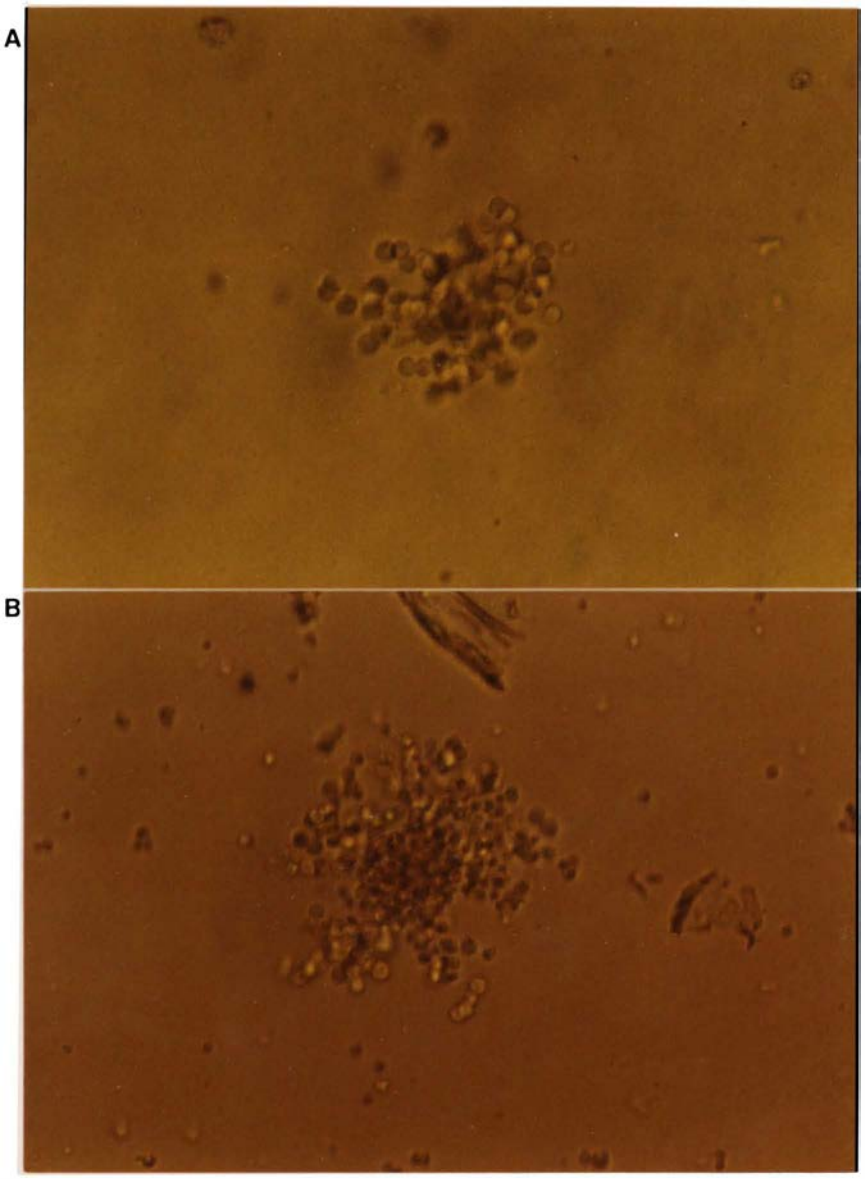
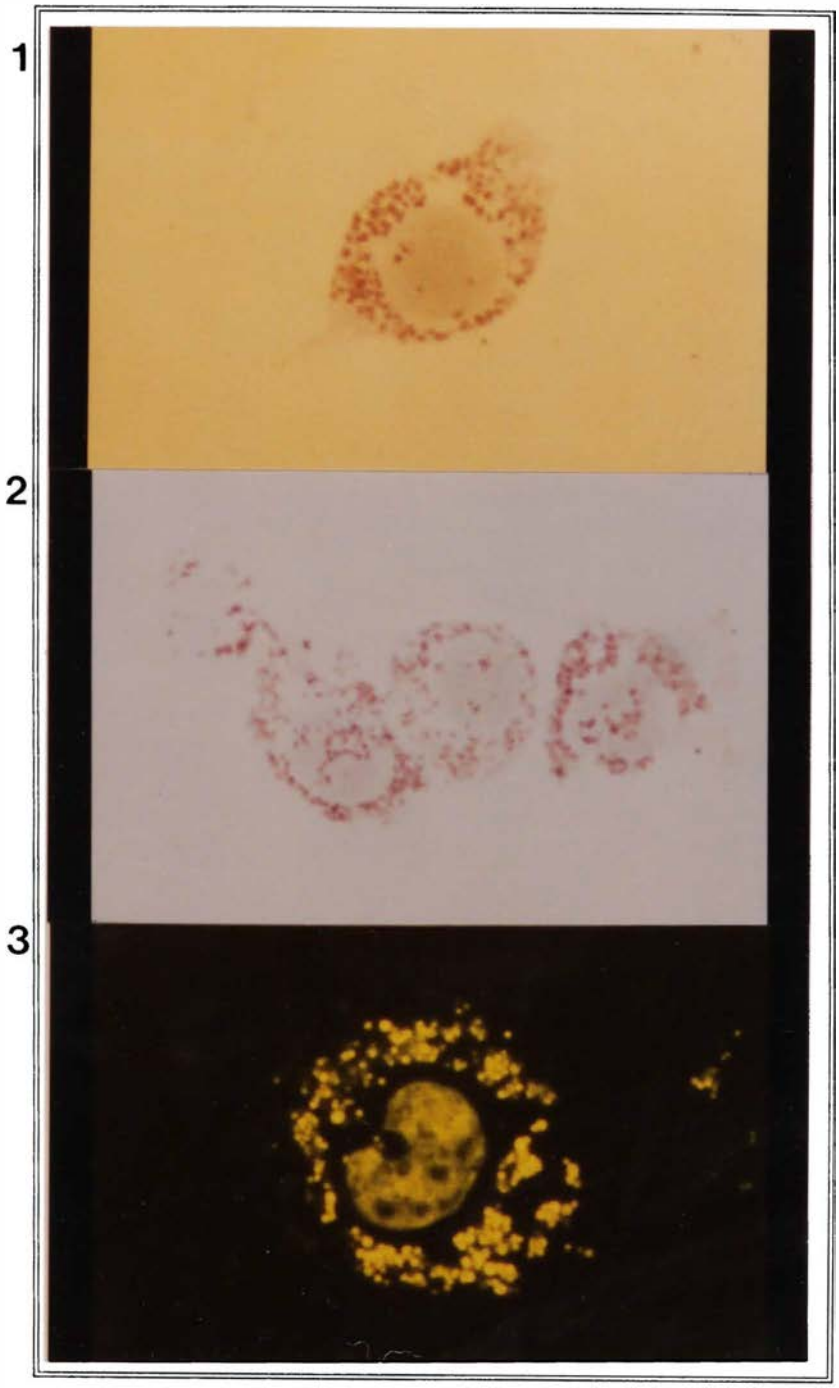

A
B

Figure 5
Phenotype of cells derived from methylcellulose culture of *Nb*-MLN cells with rmSCF. (A) Photomicrographs of cytospin preparations derived from methylcellulose culture of *Nb*-MLN cells for 13 days with 50 ng/ml rmSCF^{169}. Cells were stained with: (1) acidic toluidine blue, (2) alcian blue/safranin or (3) berberine sulfate as described in Materials and Methods. (B) Percentage of cells which stained with acidic toluidine blue, safranin, or berberine sulfate after culture of *Nb*-MLN cells with 100 ng/ml rmSCF^{164}, 15X 3T3 FCM, or PWM-SCCM. Values represent the mean of five separate experiments.


1
2
3

(B) Phenotype of Cells Derived from Culture with Stem Cell Factor

Stimulus	Toluidine Blue (Histamine)	Berberine Sulfate (Heparin)	Safranin (Heparin)
100 ng/ml SCF	99%	54%	81%
15X 3T3 FCM	90%	67%	89%
PWM-SCCM	99%	1%	0%

has long been noted concomitant to either of these noted Schwann cell neoplasia (Giorno et al., 1989). Because the findings of this laboratory have demonstrated the ability of SCF to induce mast cell development from committed progenitors, and the results of others that have indicated a role for SCF in mast cell proliferation, activation, and migration (Jozaki et al., 1991; Williams et al., 1992; Meininger et al., 1992; Bischoff and Dahinden, 1992; Nakajima et al., 1992; Wershil et al., 1992), we sought to determine if SCF could be produced by Schwann cells, offering an explanation for the mast cell hyperplasia associated with Schwann cell proliferation.

To determine if Schwann cells express SCF message, RNA extracted from the human malignant schwannoma cell line ST88-14, derived from a dermal peripheral nerve schwannoma, and an SV40-transfected neonatal rat Schwann cell line (TRSC) was subjected to RT-PCR using primers specific for rat and human SCF. As shown in Figure 6, both Schwann cell lines exhibited expression of an 830 nucleotide band after RT-PCR. This is the expected size of the SCF transcript, and a band of identical size was amplified from HT1080 RNA (Figure 6), a cell line known to express SCF (Martin et al., 1990). Thus, both ST88-14 and TRSC Schwann cell lines express SCF message.

SCF protein should be detectable in the culture supernatant of cells which express the KL-1 form of SCF, since this transcript leads to the cleavage of membrane-bound SCF and its subsequent release as a secretable product. However, cells expressing the KL-2 transcript produce only membrane-bound SCF and would have no activity in their culture supernatant (See Literature Review). To determine if either of the Schwann cells lines which have shown expression of SCF message could produce secretable SCF, conditioned medium (CM) from the transfected rat

Schwann cells and ST88-14 Schwann cell lines was concentrated 50 fold and employed in the SCF-dependent committed progenitor methylcellulose assay or an SCF-dependent proliferation assay.

Transfected rat Schwann cell-CM was able to induce the formation of mast cell colonies from *Nb*-MLN cells in a dose responsive manner (Figure 7), although this conditioned medium was less active than 3T3-CM, generating 79 mast cell colonies at its highest concentration, as compared to 108 colonies per well induced by 3T3-CM. Since only IL-3 and SCF have demonstrated activity in this assay, RT-PCR was used to determine if transfected rat Schwann cells express IL-3 message. As shown in Figure 8, RNA extracted from these cells could not be amplified with primers specific for IL-3 to yield the expected 550 base pair band expressed by the WEHI-3 positive control cell line. This same preparation of transfected rat Schwann cell RNA could, however be amplified by RT-PCR to detect SCF or actin expression (Figure 6, and data not shown). Thus the RNA extracted from this Schwann cell line appears to be intact. Because conditioned medium from transfected rat Schwann cells is able to induce mast cell colony formation from committed progenitors, and IL-3 message is not detectable in RNA extracted from this cell line, the activity of transfected rat Schwann cell-CM indicates the production of SCF by this Schwann cell line.

Since hSCF has been shown to lack activity in the committed progenitor assay, we anticipated that ST88-14-CM would also be inactive in this assay, and in fact this was the case (Data not shown). Therefore, we employed a proliferation assay to detect secreted hSCF from the ST88-14 cell line. The human megakaryocytic cell line MO7E has been shown to be dependent upon GM-CSF, IL-3 or hSCF for its

Figure 6
RT-PCR for SCF expression by Schwann cells. Total RNA was extracted from cell lines and subjected to RT-PCR using primers specific for human or rat SCF. PCR was carried out as described in Materials and Methods, with a 50°C annealing temperature for 30 cycles. Arrow head indicates location of expected 830 base pair product. Samples are (A) transfected rat Schwann cells, (B) ST88-14, (C) HT1080, (D) Control sample (-cDNA).

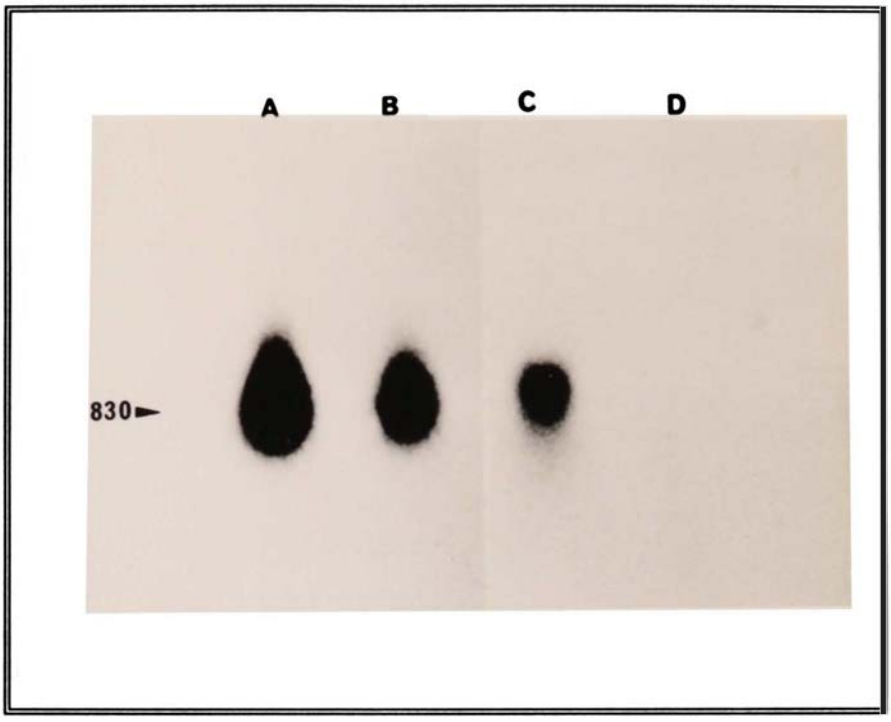

A
B
C
D
830

Figure 7
Stimulation of committed progenitors with transfected rat Schwann cell-CM. *Nb*-MLN cells were cultured in methylcellulose for 13 days in the presence of 50X transfected rat Schwann cell-CM, 15X 3T3-CM, PWM-SCCM, or Medium alone. Percent mast cells was determined by staining cytospin slides with acidic toluidine blue as described in Materials and Methods. Values represent the means and standard error measurements of duplicate wells of a 24 well plate from a representative experiment. Duplicate experiments yielded similar results.

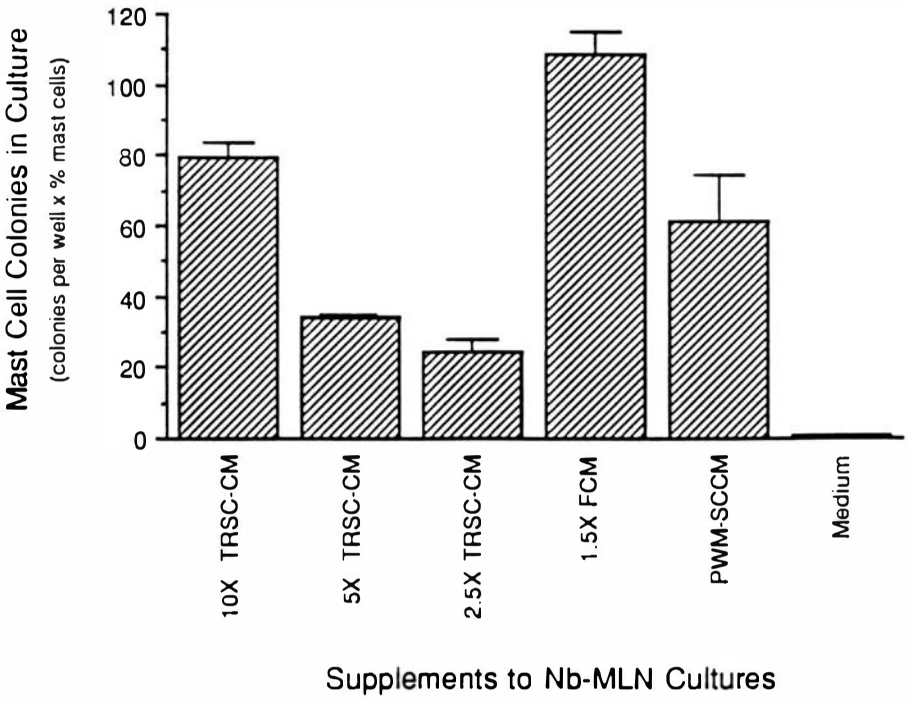
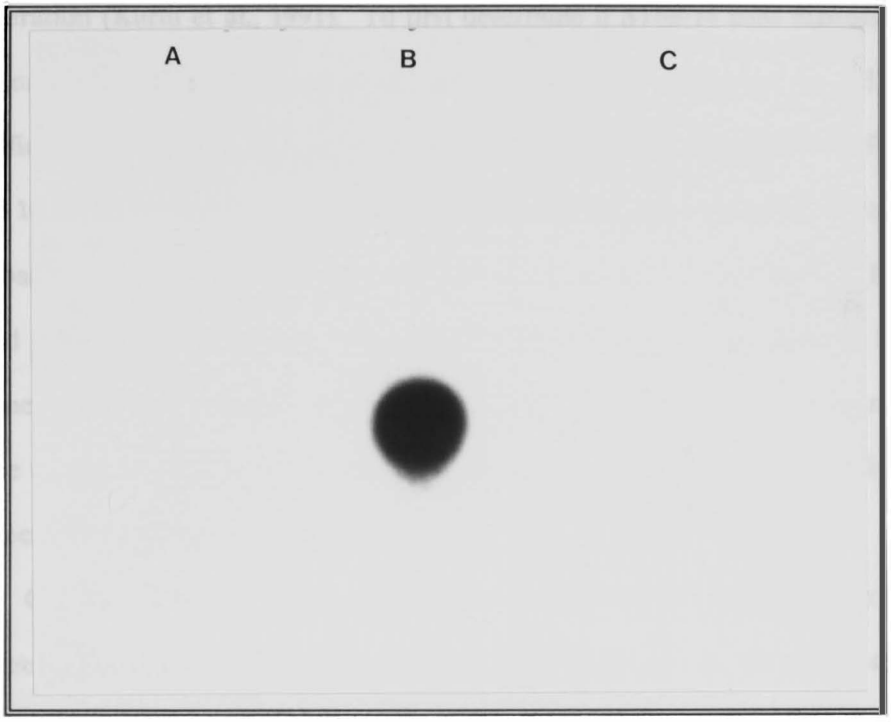

Mast Cell Colonies in Culture
(colonies per well x % mast cells)
120
100
80
60
40
20
0
10X TRSC-CM
5X TRSC-CM
2.5X TRSC-CM
1.5X FCM
PWM-SCCM
Medium
Supplements to Nb-MLN Cultures

Figure 8
RT-PCR for IL-3 from transfected rat Schwann cells. RT-PCR using primers specific for rodent IL-3 was carried out on samples containing 2μg total RNA from (A) transfected rat Schwann cells or (B) WEHI-3 cells, or (C) a sample lacking RNA, as described in Materials and Methods, using an annealing temperature of 50°C for 30 cycles. Arrowhead indicates expected 547 base pair reaction product.


A
B
C

proliferation (Kuriu et al., 1991). To first determine if ST88-14 cells express GM-CSF or IL-3, RNA extracted from this cell line was subjected to RT-PCR amplification using primers specific for these growth factors. As shown in Figures 9 and 10, ST88-14 RNA could not be amplified to detect the expected IL-3 or GM-CSF bands, althoughthis same preparation of RNA was used to detect SCF (Figure 6) and actin (Data not shown) messages. MO7E cells were then cultured in the presence of varying concentrations of ST88-14-CM. However, this medium was unable to support MO7E proliferation (Figure 11), implying that no detectable SCF was secreted by ST88-14 cells.

Our laboratory has produced antibodies to rhSCF^{169} by immunizing rabbits with recombinant proteins as described in Materials and Methods. This antibody will bind to protein extracted from bacteria induced to express the recombinant SCF protein, but not to protein extracted from uninduced bacteria (Data not shown), and recognizes both glycosylated and unglycosylated forms of rhSCF in western blot analysis (Scott Kauma, Obstetrics and Gynecology, Medical College of Virginia, unpublished observations). Using this antibody, Schwann cells were analyzed for the ability to express membrane-bound SCF as determined by flow cytometric analysis. HT1080 cells, which express SCF (Martin et al., 1990), exhibited specific binding when stained with anti-hSCF^{169} as determined by fluorescent microscopy, indicating the presence of surface-bound SCF. However, the human small cell lung cancer line H510, which does not express SCF message (Personal communication, Dr. Geoff Krystal, Hematology and Oncology, Medical College of Virginia), did not stain above background with this antibody (Data not shown). Labelling of transfected rat Schwann cells with the anti-hSCF^{169} antibody resulted in an increase in fluorescence

Figure 9
RT-PCR for IL-3 from ST88-14 schwannoma cells. Total RNA was extracted from (A) ST88-14, (B) PMA/ionomycin-stimulated Jurkat T cells (positive control) or (C) 5637 bladder cell carcinoma cells (negative control) and subjected to RT-PCR using primers specific for human IL-3. Lane (D) is a negative control containing all reactants except RNA. Reaction was carried out as described using an annealing temperature of 50°C for 30 cycles. Arrowhead indicates expected 370 base pair reaction product.

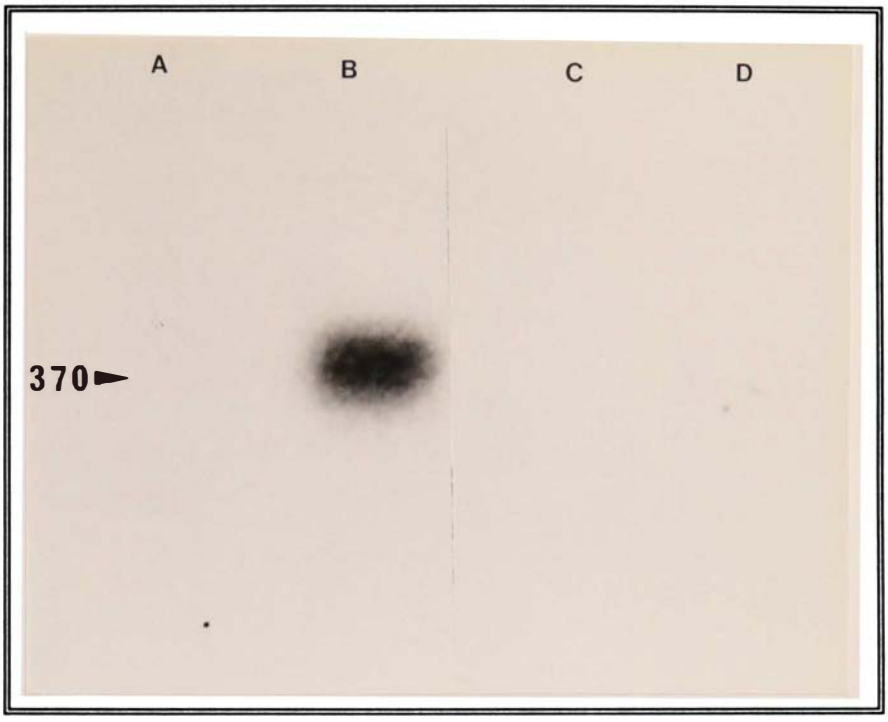

A
B
C
D
370

Figure 10
RT-PCR for human GM-CSF expression in ST88-14 schwannoma cells. RT-PCR using primers specific for human GM-CSF was carried out on samples containing 2μg total RNA extracted from (A) ST88-14 or (B) 5637 cells, or (C) a sample lacking RNA as described in Materials and Methods, using an annealing temperature of 50°C for 30 cycles. Arrowhead indicates the expected 393 base pair product.

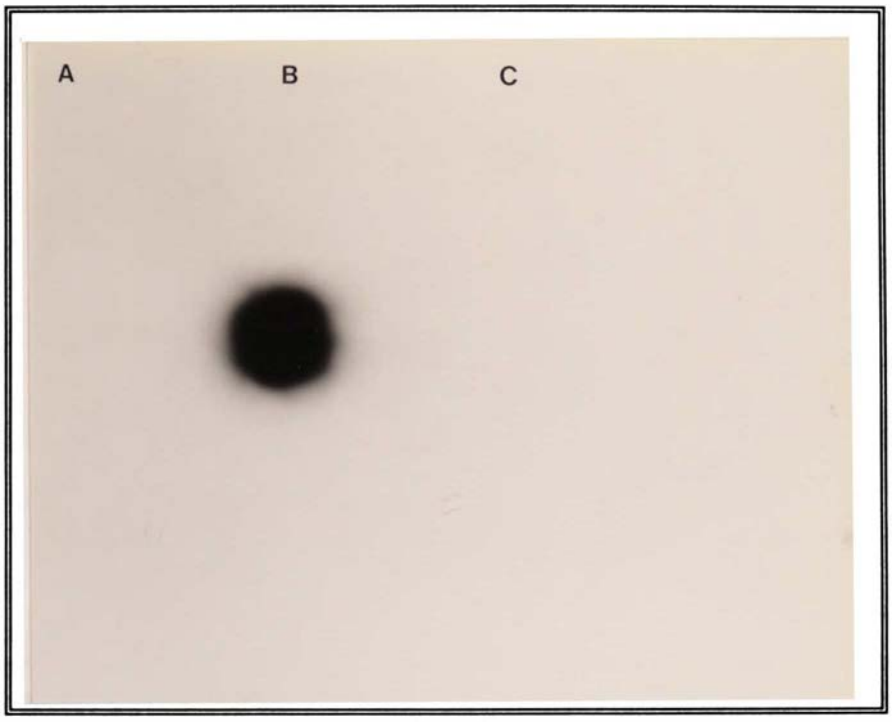
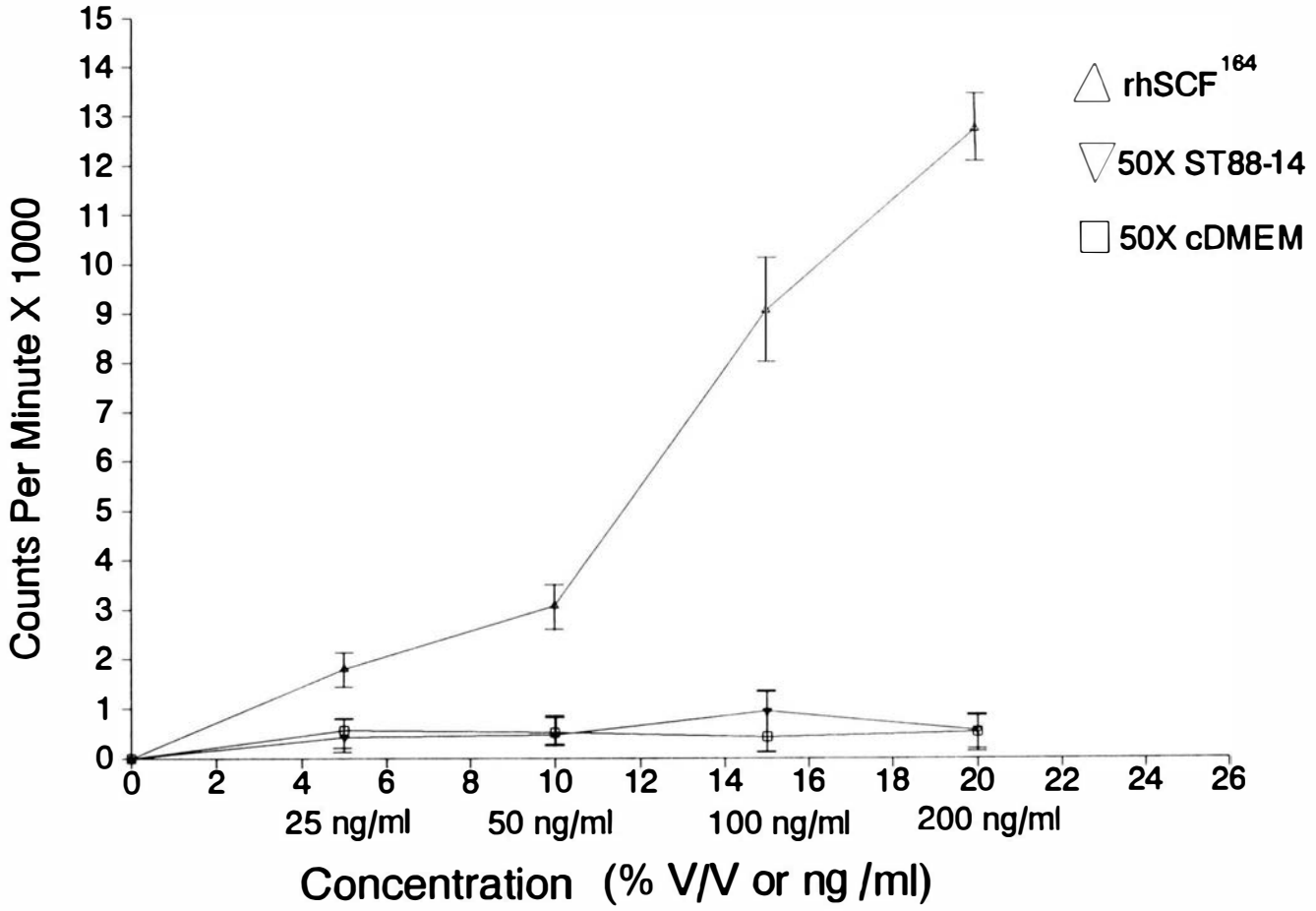

A
B
C

Figure 11
Cell proliferation assay to detect SCF. SCF-dependent MO7E cells were cultured in the presence of the indicated concentrations of rhSCF^{169}, 50X ST88-14-CM, or 50X cDMEM for 48 hours, after which time 1 μCi ^{3}H-TdR was added to each well. Wells were pulsed for 16 hours and cells harvested to absorbent glass paper with a multiport cell harvester. Radioactivity associated with cells was determined by liquid scintillation analysis. Values represent the means and standard errors of six wells from two separate experiments. Duplicate experiments gave similar results.


Counts Per Minute X 1000
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
rhSCF 164
50X ST88-14
50X cDMEM
0
2
4
6
8
10
12
14
16
18
20
22
24
26
25 ng/ml
50 ng/ml
100 ng/ml
200 ng/ml
Concentration (% V/V or ng /ml)

beyond rabbit IgG control antibody (Figure 12), as did staining of the ST88-14 Schwannoma cells (Figure 13). These results indicate that both Schwann cell lines express membrane-bound SCF. The detection of SCF protein in transfected rat Schwann cell-CM and the subsequent staining of transfected rat Schwann cells for surface SCF correlates with production of the KL-1 transcript, while surface SCF expression by ST88-14 cells and the lack of detectable SCF in culture supernatants is indicative of KL-2 expression by this cell line.

To determine if SCF is expressed by normal Schwann cells, primary neonatal rat Schwann cells (NRSC) were isolated from the sciatic nerve of two day old rats as described in Materials and Methods, cultured for several days, and stained with anti-hSCF^{169} antibody. At the time of staining, these cultures contained less than 1% contaminating fibroblasts, as determined by morphological criteria. As shown in Figure 14, the majority of this population exhibited a shift in fluorescence beyond control IgG after anti-hSCF^{169} staining, indicating SCF expression by these cells. Adult rat Schwann cells were isolated in a similar manner from sciatic nerves, and cultures of these cells contained approximately 20-25% contaminating fibroblasts. These cells were stained with anti-hSCF^{169}, as shown in Figure 15. Approximately 38% of the population exhibited fluorescence above control IgG levels, thus fibroblasts could not account for the entire SCF-expressing population. Moreover, Schwann cells can be separated from fibroblasts on the basis of their side scatter characteristics, as shown in Figure 16, which depicts a representative side scatter analysis of neonatal rat Schwann cells and 3T3 fibroblasts. Analysis of the adult rat Schwann cell cultures for fluorescence versus side scatter values was carried out to determine if the SCF-expressing cells in the adult rat Schwann cell cultures

Figure 12
FACS analysis for SCF expression by transfected rat Schwann cells. Transfected rat Schwann cells were stained with a 1:10 dilution of rabbit anti-rhSCF[169] polyclonal antiserum (light line), or a 1:10 dilution of pre-immune rabbit serum (dark line), counterstained stained with FITC-conjugated goat anti-rabbit IgG $F(ab')_2$, and analyzed using an Epics 753 flow cytometer as described in Materials and Methods.

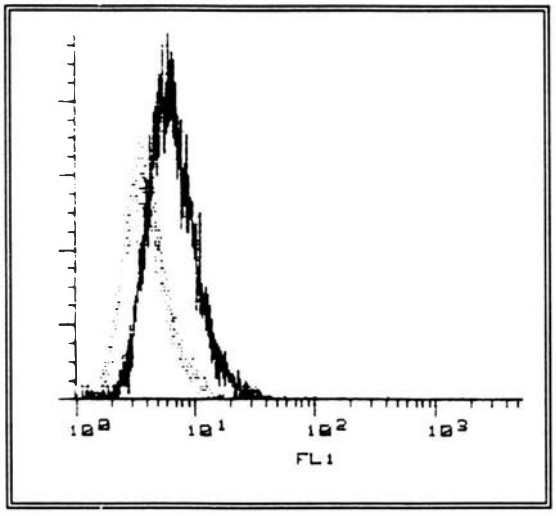
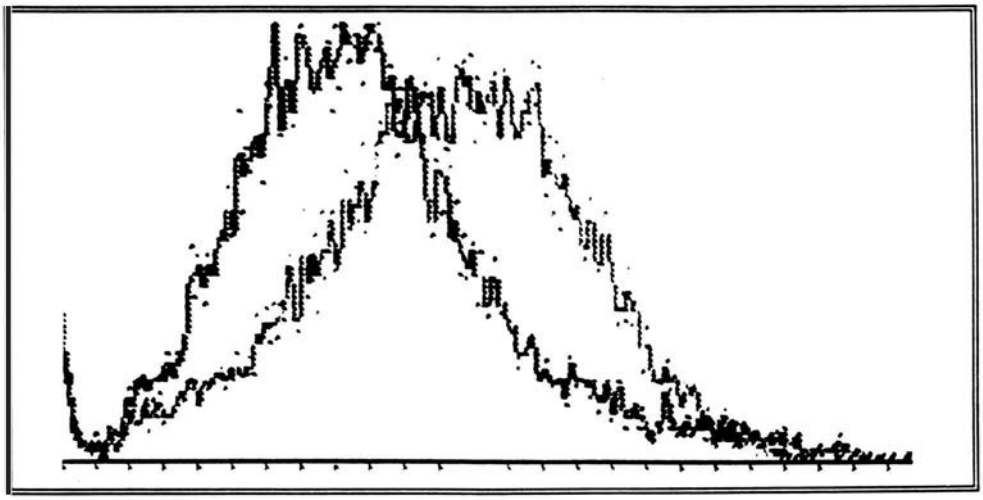

10^0
10^1
10^2
10^3
FL1

Figure 13
FACS analysis for SCF expression by ST88-14 human schwannoma cells. ST88-14 cells were stained with a 1:10 dilution of rabbit anti-rhSCF[169] polyclonal antiserum (dark line), or a 1:10 dilution of pre-immune rabbit serum (light line), counterstained stained with FITC-conjugated goat anti-rabbit IgG $F(ab')_2$, and analyzed using an Epics 753 flow cytometer as described in Materials and Methods.



Log Fluorescence (FITC)

Figure 14
FACS analysis for SCF expression by neonatal rat schwann cells. Neonatal rat Schwann cells were stained with a 1:50 dilution of rabbit anti-rhSCF^{169} polyclonal antiserum (light line), or a 1:50 dilution of pre-immune rabbit serum (dark line), counterstained stained with FITC-conjugated goat anti-rabbit IgG F(ab')$_2$, and analyzed using an Epics 753 flow cytometer as described in Materials and Methods.

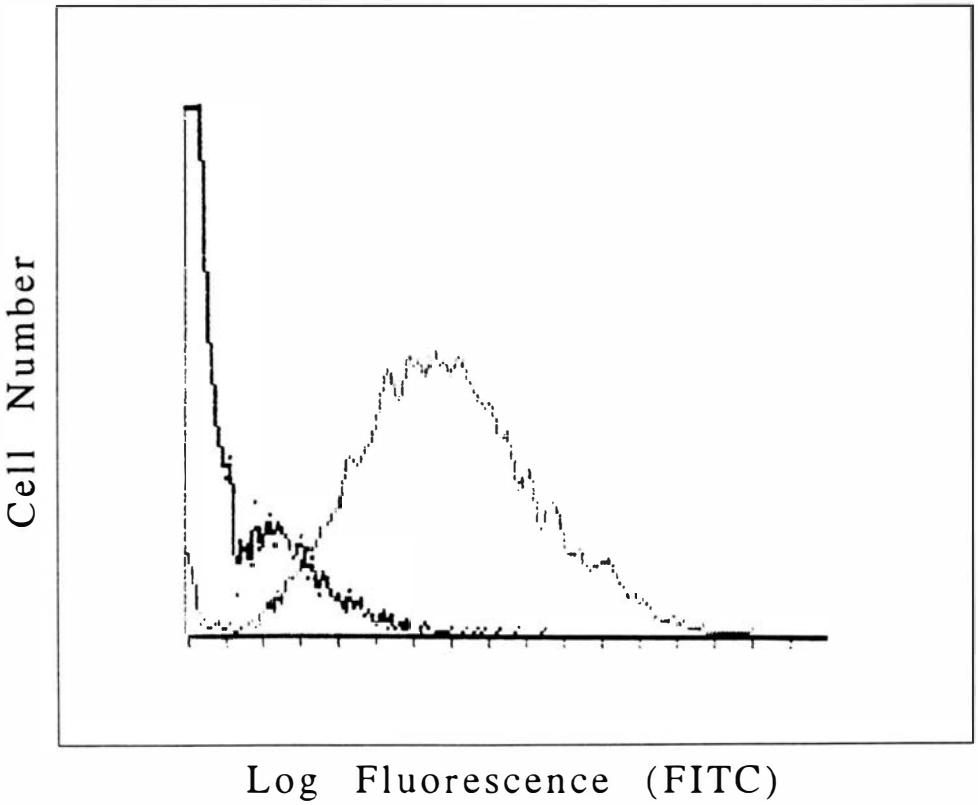
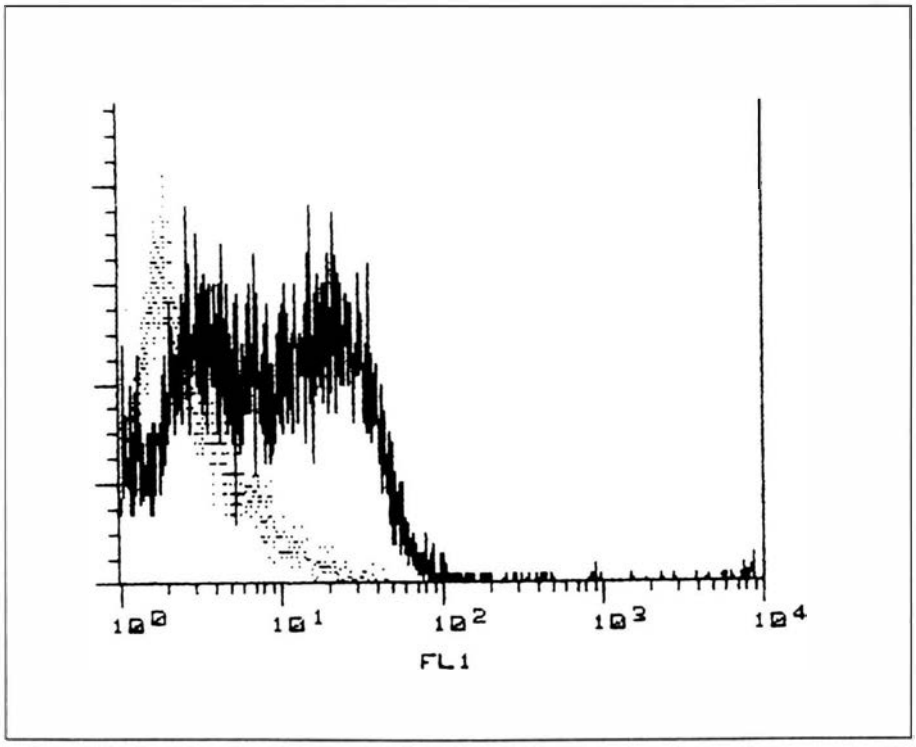

Cell Number
Log Fluorescence (FITC)

Figure 15
FACS analysis for SCF expression by adult rat schwann cells. ARSC cells were stained with 50 μg/ml rabbit anti-rhSCF[169] polyclonal antiserum IgG fraction (solid line), or 50 μg/ml rabbit IgG (dotted line), counterstained stained with FITC-conjugated goat anti-rabbit IgG $F(ab')_2$, and analyzed using a Becton-Dickinson FACScan flow cytometer as described in Materials and Methods.


Cell Number
10⁰
10¹
10²
10³
10⁴
FL1
Log Fluorescence (FITC)

conformed to Schwann cell side scatter values. As shown in Figure 17A, the majority of the cells which demonstrate a shift in fluorescence exhibit low side scatter values, similar to neonatal rat Schwann cells or the transfected rat Schwann cell line (Figure 17B and C). In fact, the side scatter values for adult and neonatal rat Schwann cells are nearly identical. Therefore, we have concluded that both neonatal and adult rat Schwann cells produce membrane-bound SCF.

Analysis of human Schwann cell cultures, which contained 40-50% Schwann cells, for SCF expression was carried out using an anti-nerve growth factor receptor (NGF-R) monoclonal antibody, which preferentially recognizes human Schwann cells. Light scatter analysis was again used to separate Schwann cells and fibroblasts (Figure 20A), and cells which showed an increase in fluorescence after staining with anti-NGF-R also exhibited a shift above background fluorescence after staining with anti-hSCF[169] (Figure 20B and 20C). Thus, we have detected SCF expression, both in secreted and membrane-bound form, in several sources of rat Schwann cells, as well as normal human Schwann cells and a malignant human schwannoma cell line.

V) c-*kit* Expression by Schwann Cells

The expression of c-*kit* in cells known to produce SCF would indicate the possible use of an autocrine growth loop. Also, increases in c-*kit* expression have been noted in neoplastic phenomena such as acute myeloblastic leukemia (Lerner et al., 1991), acute nonlymphocytic leukemia (Ikeda et al., 1991), and small cell lung cancer (Sekido et al., 1991). Since SCF expression was noted inseveral sources of Schwann cells, we have examined these cells for production of the c-*kit* receptor

Figure 16
Side scatter (SSC) analysis of neonatal rat Schwann cells (dark line) and 3T3 fibroblasts (light line), as determined using an Epics 753 flow cytometer.

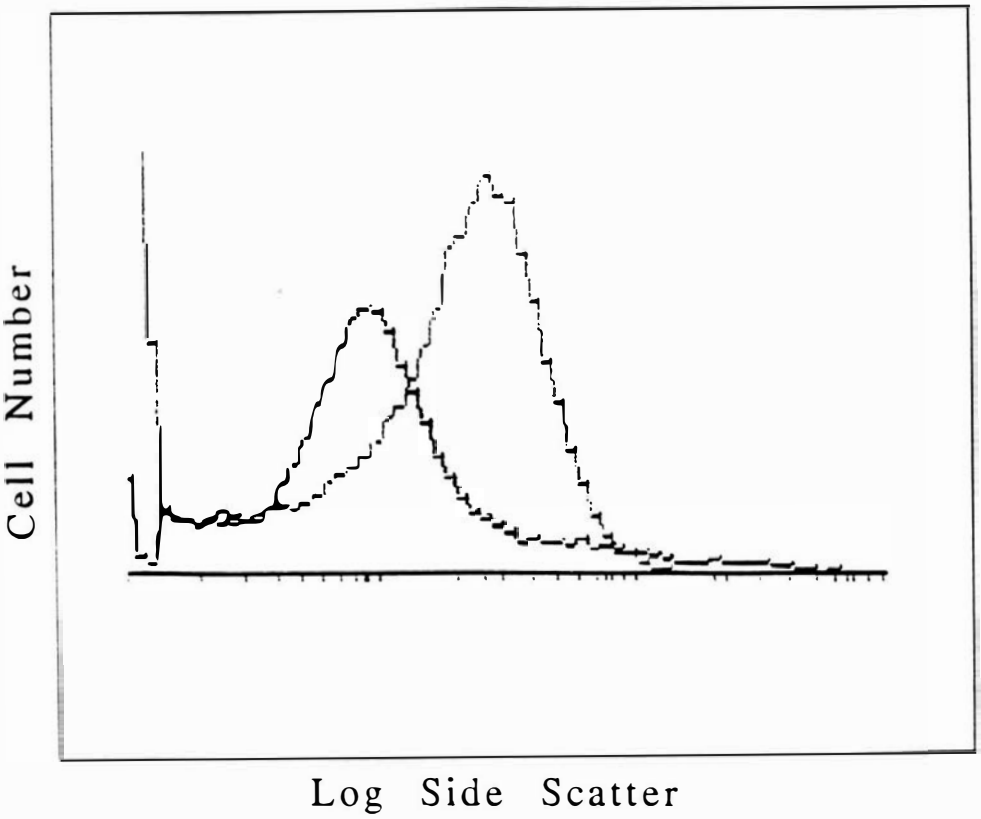
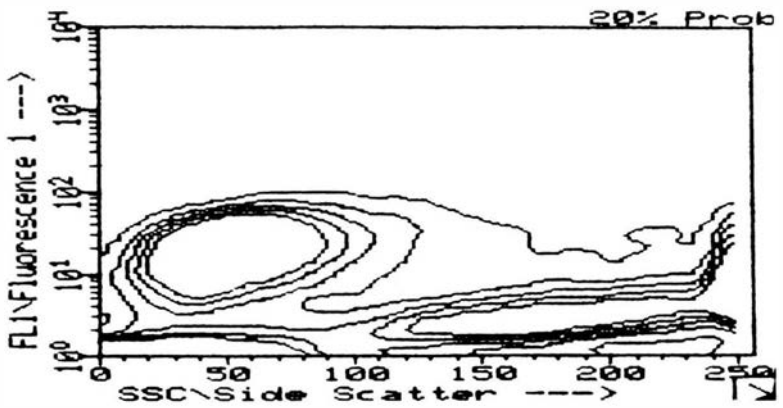
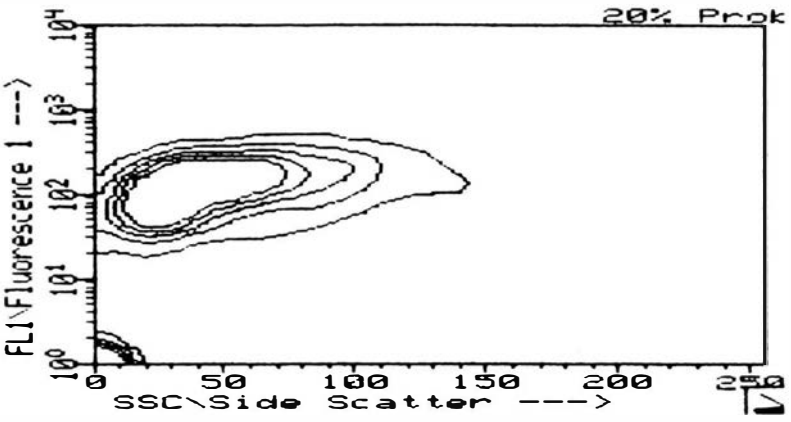

Cell Number
Log Side Scatter

Figure 17
Fluorescence-1 (FITC) versus side scatter analysis of Schwann cells. Cells were analyzed for FITC fluorescence and side scatter values after staining with 50 μg/ml rabbit anti-rhSCF[169] IgG fraction and FITC-conjugated goat anti-rabbit IgG F(ab')$_2$, using a Becton-Dickinson FACScan flow cytometer as described in Materials and Methods. Cell populations are (A) adult rat Schwann cells, (B) neonatal rat Schwann cells, and (C) transfected rat Schwann cells.

A


20% Prob
FL1\Fluorescence 1 --->
SSC\Side Scatter --->
V1

B


20% Prob
FL1\Fluorescence 1 --->
SSC\Side Scatter --->
V1

C

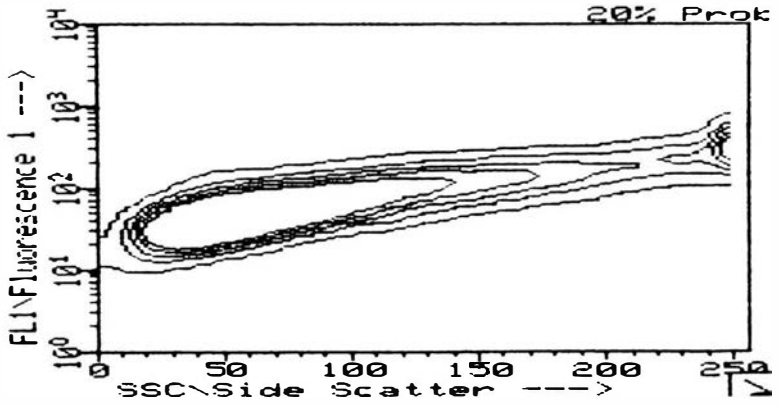
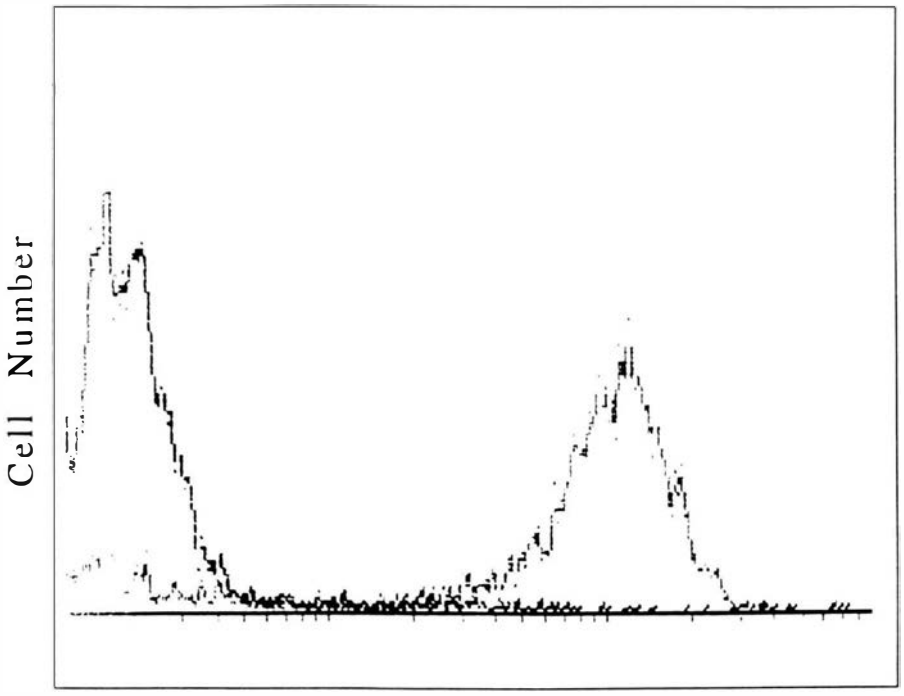

20% Prob
FL1\Fluorescence 1 --->
SSC\Side Scatter --->
V1

Figure 18
FACS analysis for SCF expression by normal human Schwann cells. A mixed population of fibroblasts and Schwann cells was stained with 50 μg/ml rabbit anti-rhSCF[169] IgG fraction and a neat solution of mouse anti-human nerve growth factor receptor, or 50 μg/ml rabbit IgG and 15 μg/ml mouse IgG, counterstained with FITC-conjugated goat anti-rabbit IgG $F(ab')_2$ and PE-conjugated goat anti-mouse IgG $F(ab')_2$, and analyzed using an Epics 753 flow cytometer as described in Materials and Methods. A population of cells which demonstrated both FITC and PE fluorescence after staining with anti-rhSCF[169] and anti-NGF-R was analyzed separately using light scatter properties and compared to a population of cells stained with control IgG-that had identical light scatter characteristics.


Cell Number
Log Fluorescence (PE)

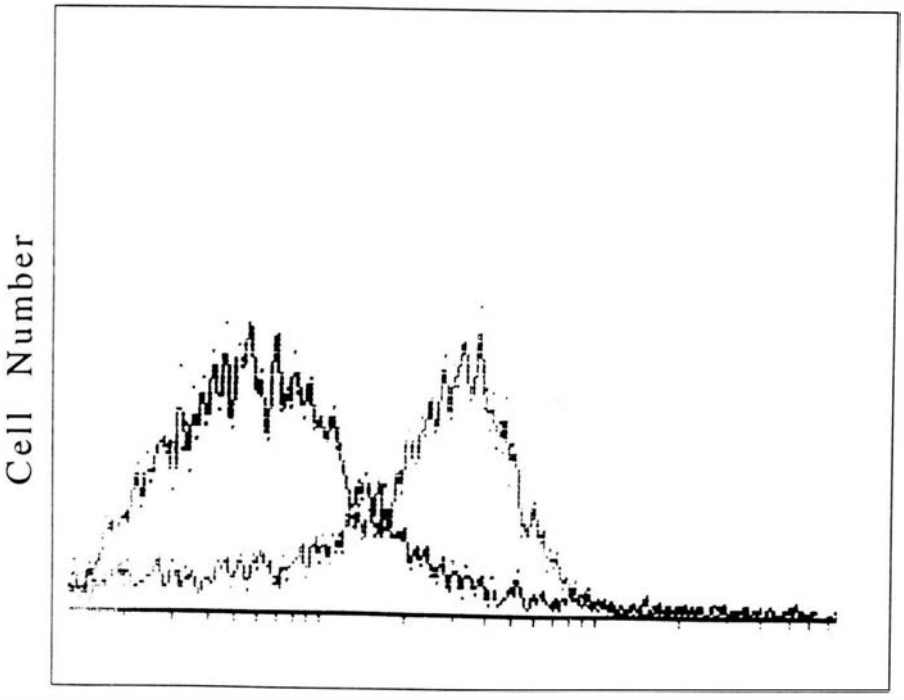

Cell Number
Log Fluorescence (FITC)

Figure 19
RT-PCR to detect expression of c-*kit* mRNA in ST88-14 human schwannoma cells. Total RNA was extracted from cells and subjected to RT-PCR as described in Materials and Methods, using primers specific for human c-*kit*, with an annealing temperature of 50°C for 30 cycles. Samples are (A) ST88-14, (B) and (C) Human AML cells, and (D) Control, lacking RNA. Arrowhead indicates expected 518 base pair PCR product.

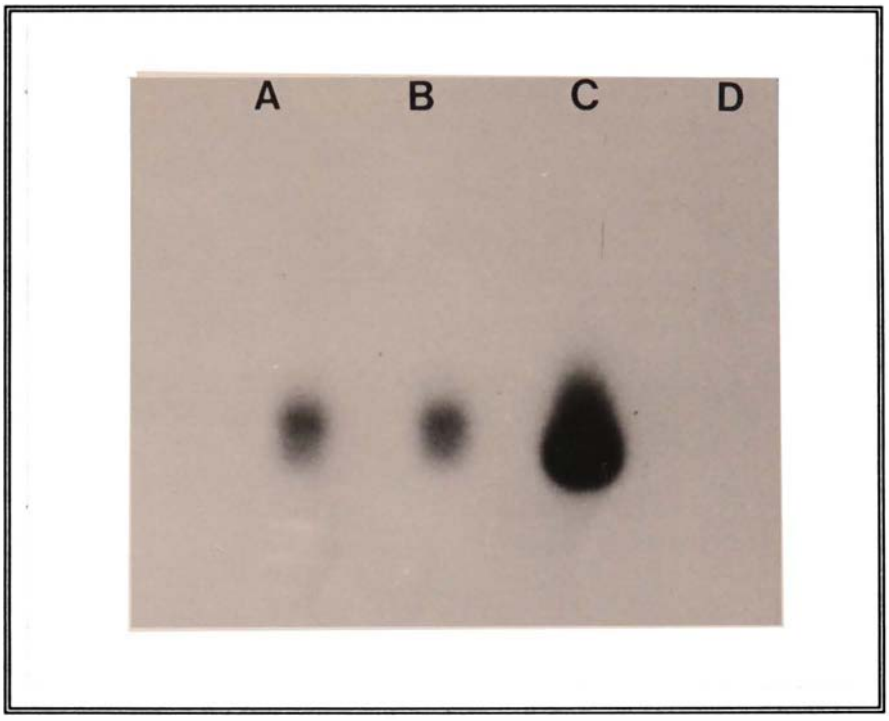
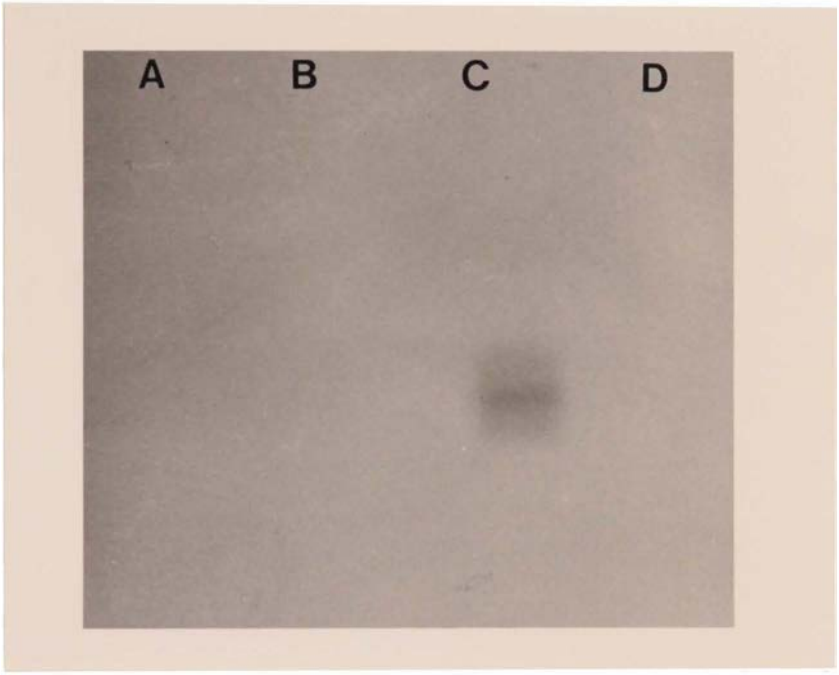

A
B
C
D

Figure 20
RT-PCR to detect expression of c-*kit* mRNA in rat Schwann cells. Total RNA was extracted from (A) NRSC, (B) transfected rat Schwann cells, or (C) RBL-2H3 cells and subjected to RT-PCR as described in Materials and Methods, using primers specific for rat c-*kit* for 30 cycles with an annealing temperature of 50°C. Lane (D) is a control lacking RNA. Arrowhead indicates expected 976 base pair PCR product. tyrosine kinase.


A
B
C
D

RT-PCR using primers specific for human c-*kit* was able to amplify the expected 500 base pair band from ST88-14 RNA, identical to the band detected from RNA extracted from two different sources of acute myelogenous leukemia cells (Figure 19), which are known to express c-*kit* (Lerner et al., 1991). However, RT-PCR amplification of RNA extracted from transfected rat Schwann cells or neonatal rat Schwann cells failed to show expression of the expected 1000 nucleotide band (Figure 20), although these same preparations of RNA could be expanded using actin primers (Data not shown).

To further examine the ability of Schwann cells to produce c-*kit*, anti-kit antibodies were used to stain and analyze Schwann cells by flow cytometry. Staining of the kit-expressing leukemic cell line MO7E resulted in a large shift in fluorescence over control IgG (Figure 21); however the small cell lung cancer line H146, which does not express c-*kit* message (Personal communication, Dr. Geoff Krystal, Medical College of Virginia), did not exhibit staining with this antibody (Figure 22). Normal human Schwann cells were labelled in a similar manner, and also showed no change in fluorescence (Figure 23), thus these cells do not appear to produce c-*kit* protein. However, the malignant schwannoma cell line ST88-14 did exhibit staining with anti-kit antibody in several experiments (Figure 24). This result, together with the detection of c-*kit* message by RT-PCR, indicates that this tumor line produces not only SCF, but its receptor as well.

Although no anti-rat c-*kit* antibodies were obtainable, the anti-human c-*kit* antibody YB5.B8 was found to cross-react with the rat c-*kit* protein, staining rat peritoneal mast cells (RPMC). However, this antibody did not successfully label

Figure 21
FACS analysis for expression of c-*kit* on human leukemic cells. MO7E cells were stained with 20 μg/ml monoclonal mouse anti-human c-*kit* antibody YB5.B8 (dark line) or mouse IgG (light line), washed and counterstained with PE-goat anti-mouse IgG $F(ab')_2$, then analyzed using an Epics 753 flow cytometer as described in Materials and Methods.

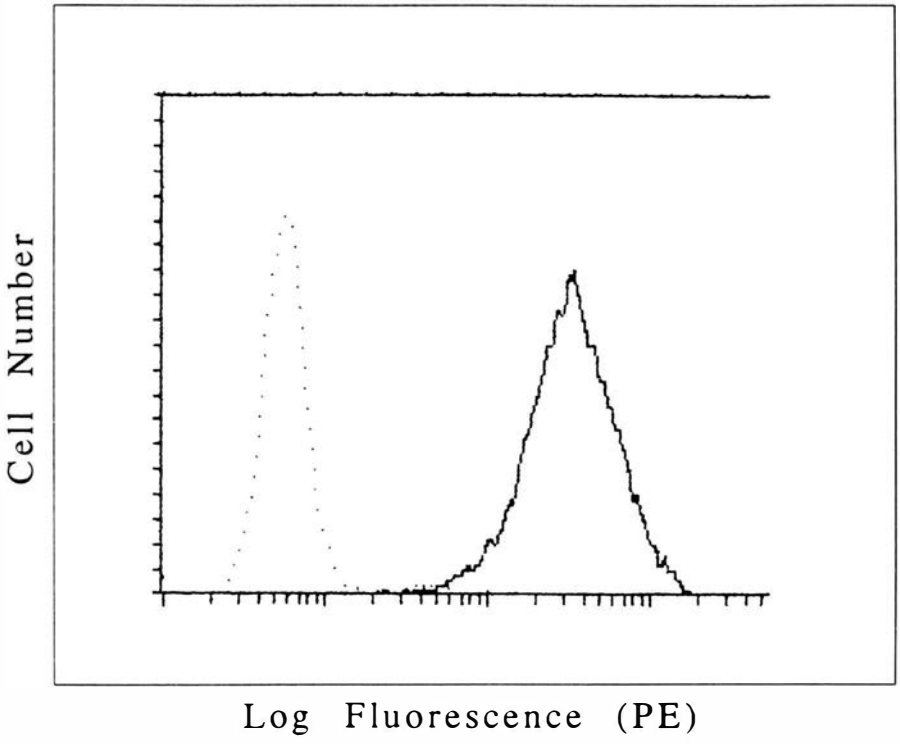

Cell Number
Log Fluorescence (PE)

Figure 22
FACS analysis for expression of c-*kit* on H146 human small cell lung cancer cells. H146 cells were stained with 15 μg/ml monoclonal mouse anti-human c-*kit* antibody (Boehringer) (dark line) or mouse IgG (light line), washed and counterstained with PE-goat anti-mouse IgG $F(ab')_2$, then analyzed using an Epics 753 flow cytometer as described in Materials and Methods.

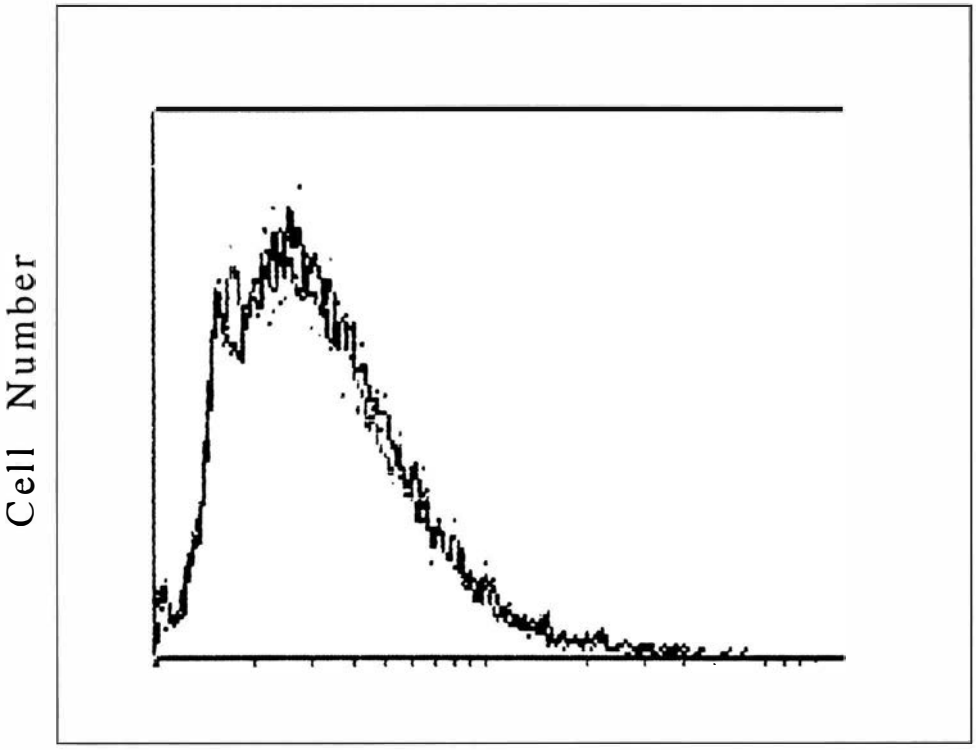
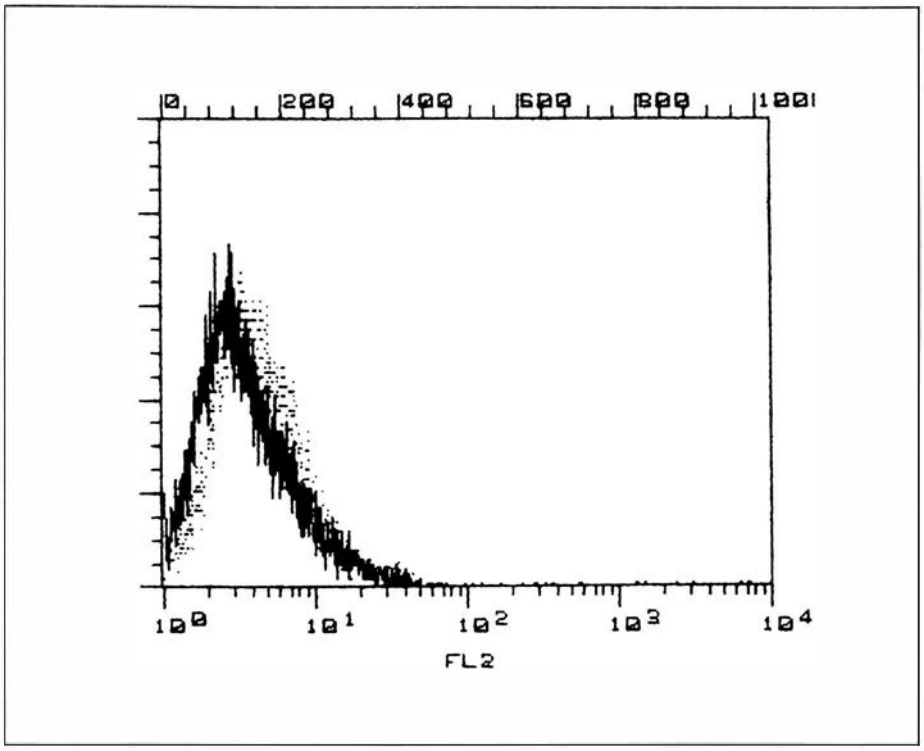

Cell Number
Log Fluorescence (PE)

Figure 23
FACS analysis for expression of c-*kit* on normal human Schwann cells. HSC cells were stained with 20 μg/ml monoclonal mouse anti-human c-*kit* antibody YB5.B8 (solid line) or mouse IgG (dotted line), washed and counterstained with PE-goat anti-mouse IgG $F(ab')_2$, then analyzed using a Becton-Dickinson FACScan flow cytometer as described in Materials and Methods.


0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4
FL2
Cell Number
Log Fluorescence (PE)

neonatal rat Schwann cells, the transfected rat Schwann cell line, or adult rat Schwann cells (Data not shown). These data correlate with the RT-PCR results above. Thus, of the Schwann cells examined, only the malignant schwannoma cells appear to express c-*kit*. Experiments aimed at slowing the proliferation of ST88-14 cells using anti-SCF or anti-kit antibodies, as well as anti-sense c-*kit* oligonucleotides have not been fruitful, but the use of an autocrine growth loop by these cells has not been excluded. Table I summarizes the extent of SCF and *c-Kit* expression by the Schwann cells examined in this study.

V) Production of SCF by the MMC-34 Mast Cell Line

Mast cells have been shown to produce an array of cytokines that roughly correlates to the TH2 subset of T lymphocytes (Plaut et al., 1989). Following from the work of this and other laboratories which has shown the importance of SCF in mast cell development and function, we sought to determine if mast cells also could produce SCF. Several mast cell lines were initially investigated for SCF gene expression using RT-PCR. These included PT-18, P815, and MMC-34. Of these, only MMC-34 reproducibly exhibited expression (Figure 25A). Although RT-PCR is not normally employed for quantitative measurements, relative expression of the expected 600 nucleotide SCF band increased after incubation of MMC-34 cells with PMA, as compared to the 500 nucleotide actin band (Figure 25B).

As described above, SCF can be produced as either a membrane-bound or secretable growth factor (Flanagan et al., 1991). To determine if MMC-34 mast cells produce the secreted form of SCF, the conditioned medium was concentrated 50 fold on an Amicon YM-10 membrane and placed in our committed progenitor assay in the presence or absence of blocking antibodies to IL-3. The use of these antibodies

Figure 24
FACS analysis for expression of c-*kit* on ST88-14 human Schwannoma cells. ST88-14 cells were stained with 15 μg/ml monoclonal mouse anti-human c-*kit* antibody (Boehringer) (light line) or mouse IgG (dark line), washed and counterstained with PE-goat anti-mouse IgG $F(ab')_2$, then analyzed using an Epics 753 flow cytometer as described in Materials and Methods.

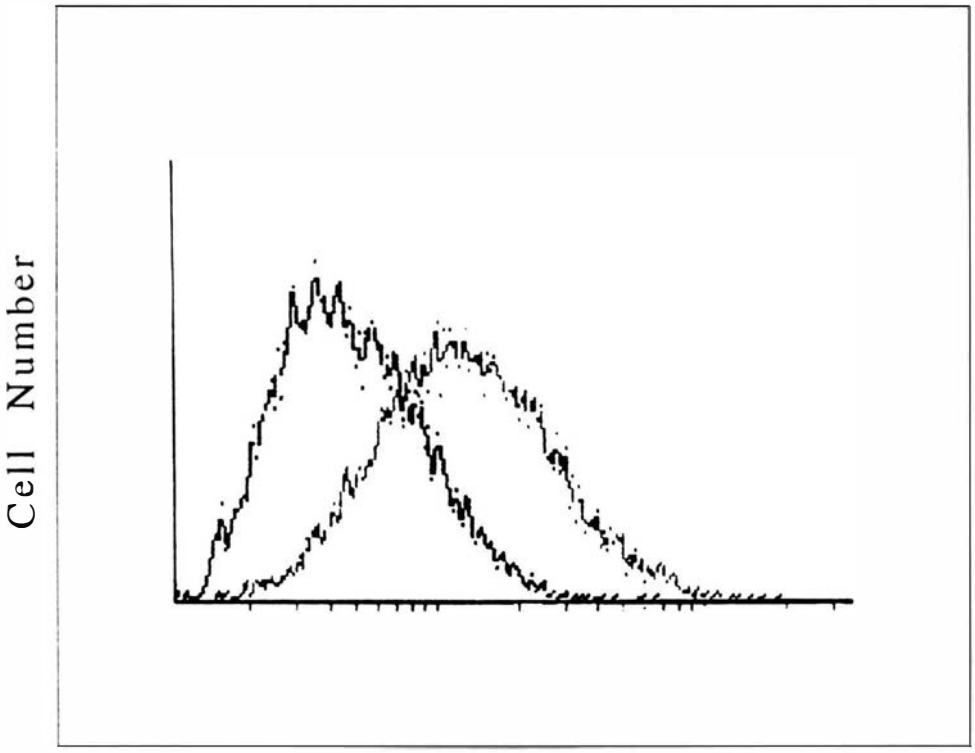
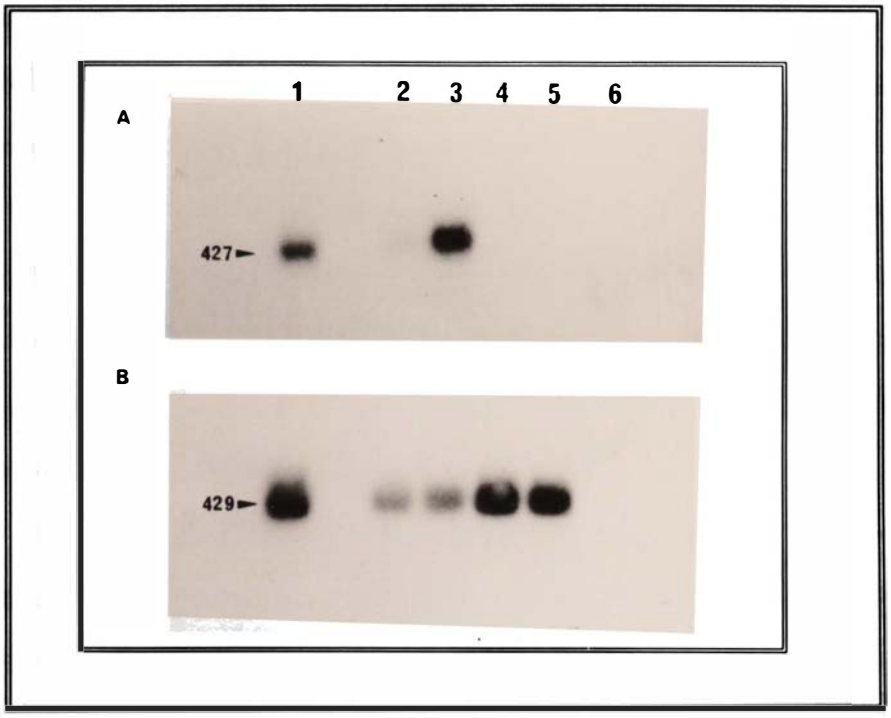

Cell Number
Log Fluorescence (PE)

Table I
Summary of SCF and c-*kit* Expression by Schwann Cells

Schwann Cell Type	SCF Expression	c-*kit* Expression
Neonatal Rat	+ + + +	-
Adult Rat	+ + +	-
Transfected Rat	+ + +	-
Normal Human	+ + +	-
Human Schwannoma	+ + +	+ +

Figure 25
RT-PCR to detect mSCF and actin expression in mast cells. Total RNA was extracted and subjected to RT-PCR with an annealing temperature of 50°C for 30 cycles using primers specific for (A) mSCF or (B) actin as described in Materials and Methods. Samples are (1) BALB/c 3T3, (2) MMC34, (3) MMC34 + 50ng/ml PMA, (4) P3Ag8.653, (5) P3Ag8.653 + 50ng/ml PMA (6) Control (-RNA). Arrowhead indicates the expected sizes of the PCR products.


1
2
3
4
5
6
A
427
B
429

was necessary, as mast cells have been shown to produce IL-3 (Plaut et al., 1989), and RT-PCR of MMC-34 RNA using primers for IL-3 message showed the presence of the expected 550 base pair (Figure 26). As shown in Figure 27, MMC-34-CM was able to generate up to 70 mast cell colonies from *Nb*-MLN cells in the presence of the anti-IL-3 antibody. Inhibition of recombinant IL-3 and PWM-SCCM activity was significantly more effective than inhibition of MMC-34-CM. Since SCF is known to synergize with IL-3 (de Vries et al., 1991), the blocking of IL-3 could be expected to decrease the activity of SCF in these cultures.

Mast cell phenotype is known to be dependent upon the microenvironment in which the cells differentiate (See Literature Review), and as supported by the above data, SCF can induce a CTMC phenotype from committed progenitors, while sources of IL-3, such as PWM-SCCM cannot. Therefore, we examined the phenotype of mast cells generated from stimulation with MMC-34-CM, using the rationale that if SCF were present in mast cell-conditioned medium, it might induce a connective tissue phenotype in newly generated mast cells. While 81% or 95% of cells derived from cultures stimulated with MMC-34-CM or 3T3 FCM, respectively, stained with safranin, cells stimulated with PWM-SCCM did not. Similar results were obtained with berberine sulfate staining, where no PWM-SCCM-stimulated cells were stained, but 98% of 3T3 FCM-stimulated and 10% of MMC-34-CM-stimulated cells were stained (Table II). Thus, *Nb*-MLN cells could be stimulated by MMC-34-CM to form colonies containing CTMC, indicative of SCF present in this conditioned medium.

Staining of MMC-34 mast cells with an antibody made against rmSCF[169] was used to determine the extent of surface-bound SCF expression. As shown in Figure 28, MMC-34 cells could be stained with this antibody above similar dilutions of pre

Figure 26
RT-PCR to detect IL-3 mRNA expression in MMC34 mast cells. Total RNA was extracted and subjected to RT-PCR with an annealing temperature of 50°C for 30 cycles using primers specific for rodent IL-3. Samples are (A) MMC34, (B) Control (-RNA), and (C) WEHI-3 cells. Arrowhead indicates the expected 547 base pair PCR product.

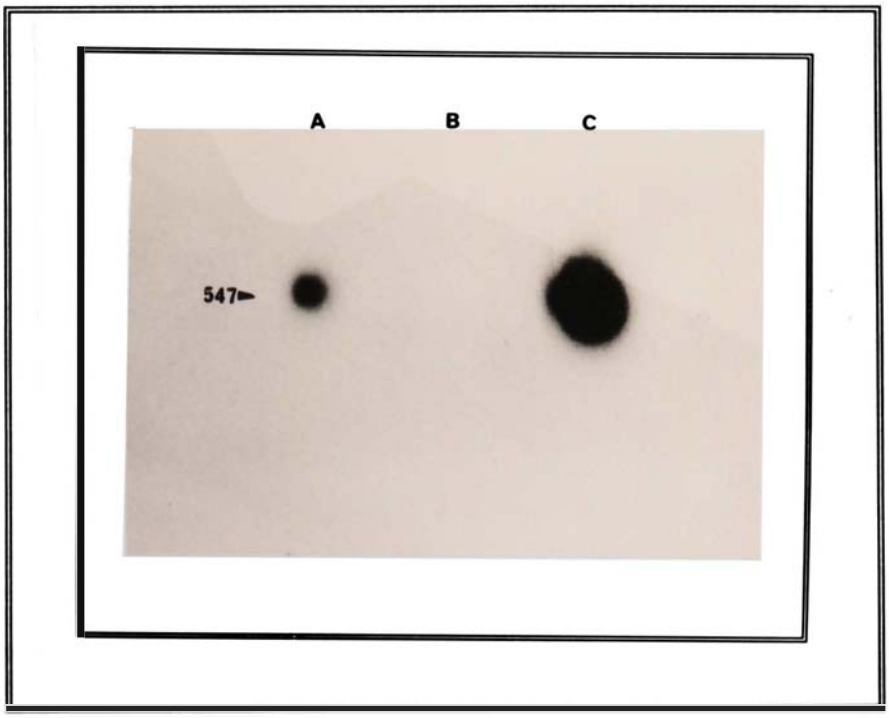

A
B
C
547

Figure 27
Activity of MMC34-CM in committed progenitor assay. *Nb*-MLN cells were cultured for 13 days in the presence of 50X MMC34-CM ± 20μg/ml anti-IL-3 antibody (diagonal-striped and open boxes, respectively), 200 u/ml recombinant mouse IL-3, or 10% v/v PWM-SCCM ±20 μg/ml anti-mouse IL-3 antibody (filled and horizontal-striped boxes, respectively). Controls are 3T3-CM and medium alone (stippled boxes). Values represent mean colony numbers and standard errors from two wells of a 24 well plate in one of two representative experiments.

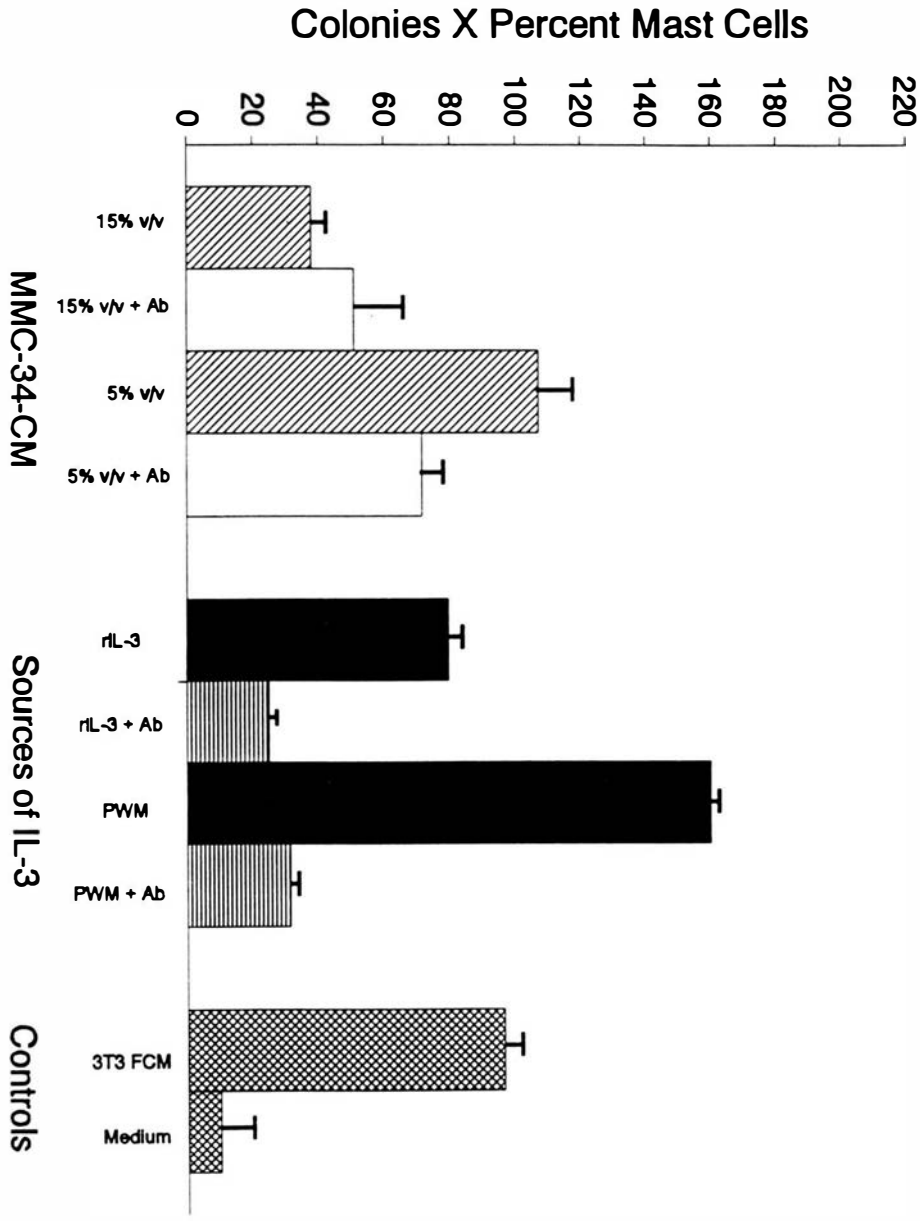
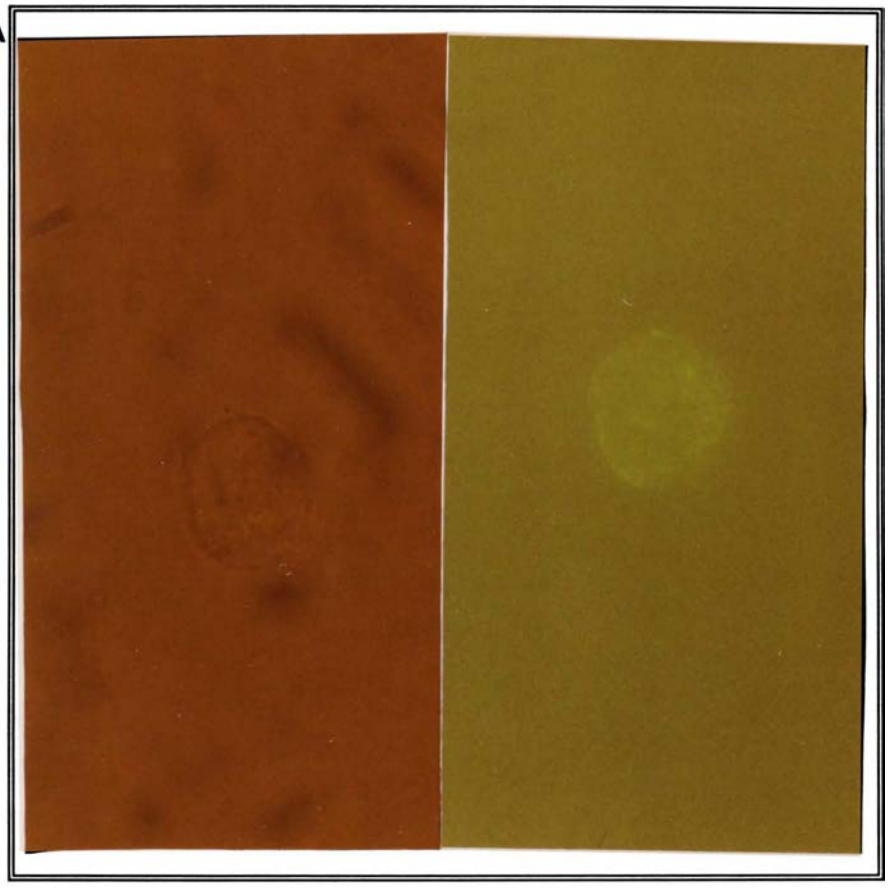

Colonies X Percent Mast Cells
0
20
40
60
80
100
120
140
160
180
200
220
MMC-34-CM
15% v/v
15% v/v + Ab
5% v/v
5% v/v + Ab
Sources of IL-3
rIL-3
rIL-3 + Ab
PWM
PWM + Ab
Controls
3T3 FCM
Medium

Table II
Phenotype of Cells Derived from Culture of *Nb*-MLN Cells with MMC-34-CM

Stimulus	Toluidine Blue (Histamine)	Berberine Sulfate (Heparin)	Safranin (Heparin)
MMC34-CM	99%	10%	81%
3T3 FCM	98%	98%	95%
PWM-SCCM	100%	0%	0%

Figure 28
Staining of MMC-34 mast cells for SCF expression. MMC-34 cells were stained with a 1:10 dilution of rabbit anti-rmSCF[169] or a 1:10 dilution of pre-immune rabbit serum and counterstained with goat anti-rabbit IgG $F(ab')_2$, then analyzed on an Olympus BH2-NIC fluorescent microscope. (A) Photomicrograph of MMC-34 cells under (1) normal, and (2) fluorescent light. (B) Percentage of MMC-34 or P3Ag8.653 (negative control) cells exhibiting fluorescence after staining with antibody or control serum.

A



B Anti-SCF Staining of MMC-34 Mast Cells

Cells	Percent Fluorescent
MMC-34 + Anti-mSCF[169]	78%
MMC-34 + Preimmune Serum	12%
P3Ag8.653 + Anti-mSCF[169]	0%
P3Ag8.653 + Preimmune Serum	17%

-immune control serum levels, and the antibody failed to stain P3Ag8.653 cells, which do not express SCF message, as determined by RT-PCR (Figure 25). However, this staining was dim, and quenched quickly, even in the presence of sodium azide. Flow cytometric analysis of these cells under similar conditions gave contrasting results. No notable change in fluorescence was detected in unstimulated cells, and only a small shift in fluorescent intensity was detected after PMA stimulation (Figure 29A and 29B).

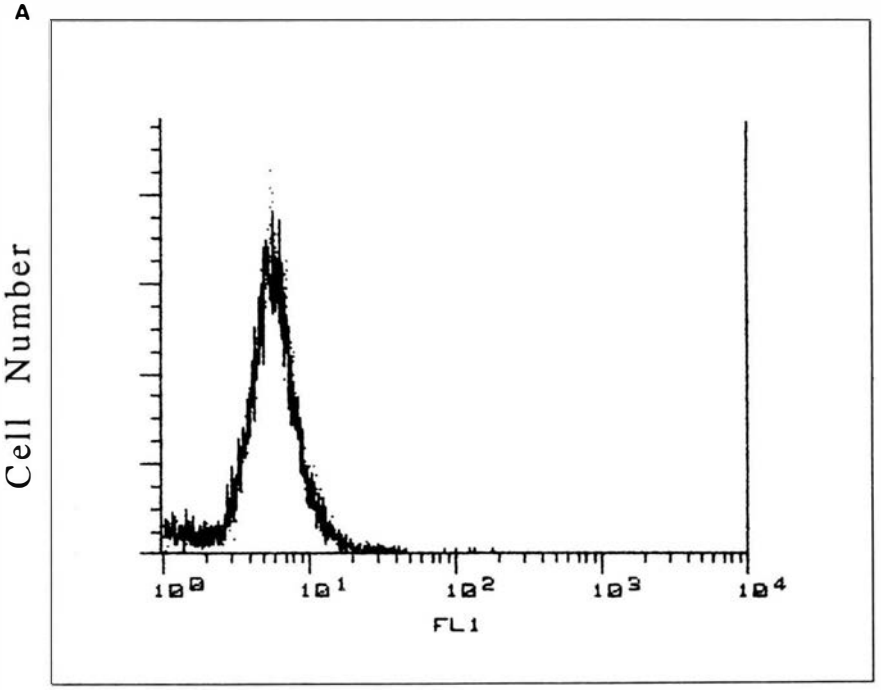
VI) The Peritoneal Mast Cell Progenitor

The committed progenitor assay exploits a local mastocytosis that occurs in the MLN during *Nb* infection. However, mast cell progenitors are present in other locations, and *Nb* infection may influence these progenitors as well. To determine what effect *Nb* infection has on peritoneal mast cell progenitors, peritoneal cells were extracted from mice on various days after infection, and cultured in methylcellulose under conditions similar to the committed progenitor assay. Peritoneal cells extracted from naive mice formed many mast cell colonies in response to stimulation with 3T3 FCM, as did cells extracted on day 3 after infection. Unexpectedly, cells harvested on days 5, 8, 11, 14 or 17 yielded essentially no mast cell colonies after stimulation with 3T3 FCM (Table III). A similar pattern of response was noted using recombinant mSCF[169], which stimulated the formation of 409 colonies from naive mice, but none on day 14 after infection (Table IV). Responsiveness seemed to return gradually 30-45 days after infection, although this has not been studied in detail (Data not shown). Thus *Nb* infection appears to have a dramatic negative effect on peritoneal mast cell precursors.

To characterize the peritoneal mast cell precursor population, peritoneal cells

Figure 29
FACS analysis of (A) unstimulated or (B) PMA-stimulated (50ng/ml, 60 minutes) MMC-34 mast cells for surface expression of mSCF. MMC-34 cells ± 50 ng/ml PMA were stained with 20 μg/ml rabbit anti-rmSCF[169] polyclonal antiserum IgG fraction (solid line) or 20 μg/ml rabbit IgG (dotted line), counterstained with FITC-goat anti-rabbit IgG, and analyzed with a Becton-Dickinson FACScan flow cytometer.

A


Cell Number
10⁰
10¹
10²
10³
10⁴
FL1
Log Fluorescence (FITC)

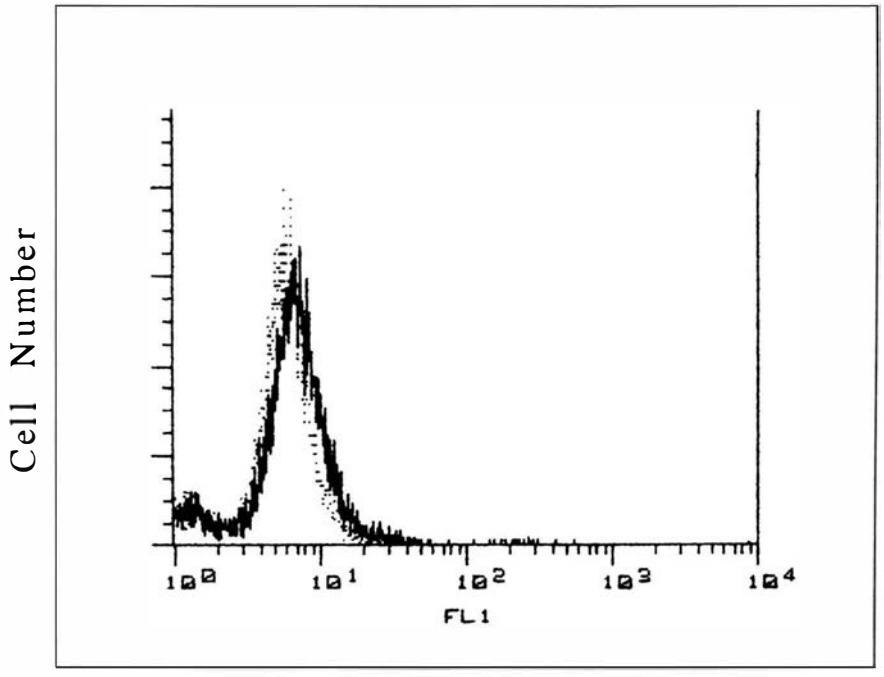

Cell Number
10⁰
10¹
10²
10³
10⁴
FL1
Log Fluorescence (FITC)

Table III
Time Course of Peritoneal Mast Cell Progenitor Response to 3T3 FCM

Day of Infection	Colonies in Response to 15x 3T3-CM ±SEM	Colonies in Response to Medium
Day 0	362 ±35.5	0
Day 5	629 ±36.0	0
Day 8	0	0
Day 11	0	0
Day 14	0	0
Day 17	0	0
Nb-MLN Day 14	139 ±18.2	0

from naive mice were separated on a discontinuous percoll gradient. Tables V shows the results of this preliminary experiment. While fraction 4 contained the highest percentage of mature mast cells (99%), fraction 3, which contained only 5% mature mast cells could be induced by 3T3 FCM to yield almost four times as many mast cell colonies as fraction 4 (84 versus 23, respectively). The density of cells in fraction 3 was 1.072-1.082 g/ml, which is slightly higher than the 1.060-1.070 g/ml density of the MCCP (Jarboe et al., 1989). The failure of the mast cell-colony forming activity to segregate with fraction 4 implies that mature peritoneal mast cells do not account for the entire proliferative capabilities found in the peritoneal mast cell population. Therefore a nongranulated, SCF-responsive mast cell progenitor appears to be present in the peritoneal cavity as well as the *Nb*-MLN.

Separation of peritoneal cells from 14 day *Nb*-infected mice gave results which contrasted with those derived from naive mice. As shown in Table VI, unseparated peritoneal cells from *Nb*-infected mice gave no mast cell colonies in response to 3T3 FCM. The same result was obtained with cells that had a density below 1.082, which includes the 3T3 FCM-responsive cells found in the naive peritoneal cavity. Unexpectedly, peritoneal cells from *Nb*-infected mice that had a density greater than 1.082 generated 40 mast cell colonies in response to 3T3 FCM. These cells were composed of 12.3% mature mast cells, and many small blast cells. The ability of this fraction and not unseparated or lower density cells to respond to 3T3 FCM implies that an active form of inhibition may be occurring among the other fractions tested.

VII) Production of SCF by Placental Tissues

As our early work on SCF expression became known among investigators in

Table IV

Time Course of Peritoneal Mast Cell Progenitor Response to SCF

Cell Population	Stimulus	Mast Cell Colonies ±SEM
Naive Peritoneum	50 ng/ml rmSCF[169]	409 ±25.0
	Medium	0
Day 14 *Nb*-Peritoneum	50 ng/ml rmSCF[169]	0
	Medium	0
Day 14 *Nb*-MLN	50 ng/ml rmSCF[169]	162 ±35.0
	Medium	0

Table V

Percoll Density Separation of Peritoneal Mast Cell Progenitors from Naive Mice

Cell Source	Stimulus	Colonies x % Mast Cells ±SEM	Percent Mast Cells at Day 0
Unseparated Peritoneum	3T3 FCM	257 ±45.2	2.2%
	Medium	0	
Frac.1 <1.062 g/ml	3T3 FCM	34 ±0.25	4%
	Medium	0	
Frac.2 1.062-1.072 g/ml	3T3 FCM	22 ±0.35	1%
	Medium	0	
Frac.3 1.072-1.082 g/ml	3T3 FCM	84 ±6.0	5%
	Medium	0	
Frac.4 >1.082 g/ml	3T3 FCM	23 ±1.5	99%
	Medium	0	
Nb-MLN	3T3 FCM	80 ±6.0	
	Medium	0	

Table VI

Percoll Density Separation of Peritoneal Mast Cell Progenitors from *Nb* Infected Mice

Cell Source	Stimulus	Colonies X % Mast Cells ±SEM	Percent Mast Cells at Day 0
Unseparated Peritoneum	3T3 FCM	0	2.2%
	Medium	0	
Frac. 1 <1.082	3T3 FCM	0	2.0%
	Medium	0	
Frac. 2 >1.082	3T3 FCM	40 ± 5.0	12.3%
	Medium	0	
Nb-MLN	3T3 FCM	80 ± 6.0	
	Medium	0	

our area, we were approached by Dr. Scott Kauma, Department of Obstetrics and Gynecology, Medical College of Virginia, with questions concerning cytokine production by placental tissues. In the course of these discussions, we suspected that SCF may play a role in placental growth and fetal hematopoiesis. As a result, Dr. Kauma agreed to provide us with RNA extracted from human, rat, or mouse placental tissues, with the purpose of detecting SCF message in these tissues. Initial experiments using RT-PCR demonstrated the expression of the expected 830 nucleotide band in many of the placental tissues examined, as is summarized in Table VII. However, the sensitivity of RT-PCR left concerns about contaminating tissue unanswered, and quantitative results could not be obtained. Therefore we employed northern analysis to detect SCF transcripts. Northern blot of rat placental tissues shows a prominent band at 6.5 kb (Figure 30), the expected size for SCF (Anderson et al., 1990; Zsebo et al., 1990; Matsui et al., 1990). There was notable variability in the levels of SCF expression, with endometrium and placenta showing strong bands, while decidual tissues had little or no expression. Expression also appeared to increase from day 15 to day 18 in the placenta. By contrast, human tissues appeared to express a 6.1 kb band in low quantities; in fact this SCF message was only detectable in human placenta after selection of polyadenylated RNA (Figure 31). Thus SCF RNA is expressed in several of the rodent reproductive tissue examined, but appears to be expressed at lower levels in equivalent human tissues.

Table VII

RT-PCR Analysis of SCF Expression by Human Reproductive Tissues

Cell Source	Stem Cell Factor Expression
1st Trimester Placenta	+ + + +
1st Trimester Decidua	+ + + +
1st Trimester Trophoblasts	+ + +
1st Trimester Villous Core	0
Term Placenta	+ + +
Term Trophoblasts	0
Term Villous Core	+ +
Term Placental Membranes	+ + +
Proliferative Endometrium	+ +
Secretory Endometrium	+ + +

Figure 30
Northern analysis of human placenta for SCF expression. Northern blot of human (A) term placental polyadenylated RNA and (B) term placental total RNA probed with a ^{32}P-labelled 539 nucleotide human SCF cDNA. Arrowheads indicate 28s and 18s ribosomal bands.

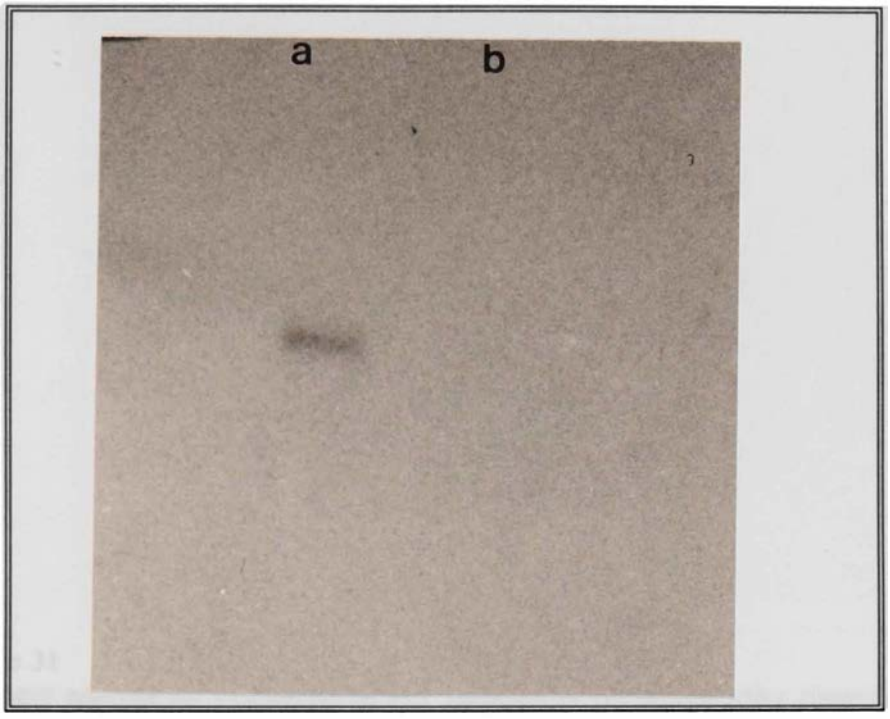
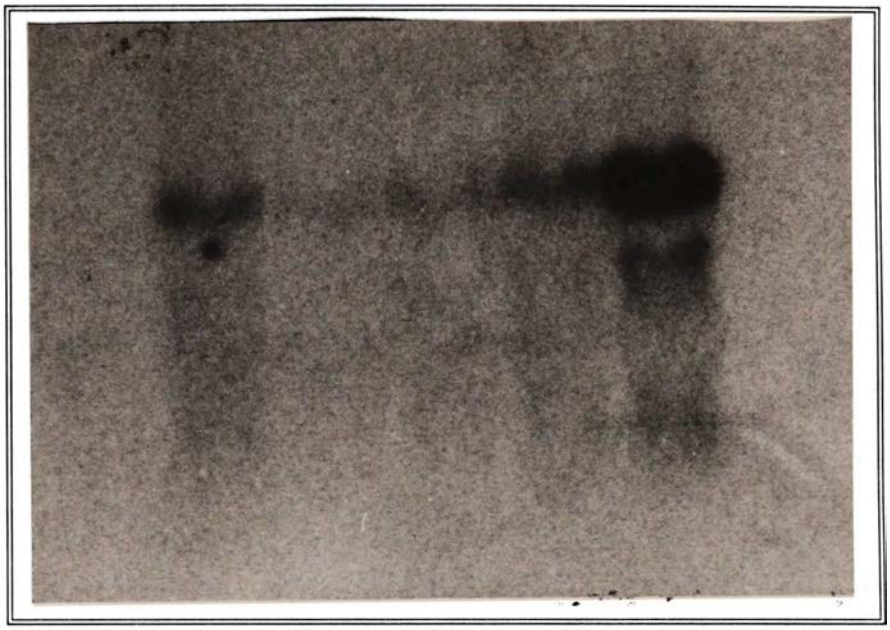
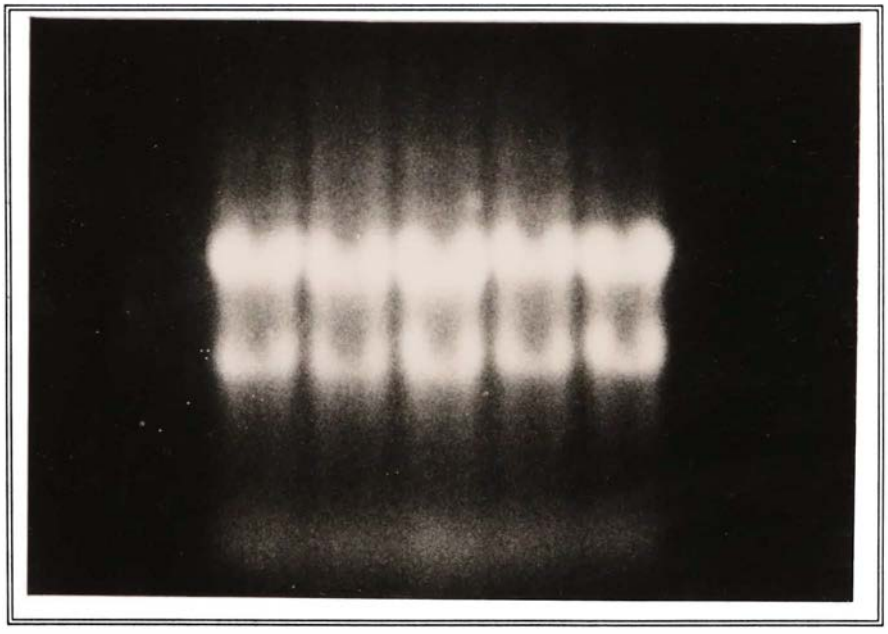

a
b

Figure 31
Northern analysis for expression of SCF message in rat reproductive tissues. (A) Northern blot of total RNA from rat (1) endometrium, (2) 15 day decidua, (3) 18 day decidua, (4) 15 day placenta, and (5) 18 day placenta probed with a ^{32}P-labelled 539 nucleotide human SCF cDNA. Arrowheads indicate 28s and 18s ribosomal bands. (B) Ethidium bromide staining of 1% formaldehyde-agarose gel in (A) to show equal loading of samples.

A.



B.



Discussion

I) Effect of rmSCF on Mast Cell-Committed Progenitors

Stem cell factor has proven to be important in many facets of mast cell biology, including proliferation (Jozaki et al., 1991), differentiation (Williams et al., 1992), migration (Meininger et al., 1992), and activation (Bischoff and Dahinden, 1992; Nakajima et al., 1992; Wershil et al., 1992). Although this growth factor cannot induce mast cell formation from bone marrow progenitors, it acts on many later stages of the lineage, including bone marrow culture-derived mast cells, and fully mature peritoneal mast cells. The mapping of SCF to the *Steel* locus and its identification as the c-*kit* ligand have proven what many investigators have long suspected: the pleiotropic abnormalities associated with mutations at the *W* or *Sl* loci are due to a growth factor-receptor interaction.

We have sought to determine the ability of SCF to induce mast cell colony formation from the mast cell-committed progenitor. This committed progenitor is poised at the stage of granulation, and can respond to either IL-3 or FCM (Jarboe et al., 1989). While the committed progenitor offers a well focused window for the study of mast cell differentiation, the signals required for this differentiation still had not been fully elucidated. This laboratory has previously shown the ability of 3T3 FCM to induce mast cell colony growth from committed progenitors, but the identity

of the fibroblast growth factor remained unknown. With the cloning of SCF came the finding that 3T3 fibroblasts produce this growth factor (Flanagan and Leder, 1990). We therefore tested to determine if recombinant SCF could mimic the effect of 3T3 FCM on committed progenitors and found that it could (Figure 3).

In fact, while an average of 90 mast cell colonies per well were generated in response to 3T3 FCM, as much as 110 colonies per well were induced by rmSCF. This result indicates that SCF alone is capable of mimicking the effect of 3T3 FCM, and may in fact be a more potent proliferative signal. This maximal response of rmSCF alone on colony formation stands in contrast to a minimal colony forming activity of rmSCF on bone marrow stem cells. Several investigators have shown that SCF acts more as a co-factor in concert with IL-3 or IL-6 to promote CFU-GEMM (de Vries et al., 1991). The ability of SCF alone to promote maximal proliferation of mast cell-committed progenitors may be important in the natural differentiation of mast cell-committed progenitors as they leave the bone marrow for fibroblast-rich sites such as dermis, which lack IL-3.

Two phenotypes of mast cells are known to exist in the mouse: connective tissue and mucosal. These cells differ in their *in vivo* location, size, life span, dependence upon IL-3, and granule contents. The difference in granule contents can be indicated by staining methods to differentiate between the two mast cell phenotypes. CTMC, but not MMC contain heparin proteoglycan. This proteoglycan can be detected by staining with either safranin or berberine sulfate. Figure 5 shows that mast cells derived from cultures stimulated with rmSCF or 3T3 FCM, but not PWM-SCCM exhibited CTMC phenotype by these staining criteria. The difference in berberine sulfate staining between 3T3 FCM and rmSCF-stimulated cells (98%

versus 63% respectively) has not been explained, although it could relate to a difference in "critical electrolyte concentration" (CEC) that exists between the two groups of cells. The CEC is determined by a cell's granule contents, and a minimum CEC is required for staining with berberine sulfate or safranin (Bernstein and Lawrence, 1990). However, this would not explain the very similar values obtained for safranin staining of 3T3 FCM versus rmSCF-stimulated cells (95% and 98% respectively). Overall, rmSCF seems to be able to promote a transition to a connective tissue phenotype.

Mast cell colonies induced by rmSCF were consistently larger than those cultured with FCM (Figure 4). While FCM stimulated formation of colonies with about 100 cells each, rmSCF generated colonies of 500 cells or more. These differences may be due to the proliferative signalling capacity of the two growth factors, i.e. rmSCF may be more mitogenic at the doses used, solely because it contains more SCF than FCM. Another possibility is that FCM and rmSCF stimulate different subsets of mast cell progenitors, as has been described for peritoneal mast cell precursors (Kanakura et al., 1988). Kanakura, et al. described the proliferative capabilities of three mast cell progenitors, stating that small (S)-CFU-Mast gave colonies containing fewer than 32 cells, while medium (M)-CFU-Mast yielded colonies with 32-499 cells, and large (L)-CFU-Mast resulted in colonies of greater than 500 cells each (Kanakura et al., 1988). While these subsets were examined in the peritoneal cavity, the authors hypothesize them to be a part of the normal mast cell lineage. Thus it could be argued that rmSCF acts on L-CFU-Mast, while 3T3 FCM stimulates M-CFU-Mast. However, we feel this is not a likely possibility for two reasons: First, colony size was directly dependent upon the

concentration of rmSCF with which the cells were stimulated. Lower concentrations of rmSCF not only yielded fewer colonies, but these colonies contained fewer cells as well. Second, the degree of differentiation seen by Kanakura, et al. varied with the progenitor subset stimulated. While S- or M-CFU-Mast yielded mast cells which stained with berberine sulfate, L-CFU-Mast did not. In our cultures this effect was not noted. As shown in Figure 5, mast cells derived from culture with rmSCF stained with berberine sulfate, as well as safranin. In fact, higher doses of SCF generally yielded greater percentages of mast cells capable of staining with these dyes (Data not shown).

We conclude that rmSCF induces both mast cell proliferation and differentiation to the CTMC phenotype from *Nb*-MLN cells, and that the increased colony size associated with rmSCF culture is due to greater proliferative capabilities possessed by this factor, rather than the stimulation of distinct mast cell subsets by rmSCF and 3T3 FCM.

II) SCF and Kit Production by Schwann cells

Mast cells are associated with normal nerves (Bienenstock et al., 1991), increase in nerve damage and repair (Isaacson, 1976; Olsson, 1971), and are hyperplastic in neoplastic phenomena that involve Schwann cells such as neurofibromas and schwannomas (Isaacson, 1976). Schwann cells are associated with peripheral nerves, functioning in myelination and nerve regeneration and repair. Therefore, it seemed logical to hypothesize a role for Schwann cells in neuron-associated mast cell hyperplasia. The role of SCF in mast cell development made it an ideal target for this hypothesis, thus we sought to examine the ability of rat and human Schwann cells to produce this growth factor. Through the kind gift of Dr.

Cynthia Morton (Brigham and Women's Hospital, Harvard), we obtained a malignant human schwannoma cell line, derived from the peripheral nerve of a neurofibromatosis patient. We also obtained a rat schwann cell line derived by SV40 large T antigen induction of neonatal Schwann cells from the laboratory of George DeVries, Virginia Commonwealth University.

As is shown in figure 6, RT-PCR of total RNA extracted from either Schwann cell line demonstrated the expression of SCF message, as determined by the presence of the expected 830 base band. Having determined that both cell lines express SCF message, these cells were then examined for the production of SCF protein. Concentrated transfected rat Schwann cell-CM placed in the committed progenitor assay (Figure 7) induced mast cell colony formation from *Nb*-MLN cells in a dose-responsive manner, and was comparable to 3T3 FCM. Of the known growth factors tested (Appendix A and Literature Review), only IL-3 has been active in this assay. However, RT-PCR for IL-3 expression failed to detect a band corresponding to IL-3 from transfected rat Schwann cell RNA in several experiments (Figure 8). Because these cells do not make IL-3, but conditioned medium from this cell line is active in the committed progenitor assay, we conclude that transfected rat Schwann cells produce secretable SCF which is active on mast cell progenitors.

Concentration of ST88-14 human schwannoma CM did not yield similar results. Use of this supernatant in a proliferation experiment with the SCF-dependent leukemic cell line MO7E was not able to support proliferation (Figure 11). This experiment was repeated several times and used multiple concentrations of ST88-14 CM. This result may be due to a selective formation of membrane-bound SCF, rather than soluble. Previous reports have shown that two transcripts for SCF

are generated by alternative splicing (Flanagan et al., 1991). One of these, KL-1 encodes the full length SCF molecule, while the other, KL-2, encodes a truncated form lacking the membrane tether region. Expression of KL-1 results in the production of a SCF protein with a proteolytic cleavage site that is hypothesized to yield secreted SCF. Expression of KL-2 results in the production of membrane-bound SCF only. Fujita and Kitamura have shown that such differential splicing may be used to phenotypically divide two cell lines as similar as BALB/c 3T3 fibroblasts and NIH 3T3 fibroblasts (Fujita and Kitamura, 1991). Thus it is possible that the transfected rat Schwann line produces predominantly KL-1, while ST88-14 cells make predominantly KL-2.

To determine if some or all Schwann cells do express SCF on their surface, a polyclonal rabbit anti-SCF^{169} antibody produced in our laboratory was used. A human fibrosarcoma cell line, HT1080, which produces SCF, could be stained with this antibody, but not pre-immune serum, an this antibody failed to stain the human small cell lung cancer cell line H510, which does not express SCF. Transfected rat Schwann cells stained with anti-SCF^{169} also demonstrated a shift in fluorescence relative to control serum when analyzed by flow cytometry. This indicates that these cells produce membrane-bound SCF in addition to the secreted SCF detected in the committed progenitor assay. This result is consistent with the expression of KL-1 hypothesized above. Normal neonatal rat Schwann cells also exhibited SCF expression by flow cytometric analysis, as did adult rat Schwann cells. This indicates that SCF expression by transfected rat Schwann cells is most probably not a function of SV40 transfection. More interestingly, the malignant human schwannoma ST88-14, which did not have SCF activity in its conditioned medium, also exhibited a

notable shift in fluorescence with anti-SCF^{169} staining versus pre-immune serum, indicating membrane-bound SCF. SCF was also detected on the surface of normal adult human Schwann cells, indicating that SCF expression by ST88-14 cells is probably not due to malignant transformation. Because all Schwann cells analyzed have demonstrated expression of SCF, we have concluded that SCF production is a normal facet of Schwann cell biology.

The consistent production of SCF by Schwann cells could explain the remarkable mast cell hyperplasia observed in many neurofibrotic lesions. A large scale increase in the number of Schwann cells would result in much higher local concentrations of SCF. This would result in local mast cell hyperplasia, which is the case. Indeed, pharmacologic agents active on mast cells (e.g. ketotifen) have proven to be efficacious in the clinical treatment of this syndrome (Riccardi, 1987). An alternative question which has not been addressed in this work is whether any of the many cytokines known to be produced in large quantity by mast cells may actually amplify the proliferation of Schwann cells in these lesions. Also not addressed in this work is the role of Schwann cell-produced SCF in the development of the nervous system. Since SCF and c-*kit* have both been shown to be expressed in the embryonic mouse nervous system during development (Keshet et al., 1991), it is possible that SCF produced by Schwann cells may regulate neuron growth or function. In fact, preliminary experiments have demonstrated strong expression of c-*kit* protein on the surface of neonatal rat neurites in culture (Neuberger and DeVries, personal communication), indicating that stimulation of neurons by SCF may be possible.

Since the receptor for SCF, c-*kit*, has been linked to AML (Lerner et al.,

1991), ANLL (Ikeda et al., 1991), and small cell lung cancer (Sekido et al., 1991), and since production of both a growth factor and its receptor would make possible the use of an autocrine growth factor loop, we sought to determine if Schwann cells produce c-*kit*. RNA isolated from ST88-14, transfected rat Schwann, or neonatal rat Schwann cells was analyzed by RT-PCR to detect c-*kit* message. While the malignant ST88-14 cells express c-*kit* message, it could not be detected in transfected rat Schwann cell and neonatal rat Schwann cell RNA, even on long exposures. These same preparations could be amplified by RT-PCR using actin primers. From these results it appears that ST88-14 cells express c-*kit* message, while normal or SV40-transfected neonatal rat Schwann cells do not.

To determine the extent of cell surface expression of the c-*kit* protein, these different Schwann cells were stained with a monoclonal mouse anti-human c-*kit* antibody, and counterstained with PE-goat anti-mouse IgG. Although this antibody was able to stain purified rat peritoneal mast cells, which express c-*kit*, no staining of either transfected rat Schwann cells or neonatal Schwann cells was detected. Coupled with the RT-PCR data, this result makes it apparent that these Schwann cells do not express c-*kit*. Similarly, normal adult human Schwann cells did not exhibit c-*kit* expression, although these cells were only evaluated in one experiment. However, the malignant schwannoma did show a shift in fluorescence, indicative of c-*kit* expression by this cell line. Thus while normal Schwann cells do not appear to produce the c-*kit* receptor tyrosine kinase, the malignant schwannoma does. This apparently aberrant expression of a protooncogene, in the presence of its ligand, could lead to the use of an autocrine growth loop by these cells. We have attempted

to investigate this possibility through the use of anti-sense oligonucleotides and antibodies against SCF and c-*kit*, however none of these agents has yet been able to consistently reduce the proliferation of this tumor line (Data not shown), although we have not ruled out this possibility.

In summary, normal Schwann cells produce SCF, and a malignant human schwannoma also produces not only this growth factor, but also the receptor for SCF, c-*kit*, which is not expressed on normal Schwann cells. The expression of both the ligand and its receptor could constitute an autocrine loop in this cell line, which may reflect the etiology of some NF-related malignant schwannomas.

III) Production of SCF by a Mast Cell Line

The ability of mast cells to produce cytokines has recently been shown (Plaut et al., 1989), allowing new insight into the role of mast cells in inflammation and immunoregulation. Since mast cells have been shown to produce such a wide array of growth factors, and SCF plays an important role in mast cell development, it is logical to hypothesize that mast cells may be able to produce SCF. To examine this possibility, we extracted RNA from the mast cell lines PT-18, MMC-34, and P815. SCF message could be amplified only from unstimulated MMC-34 mast cells. Expression appeared to increase after activation with the phorbol ester PMA. Although RT-PCR is not designed to give quantitative results, the increase in band intensity was obvious, while actin expression remained unchanged. These results agree with the data of Plaut, et al. (Plaut et al., 1989), who showed increased production of cytokines by mast cells after stimulation with phorbol esters and calcium ionophores. Thus MMC-34 mast cells express message for SCF, and appear to upregulate expression after activation with PMA.

To detect secreted SCF made by this cell line, a 50 fold concentrate of MMC-34-CM was tested in the mast cell-committed progenitor assay. Since mast cells produce IL-3, and MMC-34 mast cells expressed detectable IL-3 message by RT-PCR, the conditioned medium was incubated with a blocking antibody against IL-3 prior to using the conditioned medium in the cultures. MMC-34-CM was able to support mast cell colony development, even in the presence of blocking anti-IL-3 antibodies, from committed progenitors, although the proliferative capabilities of MMC-34-CM were less than that of 3T3-CM. Since SCF has been shown to be synergistic with many growth factors, including IL-3 (de Vries et al., 1991), we anticipated some inhibition of MMC-34-CM activity in the assay and there was some, although the inhibition seen was substantially less than that found in cultures of rIL-3 or PWM-SCCM. Thus this mast cell line apparently produces a mast cell growth factor other than IL-3. Although mast cells have not been reported to produce NGF, it is known that NGF can synergize with IL-3 to induce mast cell development from progenitors (Matsuda et al., 1991). However we do not feel that NGF is the active growth factor in MMC-34-CM, due to the presence of SCF message in MMC-34 RNA. Moreover, NGF is unable to support mast cell proliferation alone and was not active in the committed progenitor assay (Matsuda et al., 1991; Jarboe et al., 1989). Therefore it appears unlikely that NGF would be active in this system in the presence of anti-IL-3 antibodies.

While IL-3 alone can support the development of mucosal mast cells, it is unable to generate connective tissue mast cells in the committed progenitor assay (Jarboe et al., 1989). Cells derived from culture of *Nb*-MLN cells with MMC-34-CM were determined to have a connective tissue phenotype, based on staining with

safranin and berberine sulfate. Mast cells derived from culture with PWM-SCCM, a source of IL-3, did not. The difference in proportions of cells staining with berberine sulfate in cultures stimulated with MMC-34-CM was substantially less than those stimulated with 3T3-CM, but was more similar to that obtained with stimulation of *Nb*-MLN cells with recombinant SCF. The most important distinction is that any of the cells change phenotype, which may indicate the presence of SCF, in concentrations however small. The results with MMC-34-CM and the results with recombinant SCF are similar to those reported by Tsai and Galli, who showed that recombinant SCF injected into rats could enhance the proliferation of dermal connective tissue phenotype mast cells (Tsai and Galli,1991).

We then employed anti-mSCF^{169} antibodies to detect surface expression of SCF on MMC-34 mast cells. Immunofluorescent microscopy analysis showed that MMC-34 cells were stained above background levels with this antibody, however this staining was dull and quenched quickly. However, flow cytometric analysis of MMC-34 cells stained in a similar manner gave contrasting results. Unstimulated MMC-34 cells demonstrated no shift in fluorescence after staining with anti-rmSCF^{169}, although PMA stimulation of these cells resulted in a small shift in fluorescence intensity. Thus these cells appear to produce little surface-bound SCF, and the increase in SCF message correlated with an increase in surface SCF.

We conclude from these results that the MMC-34 mast cell line expresses SCF, probably of the KL-1 type, given the detectable SCF in conditioned medium from this cell line and weak surface staining of these cells for SCF. The production of SCF may not be a normal facet of mast cell biology, however, since of the four cell lines examined, only MMC-34 cells have shown SCF production.

IV) Effect of *Nb* Infection on Peritoneal Mast Cell Progenitors

The mast cell-committed progenitor is present in the *Nb*-MLN, but not naive MLN, indicating that *Nb* infection enhances mast cell progenitor populations in the region of the infection (Jarboe et al., 1989). The progenitor can be generated from naive bone marrow after stimulation with conditions mimicking the microenvironment of the MLN during infection (Ashman et al., 1991). The committed progenitor is believed to be derived from a bone marrow-derived progenitor cell, which trafficks to the MLN due to *Nb* infection (Ashman et al., 1991). These studies lead our laboratory to questions about the possible effects of *Nb* infection on mast cell progenitor populations in other areas. To approach this question, cells in the peritoneal cavity were harvested at various times after infection and were tested for their ability to function as mast cell progenitors. While mast cell colonies could be induced from peritoneal cells at days 0 and 5 of *Nb* infection, the ability of these cells to function as mast cell progenitors was completely absent at day 8, and the cells remained nonresponsive through day 17. In two separate experiments, cells cultured at days 47 and 91 after infection were again responsive to 3T3 FCM, yielding colony numbers that were 33% and 96% of Day 0 values, respectively (Data not shown). The loss of progenitor activity was not due to a dilution of the progenitor population by the influx of cells which occurs in the peritoneal cavity during *Nb* infection. Although peritoneal cell numbers increased 6.25 ± 1.43 fold from day 0 to day 14 of infection, increasing the number of peritoneal cells plated in methylcellulose to account for the increased cell numbers had no effect on progenitor development from the *Nb*-infected peritoneal cavities (Data not shown).

The mast cell progenitor found in the peritoneal cavity was found to respond to rmSCF^{169} in addition to FCM. Moreover, the pattern of response is similar to that observed with FCM during *Nb* infection: while day 0 peritoneal cells yield 404 colonies, day 14 cells gave none. Therefore this dramatic contrast in mast cell progenitor responsiveness during *Nb* infection can be reproduced with rmSCF^{169} stimulation.

Naive peritoneal cells were separated by density using percoll and assayed for FCM responsiveness. Day 0 cells exhibited responsiveness to FCM over a range of densities, however the most responsive fraction was cells with a density of 1.062-1.072. This data is important for two reasons. First, it demonstrates that mast cell colonies derived from culture of these cells with FCM are not due solely to the proliferation of mature mast cells. The greatest percentage of mast cells were found in fraction 4, which contained 99% granulated mast cells and generated 24 mast cell colonies. However, fraction 3, which contained only 5% granulated mast cells, yielded 84 colonies. Thus it is apparent that mature mast cells cannot account for the majority of the colony forming potential found in the peritoneal cavity. Second, fraction 3 possesses a similar density to that observed for the mast cell-committed progenitor, which had a range of 1.060-1.070 (Jarboe et al., 1989). Thus the cell fraction which yielded the greatest response to 3T3 FCM appears to resemble the committed progenitor found in the *Nb*-MLN.

Density-dependent separation of peritoneal cells on day 14 of *Nb* infection gave results which contrasted to the separation of day 0 cells. Day 14 cells were separated into two fractions: those with density less than 1.082 (fraction 1), and cells with density greater than 1.082 (fraction 2). As expected, unseparated day 14 cells

showed no responsiveness to FCM. However, while the lower density fraction did not respond to FCM, day 14 peritoneal cells with a density greater than 1.082 yielded 40 mast cell colonies in response to 3T3 FCM. Fraction 1 contained 2% mast cells, and a majority of large blasts and macrophages, while fraction 2 contained 12% mast cells and many small blasts. The difference in the percentage mast cells for fraction 2 of day 14 animals versus fraction 4 for naive animals, which have the same density, is not clear, but could be a result of the increased cell numbers present in the peritoneal cavity during *Nb* infection. The ability to generate mast cell colonies in response to 3T3 FCM seemed to segregate with the more dense cells in *Nb* infected animals. This suggests that some form of active suppression may be in effect to prevent mast cell progenitor proliferation in the peritoneal cavity during *Nb* infection. This data does not allow a determination of the ability of the peritoneal committed progenitor to respond, since cells of this density were pooled with the lower density cells. We therefore cannot determine if this cell is unable to respond or is no longer present in the peritoneum.

Kanakura, et al. have found in the naive animal that mast cell progenitors segregate into three groups, as discussed above (Kanakura et al., 1988). Large-CFU-Mast, which yielded the majority of mast cell proliferation, were found to possess a density less than 1.077, similar our findings. However, this group also showed that the presence of granulated peritoneal mast cells could diminish the proliferative potential of L-CFU-Mast. This finding in the naive animal contrasts with our results obtained with *Nb* infected animals, where granulated mast cells could proliferate only in the absence of lower density cells. The lower density cells may be producing INF gamma or TGFß, which have both been shown to be immunosuppressive for mast

cells and their progenitors (Huff and Justus, 1988).

Taken together these data indicate that the normal peritoneal cavity contains a mast cell progenitor that resembles the committed progenitor of the *Nb*-MLN, but this progenitor disappears from the peritoneum during *Nb* infection, perhaps due to trafficking to the MLN, or active inhibition of proliferation.

V) SCF Expression by Human and Rat Placental Tissues

Among the many tissues in which SCF expression has been detected are the murine placenta, ovaries, and testis (Matsui et al., 1990). This pattern of expression may explain the sterility associated with mutations at the *Steel* locus. In collaboration with Dr. Scott Kauma (Obstetrics and Gynecology, MCV-VCU), we attempted to determine the extent of SCF expression in human and rat placental tissues. Northern hybridization of total RNA extracted from several rat placental tissues at different time points during pregnancy showed the expression of a 6.5 kb transcript which hybridized with a SCF cDNA probe, which is similar to the published sizes of murine SCF transcripts (Anderson et al., 1990; Zsebo et al., 1990; Matsui et al., 1990). Expression of this band was observed in endometrial tissues and placenta, where it from day 15 to day 18. However, little expression was detected in day 15 or day 18 decidua. Endometrial tissues differentiate into decidua late in the menstrual cycle in preparation for egg implantation. Thus SCF expression appears to be limited to specific tissues involved in embryonic development.

Initial experiments attempting to detect SCF expression in total RNA from human placenta were unfruitful, requiring the use of poly A^+ RNA in Northern analysis. Hybridization of poly A^+ human placental RNA resulted in detection of a 6.1 kb band; no band could be detected in total RNA.

These results indicate that SCF is expressed in the rat and human placenta, similar to the findings in the murine system. However, we have found that expression in the rat is restricted to specific uterine tissues. In the human system, SCF does not appear to be as highly expressed as the murine or rat models. The expression of SCF in placental tissues from mouse, rat, and man, coupled with the sterility associated with mutations at the *Sl* locus indicates a potentially important role for SCF in embryonic development.

In summary, this work demonstrates the expression of a recently described growth factor, stem cell factor, in a myriad of tissues, including Schwann cells, mast cells, and fetal and maternal tissues during pregnancy. Since mutations which disrupt the expression of SCF and its receptor have been shown to result in deficiences in the hematopoietic and primordial germ cell lineages, and expression of SCF and c-*kit* has been detected in the nervous system, our results agree with the findings of other workers in the field. Beyond its already-described abilities as a mast cell growth factor, these results indicate that this growth factor-receptor complex may play an important, and until now undescribed, role in the development and repair of the peripheral nervous system.

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Appendix A

Human and Rat Stem cell Factor:
5' Primer: 5' ATC ATA TGA AGA AGA CAC AAA CTT GG
(Nucleotides 184-204)
3' Primer: 5' ATG GAT CCT TAC ACT TCT TGA AAC TCT CTC TCT T
(Nucleotides 1005-980)
Probe: 5' GTA GTG GCA TCT GAA ACT AGT GAT TGT GTG G
(Nucleotides 534-676)

Mouse Stem Cell Factor:
5' Primer: 5' ATG ATA ACC CTC AAC TAT GTC GCC
(Nucleotides 361-384)
3' Primer: 5' CCT ATT ACT GCT ACT GCT GTC ATT CC
(Nucleotides 788-763)
Probe: 5' AGC TTG ACT ACT CTT CTG GAC AAG TTC TCA
(Nucleotides 446-475)

Rodent interleukin-3:
5' Primer: 5' GTT CTT GCC AGC TCT ACC ACC A
(Nucleotides 1380-1402)

3' Primer: 5' TGG GCC ATG AGG AAC ATT CAG
(Nucleotides 3783-3764)
Probe: 5' GCT CTG CCT GCT GTT TTA ACA TTC CAC GGT
(Nucleotides 3724-3753)

Human Actin:
5' Primer: 5' CCA CGA AAC TAC CTT CAA CTC C
(Nucleotides 822-844)
3' Primer: 5' TCA TAC TCC TGC TTG CTG ATC C
(Nucleotides 1045-1067)
Probe: 5' GCA CCC AGC ACA ATG AAG ATC AAG ATC ATT
(Nucleotides 960-989)
Rodent Actin:
5' Primer: 5' ATC TAC GAG GCC TAT GCT CTC C
(Nucleotides 415-436)
3' Primer: 5' TCT GCA TCC TGT CAG CAA TGC C
(Nucleotides 844-823)
Probe: 5' AGA GAA GCT GTG CTA TGT TGC TCT AGA CTT
(Nucleotides 561-590)

Rat c-*kit*:

5' Primer: 5' GGG ATT CAT CAA CAT CTT CC
(Nucleotides 980-999)
3' Primer: 5' CCT TCA GTT CTG ACA TTA GGG C
(Nucleotides 1956-1935)
Probe: 5' AAG ACG TGT CTA CAT CCG TGG ACT CCA TGT
(Nucleotides 751-780)

Human c-*kit*:
5' Primer: 5' CAG CTT CAG AAT GGC ATT GTA
(Nucleotides 3868-3888)
3' Primer: 5' GGC TAC AGT CTA AAG GGT AAA
(Nucleotides 4386-4366)
Probe: 5' TCA GTA ACT TTG TCA AAC AGC ATC AAA TCC
(Nucleotides 3878-3907)

Human interleukin-3:
Primer 1: 5' CAA CTC CTG GTC CGC CCC GGA CTC (Nucleotides 40-64)
Primer 2: 5' CAA CAG ACG ACT TTG AGC CTC (Nucleotides 437-458)
Probe: 5' AAT AAC CTT CGA AGG CCA AAC CTG GAG GCA
(Nucleotides 219-248)

Human GM-CSF:
Primer 1: 5' TGA ACC TGA GTA GAG ACA CTG (Nucleotides 136-156)
Primer 2: 5' TCC CTC CAA GAT GAC CAT CCT (Nucleotides 509-529)
Probe: 5' ACC ATG ATG GCC AGC CAC TAC AAG CAG CAC
(Nucleotides 294-323)

Appendix B

Table VIII
Factors Unable to Support Committed Progenitor Development

IL-1 (1,5,20 U/ml)
IL-2 (100 U/ml)
IL-4 (100,200 U/ml)
GM-CSF (50,100,200 U/ml)
G-CSF (50 U/ml)
M-CSF (10X conc. L929-CM)
NGF (5,10,20,50,100,500 ng/ml)
EGF (0.5,1 ug/ml)
IFN-g (100 U/ml)
IFN-a/b (100 U/ml)
TNF (10 ng/ml)
Fibronectin (1,4,20,100 ug/ml)
bFGF (5,25,50 ng/ml)
TGFb (1,5,10 ng/ml)
P40 (5,10,100 u/ml)
Mixture: 100 U/ml IL-2,IL-4,G-CSF,GM-CSF; 10X CM M-CSF

Figure 32
RT-PCR for IL-3 expression. Total RNA extracted from (A) BALB/c 3T3 fibroblasts or (B) WEHI-3 myelomonocytes was subjected to RT-PCR analysis using primers specific for the mouse IL-3 RNA sequences. Lane (C) is a control sample containing all reactants except RNA. PCR was carried out as described in Materials and Methods with an annealing temperature of 50°C for 30 cycles. Arrowhead indicates expected 470 base pair product.

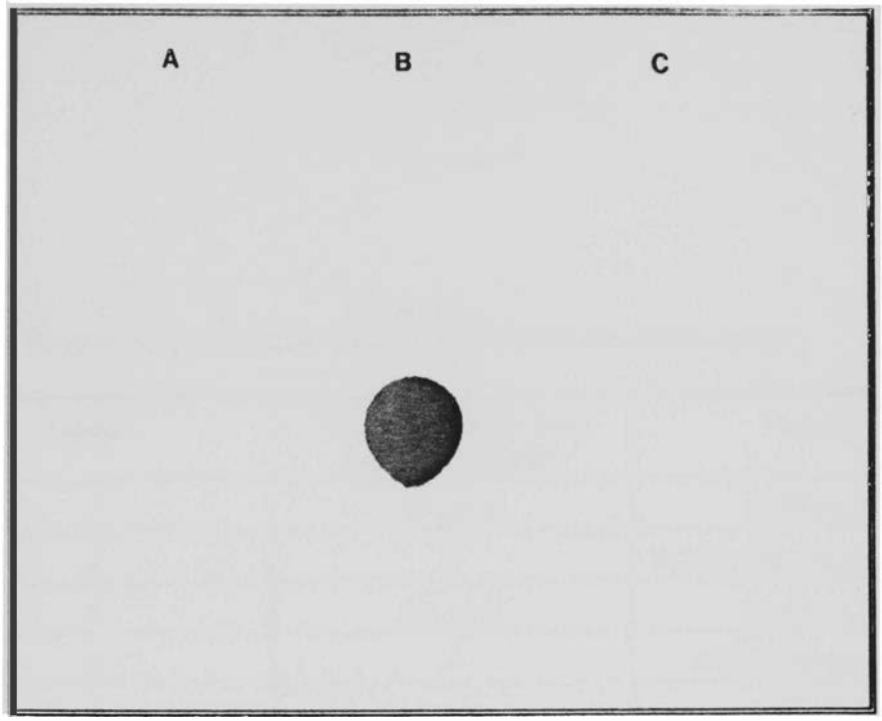
A
B
C

Table IX
Blinded Amgen Samples Used in Committed Progenitor Assay

Sample	Number of Mast Cell Colonies (±SEM)	Identity
1	16 ±1.0	1x 3T3-CM
2	0	1x 3T3-CM, No Serum
3	43 ±9.0	10x 3T3-CM
4	0	YM-5 Filtrate of #3
5	0	cDMEM
6	0	10x cDMEM
7	0	YM-5 Filtrate of #6
15x 3T3-CM	8 ±2.0	
Medium	0	

Vita