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Expression of stem cell factor and c-kit receptor in neural cells after brain injury

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Abstract Previously it has been shown that c-kit receptor (c-kitR) and its ligand, stem cell factor (SCF), are expressed in the central nervous system. We have reported that SCF in cultures regulates mouse microglial function. Here we demonstrate that SCF/c-kitR signaling also takes place in situ. We used a penetrating stab wound injury as a model and analyzed the SCF and c-kitR expression in neural cells by immunohistochemistry and in situ hybridization. We found that microglia activated by injury upregulated c-kitR expression, whereas some astrocytes in the vicinity of the wound expressed SCF mRNA in addition to neurons. This observation suggests that SCF/c-kitR signaling between neurons, astrocytes and microglia also occurs in situ.

Key words Cytokine · Inflammation · Neuroregeneration · Microglia · Astrocytes

Introduction

Stem cell factor (SCF), also termed mast cell growth factor (MGF), kit ligand (KL), and Steel factor (SLF), is a hemopoietic cytokine which structurally resembles colony-stimulating factor-1 (CSF-1) [28, 32]. Its receptor, encoded by the proto-oncogene, c-kit, is a member of the class III family of intrinsic tyrosine kinase growth factor receptors that includes receptors for CSF-1 and platelet-derived growth factor (PDGF) [4]. Both SCF and c-kit mRNAs are highly expressed in cells of the nervous system during development and in adulthood [18, 22, 24, 25, 27]. In mice, mutations at either the SCF gene (Steel locus) or c-kit gene (W locus) result in defects in the hematopoietic, melanogenic, and reproductive systems but not in the nervous system [10]. Although the role of SCF in the nervous system is so far not clear, in vitro studies indicate that SCF is trophic to certain neurons derived from neural crest [5, 16, 17] and cerebral cortex [33], and also regulates the activity of astroglia, oligodendroglia and microglia [20, 30, 33, 34]. In the present study, we investigated whether SCF/c-kitR signaling is involved in neural cell response to injury.

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Materials and methods

Stab wound lesion

Adult 2- to 3-month-old male C3H/HeJ mice were anesthetized with somnatol (25 μl/20 g body weight, i.p.) 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 (2.5–3.0 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 mice were maintained in our laboratory animal facility and had free access to water and food. At 1, 2, 4, 7, 14, and 28 days after lesioning the animals were killed and the brain tissue was examined by immunohistochemistry and, in some cases, also by in situ hybridization.

Immunohistochemistry

Normal and lesioned mice were killed with CO_2 and perfused transcardially with cold Dulbecco's phosphate-buffered saline (DPBS) followed by 4% formaldehyde in DPBS. The brain tissues were removed, cryoprotected in 25% sucrose at 4° C overnight, embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Miles Inc., Elkhare, Ind.) and rapidly frozen in liquid nitrogen. Cryostat coronal sections were cut at 8–10 μm and dried in air. For in situ hybridization, the tissues were post-fixed in 4% formaldehyde for 2–4 h before being processed for sectioning.

The immunohistochemical staining for c-kitR is described in detail elsewhere [33]. A monoclonal anti-c-kitR antibody, ACK2 (rat IgG), which recognizes the extracellular domain of the murine c-kitR (Gibco BRL, Gaithersburg, Md.; 3314SA), dilution 1 : 200, was used in the present study. The specificity of the antibody was verified by immunostaining cell lines positive for c-kitR (line MC/9) and negative for c-kitR (line WEHI-3), and by omitting the primary antibody in the immunostaining procedure [33].

To verify the cell types positive for c-kitR immunostaining, adjacent sections were immunostained with Mac-1, a monoclonal an-

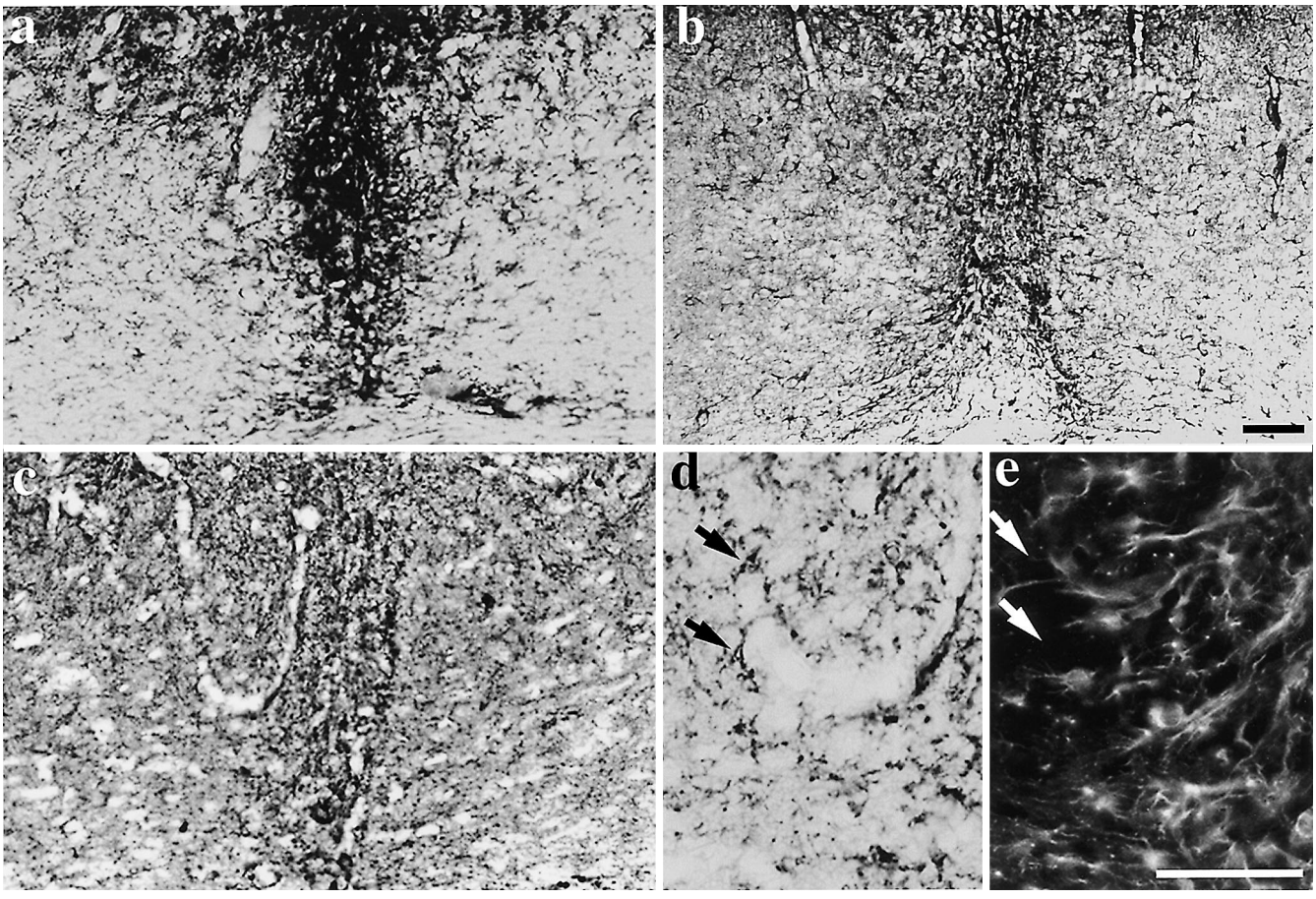

Fig. 1 a–e Images of cryostat sections through the stab wound lesion in cerebral cortex 7 days post-lesioning. **a** Stained with Mac-1 antibody for microglia; **b** adjacent section stained with anti-GFAP antibody for astroglia; **c** adjacent section stained for c-kitR. The c-kitR-positive cells follow the needle track. **d**, **e** Cryostat section double-stained for c-kitR (**d**) and GFAP (**e**). Cells positive for c-kitR appear to be negative for GFAP and vice versa (*arrows* **d**, **e**) (*GFAP* glial fibrillary acidic protein, *c-kitR* c-kit receptor). *Bars* 100 μm

tibody recognizing complement 3 receptor (CR3) in microglia, and anti-glial fibrillary acidic protein (GFAP), which is specific for astrocytes. In the case of c-kitR staining, the sections were further incubated with a rabbit polyclonal anti-GFAP antibody (1 : 200; Dimension Laboratories, Dako A/S, Denmark) for 1 h at room temperature followed by FITC-conjugated donkey anti-rabbit IgG (1 : 100) for 1 h. The c-kitR-positive cells were then matched with the GFAP-positive cells using a Zeiss fluorescence microscope III.

In situ hybridization

The cryostat sections on slides were rehydrated in PBS prepared with water treated with diethylpyrocarbonate (DEPC). They were then hybridized with digoxigenin-labeled antisense SCF RNA probe diluted in hybridization solution consisting of 50% formamide, 0.3 M NaCl, 10 mM phosphate buffer (pH 7.4), 10 mM EDTA, 10 mM TRIS-HCl pH 7.5, 10% dextran sulfate, 1 mg/ml tRNA, and 1 × Denhardt's solution, 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 2 × SSC (sodium chloride/sodium citrate) with 50% formamide at 55 °C for 30 min each, and twice in 1 × SSC, 25% formamide at 55 °C, and twice in 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 1 h in PBS with 0.1% Tween 20 (PBT) containing 0.2% bovine serum albumin (BSA) before being incubated for 1 h in alkaline phosphatase-conjugated anti-digoxigenin antibody (1 : 2000; Boehringer Mannheim, Mannheim, Germany). Before use, the antiserum was absorbed with mouse brain powder (Rockland, Gilbertsville, Pa.). 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-indolyl-phosphate (BCIP, Gibco BRL), 100 mM NaCl, 50 mM $MgCl_2$, 100 mM TRIS-HCl pH 9.5 and 0.1% Tween 20, for one to several hours to allow the color to develop.

The preparation of SCF riboprobes has been described previously [33]. Briefly, the antisense SCF RNA probe was synthesized using a Riboprobe Gemini System (Promega, Madison, Wis.; P-1121) in the presence of *Bam*HI-linearized pBSSK-MGF4 (provided by Immunex Corp., Seattle, Wash.) which contained a 1-kb insert encoding amino acid 1–47 of murine SCF [6, 31], ATP, UTP, GTP, digoxigenin-labeled CTP, T7, and RNAsin. For the sense SCF probe, the pBSSK-MGF4 was linearized with ASP718 and transcribed with T3. The probes were purified by precipitation with ethanol. A single band of about 1.2 kb of the probes was confirmed by electrophoresis.

To investigate the cell types that express SCF mRNA, the anti-digoxigenin antibody-labeled slides were further incubated with anti-GFAP for 1 h at 1 : 200 dilution, followed by incubation in FITC-conjugated anti-rabbit IgG for 45 min at 1 : 200. The slides were dehydrated, mounted in Entellan (BDH, Toronto, Canada), visualized under a Zeiss photomicroscope III and photographed using TMX3200 film.

Results

Regulation of the expression of c-kitR

We have shown previously that SCF and c-kitR are expressed in the cells of the adult mouse cerebral cortex and that SCF is expressed mainly by neurons, whereas c-kitR is expressed by glia and some types of neurons [33]. In the present study we investigated regulation of the expression of SCF and c-kitR in response to brain injury. We used needle stab wound as an injury model and immunohistochemistry and in some cases in situ hybridization to demonstrate the expression of c-kitR and SCF mRNA.

Adjacent sections through the stab wound were immunostained with Mac-1 antibody, which reacts with CR3 on microglia and macrophages, or antibody to GFAP which is specific to astroglia, or ACK2 antibody to c-kitR extracellular domain. As expected, the lesion immunostained strongly for microglia, which followed the needle track (Fig. 1 a), and less intensly for astroglia, which were more diffusely arranged (Fig. 1 b). The most intensive staining and the largest area covered by microglia occurred around 7 days after inflicting the wound and thereafter their numbers declined. After 28 days reactive microglia were still prominent in the lesion but considerably reduced in number (Fig. 2 a). Some reactive astroglia could also be seen but they were few in number and were diffusely arranged around the lesion (Fig. 2 b).

In the normal mouse cerebral cortex the expression of c-kitR in glia is barely detectable by immunohistochemistry [33]. As early as 24 h post-lesioning, however, we observed intense immunostaining for c-kitR, which similarly to microglia followed the needle track. The intensity of staining throughout the rest of the cerebral cortex, including the ipsi- and contralateral sides, was very weak, as previously reported for normal, non-injured forebrain [33]. The area of staining for c-kitR was largest and the intensity of staining was greatest from 4 to 7 days postlesioning (Fig. 1 c) and subsequently declined, although 4 weeks after injury a weak staining was still present at the site of the lesion (Fig. 2 c). An intense staining on the surface of the cortex (molecular layer) in Fig. 2 c was observed in normal uninjured mouse brain [33].

Under high power the cells close to the wound that stained intensely for c-kitR had hypertrophic cell bodies and short, stubby processes (Fig. 1 c). Cells more distant from the wound that stained positively for c-kitR stained less intensely and had smaller, irregular cell bodies and fine processes (Fig. 1 c). The transition in the morphology of c-kitR-positive cells in the area at the edge of the wound to that in more distant areas was gradual. At the wound site, the morphology of the c-kitR-positive cells was similar to that of activated (reactive) microglia and distal to the wound, the morphology was that of normal ramified microglia (Fig. 1 a). In sections that were double stained for c-kitR and antibody to GFAP, the staining profiles were different. The cells that were positive for c-kitR were negative for GFAP and vice versa (Fig. 1 d, e).

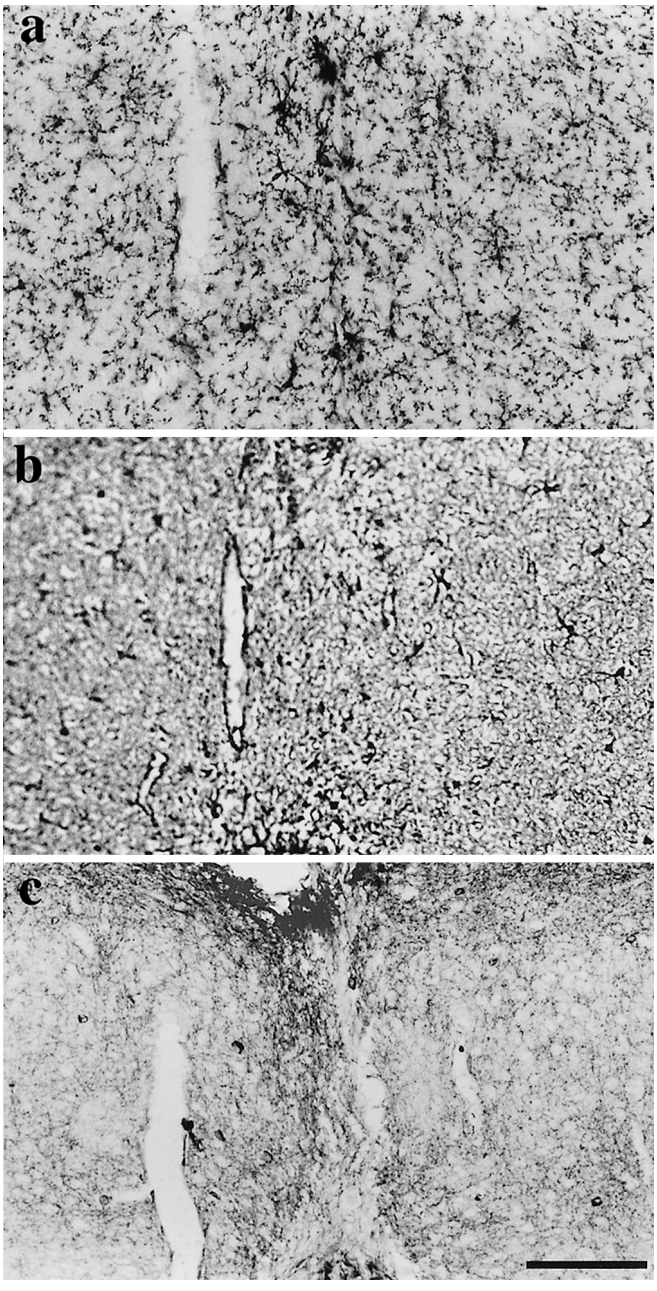

Fig. 2 a–c Images of cryostat sections through the stab wound lesion in cerebral cortex 28 days post-lesioning. **a** Stained with Mac-1 antibody for microglia; **b** adjacent section stained with anti-GFAP antibody for astroglia; **c** adjacent section stained for c-kitR. *Bar* 100 μm

Because reactive astrocytes in the stab wound lesion did not express c-kitR and the distribution of microglia was similar to that of c-kitR-positive cells, and since we had previously shown that microglia in cultures expressed c-kitR, we concluded that the c-kitR-positive cells around the wound were mostly microglia. However, more microglia than c-kitR-positive cells were present. It would seem that not all microglia had up-regulated c-kitR in response to the cerebral stab wound.

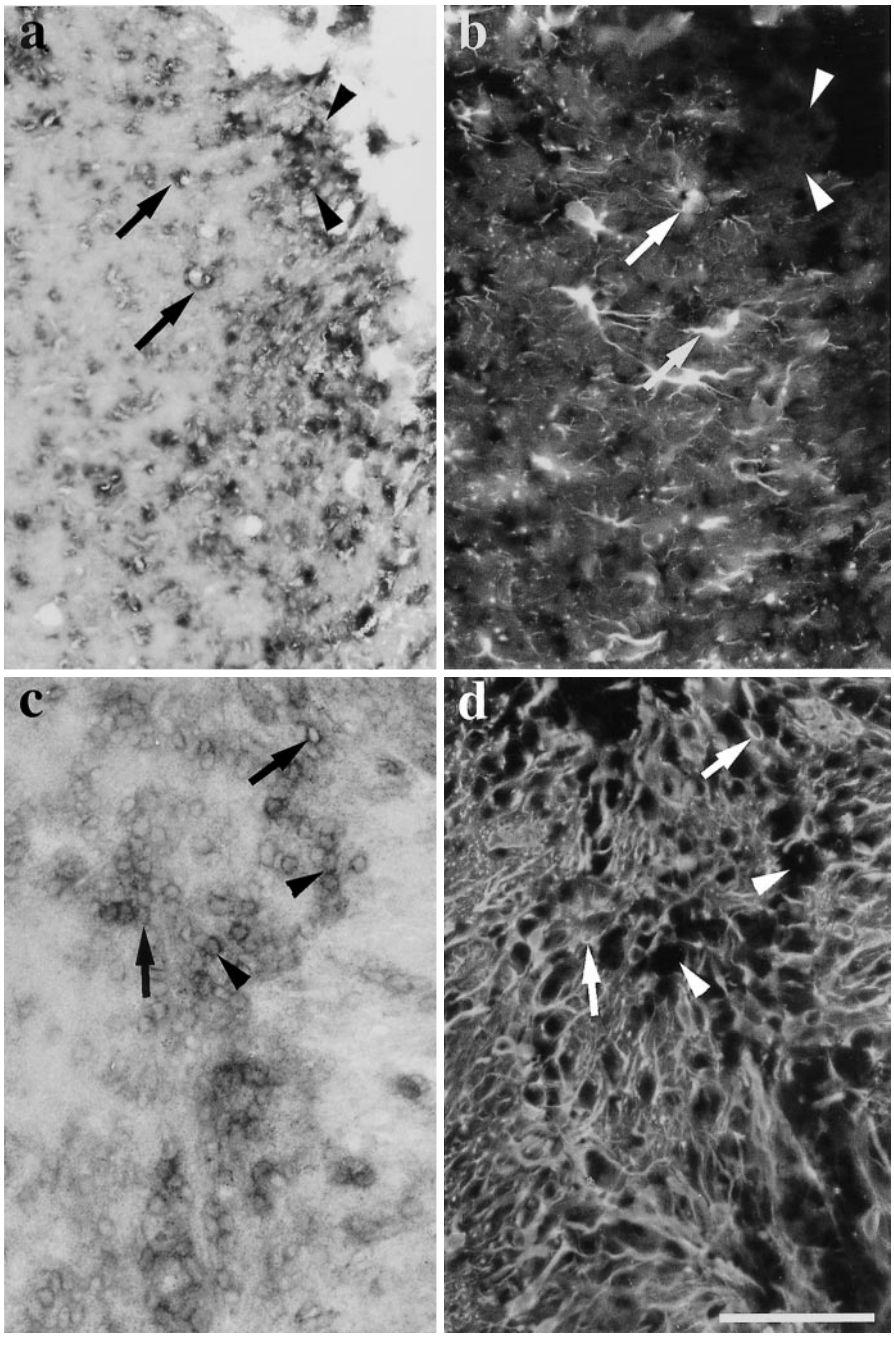

Fig. 3 a–d Images of brain sections 4 days after stab wound lesion. **a** Hybridized with antisense SCF RNA probe, **b** same section as in **a** further immunostained with anti-GFAP antibody for astrocytes. **c**, **d** Images of brain sections 7 days after stab wound lesion; **c** hybridized with antisense SCF RNA probe, **d** same section as in **c** further immunostained with anti-GFAP antibody for astrocytes. **a–d** *Arrows* indicate SCF mRNA-positive cells stained with GFAP and *arrowheads* indicate SCF mRNA-positive cells in the lesion area negative for GFAP (*SCF* stem cell factor). *Bar* 100 μm

Regulation of the expression of SCF

In normal cerebral cortex, SCF is expressed mainly in neurons [33]. In the brain, the major isoform of SCF is soluble SCF [19], which may escape detection by immunohistochemistry. Therefore, we examined the expression of SCF in stab wound injury by in situ hybridization. We found that a large number of cells surrounding the stab wound had a positive signal for SCF mRNA. Some neurons distant from the lesion also had a positive signal, as did neurons on the contralateral side, and neurons in the cerebral cortex of non-injured brains. Glia cells with positive SCF mRNA signal were observed near the wound on the 2nd day after injury. Their numbers increased and peaked at 4–7 days post-lesioning (Fig. 3), and then declined. In sections used for in situ hybridization for SCF mRNA, and then immunostained with fluorescin-tagged anti-GFAP antibody, only some of the cells with positive signal for SCF mRNA stained positively with anti-GFAP antibody. Many of the cells close to the wound that had positive signal for SCF mRNA, however, were GFAP negative (Fig. 3), suggesting that these cells were likely microglia. The hybridization signal was regarded as specific to SCF because a sense probe did not generate a positive signal in the cells in the lesion or further away from the lesion (not shown).

Discussion

The response of glia to penetrating wounds of the cerebral cortex has been well documented. The first cells to respond to the injury are microglia. Their numbers peak 2 days post-lesioning and then decline [15, 23, 29]. The microglia transform from cells with a ramified morphology to cells with hypertrophic soma and small, stubby processes and are located in the parenchyma surrounding the wound [3, 15]. The response of microglia is followed by the invasion of a few blood-borne, round, non-process-bearing macrophages situated mainly within the needle track [1, 15]. Astrocytes respond 3–4 days post-lesioning by hypertrophy. Only a few of them, if any, proliferate, depending on the age of the animal [1, 7, 21, 26]. In the present study we report that, in response to a penetrating stab wound, the neural cells up-regulate c-kitR and/or its ligand SCF. Technically, it was not possible for us to double label the sections with antibodies to c-kitR and CR3 to identify microglia and macrophages because of the animal source of antibodies available to us. However, based on the pattern of staining in adjacent sections and the location and morphology of the cells, when one section was stained with antibodies to c-kitR and the next adjacent section with Mac-1 antibody, we concluded that the cells that up-regulated c-kitR in the parenchyma surrounding the needle track are activated microglia. This conclusion was supported by the observation that GFAP-positive cells were negative for c-kitR and vice versa in double-stained preparations, indicating that astrocytes did not express c-kitR. The conclusion that microglia up-regulate the expression of c-kitR is in line with our previous observations that microglia in cultures express c-kitR and that the expression of c-kitR is up-regulated when microglia are stimulated with interferon-γ or interleukin-1 [34].

In culture, astroglia express both c-kit mRNA and c-kitR [30, 33]. In astroglia cultures initiated from neonatal neopallia, c-kitR is detected only at the early stage of culture when the cells proliferate, and the positive signal disappears when the culture becomes confluent [33], suggesting that the expression of c-kitR may relate to the proliferation of astroglia in culture. In the stab wound lesion, the proliferation of astrocytes is limited [1, 7, 21, 26]. This may explain why c-kitR is not up-regulated in astrocytes surrounding the stab wound.

SCF can appear in two distinct forms; one is membrane bound or soluble and the other is membrane bound only [2, 9]. The production of different SCF isotypes seems to be regulated in a tissue-specific manner [9, 19]. It has been reported that in the brain most of the SCF produced is in the soluble form [19] and that in the normal adult brain SCF is produced predominantly by neurons [27, 33]. We therefore used in situ hybridization to detect SCF message. In the present report we show that in response to a penetrating cerebral cortex injury expression of SCF in neurons in the parenchyma surrounding the wound was up-regulated. The expression of SCF was only weakly up-regulated in some reactive astrocytes immediately adjacent to the wound. SCF could not be detected in microglia by immunohistochemistry (not shown), but using in situ hybridization we demonstrated the presence of SCF mRNA in microglia. Based on our previous observation that the SCF mRNAs from microglia migrate faster than those from neurons [33], we suggest that microglia in situ may produce predominantly soluble SCF which is difficult to detect by immunohistochemistry. This suggests that both paracrine and autocrine SCF/c-kitR signaling is involved in neural cell interactions in the CNS.

As already noted, in normal mouse adult brain SCF is produced mainly by neurons [27, 33]. We hypothesize that the constitutive production of SCF by neurons in the steady state plays a role in maintaining microglia in a quiescent state, since SCF inhibits microglial proliferation in cultures when microglia are stimulated by CSF-1 [34]. Perforating injury caused by a needle stab in the cerebral cortex results in cell death, including that of neurons, in the needle track.

It seems reasonable to assume that in such injury the delicate balance between the concentrations of SCF, CSF-1 and probably some other cytokines, is upset [8, 11–14] and that as a result microglia and astrocytes are activated to respond to injury. The neurons in the vicinity of the stab wound, and activated microglia and astrocytes right next to the wound, up-regulate the production of SCF and the expression of its receptor, c-kitR, to facilitate signaling between the cells. This state is transitory. It lasts 7–10 days, then the production of SCF and the expression of c-kitR decline to a normal level. The activated microglia gradually disappear and then probably a new level of the steady state is established. Our present observation that the expression of SCF and c-kitR is up-regulated in response to the cerebral cortex injury, and previous observations in cultures, support the suggestion that SCF/c-kitR signaling plays an important role in the regulation of microglia as well as their activity in response to CNS injury.

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