SIGNALING OF STEM CELL FACTOR/c-kit RECEPTOR
IN THE REGULATION OF MICROGLIA

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Submitted to the College of Graduate Studies and Research
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University of Saskatchewan
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Canada

By
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SIGNALLING OF STEM CELL FACTOR/c-kit RECEPTOR IN THE REGULATION OF MICROGLIA

Signaling between stem cell factor (SCF) and c-kit receptor is required for normal hematopoiesis, melanogenesis, and gametogenesis. SCF and c-kit are also expressed in the nervous system, but their role in the nervous system is unknown. Using immunocytochemistry, I found that SCF is produced mainly by neurons, whereas the c-kit receptor is expressed by glial cells as well as some neurons, suggesting that SCF/c-kit receptor plays a role in neuron-neuron and neuron-glial interactions.

In culture, microglia express c-kit mRNA and protein. The expression of c-kit receptor in microglia is subject to regulation by cytokines such as SCF, CSF-1 and IFN-γ. Microglia also express SCF. Stimulation of microglia by IFN-γ or CSF-1 upregulates SCF mRNA expression. In situ, a stab wound in cerebral cortex upregulates c-kit receptor in reactive microglia and SCF in astrocytes and reactive microglia. Thus an autocrine and paracrine action of SCF/c-kit receptor may be involved in the regulation of some of the microglial activities.

Microglia in culture require CSF-1 for survival. I found that SCF partially substitutes for CSF-1 for microglial survival. Moreover, at a high dosage, SCF maintains microglia in a relatively quiescent state. Addition of SCF to microglia in culture increases the expression of mRNAs of NGF, BDNF, and CNTF, whereas it does not affect mRNA expression of IL-1, TNFα, and IL-6. In a neuron-microglia co-culture system, SCF-treated microglia support neuron survival and neurite outgrowth. These observations suggest that SCF maintains microglia in a quiescent state and that microglia in this state are trophic to neurons.

Stimulation of microglia in culture by CSF-1 results in proliferation. I found that SCF suppresses CSF-1-induced microglial proliferation in a dose-dependent manner. Stimulation of microglia by LPS or IFN-γ induces significant mRNA expression of IL-1, TNFα, and IL-6. Addition of SCF together with IFN-γ suppresses the induction of IL-1 and TNFα mRNAs, but addition of SCF to microglia cultures increases the production of lipocortin-1 (LC-1), an anti-inflammatory cytokine. Therefore, under pathological conditions, SCF regulates microglial reactivity, thus modulating the processes of inflammation and repair.
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ABSTRACT

Signaling between stem cell factor (SCF) produced by stroma cells and its receptor, encoded by c-kit, expressed by stem cells or progenitor cells, is required for the normal development of hematopoietic, melanogenic, and gametogenic cell lineages. SCF and c-kit are also expressed in the nervous system, but their role in the nervous system is unknown. Using immunocytochemistry, I found that SCF was produced mainly by neurons, whereas the c-kit receptor was expressed by some neurons that often make synapses with the SCF-producing neurons and by glial cells, suggesting that SCF/c-kit receptor plays a role in neuron-neuron interaction and in a paracrine interaction between the SCF-producing neurons and the c-kit-expressing glia.

Microglia, isolated from neopallial cell cultures, expressed c-kit mRNA, as shown by Northern blot analysis, and protein, shown by Western blot and immunocytochemistry. The expression of c-kit receptor in microglia in culture was found to be subject to regulation by cytokines such as SCF, colony-stimulating factor-1 (CSF-1) and interferon γ (IFN-γ). Microglia in culture also expressed SCF, as demonstrated by Northern blot and immunocytochemistry. Stimulation of microglia by IFN-γ or CSF-1 upregulated SCF expression. Thus, an autocrine action through SCF and its c-kit receptor may occur in microglia, especially when microglia are stimulated by IFN-γ or CSF-1.

Microglia in culture require CSF-1 for survival. Withdrawal of CSF-1 from culture medium causes microglial cell death, whereas I found that microglia were partially rescued by the addition of SCF. Moreover, at a high dosage, SCF maintained microglia in a bipolar or tripolar shape, a relatively quiescent state. Addition of SCF to microglia in culture increased the expression of mRNAs of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF), whereas it did not
affect mRNA expression of inflammatory cytokines, interleukin 1 (IL-1), tumor necrosis factor α (TNFα), and IL-6. Using a neuron-microglia co-culture system, I demonstrated that SCF-treated microglia supported cerebral cortical neuron survival and neurite outgrowth. These observations suggest that SCF maintains microglia in a quiescent state and that microglia in this state are trophic to neurons.

A stab wound in the mouse cerebral cortex resulted in the upregulation of c-kit receptor in reactive microglia, as detected by immunocytochemistry. Although immunocytochemistry did not detect SCF protein in reactive microglia, in situ hybridization studies suggested that reactive microglia probably produce mainly soluble SCF. Thus an autocrine action of SCF/c-kit receptor may be involved in the regulation of microglial response to injury. On the other hand, reactive astrocytes upregulated SCF expression, mainly the membrane-bound form, as shown by immunocytochemistry. Reactive astrocytes, therefore, may modulate microglial response to injury through the production of SCF.

Stimulation of microglia in culture by CSF-1 results in microglial proliferation. I found that SCF suppressed CSF-1-induced microglial proliferation in a dose-dependent manner. Stimulation of microglia by lipopolysaccharide (LPS) or IFN-γ induced significant mRNA expression of the inflammatory cytokines IL-1, TNFα, and IL-6. Addition of SCF together with IFN-γ suppressed the induction of IL-1 and TNFα mRNAs, but addition of SCF to microglia cultures increased the production of lipocortin-1 (LC-1), an anti-inflammatory cytokine. Hence, SCF in cultures of microglia suppresses the production of inflammatory cytokines and at the same time increases the production of anti-inflammatory cytokine.
Based on the data obtained, I hypothesize that in a homeostatic nervous system, neurons produce SCF which keeps microglia in a quiescent state and induces microglia to produce neurotrophic factors which in turn support neuronal survival and function. In case of injury, reactive microglia and astrocytes, as well as neurons, produce SCF which regulates microglial response to injury, thus modulating the processes of inflammation and repair.
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LIST OF ABBREVIATIONS

ABC avidin-biotin complex
ATCC American Type of Culture Collection
ATP adenosine triphosphate
BCIP 5-bromo-4-chloro-3-indolyl-phosphate
BDNF brain derived nerve growth factor
bFGF basic fibroblast growth factor
BSA bovine serum albumin
CDF cholinergic differentiation factor
cDNA complementary deoxyribonucleic acid
CFU colony forming unit
CNS central nervous system
CNTF ciliary neurotrophic factor
CR3 receptor for C3 complement component
CSF-1 colony-stimulating factor-1 or macrophage colony-stimulating factor
CTP cytidine triphosphate
DAB 3,3'-diaminobenzidine
dCTP deoxycytidine triphosphate
DEPC diethylpyrocarbonate
DIV days in vitro
DMEM Dulbecco’s modified Eagle’s Medium
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
DPBS Dulbecco’s phosphate buffered saline
DRG dorsal root ganglion
EAE experimental allergic encephalomyelitis
EDTA  ethylene diamine tetraacetic acid
EPO  erythropoietin
FBS  fetal bovine serum
G-CSF  granulocyte colony-stimulating factor
GFAP  glial fibrillary acidic protein
GM-CSF  granulocyte-macrophage colony-stimulating factor
HS  horse serum
IFN-γ  interferon-γ
IL-1  interleukin 1
IL-2  interleukin 2
IL-3  interleukin 3
IL-6  interleukin 6
IL-7  interleukin 7
IMDM  Iscove’s Modified Dulbecco’s Medium
IU  international unit
JAK  Janus kinase
kb  kilobase
kD  kilo Dalton
KL  kit ligand
LB  Luria broth
LC1  lipocortin 1
LIF  leukemia inhibitory factor
LPS  lipopolysaccharide
MGF  mast cell growth factor
MHC  major histocompatibility complex
mMEM  modified Eagle’s Minimum Essential Medium
MOPS  morpholinepropanesulfonic acid

XV
mRNA  messenger ribonucleic acid
MS    multiple sclerosis
NBT   nitroblue tetrazolium chloride
NET   sodium chloride/EDTA/Tris-HCl buffer
NGF   nerve growth factor
NTP   nucleotide triphosphate
PDGF  platelet derived growth factor
PI3'K phosphatidylinositol 3'-kinase
PKC   protein kinase C
PLC-γ1 phospholipase C-γ1
PMSF  phenylmethylsulfonfyl fluoride
RNA   ribonucleic acid
rRNA  ribosomal ribonucleic acid
SCF   stem cell factor
SDS   sodium dodecyl sulfate
SI    Steel
SLF   Steel factor
SSC   sodium chloride, sodium citrate
STAT  signal transducer and activators of transcription
STET  sodium chloride, Tris, EDTA, Triton
TBE   Tris-Boric-EDTA buffer
TE    Tris-EDTA buffer
TGFβ1 transforming growth factor β1
TIS   transcription initiation site
TNF   tumor necrosis factor
tRNA  transfer ribonucleic acid
1. INTRODUCTION

Neurons and glia of the central nervous system (CNS) form a dynamic cellular network by interacting with each other through signaling molecules which they themselves produce. Such signaling molecules include neurotransmitters, cytokines, and ions.

Cytokines are a group of polypeptides which with few exceptions are produced during immune and inflammatory responses. An individual cytokine can be produced by many different cell types in the body and have multiple effects on different cell types including neurons and glia, whereas several cytokines may mediate a common physiological event in a particular cell type. Therefore, cytokine effects display both pleiotropism and redundancy, which are determined by the distribution and level of expression of the cytokine receptors and the particular signal transduction pathways employed by the target cells.

Stem cell factor (SCF) (Martin et al., 1990; Zsebo et al., 1990a,b), also designated as mast cell growth factor (MGF) (Anderson et al., 1990; Copeland et al., 1990; Williams et al., 1990), kit ligand (KL) (Flanagan and Leder, 1990; Nocka et al., 1990) and Steel factor (SLF) (Motro et al., 1991; Williams and Lyman, 1991), is a relatively recently discovered cytokine which is encoded by the Steel locus. Its receptor (CD117) is a transmembrane protein tyrosine kinase encoded by the proto-oncogene, c-kit, an allele of the W locus (Besmer et al., 1986; Chabot et al., 1988). The similar phenotypic changes displayed by W and Steel mutants demonstrate the importance of SCF/c-kit receptor signaling in hematopoiesis, gametogenesis and melanogenesis. Extensive genetic and biochemical studies have demonstrated that SCF, including the
Extensive genetic and biochemical studies have demonstrated that SCF, including the soluble and membrane forms, supports the survival of hematopoietic stem cells and progenitor cells by suppressing their apoptosis (Cáceres-Cortés et al., 1994; Mekori et al., 1993, Yee et al., 1994b) and synergizes with other cytokines to induce proliferation and differentiation (Andrews et al., 1991; Broxmeyer et al., 1991; McNiece et al., 1991). It has also been demonstrated that SCF produced by fibroblasts in the stroma of hematopoietic tissue supports mast cell proliferation and maturation, induces mast cell adhesion to fibronectin in culture, and augments their secretion in response to IgE (Coleman et al., 1993; Dastych and Metcalf, 1994; Wershil et al., 1992; Yee et al., 1994b). Administration of anti-c-kit receptor antibody into mice induces a pattern of coat color dilution, demonstrating that SCF is required for stimulation of melanocyte proliferation, migration and maturation (Nishikawa et al., 1991). These studies demonstrate that a single cytokine, SCF, can affect almost every step in the development and function of the cells that are affected by W or Sl mutations (Besmer, 1991; Broxmeyer et al., 1991; Galli et al., 1993).

Besides the tissues or cells that are known to be affected by W or Sl mutations, SCF and c-kit genes as revealed by in situ hybridization studies, are also expressed in cells present in the lungs, digestive tract, kidneys, and brain. During embryonic development, SCF mRNA is expressed in the floor plate of the neural tube, followed by the appearance of c-kit transcripts on the dorsal aspect of the neural tube (Keshet et al., 1991), suggesting a possible role of SCF/c-kit receptor signaling in the morphogenesis of the neural tube. In the adult brain, a similar complementary pattern of expression was observed in the cerebellum in which high levels of SCF transcripts were confined to Purkinje cells, while the deep cerebellar nuclei and the basket, stellate and Golgi cells, which make synaptic connections with Purkinje cells, were c-kit positive (Hirota et al., 1992; Manova et al., 1992; Morii et al., 1992; Motro et al., 1991; Takeda et al., 1992).
In other parts of the brain, a similar yet more complex pattern of expression was found. High levels of SCF mRNA were found in the thalamus and cerebral cortex and c-kit transcripts were localized in the cerebral cortex but not in the thalamus (Matsui et al., 1990; Motro et al., 1991). Expression of c-kit was found in pyramidal neurons of the hippocampus (CA1 and CA3), whereas SCF transcripts were mainly in the entorhinal cortex and dentate gyrus (Matsui et al., 1990; Motro et al., 1991). A recent observation indicated that SCF is also expressed by hippocampal pyramidal neurons (Wong and Licinio, 1994). The fact that different groups of neurons express either SCF or c-kit receptor suggests that SCF and c-kit are involved in neuron-neuron interactions.

Although c-kit and SCF transcripts are mainly expressed by neurons, Motro et al (1991) presumed that some c-kit-expressing cells in the cerebral and cerebellar cortex might be glia since some c-kit transcript signals appeared in the molecular layer of the cortex that is relatively free from neurons. This observation pointed to a possible involvement of SCF/c-kit receptor in neuron-glial signaling.

The aim of the study reported in this thesis is to reveal the possible role of SCF/c-kit receptor in the nervous system. To this end, I investigated the cellular distribution of SCF and c-kit receptor in neural tissues in situ and in cultures. Emphasis was put on a role of SCF in the regulation of microglial activity by examining the effect of SCF on the morphology, survival, proliferation, and gene expression in microglia in culture and the expression of SCF and c-kit receptor in microglia in response to injury in situ. The data presented led to the hypothesis that SCF/c-kit receptor acts as one of the regulatory signaling pathways by which neurons regulate microglial activity.
2. LITERATURE REVIEW

2.1 Molecular Biology of SCF and c-kit

2.1.1 Allelism of SCF with Sl and c-kit with W locus

It has been known for decades that mice with mutations at either the dominant white spotting (W) locus on chromosome 5 or the Steel (Sl) locus on chromosome 10 exhibit similar phenotypes, i.e., a defect of coat colour, macrocytic anemia and sterility (Russell, 1979). The same phenotypic characteristics caused by apparently independent loci led to an assumption that they must bear a certain relationship to each other. Extensive studies, especially transplantation of the cells derived from these animals, indicated that the defect of W mutation was in the cells of the affected lineages whereas that of Sl was in the microenvironment of the affected lineages. The complementary nature of these mutations led Russell to hypothesize that the W locus might encode a receptor expressed by the cells of the affected lineages whereas the Sl locus might encode the corresponding ligand by the cells in the microenvironment (Russell, 1979).

The hypothesis was confirmed by molecular genetic studies. The proto-oncogene, c-kit, was characterized as the normal cellular homolog of the oncogene v-kit of the Hardy-Zuckermann 4 feline sarcoma virus (HZ4-FeSV) (Besmer et al., 1986) and was mapped to mouse chromosome 5 (Chabot et al., 1988; Geissler et al., 1988). The mouse c-kit gene was cloned from a mouse brain cDNA library (Qiu et al., 1988) and the predicted amino acid sequence showed extensive homology with the receptor tyrosine
kinases, especially the type III receptor tyrosine kinases (Yarden and Ullrich, 1988) such as the receptors for colony-stimulating factor-1 (CSF-1) and platelet derived growth factor (PDGF) (Qiu et al., 1988). Analysis of the W mutants demonstrated that the W mutations were associated with the alteration of c-kit gene, either rearrangement or point mutations (Bernstein et al., 1990; Geissler et al., 1988; Nocka et al., 1989, 1990; Tan et al., 1990), thus providing direct evidence that c-kit is allelic with the W locus.

Because the W locus is allelic with c-kit, i.e., encodes a transmembrane tyrosine kinase, and because there are apparent complementary phenotypic characteristics in mice carrying W and Sl mutations, it strongly suggested that Sl gene product was the ligand for the c-kit receptor. This was soon confirmed by several groups, almost at the same time, by cloning the gene (Anderson et al., 1990; Copeland et al., 1990; Huang et al., 1990; Zsebo et al., 1990a) and by purifying the protein, stem cell factor, the ligand of c-kit receptor (Flanagan and Leder, 1990; Williams et al., 1990; Zsebo et al., 1990b).

2.1.2 The structure of SCF and c-kit

The size and overall structure of the c-kit gene is highly conserved in humans and mice (André et al., 1992; Gokkel et al., 1992; Vandenbark et al., 1992; Yarden et al., 1987). The gene spans over 80 kb of genomic DNA and the coding sequence is distributed over 21 exons. Exon 1 encodes the 5' untranslated region, the initiation codon ATG and the signal peptide. Exons 2-9 encode the extracellular ligand-binding domain which forms five immunoglobulin-like loops. Exon 10 encodes the hydrophobic transmembrane domain and exons 11-20 encode the intracellular kinase domain. The kinase domain of the c-kit receptor is split into two parts by a non-kinase insert segment which is encoded by exons 14 and 15. The ATP-binding region in the kinase domain is located in exon 12. The c-kit transcription initiation site (TIS) and the promoter, however,
are not well characterized. Yasuda et al. (1993) reported that the major c-kit TIS in mouse mast cells and c-kit positive cells in the cerebellum is located 58 bp upstream from the translation start codon and the major promoter in a region between TIS and -44 bp. This promoter, however, lacks the characteristic features such as TATA or CCAAT boxes.

The genomic organization of c-kit is very similar to a group of receptor tyrosine kinase genes such as c-fms (coding for CSF-1 receptor) and PDGF receptor α and β genes, especially c-fms. The c-kit and c-fms share about 50% cDNA identity and show a striking similarity in their exon/intron structure especially in the tyrosine kinase domain, suggesting that they are closely related evolutionarily. The conservation is less striking in the extracellular domain and the kinase insert region, which reflects the difference in the specific ligand-binding property and the specific intracellular substrate association (André et al., 1992; Gokkel et al., 1992; Qiu et al., 1988; Vandenbark et al., 1992; Yarden et al., 1987).

The c-kit-encoded protein is extensively glycosylated and its molecular mass is about 145 kD. Alternative splicing of the c-kit gene gives rise to a modified form of c-kit protein that contains an in-frame insertion within the carboxy terminus of the extracellular domain in mast cells (Reith et al., 1991; Vandenbark et al., 1992). Both forms of c-kit protein showed the same affinity to SCF and similar increase in autophosphorylation and association with phosphatidylinositol 3'-kinase (PI3'K) and phospholipase C-γ1 (PLC-γ1) in response to the addition of SCF (Williams et al., 1992b). However, a low level of association with PI3'K and PLC-γ1 was detected with the short form of the receptor (without the insertion) in the absence of the exogenous SCF. Such a constitutive low level of activation may provide sufficient intracellular activation which is necessary for survival and/or differentiation. So far, the tissue distribution and the biological significance of such alternative spliced c-kit receptors remain unclear.
In addition, a soluble form of c-kit receptor was found in the media of endothelial cell cultures and hematopoietic cell lines, and in normal human serum (Broudy et al., 1994; Turner et al., 1995; Wypych et al., 1995). The soluble c-kit receptor binds to the membrane form of SCF with both high affinity (Kd = 42 pmol/L) and low affinity (Kd = 1.7 nmol/L) (Broudy et al., 1994). The high affinity binding was also demonstrated by the recombinant ectodomain of the c-kit receptor (Lev et al., 1992c). The production of the soluble c-kit receptor is likely to be the result of proteolysis since there are no mRNA splice variants corresponding to the extracellular domain although there is an insertion site in exon 6 (Reith et al., 1991). Since it can effectively compete with cell surface c-kit receptor for binding with SCF, it may thus modulate the activity and function of SCF (Broudy et al., 1994; Turner et al., 1995; Wypych et al., 1995).

The gene encoding SCF was cloned by several laboratories simultaneously, but the localization of TIS, the promoter region, and the detailed exon/intron structure were not well defined. The SCF gene cloned from both human and rat cells consists of at least 8 exons. Exon 1 encodes the 5' untranslated sequence and the first 5 amino acids of the signal peptide. Exons 2-7 encode the signal peptide and the extracellular part of the ligand, while exon 7 encodes the transmembrane domain. Exon 8 encodes the cytoplasmic domain, the stop codon, and part or all of the very long (~4 kb) 3' untranslated region of the transcript (Martin et al., 1990). The transcripts of SCF detected by Northern blot analysis in many different cells and tissues are larger than the SCF cDNA cloned so far (Anderson et al., 1990; Williams et al., 1990; Zsebo et al., 1990a).

Two alternatively spliced forms of human SCF have been reported (Anderson et al., 1990). The full length mRNA encodes the larger transmembrane protein of 248 amino acids (SCF^{248}). The alternative SCF mRNA deletes 84 bp in exon 6, thus giving
rise to a smaller transmembrane protein (SCF^{220}) which lacks 28 amino acids at the juxtamembrane region where the protease recognition site locates (Anderson et al., 1990; 1991). In mice, except for the two alternative forms, there is another alternative form of SCF mRNA with a smaller 16 amino acid deletion of the exon 6 sequence (Flanagan et al., 1991; Huang et al., 1992). The membrane forms of SCF can give rise to soluble SCF of 164 or 165 amino acids by cleaving the extracellular part at the exon 6 sequence. Recently, another proteolytic site was found in the carboxy terminus of the extracellular region encoded by exon 7 (Majumdar et al., 1994). The natural SCFs are heavily glycosylated with O-linked and N-linked sugars and exist as non-covalent homodimers (Arakawa et al., 1991; Huang et al., 1992; Lu et al., 1991, 1992; Zsebo et al., 1990b).

2.1.3 Regulation of SCF and c-kit expression

As mentioned above, there are alternate forms of c-kit receptors in mast cells (Reith et al., 1991). Although both forms show similar ligand binding affinity and intracellular substrate association, there is a subtle difference between the two forms in that the short form of c-kit receptor exhibits a constitutive low level of tyrosine kinase activation in mast cells (Williams et al., 1992b). Although the biological significance of such different forms of c-kit receptor remains unclear, it may suggest that there is a tissue- or cell-specific mechanism in regulating the expression of alternative c-kit receptors.

The expression of c-kit can be influenced by a variety of cytokines and stimuli. Treatment of bone marrow derived mast cells with SCF, interleukin-3 (IL-3), phorbol ester and calcium ionophore down-regulates c-kit mRNA and protein expression (Asano et al., 1993; Welham and Schrader, 1992; Yee et al., 1993, 1994a). The mechanisms underlying the receptor regulation are not clear. The ligand-induced downregulation of c-kit receptor involves internalization of ligand-receptor complexes, followed by
polyubiquination. It also involves the activation of certain protein kinases such as protein kinase C (PKC), which accelerates the proteolytic cleavage of the c-kit receptor to reduce ligand-binding capacity and impairs the autophosphorylation of c-kit receptor (Yee et al., 1994a). However, the c-kit receptor tyrosine kinase seems not responsible for the downregulation of c-kit receptor since abolishment of SCF-induced receptor association and activation of PI3'K and the tyrosine autophosphorylation does not affect SCF-induced internalization and ubiquitination/degradation of c-kit receptor (Yee et al., 1994) although they abolish SCF-induced cell proliferation and survival (Besmer et al., 1986). The cleavage of the extracellular domain of c-kit receptor triggered by the ligand binding may have profound physiological significance in mast cells. Since the interaction of c-kit receptor on mast cells and SCF on fibroblasts is essential for cell adhesion, proliferation, differentiation and migration, the cleavage of c-kit receptor would allow mast cells to dissociate and migrate away from fibroblasts.

The regulation of c-kit receptor by other cytokines is complicated. TNFα has been reported to upregulate c-kit receptor in acute myeloid leukemia blast cells (Brach et al., 1992) while downregulating c-kit receptor in hematopoietic progenitor cells (Jacobsen et al., 1995). Such up- and down-regulation of c-kit receptor seems to correlate with the effect of TNFα in the potentiation and inhibition of proliferation of malignant tumor cells and the hematopoietic progenitor cells, respectively (Brach et al., 1992; Jacobsen et al., 1995). The complex regulation of c-kit receptor, however, may also be contributed by other cytokines produced by certain cells under the influence of TNFα. For instance, TNFα or GM-CSF alone downregulated c-kit receptor on a GM-CSF dependent myeloid cell line while TNFα antagonized the downregulation of c-kit receptor by GM-CSF when they were added together (Oez et al., 1993a,b). The downregulation of c-kit receptor by TNFα was often accompanied by the differentiation of monocyte-macrophage-like cells
from the myeloid cell lines (Oez et al., 1993a). Therefore, the regulation of c-kit receptor by diverse cytokines may play an important role in cell proliferation and differentiation.

The regulation of SCF expression mainly involves post-transcriptional and post-translational mechanisms. As mentioned above, the SCF gene produces alternative transcripts which encode various forms of membrane-associated SCFs that can be cleaved proteolytically to generate various soluble SCFs. The pattern of SCF expression seems to be tissue-specific in that the distribution of the two major SCF mRNAs, those encoding SCF\textsuperscript{248} and SCF\textsuperscript{220}, varies with tissues, with a 1.5:1 in spleen and 26:1 in brain (Huang et al., 1992). Both SCF\textsuperscript{248} and SCF\textsuperscript{220} can be cleaved although the shorter one gives rise to the soluble SCF less efficiently because of the deletion of the major proteolytic site located in the exon 6-encoded sequence. However, in vitro studies indicate that the production of soluble SCF by COS cells transfected with SCF\textsuperscript{248} and SCF\textsuperscript{220} is enhanced by activation of PKC, at a similar rate (Huang et al., 1992). Recently, a secondary proteolytic cleavage site was found to be located in exon 7 of the murine SCF gene. This secondary site is utilized in the absence of the primary site (Majumdar et al., 1994). Therefore the regulation of the proteolytic release of soluble SCF provides another level of control over the function of SCF.

2.1.4 Signal transduction by c-kit receptor

SCF exists as a homodimer. Upon binding with SCF, c-kit receptor undergoes conformational change and dimerization. The dimerization is necessary for SCF-induced signal transduction since antibody that inhibits c-kit receptor dimerization completely blocks c-kit receptor signal transduction (Bleichman et al., 1995). The dimerization site is localized in the fourth immunoglobulin-like loop which lacks the standard intradomain
disulfide bond (Blechman et al., 1995), similar to that of CSF-1 receptor encoded by c-fms (Carlberg and Rohrschneider, 1994).

The ligand induced receptor dimerization further induces the c-kit receptor to autophosphorylate the tyrosine residue and to bind phosphatidylinositol 3'-kinase (PI3'K) and phospholipase C-γ1 (PLC-γ1). The autophosphorylation of the tyrosine stabilizes the ligand-receptor complex (Rottapel et al., 1991). The phosphorylation of tyrosine residue 719 in the kinase insert region of the c-kit receptor is required for binding the p85 subunit of PI3'K and for c-kit receptor induced PI3'K activity (Lev et al., 1992; Serve et al., 1994). The substrate specificity of phosphorylated c-kit receptor is thus different from CSF-1 receptor by virtue of its ability to bind to PLC-γ1 and distinct from PDGF receptor by its failure to bind to the Ras GTPase-activating proteins (Lev et al., 1991; Rottapel et al., 1991). However, all three receptors of the same tyrosine kinase subfamily can bind to PI3'K and Raf1. Therefore, the substrate specificity of these receptors may be achieved by the specific combination of the elements in signal transduction pathways (Lev et al., 1991).

The binding of ligand to tyrosine kinase receptors can also induce changes in early response genes such as the fos and jun families. CSF-1 can stimulate monocytes/macrophages to express c-fos and c-jun (Müller et al., 1985), and PDGF can stimulate quiescent fibroblasts to express these early response genes (Kruijer et al., 1984). SCF was shown to stimulate bone marrow derived mast cells to elevate mRNA levels for c-fos, c-jun, and jun-B (Tsai et al., 1993). In hematopoietic cell lines, SCF, as well as other cytokines such as IL-3, GM-CSF, can induce an overlapping profile of early response genes (Horie and Broxmeyer, 1993; Tsai et al., 1993). The correlation between early response gene expression and cellular functions is as yet difficult to establish.
Recently, c-kit receptor has been shown to be associated with JAK2 kinase, one of the cytoplasmic tyrosine kinases involved in the early signal transduction events (Brizzi et al., 1994a). Stimulation of hematopoietic cell lines, such as M-O7e cells, with SCF rapidly induces p130 JAK2 protein phosphorylation which is maintained for at least 60 minutes. This suggests that c-kit receptor may use such an alternative pathway of transcription activation via STAT (signal transducers and activators of transcription) proteins in certain cells. The correlation between JAK2 activation and cellular functions has not been established. Many cytokines such as IL-3, GM-CSF, SCF can induce JAK2 activation in myeloid cell lines (Brizzi et al., 1994a). However, such a convergence of signaling by various cytokines in certain cell types may explain part of the mechanisms underlying the interactions between cytokines on certain cell functions.

In order to understand the role of the various above-mentioned second messengers and signal transduction pathways in c-kit receptor mediated cellular activity, a site-directed mutagenesis study was carried out in murine bone marrow derived mast cells (Serve et al., 1994). By substituting tyrosine 719 in the kinase insert region of the c-kit receptor with phenylalanine, the PI3'K activation is abolished since the phosphorylated tyrosine residue 719 is required for binding the p85 subunit of PI3'K and for c-kit associated PI3'K activity (Lev et al., 1992a; Serve et al., 1994). At the same time, c-fos and jun B induction is diminished. The physiological consequence of such a mutation is the impaired adhesion of mast cell to fibronectin, induced by SCF whereas the activation of p21ras, and cell proliferation and cell survival is only partially affected. Substitution of tyrosine 812 in the c-kit receptor with phenylalanine impaired SCF-induced survival and proliferation without affecting cell adhesion. The signal transduction pathway(s) affected by the mutation at tyrosine 812 is not clear but the PI3'K, p21ras, and induction of c-fos or c-myc are apparently not affected (Serve et al., 1995). This observation suggests that
the signal transduction pathway(s) utilized by c-kit receptor in response to its ligand determine the functions of mast cells.

The molecular biology studies thus indicate that SCF and c-kit receptor exist in different isoforms, densities or concentrations which are regulated either developmentally, or in a tissue-specific way, or by various stimuli or cytokines. Besides, upon binding to SCF, the c-kit receptor may employ alternative signal transduction pathways in certain cell types depending on their lineage, stage of differentiation/maturation, or functional status. The diverse molecular nature of SCF and c-kit receptor may thus be responsible for the remarkable spectrum of cellular activities regulated by SCF (Galli et al., 1994).

2.2 Distribution of SCF and c-kit and Biological Consequences of SCF/c-kit Receptor Signaling

2.2.1 SCF and c-kit in hematopoietic, melanogenic, and gametogenic systems

Hematopoietic system

The hematopoietic system is one of the major systems affected by W or Sl mutation. During development in mice, hematopoiesis occurs first in the yolk sac between 8.5-11.5 embryonic days, and then in the fetal liver between embryonic days 12-17. In adult life, hematopoiesis takes place mainly in the bone marrow. Using in situ hybridization, it was found that c-kit mRNA in mice was detected in the blood islands of yolk sac at embryonic day 8.5-9, and highly expressed by the fetal liver at day 12.5 (Orr-Urtreger et al., 1990). In the adult, c-kit mRNA was detected in bone marrow cells, particularly the blast cells (Metcalf and Nicola, 1991). These findings indicate that c-kit expression corresponds to peak hematopoietic activity in these tissues. Flow cytometry,
using the anti-c-kit receptor monoclonal antibody and the erythrocyte lineage marker TER-119, demonstrated that most cells from the 8.5-day yolk sac expressed c-kit but not TER-119. The expression of c-kit receptor ceased as the cells differentiated to TER-119 positive cells at 9.5 embryonic day. At embryonic day 12, the c-kit+/TER-119- cells appeared mainly in the fetal liver (Ogawa et al., 1993). These observations indicate that hematopoietic stem cells express c-kit receptor and the expression disappears when the stem cells differentiate into lineage-specific hematopoietic cells. By injection of the anti-c-kit receptor antibody (ACK2) into fetal liver, at gestation day 12.5 or into adult bone marrow, to block the function of c-kit receptor, Ogawa et al. (1993) were able to eliminate hematopoietic stem cells or progenitor cells and eliminate the colony forming units (CFU) in fetal liver and adult bone marrow. This study suggests that signaling of SCF/c-kit receptor is essential for the self-renewal of hematopoietic stem or progenitor cells.

The analysis of SCF expression by the stromal cells in hematopoietic tissues is mainly carried out by in vitro studies. SCF is expressed by mouse fetal liver-derived stromal cells (Zsebo et al., 1990b), bone marrow stromal cells (McNiece et al., 1990), and 3T3 fibroblasts (Anderson et al., 1990, Flanagan and Leder, 1990, Nocka et al., 1990; Williams et al., 1990; Zsebo et al., 1990b).

Hematopoietic stem cells are characterized by their capacity for self-renewal and the ability to differentiate into all mature hematopoietic cells. As mentioned above, c-kit is expressed by stem cells and progenitor cells but not by the differentiated mature lymphohematopoietic cells. Thus SCF may regulate hematopoiesis by regulating stem cell activity. In vitro studies have demonstrated that SCF alone can support the survival of primitive stem cells, but requires other factors to induce their proliferation (Bernstein et al., 1991; Broxmeyer et al., 1991; Galli et al., 1993; Galli et al., 1994; McNiece et al., 1991; Metcalf and Nicola, 1990; Morrison-Graham and Takahashi, 1993; Ratajezak et
al., 1992). The survival effect of SCF is achieved by suppressing apoptosis of hematopoietic stem cells (Cáceres-Cortés et al., 1994; Mekori et al., 1993). In vitro studies using mouse bone marrow cells have indicated that SCF synergizes with other growth factors, such as erythropoietin (EPO), IL-3, IL-6, GM-CSF and IL-7, to promote the amplification of progenitor cells committed to specific hematopoietic lineages. The differentiation of stem cells to lineage-specific progenitor cells seems to be governed not by SCF but by the properties of the additional growth factors (Andrews et al., 1991; Broxmeyer et al., 1991; McNiece et al., 1991). For example, SCF synergizes with erythropoietin (EPO) to expand the population of primitive erythroid progenitors (Dai et al., 1991; McNiece et al., 1991) whereas it synergizes with GM-CSF or IL-7 to expand the myeloid lineage (Fahlman et al., 1994; Williams et al., 1992a; Wu et al., 1994). The mechanisms underlying these cytokine interactions are currently being investigated. It is possible that certain elements in the signal transduction pathways coupled to the receptors of these factors are responsible (see discussion 2.1.3). However, the fact that SCF promotes stem cell survival and expands hematopoietic lineages in synergizing with other factors in vitro has profound biological and clinical significance in that a single SCF administration in vivo can expand and/or change the distribution of hematopoietic progenitor cells, which has been demonstrated in animals as well as in clinical trials (see review, McNiece and Briddell, 1995).

Normal hematopoiesis requires the presence of both membrane and soluble forms of growth factors. As discussed above, soluble SCF can support stem cell survival in culture. However, long-term in vitro maintenance of hematopoietic progenitor cells is only achieved in the presence of stromal cells. One of the Sl mutants, Sl/Sld, has normal SCF mRNA levels as well as a comparable (to normal mice) level of soluble SCF but it completely lacks the membrane form of SCF, indicating that the membrane form of SCF is required for hematopoiesis (Brannan et al., 1991). By transflecting various forms of
SCF into immortalized stromal cell lines from Sl/Sl mutant embryos, Toksoz et al. (1992) demonstrated that either soluble SCF or membrane-associated SCF can increase the number of hematopoietic progenitor cells. However, hematopoiesis was supported for a much longer time in vitro where the stromal cell layer expressed the membrane-associated SCF. Therefore, there is a fundamental difference between soluble and membrane forms of SCFs with respect to their capacity to sustain hematopoiesis.

Mast cell biology

The virtual lack of mast cells in Sl or W mutant mice provides strong evidence that SCF/c-kit receptor signaling is required for mast cell development. Injection of recombinant soluble SCF into the skin of Sl/Sld mutant mice (daily for 21 days) results in the appearance of a large number of mast cells at the injection site (Tsai et al., 1991; Zsebo et al., 1990a). Since Sl/Sld mice produce a relatively normal amount of soluble SCF but lack membrane SCF, these experiments suggest that membrane forms of SCF and/or the SCF level in the matrix play an important role in mast cell survival and maturation.

In vitro studies have demonstrated that SCF suppresses the apoptosis of mast cells, as it does in hematopoietic stem cells (Yee et al., 1994b). However, SCF alone stimulates mast cell proliferation by driving the cells to enter the S phase from G1 phase in the absence of serum (Yee et al., 1994b), which is different from its co-stimulating effect on the proliferation of hematopoietic progenitor cells. Mature mast cells retain a high level of c-kit receptor, suggesting that SCF may also act on mature mast cells. Cultured bone marrow derived mast cells have been shown to adhere to fibronectin in response to SCF in a dose-dependent manner (Dastych and Metcalf, 1994). Since SCF is produced by stromal cells, these experiments suggest that SCF may act as a chemotactic agent to guide
mast cell migration and distribution under physiological and pathological conditions. Apart from its role in mast cell development, SCF also affects mast cell functions. The major functional characteristic of mast cell is degranulation and release of mediators such as 5-HT. Injection of recombinant SCF intradermally into normal mice triggers mast cell accumulation and degranulation and thus tissue swelling whereas injection of SCF to W/W<sup>v</sup> mutant mice does not cause tissue swelling (Wershil et al., 1992). Therefore, the SCF/c-kit receptor signaling is essential in mast cell development and functions (Valent, 1994).

Melanocyte development

White coat color is the most obvious phenotype displayed by W and Sl mutant mice, indicating that the interruption of SCF/c-kit receptor signaling results in defective melanocyte development. In situ hybridization studies showed that as early as day 10 in the mouse embryo, c-kit mRNA was detectable in presumptive melanoblasts in the cervical region. By embryonic day 13.5, c-kit mRNA-positive presumptive melanoblasts appeared to have greatly increased in number and were found in the skin from the dorsal to ventral midline (Manova and Bachvarova, 1991). From midgestation to birth in mice, c-kit mRNA was present in the dermis, epidermis and hair follicles (Keshet et al., 1990; Manova and Bachvarova, 1991; Orr-Urtregger et al., 1990). SCF mRNA was expressed in the dorsal region of day 10.5 mouse embryo somites and at late intervals in the dermis and hair follicles (Matsui et al., 1990). Such a complementary pattern of SCF and c-kit mRNA expression during development suggests that the interaction between SCF and its receptor c-kit contributes to the migration and proliferation of melanocytes. The role of SCF/c-kit receptor in melanocyte development was further substantiated by the elegant work conducted by Nishikawa et al. (1991) in which the injection of anti-c-kit antibody (ACK2) into pregnant mice resulted in depigmentation in offspring. The time of injection
seems critical in that injection later than embryonic day 15.5 or earlier than day 10.5-11.5 had little effect whereas injection on day 13.5 resulted in virtually complete depigmentation and injection on days 14.5–15.5 caused striking but variable patterns of partial depigmentation in the offspring (Nishikawa et al., 1991). Therefore, the proliferation of melanocyte precursors in the mesodermal layer, and their subsequent migration into the dermis is critically dependent on SCF.

The role of SCF isoforms, soluble and membrane forms, in the development of melanocytes was also investigated. In the null Sl or W mutant mice, no melanocyte precursors are detected in the dermatome of the embryos whereas in Sl̄d/Sl̄d mutant which lacks membrane form of SCF but produces soluble SCF, melanocytes appear in the lateral neural crest cell migration pathway between the dermatome and the overlying epithelium but subsequently disappear from the dermis (Wehrle-Haller and Weston, 1995). This observation suggests that the soluble SCF is required for melanocyte precursors to disperse on the lateral pathway whereas the membrane-bound SCF may promote melanocyte precursor survival in the dermis.

Gametogenesis

The distribution of SCF and c-kit during germ cell development has been analyzed by in situ hybridization. At mouse embryonic day 10, SCF expression was seen within the genital ridge while c-kit mRNA was present mainly along the mesentery in the vicinity of the dorsal aorta (Keshet et al., 1991). In the midgestation (embryonic day 12.5) mouse, c-kit mRNA was detected in the primordial germ cells while SCF mRNA decreased in the genital ridge (Keshet et al., 1991; Orr-Urtreger et al., 1990). Since primordial germ cells originate in the allantois and migrate through the dorsal mesentery and hindgut to the forming genital ridges between embryonic day 9.5 and 11.5 and
colonization of genital ridge happens between embryonic days 10-12.5, these data suggest that cells expressing SCF guide c-kit expressing cells toward the genital ridge. In the adult, c-kit and SCF expression is confined to the gonad. While c-kit is localized to oocytes, SCF transcript is present outside the oocytes, suggesting SCF plays a role in germ cell development. In analogy to its effect in hematopoiesis, SCF supports primordial germ cell survival but not proliferation and this effect is mainly contributed by the membrane form of SCF (Dolci et al., 1991).

In summary, c-kit and SCF are expressed complementarily by the cells of the affected lineages and their microenvironment, and SCF/c-kit receptor signaling is essential for the development of these cells. SCF acts mainly as a survival or co-stimulating factor. Soluble SCF has a limited effect although it may form a gradient for the migration of melanocytes and primordial germ cells. Membrane SCF appears to be the main actor in early hematopoiesis, gametogenesis, and melanogenesis. SCF apparently affects every aspect of mast cell biology, including survival, proliferation, migration, differentiation and function such as mediator release in response to antigen-antibody complexes. Such diverse effects exerted by SCF depend on the lineage, stage of development, isoforms of SCF and the presence of other factors.

2.2.2 Expression and functions of SCF and c-kit in the nervous system

Besides expression in the cells of the hematopoietic, gametogenic, and melanogenic systems as well as in mast cells, SCF and c-kit are also expressed by cells or tissues that are not apparently affected by W or Sl mutations, including lungs, digestive tract, kidney and brain.
During embryonic development, SCF mRNA is detectable in neural tube as early as at mouse embryonic day 9.5 while c-kit transcripts are not detectable at this stage. SCF transcripts are localized in the discrete groups of cells at the ventral boundary known as the floor plate of the hindbrain and spinal cord (Keshet et al., 1991). By day 12, SCF expression extends to the ventrolateral boundary of the neural tube in the region where the motor neurons form, whereas c-kit expression is found to be confined to the post-mitotic cells in the marginal zone at the dorsal aspect of the neural tube (Keshet et al., 1991; Matsui et al., 1990; Orr-Urtreger et al., 1990). Such a dorsoventral organization of c-kit and SCF suggests that SCF/c-kit receptor signaling may play a role in the organization of neurons along the dorsal-ventral axis.

In the adult nervous system, c-kit and SCF are expressed complementarily with some complexity. The best examined region with consensus data is the cerebellum, in which SCF mRNAs are confined to Purkinje cells whereas c-kit transcripts are expressed by basket, stellate, and Golgi cells (Hirota et al., 1992; Keshet et al., 1991; Manova et al., 1992; Morii et al., 1992; Motro et al., 1991; Orr-Urtreger et al., 1990). Such a pattern of expression was also observed recently at the protein level (Manova et al., 1992). Since basket, stellate and Golgi neurons make synapses with Purkinje cells, it is postulated that SCF-c-kit receptor signaling may act in an analogous way to synaptic signal transmission, although the information flow may not necessarily be parallel with the neurotransmitter flow from the presynaptic to the postsynaptic end. Such a "synaptically" reciprocal expression can be found in other parts of the brain. For example, a high level of SCF transcripts were found in the thalamus, whereas the c-kit transcript was scattered in the cerebral cortex, although SCF was also expressed by many neurons in the cortex (Keshet et al., 1991; Matsui et al., 1990; Orr-Urtreger et al., 1990). In hippocampal formation and the related regions, c-kit mRNA is expressed by pyramidal neurons of the hippocampus, while SCF is expressed by dentate gyrus and entorhinal cortex (Keshet et
al., 1991; Matsui et al., 1990; Orr-Urtreger et al., 1990). However, a recent in situ hybridization study showed that the pyramidal neurons of rat hippocampus also express SCF (Wong and Licinio, 1994). Yet in some other parts of the brain such as olfactory bulb, apparent contradictory data were reported although both claimed complementary expression of SCF and c-kit (Hirot a et al., 1992; Motro et al., 1991). All these observations, however, indicate that the expression of SCF/c-kit receptor in the mature brain is much more complex than that in the developing neural tube or in the hematopoietic, gametogenic and melanogenic systems. Such consideration implies that the SCF/c-kit receptor signaling between neurons may function in a different way than neurotransmission or retrogradely transported trophic factors.

At the cellular level, most of the studies described above suggest that SCF and c-kit are expressed by neurons. Motro et al (1991), however, suggested that c-kit mRNA may be also expressed by glial cells in the cerebral and cerebellar cortex based on the observation that some c-kit transcript signals appeared on the outer layer of the cortex which is relatively free from neurons. Recently, an in vitro study showed that cultured oligodendrocyte precursor cells isolated from rat cerebral cortex expressed c-kit mRNA, which disappeared with the differentiation of oligodendrocytes in culture (Ida et al., 1993). In another study, Schwann cells cultured from both rat and human sciatic nerves expressed SCF but this expression was suppressed by contact with neurites (Ryan et al., 1994). These reports suggest that SCF/c-kit receptor signaling may be involved in neuron-glial interactions.

SCF and c-kit mRNAs are highly expressed in the mature nervous system, whereas W or Sl mutations appear not to cause observable morphological changes in the nervous system (Keshet et al., 1991; Orr-Urtreger et al., 1990). These observations raise the question, what does SCF/c-kit receptor do in the nervous system? Recent reports
show that in culture recombinant SCF supports the survival and promotes neurite growth of rat and chick dorsal root ganglion neurons which express c-kit receptor (Hirata et al., 1993; Carnahan et al., 1994). This effect appears to be mediated through the c-kit receptor tyrosine kinase since SCF did not promote the neurite outgrowth of DRG neurons from such W mutants as W42/W42 which completely lack the cell surface membrane c-kit receptor (Hirata et al., 1993). Therefore, SCF may act as a neurotrophic factor for c-kit expressing DRG neurons. So far, there is no comparable report on the effect of SCF on CNS neurons. The question then is why a W or SI mutation does not result in a neurological defect. One possibility is that most homozygous W or SI mutants die in uterus or shortly after birth or that in some heterozygotes that can survive, such a subtle change as that caused by the small number of neurons in DRG may escape conventional detection. Another possibility is that the function of SCF/c-kit receptor signaling is compensated by other signaling pathways as suggested by the above experiment in which SCF supported a subgroup of NGF-responsive neurons in DRG (Hirata et al., 1993). In contrast, the majority of the neurons, such as those in the cerebral cortex, spinal cord, as well as in DRG, express a high level of SCF mRNA. What does the neuronal SCF do in the nervous system? One hypothesis, held by most researchers in the field, is that neurons produce SCF and signal to other synaptically related neurons that express c-kit receptor, although the function of such signaling is completely unknown. Yet another possibility is that SCF may act as a signaling molecule in neuron-glia interactions, as suggested by in vitro studies (Ida et al., 1993; Ryan et al., 1994).

2.3 Cytokines in Neuron-glia Signaling

Neurons and glia are implicated in a complex dynamic cellular network within which they communicate with each other through signaling molecules, including neurotransmitters, hormones, growth factors and cytokines. Such neuron-glia signaling
plays an important role in development, and maintenance of homeostasis, as well as in injury and regeneration.

The term cytokine was originally used by immunologists to refer to the soluble substances which are produced by lymphohematopoietic cells and which are involved in hematopoiesis and immune reactions. However, such "lymphohematopoietic" cytokines are also produced by and act on the cells outside the lymphohematopoietic system. Besides, recent molecular biological studies provide evidence that many membrane-associated counterparts of the soluble cytokines also play important roles in hematopoiesis and immune reactions, as exemplified by SCF. Therefore, the concept of cytokine has been expanded from its original one, but to have the term cytokine include hormones and the well-known growth factors, as well as the classical cytokines (Patterson and Nawa, 1993), seems not well accepted yet.

Generally, cytokines are a group of heterogeneous polypeptide mediators that are produced in association with the activation of the immune system and inflammatory response. Closer examination with more sensitive assays indicates that they are also expressed by normal tissues or cells in extremely low levels or in specific isoforms such as extracellular or cell surface expressions. An individual cytokine can be produced by many different cell types and have multiple effects on cells that express the relevant receptor. Different cytokines may have redundant functions, i.e., several cytokines may mediate a common event. Thus cytokines display pleiotropism and redundancy, which may be attributed to the availability of receptors or receptor subunits on different cell types as well as the signal transduction pathways used by the receptors in different cell types. Moreover, cytokines often influence both the synthesis and function of other cytokines, resulting in complex regulatory pathways.
Only recently has it been realized that cytokines also play important roles in regulating neural development, differentiation, function and cellular responses in pathological states (Benveniste, 1992; Mehler and Kessler, 1995; Patterson, 1992, 1993; Rothwell and Relton, 1993; Unsicker et al., 1992). Such a regulatory role of cytokines is obvious when the homeostasis of the CNS microenvironment is breached as in trauma or neurodegeneration. Neuronal injury initiates a glia response, which is characterized by proliferation, hypertrophy and reorganization of the cytoskeleton. The signals by which injured neurons induce the glial response are poorly understood. It is speculated that certain cytokines as well as neurotransmitters may be involved. However, the phenotypic changes of the reactive glia, including astroglia and microglia, are apparently regulated by a number of cytokines. Reactive microglia upregulate cell surface antigens such as complement receptor 3 (CR3), major histocompatibility complex (MHC) class I and II molecules, receptors for CSF-1 and GM-CSF (Graeber et al., 1988; Kreutzberg and Barron, 1978; Raivich et al., 1991; Streit and Graeber, 1993; Streit et al., 1989). The cause of such antigenic changes is largely unknown but these changes may endow microglia with the capacity to respond to certain cytokines instantly. At the same time, reactive microglia increase the production of such cytokines as IL-1, IL-6, and tumor necrosis factor-α (TNFα) (Aloisi et al., 1992; Giulian et al., 1986). These cytokines are potent mitogens for astrocytes (Aloisi et al., 1992; Giulian and Lachman, 1985; Selmaï et al., 1990). They also induce astrocytes to increase the production of trophic factors such as NGF, bFGF and cytokines such as CSF-1, IL-6, GM-CSF, which promote the survival of injured neurons, and modulate the activity of microglia and astroglia themselves (Aloisi et al., 1992; Araujo and Cotman, 1995; Fedoroff et al., 1993; Frei et al., 1992; Giulian et al., 1986; Hao et al., 1990; Malipiero et al., 1990). For example, reactive astroglia increase the production of CSF-1, which further stimulates the proliferation and reactivity of microglia which express the upregulated CSF-1 receptor. Such paracrine stimulation of astrocytes and microglia through certain cytokines finally
leads to the peak glial response to neuronal injury, and is then down-regulated to restore the homeostatic state.

The control or recession of the glial response is apparently also modulated by multiple cytokines. One such cytokine is the transforming growth factor β1 (TGFβ1). As discussed above, reactive microglia produce inflammatory cytokines, IL-1, IL-6, and TNFα and trigger an astroglial response. Reactive microglia also de novo produce TGFβ1, in virtually all kinds of brain insults (Kiefer et al., 1995), though in a latent fashion (Constam et al., 1992). TGFβ1 has been shown to exert potent effects on neural cells, such as suppression of astroglial proliferation (Lindholm et al., 1992), induction of GFAP (Yoshida and Takeuchi, 1993), and the suppression of microglial proliferation and the production of IL-1, IL-6, TNFα by reactive microglia (Suzumura et al., 1993). Thus TGFβ1 produced by reactive microglia downregulates microglial reactivity directly and the astroglial activity both directly and indirectly through the downregulated production of inflammatory cytokines by microglia. The decreased production of cytokines such as CSF-1 by reactive astrocytes may further dampen the microglial reaction by causing microglia to undergo apoptosis (Gehrmann and Banati, 1995; Gehrmann, 1995). During this process, many other cytokines are expected to be involved in order to finely adjust the cytokine network which leads to a new homeostatic neuron-glia relationship.

Although the involvement of cytokines in injury and degenerative diseases in the brain is apparent, their role in the steady state brain seems not well appreciated. One of the reasons is that the level of the majority of cytokines and their receptors is extremely low in order to function in the normal brain and such a low level of expression is often beyond conventional detection methods. Using an in vitro system, it has been shown that certain cytokine(s) act as instructive signals to orchestrate neural cell lineage commitment and differentiation from a single neural stem cell isolated from the subventricular zone.
(Mehler et al., 1993, 1995). Cytokines have also been shown to signal postmitotic neurons to alter their transmitter phenotypes. For example, leukemia inhibitory factor (LIF), also called cholinergic differentiation factor (CDF), and probably also activins, members of the TGFB family, produced by the sweat gland can induce the noradrenergic sympathetic nerves that innervate the sweat glands in the rat foot pad to switch their transmitter from adenergic to cholinergic types (Fann and Patterson, 1995; Rao et al., 1992). These observations suggest that multiple cytokines take part in neural development. In the adult brain, cytokines are also required for normal neural cell function. In a CSF-1 deficient mouse caused by an autorecessive op mutation, the number and morphology of microglia seem normal (Blevins and Fedoroff, 1995), but the function of microglia is apparently abnormal. This is demonstrated by the deficient response of microglia to ischemic insult in the cerebral cortex. Such deficiency can be remedied by providing exogenous CSF-1 (Berezovskaya et al., 1995). Therefore, cytokines are involved in neural development, maintenance of the homeostatic microenvironment, and the functions in physiological and pathological states.
3. Materials and Methods

3.1 Tissue Cultures

3.1.1 Neuronal cultures from mouse embryos

Cerebral cortical neuronal cultures were prepared from the neopallium of E15 C3H/HeJ or C3H/OuJ mouse fetuses according to the method described by Hertz et al. (1989) with modifications. Pregnant mice were euthanized by CO2, the fetuses were aseptically removed and the brains isolated. The neopallia were dissected out by carefully removing meninges, olfactory bulbs, basal ganglia, and hippocampi. The neopallia were disaggregated in 0.25% trypsin in Puck’s solution (Ca++ and Mg++ free) for 5 min at room temperature. Horse serum was added to stop the trypsin action and the tissue was tritutrated by pipetting up and down. The cell suspension was centrifuged at 260 g for 5 min. After removing the supernatant, the cells were resuspended in modified Eagle’s Minimum Essential Medium (mMEM, Gibco BRL), and filtered through a 75 μm Nitex™ mesh. The cells were plated into 60 mm petri dishes (Falcon 3002) that were pre-coated with poly-L-lysine (Sigma, P-2636, Mol Wt. 30,000-70,000) or into petri dishes containing 15x15 mm coverslips that were previously coated with poly-L-lysine, at a density of 5X10^5 cells/dish. After incubation in serum-free mMEM for 15 min, the cultures were rinsed once with fresh medium and incubated at 37°C in a humidified atmosphere of 5% CO2 in air in freshly prepared medium consisting of mMEM, 1.25 mM glutamine, 5% horse serum and 6 g/L glucose. At 3 days in vitro (DIV), 5 μg/ml of cytosine arabinoside (Sigma, C-1768) was added to the cultures to inhibit astrocyte
proliferation. The neurons cultured in dishes were used for extracting protein and mRNA while those on coverslips were used for immunocytochemistry.

3.1.2 Isolation of microglia from neopallial cell cultures

The neopallial cell cultures were prepared from newborn C3H/HeJ or C3H/OuJ mice according to the method described by Hao et al. (1992). The neopallia were dissected out as described above for cultures of neurons. The neopallia were then cut into small fragments and gently passed through a 75 μm Nitex™ mesh. The disaggregated cells were suspended in mMEM with 5% horse serum and plated into 60 mm petri dishes containing coverslips which were later used for immunocytochemical staining, or 75 cm² flasks (Corning 430720). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, with medium change every three days. After 10 to 12 days, the cultures grew to a confluent layer. The cultures were then subjected to nutritional deprivation, i.e., grown without medium change for an additional 10 to 12 days. In such cultures, microglia proliferated while astrocytes retracted and degenerated and could be easily washed away. The remaining astrocytes, if any, were removed by treatment with 0.25% trypsin for 5 min. The resulting cultures of microglia were maintained in mMEM supplemented with 5% horse serum and 2–5 % LM cell conditioned medium which contained colony-stimulating factor-1 (CSF-1).

To sub-culture the microglia, newly-formed microglia cultures in flasks were rinsed three times with Puck’s solution, which was then replaced with 0.01% ice cold Versene in Puck’s balanced salt solution (BSS). The cultures were shaken on a rotary shaker at a speed of 200–250 rpm for 10 to 15 min at 4°C. The microglia were pipetted from the flask and the cell suspension was centrifuged at 260 g for 5 min. The microglia were resuspended in mMEM containing 5% horse serum and 2-5% LM cell conditioned
medium, and plated, at desired densities, into a 96-well plate for assaying growth or into culture dishes with coverslips for immunocytochemistry or for neuron-microglia co-culture.

3.1.3 Effect of SCF on microglia survival and morphological change

Disaggregated microglia were plated on coverslips in 60 mm petri dishes and grown overnight in mMEM containing 5% HS and 2-5% LM cell conditioned medium. The microglia coverslip cultures from the same dish were divided into two groups and grown in serum-free Iscove’s Modified Dulbecco’s Medium (IMDM) containing 0.1% bovine serum albumin (BSA). Recombinant SCF (provided by Immunex Corp, Seattle, WA), at a concentration of 100 ng/ml, was added to one group of the cultures at 1, 3, and 5 DIV. The microglia in both groups were examined daily under phase contrast microscope for survival and the morphology of the control and experimental cultures was compared.

To test whether the addition of SCF to culture media affected microglial movement in culture, microglial cultures with or without the addition of SCF were recorded using TV time lapse cinematography at a speed of 240 frames/hour over a 48 hour period.

3.1.4 Assay of [³H]thymidine uptake by microglia

Microglia isolated from neopallial cell cultures were dissociated as described above and plated in a 96-well plate (Falcon 3072) with 1x10⁴ cells/well in 100 µl of IMDM with 0.5% horse serum added. The microglia were incubated for 48 hours in the medium with or without the presence of cytokines, as stated in the results. Then, 10 µl of a [³H]thymidine (NEN) solution (0.5 µCi diluted 1:10 in IMDM) was added to each of the
wells and the plate was incubated for an additional 4 hr. After removing the medium, the microglia were lysed by the addition of 100 μl of PBS containing 1% TritonX-100 to each well and the lysates were harvested to glass-fiber filters. The filters were dried in air overnight and the circles containing the cells were cut out and placed into scintillation vials with 4 ml of scintillation solution (CytoScint 882465). The radioactivity of each vial was measured using a scintillation counter. Assays were run in triplicate and repeated twice.

3.1.5 Effect of SCF-treated microglia on neurons

To test whether SCF affects microglia which in turn affect neuronal survival and neurite growth, a co-culture system, as described by Zhang and Fedoroff (1996), was used with slight modification. Neuronal cultures were prepared at a density of 5x10⁴ cells/cm² (6x10⁵ neurons were plated into a 60 mm dish containing 5 15x15 mm coverslips) and microglia cultures at a density of 1x10⁵ cells/cm², as described above. Neuron-microglia co-cultures were made by putting one coverslip from the neuronal cultures and one coverslip from the microglia cultures in the same 35 mm non-culture petri dish in a serum-free medium consisting of IMDM/mMEM (1:1), 1.25 mM glutamine, 6 g/L glucose and 0.1% BSA (Sigma, A-7888). The neuron-microglia co-cultures were divided into two groups. In one of the groups, i.e., the experimental group, recombinant SCF, at a concentration of 100 ng/ml, was added to the medium at 1 and 3 DIV. The other co-culture group without the addition of SCF was used as the control for the effect of SCF in neuron-microglia co-cultures. Two other controls were used. Both were pure neuronal cultures. While one group of neuronal cultures had an addition of 100 ng/ml of SCF the other did not. These pure neuronal cultures were used as controls for the direct effect of SCF on neurons. To assess the number of neurons present on coverslips, cells bearing processes longer than their cell body diameter in 2 randomly chosen microscopic fields in each coverslip were counted at 1, 2, 3, and 4 DIV, using a 20x objective lens on
an Olympus phase contrast microscope. Each group consisted of 6 to 12 coverslips and the experiment was repeated 3 times.

3.1.6 Effect of cytokines on neural cells in cultures

To test the effect of SCF and other cytokines, microbial cultures were starved for growth factors for 18-20 hours by being incubated in IMDM supplemented with 0.1% BSA before cytokine treatment. The optimal concentration of most of the cytokines used in the experiment was titrated using a bioassay system.

3.1.7 Cell lines

The following cell lines were selected in order to test the specificity of the anti-c-kit receptor and anti-SCF antibodies. These cells were maintained in the Tissue Culture Facility of the Department of Anatomy and Cell Biology, University of Saskatchewan, according to the protocols provided by ATCC. Briefly, cells of WEHI-3 line (myeloid cells) were cultured in IMDM containing 15% fetal bovine serum (FBS) and $10^{-5}$ M 2-mercaptoethanol. NIH 3T3 fibroblasts (ATCC, CRL-1658) were maintained in DMEM with 10% calf serum. MC/9 mast cell line (ATCC, CRL-8306) was maintained in IMDM with 10% FBS, 45% conditioned medium prepared from the X630 MIL-2 cell line (provided by Dr. Trevor Owens, Montreal Neurological Institute), which produces IL-2, and $10^{-5}$ M 2-mercaptoethanol.

3.2 Brain Lesion

Adult (2-3 month) male C3H/HeJ and C3H/OuJ mice were anesthetized with somnaton (25 μl/20g body weight) intraperitoneally and immobilized in a stereotaxic
frame. A midline incision of the skin over the skull was made and a hole of 1 mm in diameter was bored with a dental drill on the right side of the skull (2.5-3 mm caudal to the bregma and 1 mm to the right of the midline). A needle of 23 gauge was inserted to a depth of 1.5-2.0 mm under the dura mater to make a stab wound lesion, and the skin was sutured. The animals were maintained in the laboratory animal facility of the Department of Anatomy and Cell Biology and had free access to water and food. One, two, four, seven, 14, and 28 days after surgery, the animals were sacrificed and the brain tissue was examined immunocytochemically.

3.3 Histological and Immunohistochemical Methods

3.3.1 Nervous tissue preparation

Normal or lesioned mice were euthanized with CO₂ and perfused transcardially with cold Dulbecco’s phosphate buffered saline (DPBS) followed by 4% formaldehyde in DPBS. The nervous tissues were removed, cryoprotected in 25% sucrose at 4°C overnight, embedded in OCT compound and rapidly frozen in liquid nitrogen. Cryostat sections were cut at 8-10 μm thickness and dried in air. For fresh tissues, cryostat sections were fixed in methanol/acetone (1:1) for 10 min at -20°C before immunostaining. For in situ hybridization, the tissues were post-fixed in 4% paraformaldehyde for 2 to 4 h before being processed for sectioning.

3.3.2 Detection of c-kit and SCF in cryostat sections by immunocytochemistry

Before immunostaining, sections were rehydrated in DPBS and incubated for 1 hour in DPBS containing 3% skim milk powder (Carnation, Ont., Canada) and 0.1% Triton X-100. The pretreated sections were incubated at 4°C overnight with the primary
antibodies. For the detection of c-kit receptor, a monoclonal antibody, ACK2 (rat IgG), which recognizes the extracellular domain of murine c-kit receptor (Gibco BRL, 3314SA), and a monoclonal antibody, mu-c-kit-M3 (rat IgG, provided by Immunex Corp) (Williams et al., 1990), were used at a dilution of 1:100. For the detection of SCF, a monoclonal antibody, mu-MGF-M3 (rat IgG, from Immunex Corp), at a dilution of 1:100, and a polyclonal antibody, anti-mSCF, which recognizes murine SCF (R&D System, AB-455-NA), at a dilution of 1:200, were used. After being washed twice with DPBS for 10 min and incubated in DPBS containing 3% skim milk for 20 min, the sections were incubated with secondary antibodies at room temperature for 45 min (biotinylated anti-rat IgG [H+L] for monoclonal primary antibodies, biotinylated antigoat IgG [H+L] for anti-mSCF, both at dilutions of 1:500). After washing in DPBS for 20 min, the sections were exposed to 0.3% H$_2$O$_2$ in methanol for 20 min to eliminate endogenous peroxidase activity. Sections were washed in DPBS and then incubated for 1 hour with avidin-biotin complex (ABC) at a dilution of 1:150, followed by a horseradish peroxidase substrate solution prepared in 0.175M sodium acetate, containing 0.05% 3,3'-diaminobenzidine (DAB, Sigma D-5637), 1.5% NiSO$_4$ and 0.03% H$_2$O$_2$. The sections were counterstained with nuclear fast red, dehydrated and mounted in a mounting medium Entellan$^\text{®}$ (BDH, 7961). The following controls were set up to confirm the specificity of the antibodies: omitting the primary antibodies; using positive cell lines such as MC/9 cells for c-kit positivity (Flanagan and Leder, 1990; Nocka et al., 1990) and NIH 3T3 cells for SCF positivity (Nocka et al., 1990); and using negative cell lines such as WEHI-3 cells as a negative control for c-kit (André et al., 1989; Nocka et al., 1989).

To verify the cell types positive for c-kit receptor and SCF immunostaining, some of the sections were further incubated in a polyclonal anti-GFAP (Dimension Laboratory Inc., Denmark, 1:200) for 1 hour at room temperature followed by FITC-conjugated donkey anti-rabbit IgG (1:100) for 1 hour. The c-kit receptor and SCF staining positive
cells were then compared with the GFAP positive cells using a Zeiss fluorescent
microscope III.

3.3.3 Detection of c-kit receptor and SCF in cultured neural cells using
immunofluorescence

Cells cultured on the coverslips were rinsed briefly with cold DPBS and fixed in
methanol/acetone (1:1) at -20°C for 10 min. The coverslips were then washed in PBS and
incubated with anti-c-kit antibody at 4°C overnight in a humidified chamber. The slides
were then washed and incubated with goat anti-rat IgG (H+L) for 45 min, followed by
rhodamine conjugated donkey anti-goat IgG (1:200) for 1 h at room temperature.

For staining with anti-mSCF, the coverslips were incubated in anti-mSCF
overnight at 4°C, followed by incubation with rhodamine labeled donkey anti-goat IgG for
1 h at room temperature. When the mu-MGF-M3 was used, the coverslips were incubated
in the goat anti-rat IgG followed by rhodamine conjugated anti-goat IgG.

To verify the cell types of the c-kit receptor- or SCF-positive cells, the above
labeled coverslips were further labeled with anti-GFAP (astrocytes), anti-neuron specific
enolase (NSE) (neurons), or isolectin B4 (microglia). For GFAP staining, the coverslips
were incubated in rabbit anti-GFAP for 1 h, followed by incubation in fluorescein
isothiocyanate (FITC) conjugated anti-rabbit IgG for 1 h. To label neurons, the coverslips
were incubated with monoclonal antibody anti-NSE (Hybridoma Laboratory, McGill
University, Canada, 1:20) for 1 h, followed by FITC tagged anti-mouse IgG for 1 h. To
stain microglia, the coverslips were incubated with FITC labeled isolectin B4 (Sigma,
L2895, 1:200) for 1 h.
All the fluorescence labeled preparations were finally stained with nuclear dye Hoehst 33258 (Sigma) and mounted in 50% glycerol in PBS.

The fluorescent immunostaining was visualized with the aid of a Zeiss photomicroscope III equipped for fluorescence. Photographs were taken using TMAX 400 film.

3.4 Molecular Biological Methods

3.4.1 RNA extraction and Northern blot analysis

Cultured cells were rinsed with PBS prepared in diethylpyrocarbonate (DEPC) treated water, homogenized in TRIzol™ reagent (Gibco BRL, 15596-026) and total RNA was extracted according to the manufacturer's instructions (see appendix for detail). RNAs were fractionated by electrophoresis in 1.0% agarose (Promega, V-3121) gel in the presence of 1M formaldehyde. The gel was soaked in 20x sodium chloride/sodium citrate solution (SSC) for 1 hour and blotted overnight to nitrocellulose membrane (Schleicher & Schuell). To confirm that equivalent amounts of RNAs were loaded in each lane, ethidium bromide (Sigma E-1510) was added to the sample buffer before electrophoresis, and the gels were photographed under ultraviolet illumination. After transfer, both the gel and the membrane were examined under UV light to make sure that the transfer was even and complete.

3.4.2 Preparation of probes

Both RNA and DNA probes were used. The antisense RNA probes were synthesized using a Riboprobe® Gemini System (Promega, P-1121) in the presence of
linearized DNA, SP6 (or T7) polymerase, RNAsin, NTP, and [α-32P]CTP (NEN, NEG-008H). The probes were purified by precipitation with ethanol.

For digoxigenin-labeled antisense and sense RNA probes (used for in situ hybridization), digoxigenin-labeled CTP (Boehringer Mannheim), instead of [α-32P]CTP, was added to the above transcription reaction buffer.

The following plasmid DNAs were used for synthesizing riboprobes. The plasmid pBSSK-MGF4 (provided by Immunex Corp) which contained a 1 kb insert encoding amino acid 1-47 of murine SCF (Copeland et al., 1990; Williams et al., 1990) was linearized with BamHI and transcribed with T7 polymerase. For sense SCF probe, it was linearized with Asp718 and transcribed with T3. The plasmid, huIL-1β:pGEMI (provided by Immunex Corp), contained a cDNA insert of 570 bp SstI to PvuII restriction fragment of the human IL-1β gene (March et al., 1985) and was linearized with EcoRI and transcribed with SP6 polymerase. The plasmid, mTNFα:pGEM3 (provided by Dr. E. Benveniste of University of Alabama at Birmingham), contained a 1.3 kb insert of murine TNFα DNA (Beutler et al., 1985) and was linearized with HindIII and transcribed with T7. The pmNGF (provided by Dr. R. Murphy, Montreal Neurological Institute, Montreal, Canada) contained a 969 bp PCR product of mouse NGF gene cloned into pGEM3z and was linearized with EcoRI and transcribed with SP6. The pGM4BDNF (provided by Dr. F. Miller of Montreal Neurological Institute, Montreal, Canada) contained 290 bp rat BDNF DNA in pGM4z and was linearized with HindIII and transcribed with SP6. K10, IL-6GEM and R9 were provided by Dr. P. Richardson of Department of Neurosurgery, Montreal General Hospital. K10 contained the rat CNTF gene in pGEM7z and was linearized with PvuII and transcribed with T7; IL-6GEM contained a 597 bp rat IL-6 cDNA cloned into pGEM7z (Murphy et al., 1995) was linearized with HindIII and transcribed with SP6; R9 contained mouse leukemia
inhibitory factor (LIF) DNA in pGEM7z and was linearized with HindIII and transcribed with SP6. The above plasmid DNAs, the restriction enzymes to linearizing the DNAs, and RNA polymerases to transcribe the antisense or sense RNA probes are listed in Table 1.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Plasmid</th>
<th>Restriction enzyme</th>
<th>RNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>murine SCF</td>
<td>pBSSK-MGF4</td>
<td>BamHI</td>
<td>T7</td>
</tr>
<tr>
<td>murine SCF</td>
<td>pBSSK-MGF4</td>
<td>Asp718</td>
<td>T3 (sense)</td>
</tr>
<tr>
<td>human IL1β</td>
<td>huIL-1β:pGEMI</td>
<td>EcoRI</td>
<td>SP6</td>
</tr>
<tr>
<td>murine TNFα</td>
<td>mTNFα:pGEM3</td>
<td>HindIII</td>
<td>T7</td>
</tr>
<tr>
<td>rat IL-6</td>
<td>IL-6GEM</td>
<td>HindIII</td>
<td>SP6</td>
</tr>
<tr>
<td>murine CNTF</td>
<td>K10 (pGEM7z)</td>
<td>PvuII</td>
<td>T7</td>
</tr>
<tr>
<td>mouse LIF</td>
<td>R9 (pGEM7z)</td>
<td>HindIII</td>
<td>SP6</td>
</tr>
<tr>
<td>mouse NGF</td>
<td>pmNGF</td>
<td>EcoRI</td>
<td>SP6</td>
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<tr>
<td>rat BDNF</td>
<td>pGM4BDNF</td>
<td>HindIII</td>
<td>SP6</td>
</tr>
</tbody>
</table>
The cDNA probes were prepared by random priming. Briefly, the purified DNA fragment was denatured in boiling water for 10 min and cooled on ice. The denatured DNA was then added to Klenow buffer together with dNTP(without dCTP), [\(\alpha-^{32}\text{P}\)]-dCTP (NEN, NEG-013A) and Klenow enzyme and incubated at 37° for 1/2 to 1 h. The labeled DNA was then separated from the nonincorporated deoxyribonucleoside triphosphates by chromatography on a 1 ml Sephadex G50 column.

The following DNAs were used for preparing DNA probes. The plasmid, c-muckit#1 (provided by Immunex Corp), is a 3 kb murine c-kit cDNA cloned into Bluescript SK(-) (Keshet et al., 1991; Williams et al., 1990). An Asp718-DraI fragment (1.6 kb) of c-muckit#1, which encodes the extracellular domain of c-kit receptor, was used to generate a DNA probe. The plasmid, p755 (provided by Dr. L.R. Rohrschneider, Department of Cell Biology, University of Washington, Seattle, Washington), is a murine c-fms cDNA cloned into pGEM2 (Rothwell and Rohrschneider, 1987). A 3kb EcoRI-NdeI fragment of the c-fms cDNA clone was used to generate a DNA probe.

3.4.3 Hybridization, washing, and reprobing

For riboprobes, prehybridization and hybridization were carried out at 65 °C for 8-12h and overnight, respectively, in a buffer containing 50% formamide, 0.75M NaCl, 25mM Pipes, 25mM EDTA, 5x Denhardt's, 0.2% SDS, 100 μg/ml sperm DNA and 100 μg/ml transfer RNA. Posthybridization washes were done at room temperature for 30 min in 5x SSC, at 55°C for 30 min in 0.5x SSC, and at 65°C for 30-60 min in 0.05x SSC.
For DNA probes, prehybridization and hybridization were carried out at 45°C and posthybridization washes were the same as for the RNA probe except that the final wash was in 0.1x SSC for 30 min.

The filters were finally covered with saran wrap and exposed to Kodak XAR film for 1 to 3 days before the film was developed. The filters probed with c-kit DNA were exposed to the film for 10 to 14 days.

To remove the probes from the nitrocellulose filter, the filter were immersed for 15 min in 250 ml preheated stripping buffer which consisted of 0.05x SSC, 0.01M EDTA (pH 8.0) and 0.1% SDS. The procedure was repeated using freshly prepared buffer. The stripped membranes were covered with saranwrap and were exposed to x-ray film for 2 to 3 days to ensure that the original probe was removed before reprobing. The filters were then used for reprobing.

3.4.4 Examination of hybridization signals on x-ray films

The relative intensities of the hybridization signals on the x-ray films were measured using the IPLab Spectrum (Signal Analytics Corporation) and corrected by the corresponding relative intensity of the ethidium bromide-stained RNA samples on a negative. The relative signal intensities were expressed as times (or percentages) of that of the control sample in the same film.

3.4.5 In situ hybridization

The cryostat sections on slides were rehydrated in PBS prepared with DEPC-treated water. They were then hybridized with digoxigenin-labeled antisense SCF RNA
probe diluted in hybridization solution, consisting of 50% formamide, 0.3M NaCl, 10mM phosphate buffer (pH 7.4), 10mM EDTA, 10mM Tris-HCl (pH 7.5), 10% dextran sulphate, 1mg/ml tRNA, 1x Denhardt's at 55°C overnight in a humidified chamber. For control, digoxigenin-labeled sense SCF RNA probe, instead of antisense probe, was added to the hybridization solution. After hybridization, all slides were washed twice in 2x SSC with 50% formamide at 55°C for 30 min each, and twice in 1x SSC, 25% formamide, 0.5x PBS at 65°C for 30 min each. The slides were then transferred to PBS at room temperature for 10 min and blocked for 1h in PBT (PBS with 0.1% Tween 20) containing 0.2% BSA before being incubated for 1h in alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim), which had been pre-absorbed with mouse brain powder (Rockland, Gilbertsville, PA), at a dilution of 1:2000. The slides were then washed in PBT four times for 15 min each and incubated in a light-tight box in a staining solution consisting of 0.34 mg/ml nitroblue tetrazolium chloride (NBT, Gibco BRL), 0.175 mg/ml 5-bromo-4-chloro-3-indolyphosphate (BCIP, Gibco BRL), 100mM NaCl, 50mM MgCl2, 100mM Tris-HCl (pH 9.5), 0.1% Tween 20, for several hours to allow the colour to develop.

For control of the anti-digoxigenin antibody, some of the antisense SCF RNA probed slides were incubated in PBS instead of anti-digoxigenin antibody, followed by the same procedures as described above.

To investigate whether astrocytes in the lesion area express SCF mRNA, the above anti-digoxigenin antibody labeled slides were further incubated with anti-GFAP for 1h at 1:200 dilution, followed by incubation in FITC-conjugated anti-rabbit IgG for 45 min at a dilution of 1:200. The slides were visualized with the aid of a Zeiss photomicroscope III and pictures were taken using TMAX3200 film.
All slides were finally counterstained with nuclear fast red, dehydrated, and mounted in a mounting media Entellan® (BDH, 7961).

3.4.6 Western blot analysis

For Western blot analysis, cells were washed twice with cold DPBS and lysed in lysis buffer which contained 5 mM HEPES (pH 7.2), 5 mM EDTA, 50 mM NaCl, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, and 1% Triton X-100. Cell lysates were clarified by centrifugation at 12,000g for 15 min at 4°C and the protein concentrations of the supernatant were determined using Biorad protein assay kit (Bradford protein assay). About 10 to 20 µg of proteins were separated on an 8-12% gradient polyacrylamide gel and transferred to a nitrocellulose filter (Millipore). The filter was blocked with 3% skim milk powder in PBS for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies, ACK2 at a 1:1000 dilution, or Anti-lipocortin-1 antibody (provided by Dr. J.A. McKanna of University of Vanderbilt, Nashville, TN) at a 1:40,000 dilution, prepared in PBS/0.05% BSA. After repeated washings in PBS with 0.01% Tween-20 added, the filter was incubated in biotinylated secondary antibodies, anti-rat IgG at a 1:5000 dilution (for ACK2) and anti-rabbit IgG at a 1:5000 dilution (for LC-1), for 1 hour, followed by avidin-biotin complex (1:1000) for 60 min. The immunoreaction on the filters was revealed by a chemiluminescent reagent (NEN, Dupon, NEL 100) and signals were developed on an x-ray film.

For the control of anti-c-kit antibody (ACK2) in Western blot analysis, the blot containing the protein lysate prepared from MC/9 cells (positive control) and WEHI3 cells (negative control) was used.
4. RESULTS

4.1 Expression of c-kit and SCF in Nervous Tissues

4.1.1 Specificity of the antibodies for c-kit and SCF

When the project began, there were no antibodies for c-kit receptor and SCF available commercially. The specificity of antibodies, mu-c-kit-M3, and mu-MGF-M3, provided by Immunex Corp, were tested in the cell lines described below.

The anti-SCF antibodies of both sources, namely the mu-MGF-M3 from Immunex Corp and the anti-mSCF antibody purchased from R&D System Inc., stained NIH 3T3 fibroblasts which were known to produce membrane-bound SCF (Nocka et al., 1990). The mu-MGF-M3 has recently been reported to stain stem cell factor in skin tissues (Longley et al., 1993). Both antibodies stained cultured fibroblasts in the same pattern and cells in the brain tissue section similarly (Fig. 1a) although mu-MGF-M3 stained fresh tissue sections preferentially.

The c-kit antibody, mu-c-kit-M3, stained MC/9 cells, a mast cell line that express the c-kit receptor (Flanagan and Leder, 1990; Nocka et al., 1990; Williams et al., 1990) (Fig. 1b), but not WEHI-3 cells which do not express the c-kit receptor (André et al., 1989; Nocka et al., 1989) (Fig. 1c). The pattern of immunostaining of mu-c-kit-M3 on cultured cells was comparable to the now commercially available antibody, ACK2. However, mu-c-kit-M3 only stained cells of freshly frozen tissue sections as shown by the
staining of a certain group of DRG neurons but not the paraformaldehyde-perfused tissues, whereas ACK2 can be used for staining cells of paraformaldehyde-perfused tissues (without postfixation).

4.1.2 Immunocytochemical characterization of c-kit receptor and SCF in nervous tissues in situ

Cerebellum

Expression of c-kit and SCF was studied in the cerebellum by in situ hybridization (Hirota et al., 1992; Manova et al., 1992; Morii et al., 1992; Motro et al., 1991). In the present study using immunocytochemistry, I found a similar pattern of expression of c-kit receptor and SCF, as reported in the literature (Fig.2). The expression of SCF was most obvious in Purkinje cells in the cerebellar cortex (Fig. 2a). The immunostaining was mainly in the cell body and its main branches. In addition, some neurons, identified as basket cells and Golgi cells, which were between Purkinje cells and granule cells in size and which were located close to Purkinje cells, were stained, mainly in the perinuclear area (Fig. 2a).

In contrast to SCF, the c-kit receptor immunostaining was mainly in the nerve fibers, therefore the appearance of cell bodies was not very clear in the densely stained area such as the molecular layer. The most prominent c-kit receptor staining was in the axons of basket cells which, in the form of discrete nests, surround every Purkinje cell at the base of the cell body and the initial segment of the axon (Fig.2b). Almost all cells in the molecular layer were stained, suggesting that some glia were also positive for c-kit receptor. In the granular layer, light staining was interspersed among the closely packed...
Fig. 1 Immunostaining for SCF in NIH 3T3 fibroblasts (a) and for c-kit receptor in MC/9 mast cells (b), and Western blot analysis for c-kit receptor expression (c). The immunostaining for SCF in fibroblast is patchy and shows clear cell boundary (a); Strong immunostaining for c-kit receptor is over the cell body of mast cells (b); Bar = 50 µm. (c) Western blot analysis (using 7.5% SDS-PAGE) shows that MC/9 cells (lane 1), cerebral cortical neurons at 5 DIV (lane 3) and microglia (lane 4) express c-kit receptor protein of ~45 kD and a fragment of 100 kD and that WEHI-3 cells (myeloid cell line) do not express c-kit receptor (lane 2).
Fig. 2  Immunostaining for SCF (a) and for c-kit receptor (b) in the adult mouse cerebellum. The Purkinje cells and their main branches are SCF positive but are c-kit receptor negative. Almost all cells in the molecular layer are c-kit receptor positive, especially the axon terminals of the basket cells that surround the Purkinje cells. Bar = 50 μm.
granular neurons. The irregularity of the staining suggested that they are mossy fibers (Fig. 2b).

Olfactory bulb

In the olfactory bulb, the SCF signal was confined to mitral cells and some of the periglomerular cells and external tufted cells (Fig. 3a), whereas c-kit receptor immunostaining was seen mainly in the glomerular and external plexiform layer. In the glomerular layer, only fibrous staining was seen within the glomeruli and no stained cell bodies were found. In the external plexiform layer, however, distinct cellular staining continuing into the fibers was seen. The position and morphology of these cells suggest that they are tufted cells (Fig. 3b).

Forebrain

In the cerebral cortex, SCF was expressed by many neurons, especially in layers II, III, and V. The immunostaining was present to the whole cell body and its main branches. Most of the cells were identified as pyramidal neurons (Fig. 4a and c). The expression of c-kit receptor was strongest in the molecular layer, where dense fibers as well as cell bodies were stained (Fig. 4b). Under higher magnification, some small positive cells, with irregular cell bodies, pale nuclear (nuclear fast red) staining, and several fine processes, were seen among the densely stained fibers. These cells were identified as glia because of the difference in morphology from that of the nearby positive neurons which had a large cell body with a clearly stained round nucleus (Fig. 4d). In the deeper layers, stained fibers were fewer and lighter so that the discrete positive cell bodies were relatively easily recognized. The immunoreactivity was confined to the membrane of
Fig. 3 Immunostaining for SCF (a) and for c-kit receptor (b) in the adult mouse olfactory bulb. Periglomerular cells, mitral cells and some of the tufted cells are SCF positive, whereas the nerve endings of the olfactory epithelial cells in the glomeruli are positive for c-kit receptor (arrowhead). Some tufted cells are also c-kit receptor positive (arrow). Bar = 50 μm.
Fig. 4 Immunostaining for SCF (a, c) and for c-kit receptor (b, d) in the adult mouse cerebral cortex. (c) and (d) are enlargements of the insets of (a) and (b), respectively. Many neurons in layer II, III, and V are SCF positive; most are pyramidal neurons (c). Fewer neurons are c-kit receptor positive. Some glial cells, especially in the molecular layer, are also c-kit receptor positive (arrow). Bar = 50 μm.
the cells, most of which were spindle-shaped with few processes and were localized in layers II, III, and, to a lesser extent, in layer IV (Fig. 4b). The number of c-kit receptor positive neurons was much fewer than that of SCF positive neurons in the cerebral cortex.

In the hippocampal formation, SCF was expressed mainly by granule cells in the dentate gyrus but also by some pyramidal cells in the hippocampus proper (Fig. 5a). In contrast, c-kit receptor was expressed by the neurons in hippocampus proper but not in the dentate gyrus. As seen in other regions of the brain, the c-kit immunoreactivity was mainly localized in the membrane. Therefore, heavy immunostaining was located in the stratum oriens and stratum radiatum where the axons and dendrites of pyramidal neurons were located whereas only very light staining was seen in the pyramidal cell layer because of the close packing of the pyramidal cells (Fig. 5b).

In other parts of the forebrain, SCF was expressed by neurons in the habenular nucleus, thalamus, hypothalamus, amygdaloid nucleus and the choroidal plexus cells (Fig. 5c), whereas c-kit receptor immunostaining was found in the following structures: some neuronal cell bodies and numerous nerve endings surrounding the c-kit negative neurons in the putamen, glial cells and nerve endings surrounding the neurons in the ventral medial hypothalamus, fornix column, and endothelial cells in the choroid plexus (Fig. 5d).

Brain stem and spinal cord

In the brain stem, SCF positive fibers were found in the pyramidal and pontocerebellar tracts, whereas positive cell bodies were found in the area postrema, pontine nucleus, and cells in the reticular formation (Fig. 6a). The c-kit receptor positive
Fig. 5 Immunostaining for SCF (a, c, e) and for c-kit receptor (b, d, f) in the hippocampus (a, b), thalamus (c), putamen (d), and hypothalamus (e, f). SCF staining is found in granule neurons in dentate gyrus and most of the hippocampal pyramidal neurons (a), and in the majority of neurons in thalamus and hypothalamus (c, e). Staining of c-kit receptor is seen in pyramidal neurons in the hippocampus (b), some neurons and numerous nerve endings in the putamen (d), and nerve endings as well as some glia (arrows) in the hypothalamus, the enlargement of the inset in lower-left (f). Bar = 50 μm.
nerve fibers were found in the trigeminal tract, medial lamniscus, spinothalamic tract and superior colliculum in which positive fibers were arranged in layers. In addition, c-kit positive cell bodies were found in the area postrema (mainly glia) and accessory olivary nuclei (mainly neurons) (Fig. 6b).

In the spinal cord, SCF was expressed by many neurons, especially the big motor neurons in the anterior horn and some neurons in the posterior horn (Fig. 6c). The c-kit immunostaining, however, was confined to the outer layers of the posterior horn. At higher magnification, the staining was localized to nerve fibers or their endings. No positively stained cell bodies were found in the spinal cord (Fig. 6d).

Dorsal root ganglion (DRG) and peripheral nerves

In the DRG, SCF stained a majority of the DRG neurons. Some of the axons were also SCF positive as seen in the sciatic nerve (Fig. 6e). In contrast, c-kit receptor was expressed by only a small number of neurons. The immunostaining in the DRG was different from that seen in the CNS in that the cytoplasm of the DRG neurons was evenly stained. Most of the positively stained neurons were small neurons (Fig. 6f).

The expression of SCF and c-kit receptor in the nervous tissues in situ is summarized in Table 2.
Fig. 6 Immunostaining for SCF (a, c, e) and for c-kit receptor (b, d, f) in area postrema (a, b), spinal cord (c, d) and dorsal root ganglion (e, f). SCF staining is seen in some neurons in area postrema (a), in spinal cord (c), and in dorsal root ganglion (e), whereas c-kit receptor staining is seen in glial cells (arrowhead) in area postrema, the enlargement in upper-left (b), the dense nerve endings surrounding the neurons in the outer layers of dorsal horn of the spinal cord, the enlargement of the inset shown at lower-right (d), and in a few small neurons (arrows) in the adult mouse dorsal root ganglion (f). Bar = 50 μm.
Table 2. Summary of expression of SCF and c-kit receptor in neural tissues

<table>
<thead>
<tr>
<th>Region</th>
<th>SCF</th>
<th>c-kit Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory bulb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerular layer</td>
<td>++</td>
<td>+ (axons of olfactory epithelia)</td>
</tr>
<tr>
<td>Plexiform layer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mitral cell layer</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Granular layer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neocortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer I</td>
<td>-</td>
<td>++++, G+</td>
</tr>
<tr>
<td>Layer II</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Layer III</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Layer IV</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Layer V</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Layer VI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Archicortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Striatum</td>
<td>+</td>
<td>++ (nerve endings)</td>
</tr>
<tr>
<td>Thalamus</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>++</td>
<td>+ (only nerve endings), G+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular layer</td>
<td>-</td>
<td>++++, G+</td>
</tr>
<tr>
<td>Purkinje cell layer</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Granular layer</td>
<td>-</td>
<td>+ (mossy fibers)</td>
</tr>
<tr>
<td>Cerebellar nuclei</td>
<td>+ (some)</td>
<td>+ (some)</td>
</tr>
<tr>
<td>Brain stem</td>
<td>+ (some)</td>
<td>+ (some), G+</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>++</td>
<td>++ (only nerve endings)</td>
</tr>
<tr>
<td>Dorsal root ganglion</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: (+) indicates positive neurons; (-) indicates negative; G+ indicates positive glia.
4.1.3 Expression of SCF and c-kit in neural cells in cultures

In culture, almost all cerebral cortical neurons in 4-day old cultures expressed SCF. The immunofluorescence was distributed throughout the cell body and the main branches of the neurites (Fig 7a). Under higher magnification, the immunostaining in neurons was patchy. Most cerebral cortical neurons were also c-kit receptor positive. The immunofluorescence was localized in the cell membrane. Two types of staining patterns which differed in fluorescent intensity were found. The majority of the cortical neurons stained weakly with anti-c-kit receptor antibody, while a small number of neurons (about 2-5% of the neurons in the 4-day-cultures) exhibited a bright fluorescent signal. In most cases, the brightly stained neurons had specific morphological characteristics, i.e., they had long branched neurites with numerous bead-like formations on the neurites (Fig.7b).

Microglia maintained in LM-cell conditioned medium which contains CSF-1 expressed a low level of SCF. The staining pattern was different from neuronal staining in that the staining in microglia was weak and diffuse around the nuclei without showing clear boundaries of the cells (Fig. 8e). Interestingly, strong membrane staining of SCF was found in small round microglia formed in neopallial cell cultures (Fig. 8a). The c-kit receptor was expressed by many microglia maintained in LM-cell conditioned medium. The immunofluorescent signal was distributed over the cell body with clear staining of the cell membrane (Fig. 8f). When the microglia, which were recently formed in neopallial cell cultures were stained, bright staining of cell membrane, similar to the bright staining in neurons, was found (Fig. 8b).

The expression of c-kit receptor and SCF in neurons and microglia in culture is summarized in Table 3.
Fig. 7 Double immunostaining for NSE and for SCF or c-kit receptor in the cerebral cortical neurons in culture (5 DIV). Immunostaining with rhodamine-conjugated antibodies to SCF (a) or c-kit receptor (b), and immunostaining with FITC-conjugated antibody to NSE (c, d). All neurons are SCF positive (a). Most neurons are weakly positive for c-kit receptor (arrows in b) except a few strongly positive neurons (arrowhead in b). Bar = 50 μm.
Fig. 8 Double staining for isolectin B4 and for SCF or c-kit receptor in the microglia formed in neopallial cell cultures (a, b, c, d) and in microglia maintained for 3 days in medium conditioned by LM cells (e, f, g, h). Immunostaining with rhodamine conjugated antibody to SCF (a, e) and with rhodamine-conjugated antibody to c-kit receptor (b, f). Staining with FITC-conjugated isolectin B4 (c, d, g and h). The small, round, newly formed microglia were strongly stained with SCF in the membrane (a) whereas those maintained in the LM cell conditioned medium had very weak staining (e). Staining of c-kit receptor was localized in the membrane of the newly formed microglia (b) and in the perinuclear area as well as in the membrane of the microglia maintained in the LM cell conditioned medium (f). Bar = 50 μm.
Table 3. Expression of SCF and c-kit receptor in neurons and microglia in culture

<table>
<thead>
<tr>
<th>Cell</th>
<th>SCF</th>
<th>c-kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons (4 DIV)</td>
<td>++</td>
<td>++(few), ± (majority)</td>
</tr>
<tr>
<td>Microglia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In LM cell CM</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Newly isolated</td>
<td>++(membrane staining)</td>
<td>++(membrane staining)</td>
</tr>
</tbody>
</table>

Note: (±) indicates weakly positive; (+) indicates positive; (++) indicates strongly positive.

4.1.4 Analysis of mRNA expression in cultured neural cells.

Northern blot analysis demonstrated that cultured cortical neurons and microglia express a c-kit transcript about 5.5 kb in size (Fig. 9b). They also express SCF mRNAs. Neurons expressed mainly the 4.5 kb and 6.5 kb SCF transcripts. In addition, neurons, mainly those maintained in culture for 5 DIV and well differentiated, expressed another transcript of about 9 kb in size. Microglia in culture expressed predominantly the 4.5 kb and the 3.5 kb SCF mRNAs (Fig. 9a).
Fig. 9 Expression of SCF (A) and c-kit (B) mRNAs in cerebral cortical neurons (5 DIV) and microglia (one day after isolation) demonstrated by Northern blot analysis using total RNA. Cortical neurons express the 4.5 kb and 6.5 kb SCF mRNAs and in addition, they express a large SCF transcript (~9 kb) (lane 1 in A). Microglia express the 4.5 kb and 3.5 kb SCF mRNAs and only slightly the 6.5 kb transcript (lane 2 in A). Both cortical neurons (lane 1) and microglia (lane 2) express the 5.5 kb c-kit mRNA (B). The ethidium bromide-stained rRNAs are shown to indicate the relative amount of total RNA in each sample.
4.2 Cytokine regulation of c-kit and SCF expression in microglia

The regulation of c-kit receptor in microglia was studied by Western blot analysis (Fig. 10). Microglia in culture expressed the 145 kD c-kit protein and the 100 kD fragment. Treatment of the microglia for 24 hours with c-kit ligand, SCF (100 ng/ml), greatly downregulated the 145 kD c-kit protein but increased the 100 kD fragment. Other cytokines, such as CSF-1 (800 U/ml), PDGFαβ (50 ng/ml), and lipopolysaccharide (LPS, 1 μg/ml) also downregulated 145 kD c-kit receptor expression. However, interferon-γ (IFN-γ, 250 u/ml) and interleukin-1 (IL-1, 0.1ng/ml) significantly increased the 145 kD c-kit protein and the 100 kD fragment in microglia. Interestingly, the ligand for flt3 (Immunex Corp, 100ng/ml), a new member of the type III tyrosine kinase receptor subfamily (Lyman et al., 1993), also upregulated the c-kit receptor in microglia.

The regulation of SCF expression in microglia was investigated at the mRNA level. The maximum SCF mRNA expression was obtained 12 to 24 hours after treatment of the microglia with cytokines, IFN-γ or CSF-1. IFN-γ increased mainly the 6.5 and 4.5 kb SCF mRNAs, CSF-1 upregulated all 3 transcripts, especially the 3.5 and 4.5 kb SCF mRNAs (Fig. 11). The ligand SCF potentiated the expression of SCF mRNAs in microglia, mainly the smaller 3.5 kb transcript.
Fig. 10 Effect of cytokines on the expression of c-kit receptor protein in microglia in culture demonstrated by Western blot analysis. Microglia were maintained in mMEM supplemented with 0.5% HS. The protein was extracted and quantified 24 hours after cytokine treatment. The protein was separated in a 10% SDS-acrylamide gel and Western blot analysis was done. A 145 kD and a 100 kD protein was revealed by the anti-c-kit receptor antibody, ACK2. Treatment of the microglia for 24 hours with SCF, 100 ng/ml (lane 2), CSF-1, 1000 U/ml (lane 3), PDGF, 50 U/ml (lane 5), and LPS, 1 μg/ml (lane 7) decreased the 145 kD protein but increased the 100 kD protein, whereas treatment with fliL, 100 ng/ml (lane 4), IFN-γ, 250 U/ml (lane 6), and IL-1, 0.1 ng/ml (lane 8) increased both the 145 kD and the 100 kD proteins as compared with the control (lane 1).
Fig. 11 Effect of SCF, CSF-1, and IFN-γ on the expression of SCF mRNA in microglia in culture as analysed by Northern blot. Treatment of the microglia with IFN-γ (250 U/ml) for 0 h (lane 1), 1 h (lane 2), 6 h (lane 3), 12 h (lane 4) and 24 h (lane 5) increased the SCF mRNAs, mainly the 4.5 kb and 6.5 kb transcripts, with its peak effect after 12 h of treatment. Treatment of the microglia with CSF-1 (800 U/ml) for 12 h increased all three transcripts of SCF, especially the 4.5 kb and 3.5 kb SCF mRNAs (lane 6). SCF at a dosage of 100 ng/ml slightly increased the 3.5 kb SCF mRNA and did not affect the 4.5 kb and 6.5 kb transcripts (lane 7). The ethidium bromide-stained rRNAs are shown to indicate the relative amount of total RNA in each sample.
4.3 Effects of SCF on Microglial Morphology, Survival, and Gene Expression

4.3.1 Effect of SCF on microglial survival and morphology

When microglia were cultured in mMEM supplemented with 5% horse serum and 2-5% medium conditioned by LM cells, they protruded processes to become bipolar or tripolar cells and could survive for over a week without medium change. After replacement of the serum-containing medium with serum-free medium, IMDM, without the addition of growth factors, the microglia gradually retracted their processes. At 3 DIV, the cell bodies and nuclei of some microglia began to shrink, and at 7 DIV some dead cells were observed. Addition of 100 ng/ml SCF to microglia cultures in serum-containing medium did not cause an observable change in microglial morphology or survival time. However, addition of 100 ng/ml of SCF into serum-free IMDM at 1, 3, and 5 DIV consecutively supported microglia survival and changed their morphology. To increase the survival time of microglia, SCF had to be added to the culture every other day, but even so some microglia still degenerated (Fig 12).

The morphological change was discernable after 3 days of treatment with SCF and was most obvious at 4 and 5 DIV. The microglia maintained in IMDM with SCF added retained the bipolar shape although the cytoplasm shrank slightly compared with those grown in medium conditioned by LM cells. After 3 days of treatment with SCF, about half of the microglia grew out very long, fine processes with a bulb-like formation at or near the end of the processes (Fig. 12). These processes usually did not branch and most microglia had only one or two such processes. This phenomenon was rarely observed in the control microglia.
Fig. 12  Effect of SCF on microglial survival and morphology. When microglia were maintained in serum-free IMDM for 7 days without the addition of growth factors, processes were retracted, cell bodies shrunk, and some microglia died (b). When SCF was added to the culture at a dosage of 100 ng/ml every other day, most microglia retained bipolar morphology with very long fine processes (a). Bar = 50 μm.
Fig. 13 Effect of SCF on the uptake of $[^3H]$ thymidine by microglia in culture. Microglia were maintained in a 96-well plate in IMDM with 0.5% horse serum, and treated with the growth factors as indicated for 48 hours, followed by the addition of 0.5 µCi of $[^3H]$ thymidine to each well for another 4 hours. The samples were triplicated, and the radioactivity was expressed as mean (cpm) ± standard error. Treatment of the microglia with SCF at dosages of 1, 10, and 100 ng/ml did not affect $[^3H]$ thymidine uptake as compared with the control group, i.e., the culture without the addition of growth factors. CSF-1, at a dosage of 1000 U/ml, increased the $[^3H]$ thymidine uptake by a factor of 4. Addition of SCF together with CSF-1 decreased the $[^3H]$ thymidine uptake as compared with the group treated with CSF-1 alone.
4.3.2 Effect of SCF on microglia proliferation

The proliferation of microglia was measured by [³H] thymidine incorporation. CSF-1, a known proliferative stimulant of microglia, was used as a positive control. CSF-1 (Sigma M-9667), at a dosage of 1000 IU (international unit)/ml, increased [³H] thymidine incorporation by microglia by a factor of 4. Addition of SCF from 1 ng/ml to 100 ng/ml, however, did not affect [³H] thymidine uptake by microglia. When SCF was added to the cultures together with CSF-1, SCF suppressed, in a dose-dependent manner, the increment of [³H] thymidine uptake stimulated by CSF-1 (Fig. 13).

4.3.3 Effect of SCF on gene expression by microglia

To compare cytokine mRNA expression in microglia affected by SCF and other cytokines, the time course of mRNA expression after the treatment with SCF was assessed. As exemplified by the mRNA expression of CNTF and BDNF after treatment with SCF (Fig. 14) and SCF mRNA expression after treatment with IFN-γ (Fig. 11), the peak of mRNA expression in microglia was induced between 12 and 24 hours after treatment with SCF and other cytokines. Thus the effect of SCF on gene expression in microglia described below was investigated after 12 to 18 hours of cytokine treatment.

The proto-oncogene, c-fms, encodes the receptor for CSF-1. Microglia maintained in serum-free medium expressed a low level of c-fms mRNA of 4.5 kb in size. Treatment of the microglia with 1000 IU (international unit)/ml CSF-1 for 12 hours upregulated c-fms mRNA. Addition of SCF (100 ng/ml) to microglial cultures did not affect c-fms mRNA expression. Addition of SCF together with CSF-1 to cultures inhibited the upregulation of c-fms expression (Fig. 15).
Fig. 14  Time course of BDNF and CNTF mRNA expression in microglia in culture. Lane 1 (0 hr), lane 2 (1 hr), lane 3 (6 hr), lane 4 (12 hr), and lane 5 (24 hr), after treatment with SCF at 100 ng/ml. Both BDNF and CNTF mRNAs were detected after 6 hours of treatment with SCF and peaked between 12 and 24 hours. The rRNAs were stained with ethidium bromide to show the relative amount of total RNA that was loaded in each sample.
Fig. 15 Effect of SCF and CSF-1 on the expression of c-fms mRNA in microglia, detected by Northern blot analysis. Microglia maintained in serum-free IMDM express detectable 4.5 kb c-fms mRNA (lane 1). Treatment of the microglia with CSF-1, at a dosage of 1000 U/ml, for 12 hours increases c-fms mRNA (lane 3). SCF (100 ng/ml), when added together with CSF-1 (lane 4), decreases c-fms mRNA as compared with the CSF-1-treated group but SCF by itself does not affect c-fms mRNA expression (lane 2). The ethidium bromide stained rRNAs are shown to demonstrate the relative amount of total RNA in each sample.
Interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) are representative of the main inflammatory cytokines produced by stimulated microglia. Microglia maintained in vitro expressed low levels of TNF-α and IL-1β mRNAs. Stimulation of microglia with either LPS or IFN-γ substantially elevated both TNF-α and IL-1β mRNAs. Recombinant SCF itself did not affect, or slightly downregulated, either TNF-α or IL-1β mRNA expression. However, SCF antagonized IFN-γ’s effect in the induction of TNF-α and IL-1β mRNA, especially the latter, whereas it did not affect the effect of LPS' in the induction of TNF-α and IL-1β mRNA expression. The induction of IL-1β mRNA by IFN-γ was inhibited by SCF by a factor of 2. CSF-1 did not change the expression of IL-1β and TNF-α mRNA expression. Addition of SCF together with CSF-1 only slightly increased TNF-α mRNA expression (Fig. 16 and 17).

In contrast to IL-1β and TNF-α, lipocortin-1 (LC-1) is an anti-inflammatory cytokine and is also expressed by microglia (McKanna, 1993). Western blot analysis demonstrated that cultured microglia expressed the 38 kD and a 35 kD LC-1 fragment. Treatment of the microglia with SCF (100 ng/ml) for 24 h increased the 38 kD LC-1 by a factor of 2 while only slightly increasing expression of the 35 kD fragment. Other cytokines such as CSF-1 (800 IU/ml) and IFN-γ (250 U/ml) also increased expression, mainly the 38 kD form of LC-1. In contrast, LPS had a slight effect on expression of either the 38 kD or 35 kD LC-1 fragments. However, when SCF was added to the microglia cultures together with CSF-1, IFN-γ or LPS, both the 38kD and the 35 kD fragment were substantially increased (Fig. 18).

Microglia in culture expressed undetectable or very low levels of the mRNAs of neurotrophins such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF). LPS is the best known stimulant for induction of NGF in microglia (Mallat et
Fig. 16  Effect of SCF and other cytokines on IL-1β mRNA expression in microglia in culture. Microglia were maintained in serum-free medium and Northern blot analysis was done after 12 hours of treatment of the microglia with cytokines. The rRNAs were stained with ethidium bromide to show the relative amount of total RNA in each sample. IL-1β mRNA was undetectable in untreated microglia (lane 1), in the microglia treated with SCF (100 ng/ml, lane 2), CSF-1 (800 U/ml, lane 3) or CSF-1 plus SCF (lane 4). Both LPS (1 μg/ml, lane 5) and LPS plus SCF (lane 6) potently increased 1.9 kb IL-1β mRNA similarly. IFN-γ (250 U/ml) stimulated IL-1β mRNA (lane 7) which was decreased by the addition of SCF together with IFN-γ (lane 8), based on the slightly larger amount of total RNA in lane 8 than in lane 7 as measured by densitometry.
Fig. 17  Effect of SCF and other cytokines on TNFα mRNA expression in microglia in culture. Microglia were maintained in serum-free medium and Northern blot analysis was done after 12 hours of treatment of the microglia with cytokines. The rRNAs were stained with ethidium bromide to show the relative amount of total RNA in each sample. Microglia expressed detectable 1.8 kb TNFα mRNA in untreated microglia (lane 1). Microglia treated with SCF (100 ng/ml) had undetectable TNFα mRNA (lane 2). CSF-1 at 800 U/ml (lane 3) or CSF-1 plus SCF (lane 4) did not affect TNFα mRNA. Both LPS (1 μg/ml, lane5) and IFN-γ (250 U/ml, lane 7) potently increased TNFα mRNA. The TNFα mRNA stimulated by LPS was not affected by the addition of SCF together with LPS (lane 6) as compared to the larger amount of total RNA in lane 6 than in lane 5, but the TNFα mRNA stimulated by IFN-γ was decreased by the addition of SCF together with IFN-γ (lane 8) based on the relative amount of total RNA in lane 7 and lane 8.
Fig. 18 Effect of SCF and other cytokines on the expression of lipocortin-1 (LC-1) in microglia in culture. Microglia were maintained in mMEM supplemented with 0.5% horse serum. The protein was extracted and quantified after 28 hours of cytokine treatment. The protein was extracted, quantified and electrophoresed in a 10% SDS-acrylamide gel. Untreated microglia expressed a major 38 kD and a minor 35 kD LC-1 protein (lane 1). SCF (100 ng/ml, lane 2), CSF-1 (800 U/ml, lane 3), and IFN-γ (250 U/ml, lane 7) increased the LC-1, mainly the 38 kD fragment, in microglia. LPS (1 μg/ml) alone had no effect (lane 5). Addition of SCF together with CSF-1 (lane 4), LPS (lane 6) and IFN-γ (lane 8) increased both the 38 kD and the 35 kD LC-1 proteins, especially the latter one.
al., 1989). I demonstrated here that LPS induced both NGF and BDNF mRNA expression by microglia. SCF itself moderately induced NGF and BDNF mRNAs and exhibited an additive effect with LPS in the induction of NGF and BDNF mRNAs. The expression of BDNF mRNA induced by LPS was increased by 70% when SCF was added. CSF-1 or INF-γ did not induce the expression of either NGF or BDNF. Interestingly, addition of CSF-1 or INF-γ together with SCF to microglia eliminated the expression of NGF and BDNF mRNAs induced by SCF (Fig. 19 and 20).

In addition, SCF was very potent in inducing the mRNA expression of ciliary neurotrophic factor (CNTF) (Fig. 14 and 23). However, SCF itself did not affect the expression of other cytokines such as IL-6 and leukemia inhibitory factor (LIF). Although SCF did not affect IFN-γ induced LIF expression, it synergized with IFN-γ in the induction of IL-6 mRNA expression (Fig. 21, 22).

Since SCF moderately induced the expression of such genes as CNTF, NGF and BDNF, anti-c-kit antibody (ACK2, 1µg/ml) was added to the culture together with SCF to verify whether SCF acted on microglia through its cognate receptor. As shown in Fig. 23, the SCF-induced CNTF mRNA expression was inhibited by ACK2 whereas ACK2 itself did not affect CNTF expression.

The microglial gene expression affected by SCF and other cytokines is summarized in Table 4.
Fig. 19 Effect of SCF and other factors on NGF mRNA expression in microglia in culture. Microglia were maintained in serum-free medium and treated with factors for 18 hours before RNA extraction and Northern blot analysis. Ethidium bromide-stained rRNAs were shown to indicate the relative amount of total RNA in each sample. Untreated microglia had barely detectable 1.3 kb NGF mRNA (lane 1). SCF at 100 ng/ml (lane 2), increased NGF mRNA by a factor of 2, after subtracting the higher density in lane 2 over lane 1. LPS at 1 μg/ml (lane 5) increased NGF mRNA by a factor of 5 as compared with the control (lane 1), CSF-1 at 800 U/ml (lane 3) and IFN-γ at 250 U/ml (lane 7) did not give detectable NGF mRNA expression. SCF potentiated the NGF mRNA expression induced by LPS (by 50%) when SCF and LPS were added to the culture together (lane 6). When SCF was added to the culture together with CSF-1 (lane 4) or with IFN-γ (lane 8), no NGF mRNA was detected.
Fig. 20  Effect of SCF and other factors on BDNF mRNA expression in microglia in culture. Microglia were maintained in serum-free medium and treated with the factors indicated for 18 hours before RNA extraction and Northern blot analysis. Ethidium bromide-stained rRNAs were shown to demonstrate the relative amount of total RNA in each sample. Untreated microglia had barely detectable BDNF mRNA (lane 1). BDNF mRNAs (4 kb and 1.5 kb) were increased, by a factor of 3, after treatment with SCF at 100 ng/ml (lane 2) and LPS at 1 μg/ml (lane 5), while no signal was detected after treatment with CSF-1 at 800 U/ml (lane 3) and IFN-γ at 250 U/ml (lane 7). SCF potentiated the BDNF mRNA expression induced by LPS (by 70%) when SCF and LPS were added to the culture together (lane 6). When SCF was added to the culture together with CSF-1 (lane 4) or with IFN-γ (lane 8), no BDNF mRNA was detected.
Fig. 21 Effect of SCF and other factors on IL-6 mRNA expression in microglia in culture. Microglia were maintained in serum-free medium and treated with the factors indicated for 12 hours before RNA extraction and Northern blot analysis. Ethidium bromide-stained ribosomal RNAs were shown to indicate the relative amount of total RNA in each sample. Untreated microglia had undetectable IL-6 mRNA (lane 1). No signal was detected in the samples treated with SCF at 100 ng/ml (lane 2), CSF-1 at 800 U/ml (lane 3), or SCF plus CSF-1 (lane 4). LPS at 1 μg/ml (lane 5) and IFN-γ at 250 U/ml (lane 7) potently induced 1.3 kb IL-6 mRNA. SCF did not affect the IL-6 mRNA expression induced by LPS when SCF and LPS were added to the culture together (lane 6), after subtracting the higher density in lane 5 over lane 6, whereas it potentiated the IL-6 mRNA induced by IFN-γ (by 50%) when SCF was added to the culture together with IFN-γ (lane 8).
Fig. 22  Effect of SCF and other factors on leukemia inhibitory factor (LIF) mRNA expression in microglia in culture. Microglia were maintained in serum-free medium and treated with the factors indicated for 12 hours before RNA extraction and Northern blot analysis. Ethidium bromide-stained rRNAs were shown to indicate the relative amount of total RNA in each sample. No signal was detected in the untreated (lane 1) or the samples treated with SCF at 100 ng/ml (lane 2), CSF-1 at 800 U/ml (lane 3), or SCF plus CSF-1 (lane 4). LPS at 1 μg/ml (lane 5) and IFN-γ at 250 U/ml (lane 7) induced 4.5 kb LIF mRNA. SCF did not affect the LIF mRNA expression induced by LPS or by IFN-γ when SCF was added to the culture together with LPS (lane 6) or with IFN-γ (lane 8).
Fig. 23 Effect of anti-c-kit antibody on the expression of CNTF mRNA in microglia in culture induced by SCF. Microglia were maintained in serum-free medium and treated with the factors indicated for 12 hours before RNA extraction and Northern blot analysis. Ethidium bromide-stained rRNAs were shown to demonstrate the relative amount of total RNA in each sample. Microglia grown in serum-free medium had detectable 1.2 kb CNTF mRNA (lane 1) and SCF (100 ng/ml) increased CNTF mRNA (lane 2). Addition of the anti-c-kit antibody, ACK2, at a dosage of 1 μg/ml, together with SCF decreased the CNTF mRNA (lane 3) whereas ACK2 alone did not affect CNTF mRNA (lane 4).
Table 4. Summary of the microglial mRNAs affected by treatment with SCF and other cytokines

<table>
<thead>
<tr>
<th>mRNA</th>
<th>NGF</th>
<th>BDNF</th>
<th>CNTF</th>
<th>LIF</th>
<th>IL-6</th>
<th>IL-1β</th>
<th>TNFα</th>
<th>c-fms</th>
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<tr>
<td>Control</td>
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<td>±</td>
<td>-</td>
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<td>±</td>
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<td>+</td>
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<tr>
<td>SCF</td>
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<td>+</td>
<td>++</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
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<tr>
<td>CSF-1</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>SCF/CSF-1</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
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<tr>
<td>LPS</td>
<td>++</td>
<td>++</td>
<td>N</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>N</td>
</tr>
<tr>
<td>SCF/LPS</td>
<td>+++</td>
<td>+++</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<td>IFNγ</td>
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<td>SCF/IFNγ</td>
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Note: (-) represents negative; (±) weakly positive; (+) positive; (++) strongly positive; (+++) most strongly positive; (N) "not done".
4.4 Effect of SCF on Neuron-Microglia Interaction

As shown above (4.3.3), SCF affected mRNA expression of a variety of genes in microglia, including some neurotrophins and cytokines. To test whether SCF-treated microglia have any physiological effect on neuronal survival, a neuron-microglia co-culture system was used. In this system, cortical neurons, at a density of 5x10^4 cells/cm^2, and microglia, at a density of 1x10^5 cells/cm^2, were cultured on separate coverslips in the same petri dish. Thus the interaction between neurons and microglia took place by means of soluble factors released into the common culture medium. In this experiment, serum-free medium, IMDM/mMEM (1:1) supplemented with 6 g/L glucose, 1.25 mM glutamine, and 0.1% BSA was used.

The effect of SCF-treated microglia versus untreated microglia on neurons was assessed by comparing the number of neurons that survived and their morphology in each group after 1, 2, and 4 days of co-culturing. After 24 hours of incubation, it was observed that the number of process-bearing neurons was higher in co-culture groups (both SCF treated and non-SCF-treated co-culture groups) than in pure neuronal culture groups. The neurons in co-culture groups also bore longer neurites. When comparing the co-culture groups, i.e., the neuron-microglia co-culture versus neuron-SCF treated microglia co-culture, there was hardly any difference in neuronal numbers at this stage (Fig. 24). However, the growth cones in the SCF treated microglia-neuron co-culture group were fan-shaped. Forty-eight hours after incubation, the SCF treated microglia-neuron co-culture group had approximately 1.4 times as many neurons as in the untreated neuron-microglia co-culture group. This ratio went up to 1.6 at 3 DIV and 4 DIV. Morphologically, neurons in both co-culture groups bore much longer neurites than those
Fig. 24 Effect of factors derived from microglia, under the influence of SCF, on the survival of cerebral cortical neurons. □ indicates the control culture that consists of two coverslips of pure neuronal cultures, □ two neuronal coverslip cultures with the addition of 100 ng/ml SCF, ■ one neuronal coverslip culture and one microglia coverslip culture, and ■ one neuronal coverslip culture and one microglia coverslip culture with the addition of 100 ng/ml of SCF. The neurons with neurites longer than the cell body were counted at 2 DIV and 4 DIV and the values were expressed as mean ± standard error. Asterisks indicate the difference (p<0.001), between the neuron-microglia co-culture group with the addition of SCF and the co-culture group without the addition of SCF.
Fig. 25 Phase-contrast photographs of neurons in culture at different conditions (2 DIV). (a) shows neurons in control cultures (without the presence of microglia), (b) shows the neurons in pure culture with the addition of 100 ng/ml of SCF, (c) shows neurons in neuron-microglia co-culture, and (d) neurons in the neuron-microglia co-culture with the addition of SCF. Bar = 50 μm.
in pure neuronal culture groups. However, more branches, especially at the growth cones, were found in neurons of the SCF treated microglia-neuron co-culture group at 2 DIV (Fig. 25).

Since cortical neurons bear c-kit receptor (4.1.1), I tested whether SCF affected neurons directly. The addition of SCF to pure neuronal cultures resulted in a slight increase (about 10%) in the number of neurons compared with control pure neuronal cultures (Fig. 24). Some neurons, however, bore much longer neurites than those in the control group (Fig. 25). This observation suggested that the effect of SCF on neuronal survival in neuron-microglia co-culture was mediated, at least partly, by microglia.
4.5 Expression of c-kit and SCF in nervous tissues after injury

In culture, microglia express c-kit receptor and its ligand SCF (4.1.3), whereas in normal brain in situ the expression of c-kit receptor in microglia is hardly detectable immunocytochemically, suggesting that the expression of c-kit receptor in microglia in culture may be due to some kind of stimulation of microglia. Therefore, I investigated whether a stab wound in the brain would cause microglia and astroglia to express c-kit receptor and SCF.

Using immunocytochemistry, I found that c-kit immunostaining was seen 24 hours after a stab wound lesion was made. The c-kit receptor expression occurred around the needle track. The intensity of the immunostaining peaked at 4 to 7 days after surgery and then decreased. The c-kit receptor staining was localized mainly in the cell membranes, especially in the processes in cells which, under higher magnification, exhibited irregular cell bodies with branched fine processes. The morphology of these cells gradually changed from the lesion center to the periphery. The cells closer to the lesion center often had thicker processes while those distant from it had thinner ones, a situation parallel to that of the cells which were positive for Mac-1 in the adjacent sections. These observations suggested that the c-kit receptor positive cells were microglia (Fig. 26). To verify whether astrocytes also expressed the c-kit receptor in the lesion area, the DAB labeled sections were further stained with fluorescent conjugated antibody to GFAP. In Fig. 27, the profile of GFAP immunostaining did not correspond to the c-kit staining, suggesting that reactive astrocytes in stab wound lesion did not express c-kit receptor.

SCF was expressed mainly by neurons in normal brain as determined by immunocytochemistry. The change of SCF expression in a cortical stab wound lesion
Fig. 26 Photographs of the brain sections 7 days after stab wound lesion immunostained with anti-c-kit receptor antibody (a), anti-mSCF antibody (b), Mac-1 antibody (c), and anti-GFAP antibody (d). Bar = 50 μm.
Fig. 27 Photographs of brain section 7 days after lesion double immunostained for c-kit receptor and GFAP. Immunostaining for c-kit receptor (a), (b) the enlargement of the inset in (a), and (c) the same area of (b) but immunostained for GFAP. Arrows indicate the microglia stained with c-kit receptor (b) but not GFAP (c). Bar = 50 μm.
was observable 2 days later. The neurons surrounding the stab track stained for SCF more strongly than those distant from the wound, whereas cells in the center of the needle track were negative. The upregulated staining of SCF on neurons peaked at 7 days postsurgery and gradually decreased to normal staining after 2 weeks. In addition to the transient upregulation of SCF staining in neurons, the hypertrophic astrocytes which stained for GFAP, were seen as early as 2 days after surgery. At that time, only a few SCF positive astrocytes, close to the needle track, were found. The strong staining of SCF in astrocytes was seen at 7 to 14 days after surgery and then declined, but even 28 daysofter lesion, the staining of SCF still could be seen (Fig. 28).

Microglia in culture express SCF mRNAs and protein as shown by Northern blot analysis and immunocytochemistry (Fig. 8 and 9), but in situ SCF is expressed mainly by the reactive astrocytes, as revealed by immunocytochemistry (Fig. 28). It is possible that the reactive microglia produce mainly the soluble SCF, which may escape detection by conventional immunocytochemistry. To test this possibility, I conducted in situ hybridization of the brain sections with an SCF RNA probe. I found that a large number of cells surrounding the needle track were stained except for the neurons distant from the lesion (Fig. 29). The expression of SCF mRNA in glial cells was seen as early as 2 days after surgery, peaked at 4 to 7 days after surgery and then declined. The number of SCF mRNA positive glial cells in the lesion area was apparently larger than that of the cells positive for SCF as revealed by immunocytochemistry in similar sections at the same stages after surgery (comparing Fig. 26 with Fig. 29, 30). When the sections were further labeled with fluorescent tagged antibody to GFAP, it was found that only some of the SCF mRNA positive cells in the lesion area were also GFAP positive, suggesting that microglia in this area expressed SCF mRNA. The hybridization signal was regarded as specific to SCF mRNA because the sense probe gave rise to only background staining and
the omission of anti-digoxigenin antibody in the labeling procedure resulted in no staining in the antisense RNA probed sections (Fig. 30).

Although the reactive microglia, characterized by their hypertrophied cell bodies and thickened processes seen in Mac-1 stained preparations, persisted for over 1 month, the expression of c-kit receptor in microglia was mainly seen in the first week after the stab wound. Moreover, although immunocytochemistry did not reveal the expression of SCF in reactive microglia, the fact that they expressed the SCF mRNA, as shown by in situ hybridization, suggests that reactive microglia may produce soluble SCF. Astrogliosis around the wound, characterized by hypertrophied processes and upregulated GFAP staining, was obvious 4 days after lesion; however, the expression of SCF in astrocytes was most obvious 7 days after surgery and persisted as long as 28 days after surgery.
Fig. 28  Photographs of the brain sections 28 days after lesion immunostained with anti-c-kit receptor antibody (a), anti-mSCF antibody, enlargement of the inset shown in upper-right (b), Mac-1 antibody (c), and anti-GFAP antibody (d). Arrows indicate the SCF positive astrocytes. Bar = 50 μm.
Fig. 29  Photographs of brain section 4 days after stab wound lesion, hybridized with antisense (a) SCF RNA probes. This section was further immunostained with anti-GFAP antibody (b). Arrows indicate the SCF mRNA positive cells stained with GFAP and arrowheads indicate the SCF mRNA positive cells in the lesions area but negative for GFAP. Bar = 50 μm.
Fig. 30 Photographs of brain section 7 days after stab wound lesion, hybridized with sense (a) and antisense (b) SCF RNA probes. (c): enlargement of the inset in (b); (d): same area of (c) stained with anti-GFAP antibody. Arrows indicate the SCF mRNA positive cells stained with GFAP and arrowheads indicate the SCF mRNA positive cells in the lesions area but negative for GFAP. Bar = 50 μm.
5. DISCUSSION

Microglia constitute about 20% of the total glial cell population in the brain (Perry and Gordon, 1988). They are ubiquitously distributed in nonoverlapping territories throughout the CNS. Microglia are the first cells to respond to neural insults. For example, microglial reaction, as shown by upregulated B4 isolectin staining, can be detected as early as minutes after transient forebrain ischemia (Morioka et al., 1991). In peripheral nerve lesion models, such as facial nerve axotomy, where the blood-brain barrier is intact, microglial response is demonstrable within 24 hours after axotomy. Microglia proliferate and upregulate cell surface antigens such as complement receptor 3 (CR3), major histocompatibility complex (MHC) I and II, the receptors for colony-stimulating factor-1 (CSF-1) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Kreutzberg and Barron, 1978; Raivich et al., 1991; Streit et al., 1989; Streit and Graeber, 1993). They also protrude cellular processes to ensheathe the injured neuronal cell bodies, a process often referred to as synaptic stripping (Blinzinger and Kreutzberg, 1968). These observations indicate that microglia have a close relationship with neurons and that microglial responses are determined by signals from the environment (e.g., neurons).

The nature of the signaling molecules that mediate neuron-microglia interactions is so far unknown. The fact that reactive microglia upregulate receptor molecules such as GM-CSF and CSF-1 receptor in response to injury suggests that cytokines are involved in the regulation of microglial responses to neuronal injury. The present thesis deals with SCF/c-kit receptor signaling as one such possible signaling pathway.
5.1 Signaling of SCF/c-kit Receptor in the Nervous System in Steady State

5.1.1 Signaling of SCF/c-kit receptor in neuron-neuron interactions

The present immunocytochemical study, summarized in Table 2, reveals a complementary expression of SCF and c-kit receptor in neurons of many parts of the brain such as the cerebellum and olfactory bulb, and a more complex pattern of expression in neurons of other areas of the nervous system, such as the hippocampus and cerebral cortex. The structural arrangement of neurons in the cerebellum is strictly geometric, which helps to identify neuronal types fairly easily. The characteristic pattern of expression, i.e., SCF produced by Purkinje cells and c-kit receptor expressed by basket and stellate cells, suggests that SCF/c-kit receptor is involved in some way in synaptic structure and/or function. Stellate and basket cells are known to make inhibitory synapses with Purkinje cells. Therefore, the signaling flow from the ligand to c-kit receptor would be the opposite of the synaptic transmission through neurotransmitters, which argues against a role of SCF/c-kit receptor signaling in synaptic transmission. SCF and c-kit receptor have been shown to act as adhesion molecules in the interaction between mast cells and fibroblasts. In culture, mouse mast cells have been shown to adhere to COS cells that are transfected with membrane-associated but not the soluble form of SCF (Flanagan et al., 1991). In contrast, the mast cells derived from W mutant mice which do not express c-kit receptor on their surface are markedly impaired in their ability to bind to fibroblasts. If, however, the mast cells are derived from W mutant mice (e.g., W^V/W^V) which express the ectodomain of the c-kit receptor on their surface, they can still bind to fibroblasts (Adachi et al., 1992). Indeed, the present immunocytochemical study demonstrates that the c-kit receptor is localized predominantly in the cell membrane and the
SCF is probably also membrane-associated in neurons because of its patchy staining over both cell bodies and axons. SCF has been postulated to act as a chemoattractant molecule to attract c-kit-expressing neurons or axons in the dorsal region of the neural tube to the ventral target because of their reciprocal ventrodorsal expression pattern in the developing neural tube (Keshet et al., 1990). It is thus possible that the expression of SCF and c-kit receptor at the apposing sides of the synapses in the adult nervous system may play a role in maintaining the synapse structure.

However, in some areas, the expression of SCF and c-kit receptor is somewhat more complicated than the complementary pattern in synaptically related neurons. For example, neurons in the hippocampus express both SCF and c-kit receptor (Fig. 5). This interpretation is based on the fact that a majority of the hippocampal pyramidal neurons express c-kit receptor in one section and SCF in the adjacent section (Fig. 5a and b). The immunocytochemical result is also supported by a recent in situ hybridization study in which hippocampal pyramidal neurons have been shown to express SCF mRNA (Wong and Licinio, 1994). Yet in other areas where neurons do not form synapses, e.g., DRG, neurons express either SCF or c-kit receptor (Fig. 6c and d). Such a cellular distribution of SCF and c-kit receptor suggests an autocrine and/or a paracrine mechanism for SCF action between the neurons that are not necessarily synaptically related.

Although no SCF receptor binding assay has ever been done in neural cells in situ or in culture, some of the immunocytochemical observations indirectly suggest that SCF may actually bind to c-kit receptor and be internalized. Using the SCF antibodies from both sources (i.e., ACK2 purchased from Gibco BRL, and mu-c-kit-M3 provided by Immunex Corp), I have found that stellate cells and Golgi cells in the cerebellum, which are c-kit receptor positive but do not express SCF mRNA are stained with SCF in the perinuclear area (Fig. 2). Such a phenomenon was also observed by Manova et al. (1992)
using the antibody they developed. Using an antibody directed against the extracellular domain of SCF, Manova et al. have demonstrated that the perinuclear staining is attributable to soluble SCF. Because stellate cells and Golgi cells express the c-kit receptor but do not express SCF mRNA, it is assumed that the SCF in these cells is probably internalized after binding to the cell surface c-kit receptor (Manova et al., 1992). Recently, SCF has been shown to support the survival of a subpopulation of small DRG neurons isolated from mice and chicks in culture (Carnahan et al., 1994; Hirata et al., 1993). I have also found that recombinant SCF promotes neurite growth from a group of cerebral cortical neurons in culture (data not shown). Therefore, SCF may act as a trophic factor for c-kit receptor-expressing neurons. The concentrated localization of c-kit receptor in synaptic ends (Fig. 2) and growth cones in culture (Fig. 7) implies that neurons may take up SCF as a retrograde-transported trophic factor, analogous to classic neurotrophins. A recent preliminary report has demonstrated that the regeneration of nociceptive nerve fibers in W and Steel mutant mice is impaired after nerve crush (Lourenssen et al., 1995), suggesting that SCF is a neurotrophin in vivo.

5.1.2 Role of SCF in neuron-glial interactions

The possibility that c-kit receptor is expressed by glial cells was postulated by Motro et al. (1991) based on their in situ hybridization study. They observed that some c-kit mRNA signals were detected in the molecular layer of the cerebral cortex that is relatively free from neurons. In culture, oligodendrocyte progenitor cells have been shown to express c-kit receptor, which is downregulated when the progenitor cells differentiate into mature oligodendrocytes (Ida et al., 1994). I found that microglia and astrocytes in culture express c-kit mRNA and c-kit encoded protein. Therefore, glial cells may be the target of SCF produced by neurons. From this point of view, the in vitro experiments correlate to the in vivo observation in which glial cells in the cerebral cortex,
cerebellum, hypothalamus, and area postrema are found to express c-kit receptor although the specific glia types identified in situ are not easily distinguished.

There is some discrepancy between the in vivo and in vitro observation as to the expression of c-kit receptor in glia, i.e., microglia in culture express a high level of c-kit receptor while microglia in the cerebral cortex are not clearly stained. Such a discrepancy is likely attributable to the regulation of c-kit receptor in glial cells. As mentioned above, the expression of c-kit receptor in oligodendrocyte precursors seems to be developmentally regulated (Ida et al., 1994). In culture, microglia develop from progenitor cells that exist in neopallial cell cultures and proliferate in response to CSF-1 produced by astrocytes (Hao et al., 1990). After being isolated and maintained in medium conditioned by CSF-1-producing LM cells, microglia become bipolar or tripolar in shape. Microglia with such morphology are generally regarded as relatively "quiescent" in comparison with the microglia which are small, round and proliferative (Glenn et al., 1992). Therefore, the strong membrane staining of c-kit receptor in small round microglia versus weak staining in bipolar microglia suggests that the expression of c-kit receptor in microglia relates to their physiologically activated state. In fact, microglia in situ upregulate c-kit receptor expression in response to stab wound (see 5.2.1). Therefore, it seems that the expression of c-kit receptor in microglia in situ and in culture is functionally regulated and in normal brain the expression of c-kit receptor is downregulated. This consideration in turn suggests that the c-kit receptor in microglia may play an important role in microglial response to brain injuries.

Besides the c-kit receptor, its ligand SCF is also expressed by microglia in culture. Furthermore, the expression of SCF mRNA in microglia in culture is subject to the regulation of a number of cytokines (Fig. 11). That c-kit receptor and SCF can be
expressed by the same cells indicates that autocrine signaling can also exist in microglia under certain conditions.

The immunohistochemical observations point to a possible SCF/c-kit receptor signaling between neurons and microglia. In order to prove that the c-kit receptor in microglia is functional, I tested whether recombinant SCF can affect microglia activity. In Fig. 12 and 13, it is shown that SCF supports microglia survival and by itself has no effect on microglia proliferation. SCF is structurally similar to CSF-1, which is a growth factor for microglia. The receptors of SCF and CSF-1, encoded by c-kit and c-fms respectively, are structurally and evolutionally closely related. Both receptors are expressed by microglia. CSF-1 is required for microglia survival in culture (Hao et al., 1990). Withdrawal of CSF-1 from culture medium results in DNA fragmentation and apoptosis of microglia (Gehrmann, 1995). SCF seems also to support microglial survival, probably by suppressing apoptosis. SCF acts similarly in hematopoietic, gametogenic and melanogenic systems (Cáceres-Cortés et al., 1994; Mekori et al., 1993; Yee et al., 1994b). This observation also suggests that microglia can survive in the presence of cytokines other than CSF-1, indicating the redundancy of cytokine effect. It is interesting to note that in op/op mice that lack CSF-1, the number of microglia in the brain is similar to that in the heterozygous or dominant homozygous littermates (Blevins and Fedoroff, 1995). In these mice, SCF is expressed by neurons. It is thus reasonable to speculate that SCF is one of the microglial survival factors, especially in its continual presence in situ, as suggested by the in vitro experiments in which frequent addition of SCF into culture medium is required. In contrast, CSF-1, but not SCF is required for microglial proliferation. This in situ observation corresponds to the effect of SCF and CSF-1 on microglia in culture. In fact, SCF suppresses CSF-1-induced microglial proliferation in culture (Fig. 12). Besides, the morphology of microglia in the presence of SCF is very similar to that seen in low concentration of CSF-1. Microglia under such
conditions are "quiescent-like". From the above comparison between the effects of SCF and CSF-1 on microglia in situ and in culture, together with the fact that a high level of SCF exists in the normal brain, it is reasonable to conclude that one of the effects of SCF is to maintain microglia in a quiescent state, which can be regarded as the result of the SCF produced by neurons in the normal nervous system.

The functions of quiescent ramified microglia in the normal brain in situ are largely unknown (Thomas, 1992). As discussed above, SCF maintains microglia in a "quiescent-like" state in culture and I hypothesize it does so also in situ. If so, what is the signal which emanates from quiescent microglia to neurons? One of the most important findings in the present study is that SCF by itself induces the expression in microglia of neurotrophins or cytokine genes such as NGF, BDNF and CNTF (Fig. 14, 19, 20). To the best of my knowledge, SCF is the only cytokine that is produced within the nervous system that is found to have such an effect on microglia. This would suggest that quiescent microglia in the normal nervous system may play an active role such as trophic support for neuronal survival and/or functions.

Microglia have the capacity to produce both trophic and potentially toxic substances in response to different stimuli. The biological effect of microglia on neurons thus depends on what factors microglia produce. Therefore, I tested the biological effect of SCF-treated microglia on neurons using a co-culturing system (Zhang and Fedoroff, 1996). I have found that SCF potentiates the effect of microglia in support of neuronal survival by as much as 60% at day 3 in co-cultures as compared to the co-culture group without the addition of SCF, whereas SCF by itself has only a slight (10%) supportive effect on neuronal survival when SCF was added to pure neuronal cultures (Fig. 24). Neurons in the presence of SCF-treated microglia seem to have more branches on neurites than control groups (Fig. 25). This suggests that the effect of SCF on neuronal survival
in co-cultures is, at least partly, mediated by released substances from microglia. Our coculture system, however, does not exclude the possibility that SCF induces microglia to produce trophic factors and at the same time synergizes with these factors in promoting neuronal survival. In any case, the consequential effect of microglia in response to SCF stimulation is beneficial to neuronal survival. This conclusion is also supported by other lines of evidence that SCF upregulates the transcripts of NGF, BDNF and CNTF and at the same time cytokines such as IL-1, TNF-α, and IL-6 are unaffected (Fig. 16, 17, 19-22).

5.2 Role of SCF/c-kit Receptor Signaling in the Nervous System After Injury

5.2.1 Expression of SCF/c-kit receptor in microglia in response to brain injury

Penetrating trauma to the cerebral cortex triggers a number of endogenous host defense mechanisms that always leads to inflammation. The earliest inflammatory response in the brain is the response of microglia. The microglial reaction is characterized by proliferation, upregulation of cell surface antigens, and the release the pro-inflammatory cytokines, IL-1, TNFα, and IL-6, which in turn elicit an astroglial response (Gehrmann and Kreutzberg, 1995). The reactions of microglia and astroglia thus determine the inflammatory process and the healing process.

Accompanied by these responses is an upregulated expression of c-kit receptor in the cells surrounding the wound. The c-kit receptor positive cells are identified as reactive microglia because the morphology of the c-kit receptor-expressing cells is strikingly similar to those stained with Mac-1, and because these cells are GFAP negative (Fig. 26, 27). The c-kit receptor staining also displays a gradient pattern with the most intense
staining close to the wound, paralleling that of Mac-1 staining. Temporally, the expression of c-kit receptor in microglia is seen mainly in the first week after injury, corresponding to the reaction of microglia seen after a stab wound (Giulian et al., 1989). That the c-kit receptor in microglia is upregulated in response to injury indicates that there are some, yet unknown, signals released which cause c-kit receptor upregulation.

In culture, the expression of c-kit receptor in microglia is subject to the regulation of a number of cytokines. IL-1 is produced predominantly by reactive microglia during inflammation and IFN-γ is mainly produced by activated T-lymphocytes during immune responses. Both cytokines stimulate microglia to produce inflammatory cytokines, and at the same time stimulate the expression of c-kit receptor. The upregulation of c-kit receptor seems attributable to the increased synthesis of the protein because both the full-length 145 kD c-kit receptor and the 100 kD fragment are increased (Fig. 16). In situ, the expression of c-kit receptor corresponds to the reaction of microglia. It is possible that the expression of c-kit receptor in reactive microglia results from the cytokines produced by reactive glia, especially microglia. The inflammatory cytokines, IL-1, TNFα, and IL-6 produced by reactive microglia stimulate astrocytes. Reactive astrocytes produce cytokines, including the increased production of CSF-1 (Giulian and Ingeman, 1988). CSF-1 potently stimulates microglia in culture to express SCF mRNAs (Fig. 17). It is interesting to note that both CSF-1 and SCF downregulate c-kit receptor expression (Fig. 16). These cytokines may thus attribute to the downregulation of c-kit receptor seen 1 week after stab wound. The downregulation of c-kit receptor in microglia in culture seems to be mainly at the posttranslational level since SCF as well as CSF-1 decreases the full length 145 kD c-kit receptor greatly while it increases the 100 kD fragment (Fig. 16). The 100 kD fragment detected by Western blot analysis has been shown to be equivalent to the extracellular and the transmembrane part of the full length c-kit receptor and to be easily
released from the cell membrane. It also retains the ability to bind SCF with high affinity and may thus act as an antagonist of SCF action (Brizzi et al., 1994b).

The regulation of c-kit receptor expression in microglia by cytokines produced by reactive microglia themselves and astrocytes therefore finely adjusts the responsiveness of microglia to SCF and thus may modulate the reactivity of microglia.

After stab wound, the SCF immunostaining within the needle track disappears. This is probably due to the death of neurons in this area. In contrast, SCF is upregulated in the neurons in the periphery of the wound which may represent the response of neurons to injury. The biological significance of the upregulated neuronal production of SCF to injury is not clear, but it may be one of the mechanisms by which microglial reactivity is modulated (5.2.2). In addition, reactive astrocytes surrounding the wound also express SCF (Fig. 27). Therefore, reactive astrocytes as well as neurons are the sources of SCF after injury. The reciprocal expression of SCF in neurons and reactive astrocytes, and c-kit receptor in reactive microglia suggests a possible paracrine signaling after injury from neurons and astrocytes to microglia through SCF.

Immunocytochemical investigation did not reveal the positive SCF signal in reactive microglia; however, microglia in culture express SCF mRNAs. In situ hybridization experiments indirectly indicated that reactive microglia may express SCF mRNA, because the hybridization signal was located on the edge of the stab wound 4 days after lesion, when astrocytic elements in this area were still minimal. In view of the differential SCF transcripts, and differential immunostaining pattern in neurons and microglia in culture, it is possible that reactive microglia produce soluble SCF. Further investigation is needed to elucidate the isoforms of SCF that reactive microglia produce.
Taken together, injury to the brain triggers the reaction of microglia and astrocytes. Reactive microglia upregulate the expression of c-kit receptor, possibly via the cytokines produced by microglia, astrocytes and neurons. The increased production of SCF by neurons, astrocytes, and possibly also by reactive microglia, may act as a neurotrophic factor, supporting the survival of injured neurons which express the c-kit receptor, in an analogy with a role of SCF on c-kit receptor-expressing DRG neurons (Carnahan et al., 1994; Hirata et al., 1993), and/or supporting axon regeneration. The sustained expression of membrane SCF in astrocytes may support axonal regeneration since in embryonic development, SCF seems to guide neuron or axon growth (Keshet et al., 1990). On the other hand, the SCF produced by neural cells after injury may play a role in regulating microglial reactivity.

5.2.2 Modulation of microglial response to injury by SCF

Brain injury triggers the reaction of microglia and astrocytes and the release of cytokines from these glial cells. One of the cytokines, CSF-1, is released by astrocytes and reactive microglia in vitro (Hao et al., 1990; Suzumura et al., 1990) and is required for the proliferation of microglia in culture (Hao et al., 1990) and in situ (Berezovskaja et al., 1995). CSF-1, on the other hand, potently stimulates the expression of SCF mRNAs by microglia in culture (Fig. 11). The increased production of SCF may in turn inhibit the effect of CSF-1 on microglial proliferation as suggested by the in vitro observation (Fig. 13). The mechanism underlying the inhibition of microglial proliferation by SCF is not clear. It is possibly achieved by inhibiting the expression of c-fms (Fig. 15). Furthermore, both SCF and CSF-1 seem to stimulate the production of LC-1 by microglia in culture and SCF and CSF-1 together have a synergistic effect (Fig. 18). LC-1 is a potent anti-inflammatory cytokine (Flower and Rothwell, 1994) and is expressed by ramified microglia in situ (McKanna, 1993). Both the 38 kD LC-1 and the 35 kD
fragment have a similar anti-inflammatory effect (Flower and Rothwell, 1994). LC-1 is also reported to support neuronal survival in culture although the mechanism whereby this occurs is unknown (Flower and Rothwell, 1994). Thus, the inflammatory cytokines produced by reactive microglia and astroglia, at the same time, elicit an anti-inflammatory response: SCF inhibits microglia proliferation in response to cytokine CSF-1 and the release of proinflammatory cytokines IL-1, TNFα; LC-1 inhibits the synthesis of prostaglandin E2 thus prohibiting the release of inflammatory mediators. In situ, many other cytokines including the TGFβ1 produced by the reactive microglia (Kiefer et al., 1995) are involved in the inflammatory process. The cells in the nervous system are equipped with an extraordinary capacity to regulate anti-inflammatory reactions.

Lipopolysaccaride (LPS) is an endotoxin derived from the cell wall of Gram negative bacteria and has been widely used to stimulate monocytes/macrophages. In the present study, LPS has been shown to potently stimulate the production of inflammatory cytokines IL-1, TNFα, and IL-6 by microglia (Fig. 16, 17, and 21) but not to affect LC-1 production (Fig. 18), indicating that LPS is a potent stimulator of inflammation. Addition of SCF together with LPS did not affect the production of IL-1, IL-6 and TNFα, but increased the production of LC-1, suggesting that SCF, in the case of bacterial infection, modulates inflammation mainly through the production of anti-inflammatory cytokines. Both SCF and LPS induce microglia to express mRNAs of NGF and BDNF and they exhibit an apparent additive effect on microglia in culture (Fig. 19 and 20). Such an additive effect of SCF and LPS in the induction of NGF and BDNF mRNAs contrasts to the effect of IFN-γ which eliminates the effect of SCF in the induction of neurotrophin mRNAs when they are both present (Fig. 19 and 20). Both LPS and IFN-γ are potent stimulators of the production of inflammatory cytokines IL-1, TNFα and IL-6 by microglia. Since LPS downregulates c-kit receptor expression in microglia while IFN-γ potently stimulates the expression of c-kit receptor as well as SCF mRNA expression
(Fig. 10 and 11), the above observations appear paradoxical. Such a paradox is probably due to the activation of different elements of the signal transduction pathways utilized by microglia treated with SCF/LPS and SCF/IFN-γ, or the different signal transduction pathways utilized by c-kit receptor in microglia when there are different numbers of active c-kit receptors available on the microglial cell surface and/or a different level or isoform of SCF present in the microglial surroundings. The latter mechanism has been postulated for many cytokines based on a series of in vitro experiments (see review Marshall, 1995). For example, NGF elicits either differentiation or proliferation of PC-12 cells depending on the number of trkA receptors available on their surface (Schlessinger and Bar-Sagi, 1995). As discussed above (2.1.4), activation of c-kit receptor leads to proliferation, migration, adhesion, or mediator release of mast cells, depending on the specific signal transduction pathways used, and different isoforms of SCF (soluble versus membrane-bound) result in different biological consequences even though they use the same c-kit receptor. In the present study, IFN-γ, in addition to upregulating the expression of c-kit receptor, stimulates SCF mRNA expression, mainly the 6.5 kb transcript. Microglia in the relatively quiescent state express predominantly the 3.5 kb and 4.5 kb SCF mRNAs. Furthermore, following prolonged treatment with IFN-γ (12 to 24 hours), microglia express another SCF mRNA that is larger than 6.0 kb and is similar to that observed in neurons (Fig. 11), suggesting that microglia may produce membrane-associated SCF and intracrine action of SCF may occur in microglia when they are stimulated by IFN-γ, which may result in different biological consequences.

Although the mechanism underlying the interaction of SCF and LPS or SCF and IFN-γ is not known, the biological consequence may differ. In the case of infection with microorganisms, microglia are the first and the major cellular components in the CNS to be stimulated to remove foreign invaders by phagocytosis in order to restore the CNS microenvironment. To do this, certain humoral factors, probably mainly produced by
reactive microglia themselves, such as IL-1, TNFα, and IL-6, are required. At the same time, reactive microglia produce trophic factors, such as NGF and BDNF, to support the survival and/or function of neurons in the "temporarily" disturbed CNS environment. From this point of view, the effect of SCF, which is produced by the neighbouring neurons and/or reactive microglia themselves, seems to fit the requirement of microglia to attack the microorganism and to support neuronal survival at the same time. This is the hypothetical view based on in vitro observations, but the different behaviours of microglia stimulated by either LPS or IFN-γ, as judged by their production of inflammatory cytokines and neurotrophins, points to the simple fact that the reaction of microglia, though universal to all kinds of insults to the brain, may have different biological consequences to the CNS, depending upon the presence of specific factors (or cytokines) in individual pathophysiological conditions. Accordingly, we may intervene in the inflammatory and wound healing processes with a better understanding of the regulation of microglial reactivity.

Microglia respond to virtually all neurological insults, from trauma, infection, to acute or chronic diseases (Thomas, 1992). The reaction of microglia, through production of cytokines and phagocytosis, is required to stimulate tissue repair and wound healing, since ischemic lesion to the cerebral cortex of the op/op mice that are deficient in microglia reaction because of the lack of CSF-1 causes extensive neuronal death whereas restoration of microglial reaction by exogenously providing CSF-1 substantially rescues neurons from death (Berezovskaya et al., 1995). Nevertheless, overproduction of cytokines or inflammatory mediators by the reactive microglia, especially in the persistent presence of immunogenic stimulus, e.g., antigen presentation, may lead to chronic inflammatory diseases in which T lymphocytes may become activated.
IFN-γ is a cytokine that is produced predominantly by T lymphocytes during an immune reaction (Farrar and Schreiber, 1993). It has been shown to inhibit microglial proliferation, stimulate the expression of MHC molecules and the production of inflammatory cytokines (Chao et al., 1992; Frei et al., 1987; Meda et al., 1995; Suzumura et al., 1987; Yoon et al., 1994). In the present study, IFN-γ stimulated the expression of IL-1, TNFα, and IL-6 mRNAs, greater than LPS, at least at the transcription level (Fig. 16, 17, 21). Interestingly, SCF, apparently, though only partially, inhibits expression of IL-1 and TNFα mRNAs induced by IFN-γ. Since a large amount of TNFα has been shown to be cytotoxic to oligodendrocytes in culture (Perez et al., 1990; Selmaj et al., 1991), the overproduction of TNFα by reactive microglia has been proposed as being involved in the progression of demyelination as in experimental allergic encephalomyelitis (EAE) and multiple sclerosis in humans (Owens et al., 1994; Williams et al., 1994). IL-1 and TNFα are also stimulators of microglial reactivity. Thus, the inhibition by SCF of the production of IL-1 and TNFα by microglia may act as a feedback mechanism to suppress inflammation elicited by IFN-γ. As mentioned above, IFN-γ upregulates the expression of SCF mRNA and c-kit receptor in microglia, and this may further strengthen the anti-inflammatory action of SCF.

IL-6 is often regarded as an inflammatory cytokine; it has pleiotropic effects in the CNS. It has a mitogenic effect on astrocytes (Selmaj et al., 1990) and stimulates astrocytes to produce NGF (Frei et al., 1989). IL-6 is also regarded as a neuroproietic cytokine as it can induce PC12 cell differentiation (Satoh et al., 1988). The effect of IL-6 on microglia is not clear. However, IL-6 has been shown to suppress TNFα production by activated monocytes (Aderka et al., 1989). It is tempting therefore to speculate that the potentiation of IL-6 production by SCF (Fig. 21) may provide another feedback mechanism to suppress the inflammatory response.
5.3 SCF/c-kit Receptor as Part of the Cytokine Network in the Regulation of Neuron-Microglia Interactions

Microglia are part of the neural cell network. The ubiquitous and non-overlapping distribution in the brain parenchyma and the presence of various receptor molecules in microglia enable them to respond to diverse signals from other cells and the extracellular matrix. They also have the capacity to produce a profile of bioactive molecules including cytokines in response to stimuli. Therefore, microglia are the regulators of homeostasis and the effector cells in pathological conditions in the nervous system. Because of these characteristics, the activity of microglia needs to be scrupulously regulated. Cytokines are among the known molecules that take part in the regulation of microglial activity.

The present study indicates that SCF may be employed by neurons to signal microglia because of the reciprocal expression of SCF and c-kit receptor in neurons and microglia. In vitro experiments using recombinant SCF indicate that SCF maintains microglia in a "quiescent-like" state and induces them to express mRNAs of NGF, BDNF, CNTF, and possibly other trophic factors as suggested by the neuron-microglia co-culture experiment. In normal brain, SCF is produced predominantly by neurons, as shown by the immunocytochemistry study. I thus hypothesize that in the homeostatic nervous system, neurons produce SCF which maintains microglia in a quiescent state. The resting microglia, in response to SCF, produce trophic factors which support neuron survival and/or function (Fig 31A). This hypothesis indicates that resting microglia play an active role in the normal nervous system.

In the case of injury, microglia are stimulated. The reaction of microglia is accompanied by the increased production of inflammatory cytokines, IL-1 and TNF-α, which potently stimulate astrocytes to produce trophic factors such as NGF and BDNF,
supporting the survival of injured neurons. Reactive astrocytes also increase the production of other cytokines such as CSF-1, which stimulate microglial proliferation. CSF-1, at the same time, stimulates microglia to produce SCF which in turn suppresses microglial proliferation. Thus, CSF-1 and SCF produced by the astrocytes and reactive microglia form a feedback cytokine loop to control microglial proliferation. Furthermore, SCF produced by the reactive glia potentiates the production of other anti-inflammatory cytokines such as LC-1 by reactive microglia, perhaps through the action of the inflammatory cytokines, as suggested by the in vitro observations, which downregulate microglial reactivity. In addition, the c-kit receptor appearing in the reactive microglia is also tightly regulated by the above-mentioned cytokines at multiple levels. Together with the possible different isoforms of SCF produced in different pathological conditions, the responsiveness of microglia to SCF is dynamically tuned until a new balance of cytokine effect is reached. Taking together the in vivo and in vitro observations made in this study, I hypothesize that in response to injury, SCF produced by reactive microglia and astrocytes, as well as by neurons, modulates the inflammatory process by regulating the proliferation and production of inflammatory and anti-inflammatory cytokines (Fig. 31B). This hypothesis suggests that we may intervene in the inflammatory and healing processes by modulating SCF/c-kit receptor signaling.
Fig. 31 Schematic representations showing the hypothetical role of SCF in neuron-glial interactions in steady state (A) and under pathological conditions (B). Under normal conditions, neurons produce SCF which keeps microglia in a quiescent state and the resting microglia produce trophic factors supporting neuronal survival and function (A). In case of injury, microglia are stimulated, upregulate c-fms, and increase the production of IL-1 and TNFα which stimulate astrocyte proliferation and production of trophic factors. Reactive astrocytes increase the production of CSF-1 which stimulates microglial proliferation. Reactive microglia, as well as reactive astrocytes produce SCF which suppresses microglial reactivity through the decreased production of inflammatory cytokines IL-1 and TNFα and the increased production of anti-inflammatory cytokines such as LC-1 (B). NTs: neurotrophins; trks: trk receptors; IL-1R: IL-1 receptor; IL-6R: IL-6 receptor; TNFR: TNF receptor; (+): potentiation; and (-): inhibition.
5.4 Scientific Contributions and Future Directions

This study has revealed the cellular localization of stem cell factor and its receptor encoded by c-kit in the nervous system. The characteristic distribution of SCF and c-kit receptor in the nervous system reported in this thesis leads to the hypothetical role of SCF in neuron-neuron and neuron-gliala interactions. Special attention has been paid to the possible role of SCF in neuron-microglia interaction because the expression of c-kit receptor and SCF in microglia is regulated in response to brain injury in situ and in response to cytokine treatment in culture. The effect of SCF on microglia in culture, including survival, morphology, proliferation, and gene expression, strongly suggests that SCF maintains microglia in a quiescent state, and modulates the inflammatory process when microglia are stimulated, as in the case of injury.

The hypothesis is made mainly based on the in vitro evidence. Direct evidence of a role of SCF on microglia in situ is still lacking. This lag of research on the role of SCF in the nervous system is hindered by the lack of viable homozygous W or Sl mutants in adulthood and the lack of transgenic mice that are phenotypically comparable to the natural mutants. One approach that has been adopted is to look into the signaling of SCF/c-kit receptor in wild animals, i.e., by using neutralizing antibody to block the function of SCF/c-kit receptor signaling. Nishigawa et al. (1991) successfully induced nonpigmented mice by introducing blocking anti-c-kit antibody to pregnant mice. As for the CNS, a new delivery system has been developed, i.e., encapsulated protein as reported by Berezovskaya et al. (1995). By delivering SCF or neutralizing anti-SCF or anti-c-kit receptor antibodies into the brain under certain circumstances, one may gain insight into the role of SCF/c-kit receptor signaling in the nervous system in situ.

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In the present study, the effect of SCF on neurons and microglia was investigated using (soluble) recombinant SCF. SCF exists in at least two forms, soluble and membrane-associated forms (Flanagan and Leder, 1990; Huang et al., 1992). Both forms are active but with different biological functions. By delivering the genes encoding the specific isoforms of SCF into neural cells (possibly isolated from Steel mutant mice), it is hoped to determine the role of membrane-associated SCF in neural cell interactions.

This study has also generated a series of questions about the mechanisms underlying the actions of SCF in neural cells. Of special interest is the interaction between SCF and other cytokines in the regulation of microglial activity. As mentioned above, SCF induced microglia to express neurotrophin mRNAs but this effect was inhibited by CSF-1 and INF-γ. IFN-γ stimulated microglia to express inflammatory cytokines IL-1, TNFα and IL-6 but SCF suppressed IL-1 and TNFα expression induced by IFN-γ and potentiated the IFN-γ-stimulated IL-6 expression. Such cytokine interactions may be due to the signal transduction pathways used by the cells from the receptor level to gene transcription. Study in the field of cytokine signal transduction is very active recently and novel signal transduction pathways such as the JAK-STAT pathway (Larner and Finbloom, 1995) have been discovered. A better definition of cytokine signal transduction pathways in neural cells and more importantly, of biological responses regulated by the specific pathways, will certainly help our understanding of the mechanisms underlying the cytokine regulation of neural cell activity.
6. APPENDICES

6.1 Media for Cell Cultures

Preparation of media for cell cultures

Powdered media, modified Eagle's Minimum Essential Medium (mMEM), Dulbecco's Modified Eagle Medium (DMEM, #23800), and Iscove's Modified Dulbecco's Medium (IMDM, #12200), were purchased from Gibco BRL and dissolved in triple distilled water, filtered through a 0.2 µm Millipore filter and stored at 4°C in aliquots. Before using the media, 2.6 ml of 1.0 M sodium bicarbonate (NaHCO₃) was added to every 100 ml of media and the pH was adjusted to 7.2 by gasing with 5% CO₂. For mMEM and DMEM without glutamine, 1.25 ml of 0.2 M glutamine was added to each 100 ml of media. For neuronal cultures, 0.625 ml of 0.2 M glutamine was added.

Nigrosin (0.3%)

Nigrosin dye (0.75 gm, Sigma N4763) was dissolved in 250 ml of 0.85% saline. The solution was filtered through Whatman #1 paper, autoclaved and stored at 4°C.

Puck's Solution

The following components were dissolved in a total volume of 1 liter triple distilled water:
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>8 gm</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.06 gm</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.$7$H$_2$O</td>
<td>0.09 gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4 gm</td>
</tr>
<tr>
<td>phenol red (1%)</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

The solution was filtered through a 0.2 $\mu$m Millipore filter and stored at 4 °C in aliquots.

**Trypsin (1%)**

Trypsin (1 gm, Difco 1:250 #0152-15-9) was dissolved in 100 ml of Puck's solution. The solution was filtered through a 5.0 $\mu$m and a 1.2 $\mu$m filter, stacked, and a 0.45 $\mu$m filter followed by a sterile 0.2 $\mu$m filter. The solution was stored at -20 °C in aliquots.

**Preparation of poly-L-lysine coated coverslips**

The poly-L-lysine (2.5 mg, Sigma, P-9155) was dissolved in 10 ml of sterile triple distilled water, and stored at -20 °C as a stock solution (250 $\mu$g/ml). To coat the coverslips, 100 $\mu$l working solution (25 $\mu$g/ml) was spread over the 15x15mm coverslips, and the coverslips were incubated at 37 °C for 1 h to overnight. The coverslips were then washed with distilled water three times and dried in a laminar flow hood. Before being used for culture, the coverslips were incubated in the culture medium (same as that used for culturing next day) at 37 °C overnight.
6.2 Histochemical Solutions

Dulbecco’s Phosphate Buffered Saline (0.1M, pH 7.4)

The following ingredients were dissolved in 500 ml triple distilled water:

\[
\begin{align*}
KCl & \quad 0.2 \text{ gm} \\
K_2HPO_4 & \quad 0.2 \text{ gm} \\
NaCl & \quad 8.0 \text{ gm} \\
Na_2HPO_4 & \quad 1.15 \text{ gm}
\end{align*}
\]

CaCl$_2$ (0.1 gm) was dissolved in 250 ml distilled water and 0.1 gm of MgCl$_2$.6H$_2$O in 250 ml of distilled water separately.

The above three solutions were mixed by gently pouring the CaCl$_2$ and MgCl$_2$ solutions into the remaining solution and the DPBS was stored at 4 °C.

Sucrose (25%)

Sucrose (12.5 gm) was dissolved in 50 ml DPBS.

Paraformaldehyde (4%)

Powdered paraformaldehyde (8.0 gm) was dissolved in 100 ml of triple distilled water by heating to 60–80 °C in a fume hood. Sodium hydroxide (1.0 N) was added dropwise to clear the solution. After the solution was cooled to room temperature, it was filtered through Whatman #1 paper, mixed with 100 ml 2x DPBS and stored at 4°C.
6.3 Molecular Biological Stock Solutions and Protocols

Agarose gel (1%)

(1) Non-denaturing agarose gel

Agarose (Promega, V-3121), 0.5 gm, was dissolved in 50 ml of 0.5x TBE in an erlenmeyer flask by heating in a microwave oven. The flask was weighed before and after microwave boiling and after cooling down it was brought up to the same weight as before boiling by adding TBE. One µl of 10% Ethidium bromide (Sigma, E-1510) was added to the solution and mixed well before pouring into the gel apparatus.

(2) Denaturing agarose gel

Agarose (0.6 gm) was dissolved in 50 ml of MilliQ water in the same way as for non-denaturing gel. After cooling down to 65 °C, 6 ml of 10x MOPS and 5 ml formaldehyde (Sigma, 37%) were added in the fume hood. The solution was mixed well and poured into the gel apparatus.

Ammonium acetate (10M)

NH₄Ac, 77 gm, was dissolved in 100 ml DEPC water.

Bromophenol blue (10%)

Bromophenol blue (Bio-Rad, M-2324), 1 gm, was dissolved in 10 ml DEPC-treated water.
**Denhardt's (50x)**

Five gm Ficoll (Sigma, F-2637), 5 gm polyvinylpyrrolidone (MWt 360,000, Sigma P-5288) and 5 gm bovine serum albumin (BSA, fraction V, Sigma A-7888) were dissolved in 500 ml Milli Q water. The solution was stored in aliquots at -20°C.

**DEPC treated water**

One ml Diethylpyrocarbonate (Sigma, D-5758) was added to 1000 ml of Milli Q water in a fume hood. The flask was covered and shaken vigorously. The solution was stirred using a magnetic stirrer overnight and autoclaved on liquid cycle for 15 min.

**EDTA (0.5 M, pH8.0)**

EDTA (disodium, dihydrate, Sigma E-5134), 18.6 gm, was added to 80 ml of DEPC-water and stirred vigorously. The pH of the solution was adjusted to 8.0 by adding NaOH pellets (about 20 gm) and the volume was brought to 100 ml by adding H₂O.

**High salt NET buffer**

- NaCl 1 M
- EDTA (pH 8.0) 0.1 mM
- Tris-HCl (pH 8.0) 20 mM
LB medium

Luria broth base (Sigma L-3522), 25 gm, was dissolved in 1 liter triple-distilled water and autoclaved in a liquid cycle for 15 min.

MOPS (3-(N-morpholino)-propanesulfonic acid) buffer (10x)

MOPS, 20.93 gm, NaAc, 2.05 gm, and EDTA (free acid, BDH, ACS 342), 1.46 gm, were dissolved in 300 ml DEPC water and the pH of the solution was adjusted to 7.0 with (~2 gm) NaOH and the final volume was brought to 500 ml.

NET buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.15 M</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Tris-HCl (pH 8.0)</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

PIPS (Piperazine-N,N'-bis[2-ethanesulfonic acid]) 20x

NaCl, 17.53 gm, Pipes (Sigma P-9291), 3.62 gm, EDTA (free acid), 2.92 gm, were dissolved in 100 ml DEPC water, and the pH to 6.8 with NaOH (~1.6 gm). The solution stored at 4 °C.

Salmon sperm DNA

The salmon sperm DNA (Sigma, D-1626) was dissolved in Milli Q water at a concentration of 10 mg/ml. The solution was stirred for 2 to 4 hours at room temperature.
to help to dissolve the DNA. The DNA was sheared by passing it several times through an 18-gauge hypodermic needle. The DNA was boiled for 10 min and stored at -20°C in small aliquots.

**SDS (10% and 20%)**

SDS (sodium dodecyl sulfate, Bio-Rad M3356), 10 and 20 gm, was dissolved in a final volume of 100 ml Milli Q water.

**Sodium acetate (3M, pH 5.2)**

NaOAc.3H₂O, 40.81 gm, was dissolved in DEPC water, the pH was adjusted to 5.2 with glacial acetic acid and the volume was brought to 100 ml. The solution was stored at room temperature in aliquots.

**SSC (20x)**

NaCl, 701.28 gm, and sodium citrate (C₆H₅Na₃O₇.2H₂O, Sigma, S-4641), 402.42 gm, were dissolved in Milli Q water, the pH was adjusted to 7.0 and the volume was brought to 4 liters.

**STET**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Tris-HCl (pH8.0)</td>
<td>10 mM</td>
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<tr>
<td>EDTA (pH 8.0)</td>
<td>1 mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>5%</td>
</tr>
</tbody>
</table>
**Tris-HCl (1 M, pH 7.4, 7.6, 8.0)**

Trisma base (Sigma, T-8524), 12.1 gm, was dissolved in 80 ml DEPC water, the pH was adjusted to either 7.4, 7.6, or 8.0 by adding approximately 7, 6, and 4.2 ml HCl, respectively, and the volume was brought to 100 ml.

**TBE (1x)**

Tris base, 12.10 gm, boric acid, 6.17 gm, and 4 ml of 0.5M EDTA were dissolved and brought to 2 liters with Milli Q water.

**TE (pH 7.6)**

100 µl of 1M Tris-HCl and 20 µl of 0.5M EDTA were added to DEPC water in a final volume of 10ml.

**Preabsorption of anti-digoxigenin antibody**

The alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim) was diluted in PBT at 1:100 and incubated with 6 mg/ml of mouse brain powder (Rockland, Gilbertsville, PA) at 4 ° overnight. The absorbed antibody was recovered by centrifugation and stored at 4 °C.

**Total RNA extraction from cultured cells**

The cells in culture were rinsed with PBS prepared in DEPC water and homogenized in 1 ml of Trizol reagent (Gibco BRL). The homogenate was allowed to sit
at room temperature for 5 min, then 200 µl of phenol/chloroform was added and the microtube was capped tightly, shaken vigorously and incubated at room temperature for 5 min. The mixture was centrifuged at 12,000g at 4°C for 15 min and the transparent supernatant was transferred to a new tube. 150 µl of isopropanol was added to the supernatant, mixed well, incubated at room temperature for 10 min and then centrifuged at 12,000 for 10 min at 4°C. The RNA pellet was washed by adding 1 ml of 70% ethanol and centrifuging at 7,500 g for 5 min at 4°C. The supernatant was removed and the pellet was dried in air and finally dissolved in 10 to 20 µl of TE and stored at -20°C.

**Amplification of plasmid DNAs**

The plasmids which contained specific DNAs were amplified in Subcloning Efficiency DH5α™ Competent Cells purchased from Gibco BRL (8265SA) according to the following protocol.

The competent cells aliquoted as 50 µl per tube were removed from the -70°C freezer and thawed on wet ice. The plasmid DNA (5-10 ng in 3-5 µl) was added to the competent cells by moving the pipette through the cells while dispensing. After incubating the cells on ice for 30 min, the cells were heat shocked for 20 seconds by placing the tube into a 37°C water bath, then placing the tube on ice for 2 min with an opened lid. Then 0.95 ml of LB was added and the tube was shaken at 225 rpm for 1 hour at 37°C for expression of the plasmid in bacterial cells. After expression, the cell suspension was diluted 1:10 and 100 µl of the diluted suspension was spread on an LB plate with 100 µg/ml ampicillin added. The plate was incubated overnight at 37°C.

When the bacterial colonies formed, a single bacterial colony was transferred into 5 ml of LB medium containing 50 µg/ml ampicillin, which was incubated overnight at
37°C with vigorous shaking. The bacteria were then used to extract plasmids according to the method (small-scale preparations of plasmid DNA) described by Sambrook et al (1989).

**Preparation of plasmid DNA (small-scale)**

The bacterial culture was poured into a microtube and the tube was centrifuged at 12,000g for 30 sec at 4°C. The medium was removed by aspiration and the bacterial pellet was left to dry as much as possible. The pellet was resuspended in 350 µl of STET. Then 25 µl of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0) was added and the tube was vortexed for 3 sec. The tube was then placed in a boiling-water bath for exactly 40 sec, followed by centrifugation at 12,000g for 10 min at room temperature. The pellet of bacterial debris was removed with a sterile toothpick. To the supernatant was added 30 µl of 3.3 M sodium acetate (pH5.2) and 420 µl of isopropanol. The solution was mixed well and stored at room temperature for 5 min. The pellet of nucleic acids was recovered by centrifugation at 12,000g for 5 min at 4°C. The supernatant was removed and the pellet was washed by adding 1 ml of 70% ethanol and recentrifuging at 12,000g for 2 min at 4°C. The supernatant was removed and the pellet dried. The nucleic acids were finally dissolved in 50 µl of DEPC water.

**Isolation of DNA fragment from agarose gel**

The plasmid DNA was incubated with specific restriction enzymes in a relevant buffer(s) for 1 hour at 37°C. The reaction was stopped by adding 1 µl of EDTA and the solution was electrophoresed in 1% non-denaturing agarose gel. Under UV illumination, using a clean razor blade, a cut was made on the gel just ahead of the desired band, a strip of NA-45 DEAE membrane (Schleicher & Schuell, #23290) was put in place and
electrophoresis was resumed. When a clear fluorescence band was observed on the membrane under UV light, the membrane was put immediately into 10 ml NET buffer and shaken thoroughly to get rid of the excess gel. The membrane was transferred to a new tube containing 150 µl of high salt NET and incubated at 65°C for 15 min. The supernatant was withdrawn and the ethidium bromide in the supernatant was extracted with water-saturated 1-butanol 3 times. The high salt buffer was precipitated with 2.5 volumes of 100% ethanol by incubation for 1 hour at -70°C followed by centrifugation at 12,000g for 15 min at 4°C. The DNA pellet was dissolved in 100 µl of DEPC water and reprecipitated with 1/10 volume of 3M NaOAc (pH 5.2) and 2 volumes of ethanol. The pellet was washed with 70% ethanol and dissolved in 50 µl of DEPC water. The DNA was then ready for probe preparation such as random priming.

**Linearization of Plasmid DNA**

The plasmid DNA (5 µl) with the desired enzyme (2 µl) in a reaction buffer (2 µl) specific to the enzyme was incubated in a solution of 20 µl at 37°C for 2 hours. One µl of 0.5M EDTA was added to the reaction mixture to stop the reaction. The mixture (1-2 µl) was electrophoresed in 1% non-denaturing agarose gel to check whether the plasmid DNA was completely linearized. The mixture was extracted twice with phenol/chloroform and the DNA was precipitated by incubating the supernatant with 1/10 volume of 3 M NaAc and 2 volume of ethanol at -70 °C for 1 h, followed by centrifugation at 12,000g for 15 min at 4 °C. The procedure was repeated, the DNA pellet was dried and resuspended in 20 to 50 µl of TE.
Preparation of RNA probes by in vitro transcription

The following components were incubated in a total volume of 10 µl at 37 °C for 1h: 1-2 µl of linearized DNA, 2 µl of 5x TSE buffer, 2 µl of mixture of ATP, GTP and UTP, 0.6 µl of DTT, 1 µl of nuclease-free water, 0.3 µl of RNASin, 2.5 µl [³²P]CTP, and 1 µl of RNA polymerase.

The synthesized RNA probe was separated from the unincorporated nucleotides by precipitation according to the following procedures. To the reaction solution 1/4 volume of 10M NH₄Ac and 2 volumes of isopropanol were added, mixed well, incubated at room temperature for 15 min, and centrifuged at 12,000g for 15 min. The supernatant was removed and the radioactivity in the supernatant and the pellet was checked. The pellet was dissolved in 10 µl TE and the precipitation procedures were repeated. The pellet was then dissolved in 50 µl TE which was ready for use.
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