The Rat c-kit Ligand, Stem Cell Factor, Induces c-kit Receptor-dependent Mouse Mast Cell Activation In Vivo. Evidence that Signaling through the c-kit Receptor Can Induce Expression of Cellular Function

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Summary

Interactions between products of the mouse W locus, which encodes the c-kit tyrosine kinase receptor, and the Sl locus, which encodes a ligand for c-kit receptor, which we have designated stem cell factor (SCF), have a critical role in the development of mast cells. Mice homozygous for mutations at either locus exhibit several phenotypic abnormalities including a virtual absence of mast cells. Moreover, the c-kit ligand SCF can induce the proliferation and maturation of normal mast cells in vitro or in vivo, and also can result in repair of the mast cell deficiency of Sl/Sl^d mice in vivo. We now report that administration of SCF intradermally in vivo results in dermal mast cell activation and a mast cell–dependent acute inflammatory response. This effect is c-kit receptor dependent, in that it is not observed when SCF is administered to mice containing dermal mast cells expressing functionally inactive c-kit receptors, is observed with both glycosylated and nonglycosylated forms of SCF, and occurs at doses of SCF at least 10-fold lower on a molar basis than the minimally effective dose of the classical dermal mast cell–activating agent substance P. These findings represent the first demonstration in vivo that a c-kit ligand can result in the functional activation of any cellular lineage expressing the c-kit receptor, and suggest that interactions between the c-kit receptor and its ligand may influence mast cell biology through complex effects on proliferation, maturation, and function.

Several lines of evidence indicate that interactions between the c-kit tyrosine kinase receptor and its ligand critically regulate mouse mast cell development (reviewed in references 1–3). Mice with a double dose of mutant genes at either the W/c-kit locus on chromosome 5 or the Sl locus on chromosome 10, which encodes a ligand for the c-kit receptor that has been named stem cell factor (SCF)[1] (4–6), mast cell growth factor (7–9), kit ligand (10), and Steel factor (11), are virtually devoid of mature mast cells (reviewed in references 1 and 12). Recombinant forms of the c-kit ligand induce proliferation of in vitro–derived immature IL-3-dependent mast cells (4, 5, 7, 9) or mature mast cells purified from

the peritoneal cavity of mice (3). Recombinant rat SCF^{164} ($rrSCF^{164}$), representing the soluble form of the c-kit ligand, secreted by a Buffalo rat liver cell line (4–6), not only induces proliferation of immature or mature mouse mast cells in vitro (3, 4), but also promotes the maturation of IL-3-dependent, bone marrow–derived cultured mouse mast cells (BMCMC) in vitro (3) and induces the proliferation and maturation of mouse or rat mast cells when administered in vivo (2). Administration of $rrSCF^{164}$ in vivo also can repair the macrocytic anemia and the mast cell deficiency expressed by Sl/Sl^d mice (2, 6).

The effects of $rrSCF^{164}$ on mouse or rat mast cell proliferation and maturation, when taken together with the profound mast cell deficiencies expressed by W or Sl mutant mice, indicate that interactions between the c-kit receptor and its ligand importantly regulate normal mast cell development. However, certain other hematopoietic growth factors, in addition to regulating the proliferation of cells that express the

[1] Abbreviations used in this paper: BMCMC, IL-3-dependent, bone marrow–derived cultured mast cells; CHO, Chinese hamster ovary cell; CTMC, connective tissue type mast cell(s); DNP_{30-40} HSA, DNP human serum albumin; ^{125}I-GPF, ^{125}I-guinea pig fibrinogen; $rrSCF^{164}$, recombinant rat stem cell factor^{164}; SCF, stem cell factor.

appropriate receptors, can also significantly influence the functional activity of their target cells. For example, IL-3 represents a major growth factor for human basophils (13, 14), but also both can function as an agonist that induces basophils to release histamine and other mediators, and can enhance the ability of basophils to release mediators in response to other secretagogues (15-20).

We have reported that rrSCF^{164} can induce the release of serotonin (5-hydroxytryptamine) from BMCMC in vitro, but the magnitude of serotonin release is modest, and mediator release is observed only at concentrations of rrSCF^{164} at least 10-fold higher than those that can induce proliferation of these cells (21). However, different populations of mast cells can express multiple differences in phenotype, notably including variation in their sensitivity to the same agonists of mediator release (reviewed in reference 22). Moreover, certain agents that are relatively weak agonists of mast cell mediator release in vitro, such as PMA, represent potent stimuli of mast cell mediator release in vivo (23). We therefore investigated the ability of rrSCF^{164} to induce dermal mast cell activation when injected into the skin of mice in vivo, using approaches that permitted us to test dermal mast cells expressing either normal or functionally inactive forms of the c-kit receptor. Our results clearly indicate that rrSCF^{164} represents a potent agonist of dermal mast cell activation, and show that this effect of rrSCF^{164} requires that the mast cells express a functionally active c-kit receptor.

Materials and Methods

Recombinant SCF. Recombinant rat SCF^{164} (rrSCF^{164}) was purified from either *Escherichia coli* (*E. coli*-SCF) or Chinese hamster ovary cells (CHO-SCF) (5, 6). In some experiments, we used *E. coli*-derived rrSCF^{164} that had been modified by the covalent attachment of polyethylene glycol (6). The various forms of rrSCF^{164} were kept as stock solutions at 4°C in PBS and were diluted for intradermal injection in Hanks' MEM (HMEM) containing piperazine-N,N'bis[2-ethane sulfonic acid] (HMEM/Pipes) just before use (24). Similar dilutions of PBS alone in HMEM/Pipes were used for control injections. In most experiments, the left ears received injections of rrSCF^{164} in 0.02-ml volumes (challenged ears), whereas the right ears received 0.02 ml of the vehicle (control ears). *E. coli*-derived rrSCF^{164} was used for the most of our experiments. According to the limulus amebocyte lysate chromogenic assay (LAL Assay; Whitaker Bioproducts Inc., Walkersville, MD), the endotoxin content of the highest concentration of this preparation used for our studies (51 μg/ml in HMEM/Pipes, to provide 30 μg/kg/dose), was 13.1 EU/ml ($\sim$1.5 ng/ml), whereas the value for the HMEM/Pipes alone was 2.3 EU/ml (0.27 ng/ml). Based on these values and the volume of the reagents injected (20 μl), the amounts of endotoxin that were administered in these preparations were at least 30- to 200-fold less than that required to elicit significant inflammation when injected into the skin of mice (25).

Mice. Genetically mast cell-deficient W/W^v or Sl/Sl^d mice and the congenic normal (+/+) mice ([WB/ReJ-W/+ × C57Bl/6J-W^v/+]F$_1$[W/W^v, +/+], designated here WBB6F$_1$[W/W^v, +/+], and [WC/ReJ-Sl/+ × C57Bl/6J-Sl^d/+]F$_1$[Sl/Sl^d, +/+], designated here WCB6F$_1$[Sl/Sl^d, +/+]) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from the Charles River Laboratories (Wilmington, MA).

The skin of adult W/W^v (26) or Sl/Sl^d (27) mice contains <1.0% the number of dermal mast cells present in the skin of the congenic normal (+/+) mice. All mice were used at 2-7 mo of age unless stated otherwise.

Measurement of Tissue Swelling and Assessment of Mast Cell Activation. Ear swelling was determined by measurements of ear thickness with a micrometer before and at various intervals after the injection of rrSCF^{164} or vehicle; swelling was expressed as the increment (Δ) of thickness (postinjection value − preinjection baseline value) in units of 10^{-4} in (23, 24).

In all experiments, tissue from the injection sites was obtained after the mice were killed by cervical dislocation and were processed for 1-μm Epon-embedded, Giemsa-stained sections (28, 29). Sections were coded and then were examined by observers who were unaware of the identity of individual sections. The number of mast cells per square millimeter of dermis in each biopsy specimen was determined as previously described (23, 24), and the mast cells were classified morphologically as exhibiting extensive degranulation (50% of the cytoplasmic granules exhibiting fusion, staining alterations, and/or extrusion from the cell), moderate degranulation (10-50% of the granules exhibiting altered or discharged), or no significant degranulation ("none"; <10% of the granules exhibiting alternatives (23, 24).

Assessment of SCF-Induced ^{125}I-Fibrin Deposition. In most experiments, we assessed altered vascular permeability using a very sensitive assay for detecting extravasation of ^{125}I-guinea pig fibrinogen (^{125}I-GPF) and deposition of crosslinked ^{125}I-fibrin (30, 31). At 20-30 min before challenge with SCF, the mice were injected intravenously with $\sim$2-3 × 10^6 cpm of ^{125}I-GPF. The swelling responses in SCF- and control-injected ears were measured with a micrometer 1 and 2 h after challenge as described above. The mice then received a mixture of anticoagulants and antifibrinolytic agents intravenously to impede clotting or fibrinolysis during specimen preparation: each mouse received 0.2 ml of a mixture containing 80 U heparin, 20 μg D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, 2 μg ϵ-aminocaproic acid, and 56 U trasylol, all in 0.15 M NaCl. Immediately thereafter, the mice were lightly anesthetized with ether, and a sample of retroorbital blood was obtained. These samples, which exhibited no clotting during an observation period of 3 h, were centrifuged at 10,000 g at room temperature to prepare platelet-poor plasma for radioactive counting. The mice were killed by cervical dislocation immediately after retroorbital bleeding, and both ears were amputated and trimmed. A small full-thickness biopsy of each ear was fixed as described above for histologic studies. The rest of each ear was then promptly minced into fine fragments in 2-ml 0.01 M phosphate buffer (pH 7.5) containing a mixture of proteolytic inhibitors: heparin (10 U/ml), D-phenylalanyl-L-arginine chloromethyl ketone (5 mg/ml), EDTA (2 mg/ml), ϵ-aminocaproic acid (0.1 M), trasylol (10 U/ml), 2 mM PMSF, 2 mM iodoacetate, and 2 mM N-ethylmaleimide. The minced ears were then extracted in the buffer by keeping the tubes for 18 h at 4°C. Each tube was centrifuged (1,000 g for 20 min at 20°C), and supernatant was decanted. The pellet containing the tissue fragments was resuspended vigorously in 2 ml of the same buffer and was centrifuged again, as above. The supernatant was aspirated and pooled with the earlier extract for radioactive counting as the "aqueous extract." The pelleted tissue fragments were suspended in 2 ml of freshly prepared 3-M urea, extracted for 2 h at 37°C, centrifuged (1,000 g for 20 min at 20°C), and the urea-soluble supernatant and the urea-insoluble pellet counted for radioactivity in a Tm Analytic 1193 gamma counter. ^{125}I-GPF-derived species present in mouse tissues consist of a mixture of fibrinogen, fibrin, and fibrinogen-fibrin degradation products (31). The "urea-insoluble" residue consists

of crosslinked fibrin as well as some early degradation fragments of crosslinked fibrin.

Bone Marrow Transplantation of Mast Cell–deficient (W/W^v) Mice. Mast cell deficient W/W^v mice received an injection of 2.0 × 10^7 i.v. femoral bone marrow cells pooled from their congenic (+/+) littermates. Kitamura et al. (26) demonstrated that 10 wk after they received such transplantation of congenic +/+ bone marrow cells, the recipient W/W^v mice had undergone correction of both their macrocytic anemia and their mast cell deficiency.

Mast Cell Reconstitution of Mast Cell–deficient W/W^v Mice. W/W^v mice were repaired of their mast cell deficiency selectively and locally by the injection of growth factor-dependent, BMCMC into one ear (23, 24, 31). We reported that IL-3–dependent BMCMC of WBB6F$_1$-+/+ origin maintained in suspension culture represent immature mast cells that express certain phenotypic similarities to mucosal mast cells, but that upon injection into the skin (23, 24, 31, 32) of WBB6F$_1$-W/W^v mice, these mast cell populations gradually (over a 10-wk period) acquire multiple phenotypic characteristics of the mature connective tissue-type mast cells (CTMC) present in the dermis of normal mice. Moreover, injection of BMCMC into one ear of W/W^v mice repairs the mast cell deficiency in that ear alone. The mice remain mast cell deficient at other cutaneous sites and in other organs, and remain anemic as well (23, 24, 31, 32). Briefly, bone marrow cells from WBB6F$_1$ +/+ mice were grown in vitro for 3–5 wk in concanavalin A–stimulated mouse spleen cell–conditioned medium until mast cells represented >95% of the total cells as determined by neutral red staining. Mast cells (0.5 × 10^6) in 20 μl of DMEM (Gibco Laboratories, Grand Island, NY) were injected into the left ears and 20 μl of medium alone into the right ears. The mice were used for experiments 10 wk after injection of mast cells. In each experiment, we confirmed histologically that local reconstitution of dermal mast cell populations had occurred and determined that the W/W^v mice locally reconstituted with mast cells remained anemic.

Induction of Cutaneous Mast Cells in W/W^v and Congenic +/+ Mice by the Chronic Application of PMA. We previously showed that the chronic epicutaneous application of PMA to the skin of genetically mast cell–deficient W/W^v mice induces the development of mature dermal mast cells that morphologically and histochemically resemble the dermal CTMC of normal mice and that are fully competent to orchestrate IgE-dependent passive cutaneous anaphylaxis responses (33). Moreover, these PMA-induced W/W^v mast cell populations persist at the treated sites even after PMA treatment has been stopped to permit the inflammatory reactions at these sites to wane (34). For these experiments, W/W^v mice and congenic +/+ mice received 5 μg of PMA in 5 μl of acetone to the inner surface of the left ear and acetone alone to the inner surface of the right (control) ear, three times a week for 6 wk. The mice received no treatment for an additional 16 d and then were used in an experiment. Some mice from both W/W^v and congenic +/+ groups received E. coli–derived SCF (10 μg) into both ears, whereas other mice from both groups received a similar dilution of PBS in HMEM/PIPES into both ears. The latter groups represented controls for any possible effects of the chronic PMA treatment alone on cutaneous vascular permeability. Ear swelling measurements, assessment of ^{125}I-fibrin deposition, and histology were performed as described above. To test the ability of W/W^v mast cells to orchestrate passive cutaneous anaphylaxis responses, reactions were elicited as previously described (31) by injecting a monoclonal anti-DNP IgE (35) or vehicle alone into the ears and then challenging the mice ~24 h later by injecting 100 μg of DNP human serum albumin (DNP$_{30-40}$HSA) (Sigma Chemical Co., St. Louis, MO) intravenously in 0.1 ml of sterile

0.9% NaCl containing 1.0% Evans blue dye (Sigma Chemical Co.). The reactions were assessed 2 h after antigen challenge by visualization of the blue dye as well as by assessment of tissue swelling, ^{125}I-fibrin deposition, and histology, as noted above.

Statistical Analysis. The results of ear swelling assays, and differences in mast cell counts in different groups of mice, were analyzed for statistical significance ($p < 0.05$) by the unpaired Student's t test (two-tailed); comparison of values in the left (challenged) versus right (control) ears of the same mice was performed using the paired Student's t test (two-tailed). Ratios of radioactivity in left (challenged) versus right (control) ears were analyzed by the Mann-Whitney U test (two-tailed). The extent of mast cell activation (marked degranulation, moderate degranulation, normal) was assessed using the χ^2 test. Each experimental group contained four to five mice. All results are expressed as the mean ± SEM.

Results

SCF Induces Cutaneous Inflammation and Mast Cell Activation In Vivo. We first determined whether rrSCF^{164} could induce mast cell activation and an acute inflammatory response when injected at doses similar to those that induce mast cell proliferation in vivo (2). Fig. 1 shows that all three forms of rrSCF^{164} tested, including CHO-derived (glycosylated) rrSCF^{164}, E. coli–derived (nonglycosylated) rrSCF^{164}, and PEG-rrSCF^{164}, induced the rapid development of tissue swelling and ^{125}I-fibrin deposition when injected into the skin of normal mice at 30 μg/kg/site.

We then evaluated the effect of injecting different doses of E. coli–derived rrSCF^{164} into the skin of C57BL/6 mice. rrSCF^{164} induced significant ^{125}I-fibrin deposition (Fig. 2 A)
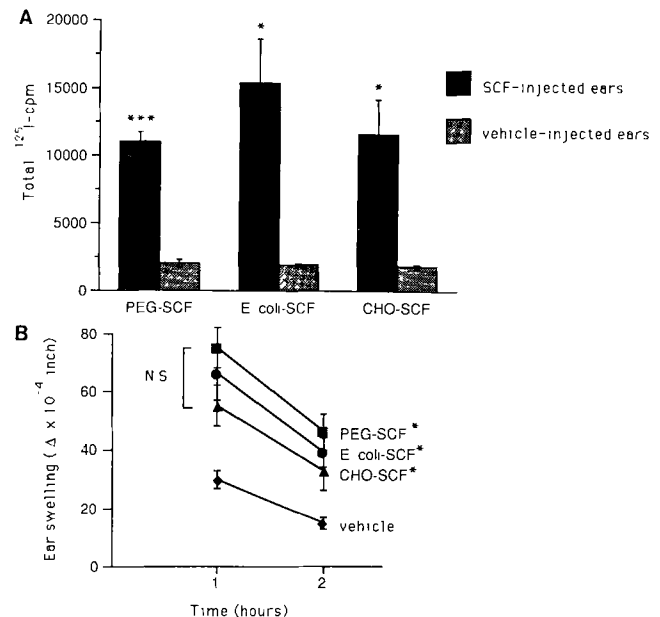

Figure 1. (A) Total ^{125}I-GPF–derived cpm 2 h after injection of various preparations of rrSCF^{164} (left ears, 30 μg/kg/site) or vehicle alone (right ears) into C57BL/6 mice. (B) Ear swelling (postinjection minus baseline values) determined in the same mice 1 and 2 h after injection. *$p < 0.05$ and ***$p < 0.001$ vs. value for contralateral vehicle-injected ears by the paired Student's t test (two-tailed).
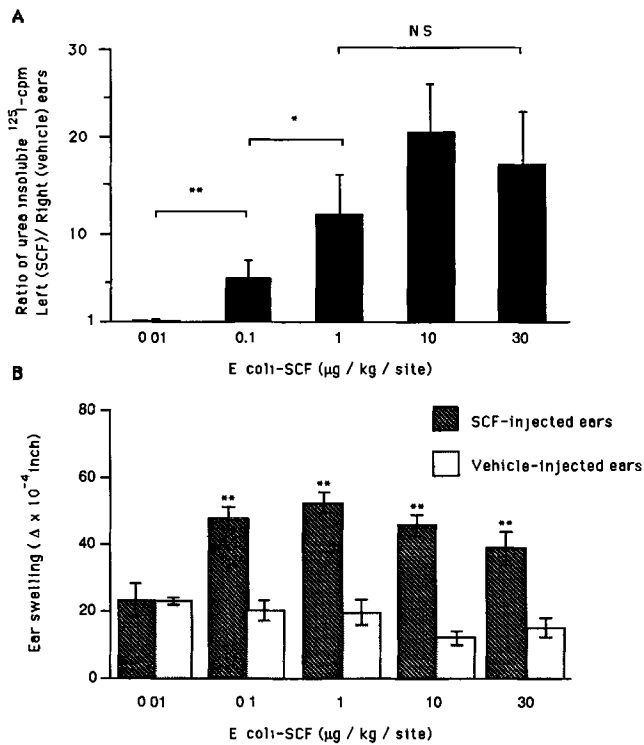
Figure 2. (A) Ratio of crosslinked ^{125}I-fibrin–associated cpm (urea-insoluble ^{125}I-cpm) in left vs. right ears determined 2 h after injection of various doses of E. coli–rrSCF[164] into left ears and vehicle alone into right ears. $^{*}p < 0.05$ and $^{**}p < 0.01$ for indicated comparisons by the Mann-Whitney U test (two-tailed). All values for sites injected with rrSCF[164] at doses of $\geqslant 0.1$ µg/kg/site are significantly greater ($p < 0.01$–0.001) than that at sites injected with 0.01 µg/kg/site. (B) Ear swelling in rrSCF[164]-injected left ears vs. vehicle-injected right ears, determined 2 h after injection in the same mice shown in A. $^{**}p < 0.01$ vs. value in contralateral vehicle-injected ears by the paired Student's t test (two-tailed).

and tissue swelling (Fig. 2 B) in doses as low as 0.1 µg/kg (∼140 fmol) per site. When the results were expressed as the ratio of the crosslinked ^{125}I-fibrin–associated cpm in rrSCF[164]-challenged as opposed to vehicle-injected mice (Fig. 2 A), the amount of ^{125}I-fibrin at rrSCF[164] injected sites was ∼21 times that at control sites at a dose of 10 µg/kg and ∼5 times that at control sites at a dose of 0.1 µg/kg.

Histological analysis demonstrated extensive mast cell activation at the sites that had been injected with all three forms of rrSCF[164], but not at control sites injected with vehicle (Table 1). Moreover, the intradermal injection of various doses of E. coli–SCF in the experiments reported in Fig. 2 induced a dose-dependent activation of dermal mast cells (Table 1). Mast cell activation was extensive at the higher doses tested (e.g., 75 ± 2% and 73 ± 10% extensively degranulated mast cells at sites injected with E. coli–SCF at 30 and 1.0 µg/kg/site, respectively). However, even at doses at low as 0.1 µg/kg/site, activation of mast cells at rrSCF[164] injection sites was significantly greater than that at contralateral control sites injected with vehicle alone. Comparison of these findings to the results of our previous study of the effects of substance P on dermal mast cell activation (24) indicates that rrSCF[164]

is at least 10 times more potent on a molar basis as a dermal mast cell activating agent than is substance P.

Cutaneous Inflammation Induced by SCF is Mast Cell-Dependent. To determine the extent to which the acute inflammation induced by intradermal rrSCF[164] is mast cell dependent, we injected rrSCF[164] into the skin of genetically mast cell–deficient mice and the congenic normal mice. We first tested W/W^v mice and their normal $(+/+)$ littermates. Fig. 3 A shows that injection of E. coli–rrSCF[164] at 30 µg/kg/site produced neither significant tissue swelling nor significant ^{125}I-fibrin deposition in the skin of W/W^v mice, whereas the identically challenged normal littermates exhibited the expected inflammatory responses. In other experiments, W/W^v mice gave no significant response to doses of rrSCF[164] as high as 100 µg/kg/site (data not shown).

The results obtained with W/W^v mice are consistent with the possibility that mast cells are required for the proinflammatory effects of rrSCF[164]. However, these experiments are also consistent with a second possibility: rrSCF[164]-induced inflammation is mast cell independent but requires an intact c-kit receptor. To address this possibility, we injected rrSCF[164] into genetically mast cell–deficient Sl/Sl^d mice. These animals express a normal c-kit receptor, but virtually lack cutaneous mast cells. As shown in Fig. 3 B, rrSCF[164] (30 µg/kg/site) produced no detectable inflammation when injected intradermally into the skin of Sl/Sl^d mice. By contrast, the normal $(+/+)$ littermates gave a strong response (Fig. 3 B). The failure of even high doses of rrSCF[164] to induce any detectable augmented vascular permeability, tissue swelling, or ^{125}I-fibrin deposition in W/W^v or Sl/Sl^d mice indicates that it is the lack of mast cells in these mutants, not abnormalities in the c-kit receptor per se, that is responsible for the animals' unresponsiveness to the proinflammatory effects of rrSCF[164]. This conclusion is supported further by the demonstration that reconstitution of W/W^v mice with mast cells of congenic $+/+$ origin permits these mice to develop an inflammatory response to intradermal challenge with rrSCF[164] (see below).

Proinflammatory Effects of Intradermal Injection of rrSCF[164] Require Mast Cells with a Functionally Active c-kit Receptor. To evaluate whether the proinflammatory effects of rrSCF[164] reflected a c-kit-dependent action of the cytokine on mast cells, we evaluated the effects of injecting rrSCF[164] (10 µg/kg site), or vehicle, into W/W^v mice containing either normal $(+/+)$ mast cells or mast cells of W/W^v origin that express a defective c-kit receptor. Fig. 4 shows that W/W^v mice that contained adoptively transferred dermal mast cell populations of $+/+$ origin developed tissue swelling and extensive deposition of ^{125}I-fibrin at sites of rrSCF[164] injection. Note that rrSCF[164] induced an inflammatory response only in the left ears of these W/W^v mice, i.e., in those ears in which dermal mast cells had been selectively reconstituted by local injection of BMCMC of WBB6F₁-+/+ origin. The number of mast cells in these ears was 34 ± 3/mm² of dermis, of which 34 ± 4% or 34 ± 4% exhibited extensive or moderate degranulation, respectively. By contrast, the mast cell–deficient right ears of these same mice (0 mast cells/mm²) developed little or no reaction in response to

Table 1. *Extent of Dermal Mast Cell Activation in the Skin of C57BL/6 Mice Injected with rrSCF[164] and Examined 2 h Later*

Agent	rrSCF[164]	Ear	Mast cell degranulation			p Value: L vs. R
			None	Moderate	Extensive	
	$\mu g/kg$		%	%	%	
CHO-SCF	30	L	12 ± 2	20 ± 4	68 ± 2	$p < 0.01$
Vehicle	0	R	76 ± 7	13 ± 3	10 ± 4	
Polyethylene glycol-SCF	30	L	7 ± 2	11 ± 1	82 ± 1	$p < 0.01$
Vehicle	0	R	95 ± 2	5 ± 2	0	
E. coli-SCF	30	L	8 ± 2	18 ± 3	75 ± 2	$p < 0.01$
Vehicle	0	R	87 ± 4	8 ± 2	5 ± 2	
E. coli-SCF	1	L	14 ± 6	13 ± 5	73 ± 10	$p < 0.01$
Vehicle	0	R	87 ± 2	10 ± 1	3 ± 2	
E. coli-SCF	0.1	L	62 ± 2	28 ± 6	10 ± 3	$p < 0.01$
Vehicle	0	R	97 ± 2	2 ± 1	1 ± 1	
E. coli-SCF	0.01	L	90 ± 2	7 ± 2	3 ± 1	NS
Vehicle	0	R	97 ± 1	3 ± 1	0	

These data are from the same experiments depicted in Figs. 1 and 2.
NS, not significant ($p > 0.05$).

rrSCF[164] injection. We also injected rrSCF[164] (10 μg/kg/site) into the ears of W/W^v mice that had undergone repair of their mast cell deficiency as a result of intravenous injection of bone marrow cells of WBB6F$_1$-+/+ origin. The extent of mast cell degranulation, and the inflammatory responses, observed in these mice were similar in magnitude to those in identically challenged +/+ mice (data not shown).

In contrast to W/W^v mice containing adoptively transferred mast cell populations of +/+ origin, W/W^v mice that contained PMA-induced dermal mast cells of W/W^v origin developed no significant tissue swelling or ^{125}I-fibrin deposition at sites injected with 10 μg/kg of rrSCF[164] (Fig. 5 A). This finding reflected neither a general unresponsiveness of the W/W^v dermal mast cells nor a suppression of mast cell reactivity at sites of PMA treatment. Thus, W/W^v mast cells at sites of PMA treatment were able to orchestrate passive cutaneous anaphylaxis reactions which were similar in magnitude to those observed in the PMA-treated ears of the congenic +/+ mice ($p > 0.05$ by the Student's t test, two-tailed) (Fig. 5 C). Moreover, in WBB6F$_1$-+/+ mice, sites treated chronically with PMA developed amounts of rrSCF[164]-induced (Fig. 5 B), or IgE and antigen-induced (Fig. 5 C) tissue swelling and ^{125}I-fibrin deposition that actually exceeded those recorded at identically challenged contralateral sites that had been treated with acetone alone. This result indicates that chronic PMA treatment does not reduce, and actually appears to enhance, mast cell-dependent responses to challenge with either rrSCF[164] or IgE and antigen in

normal (+/+) mice. Furthermore, comparison of the results obtained at PMA-treated or acetone-treated sites that had been challenged with vehicle indicated that chronic treatment with PMA did not significantly augment baseline levels of vascular permeability at these sites either in W/W^v mice (Fig. 5 A) or in +/+ mice (Fig. 5 B). Fig. 5, A and B report the results of one of two independent sets of experiments, both of which gave similar results.

Histological analysis of the injection sites of the mice reported in Fig. 5 confirmed that mast cells had developed at sites of PMA treatment in W/W^v mice, but not at contralateral control sites treated with acetone alone, and also showed that PMA treatment increased numbers of dermal mast cells in +/+ mice (Table 2). Dermal mast cells of +/+ origin in the PMA- or acetone-treated skin of +/+ animals exhibited extensive degranulation when examined 2 h after challenge with rrSCF[164] (Table 2). By contrast, little or no mast cell activation was exhibited at sites of rrSCF[164] challenge in the PMA-treated skin of W/W^v mice (Table 2). Indeed, the extent of mast cell activation observed at sites of rrSCF[164] injection in the PMA-treated skin of W/W^v mice was not significantly different than that at sites of vehicle injection ($p > 0.05$ by the χ^2 test). However, extensive degranulation of W/W^v dermal mast cells was observed at sites of passive cutaneous anaphylaxis (Table 2). This result confirmed our previous work (33) indicating that W/W^v mast cells can degranulate in response to challenge with IgE and antigen.
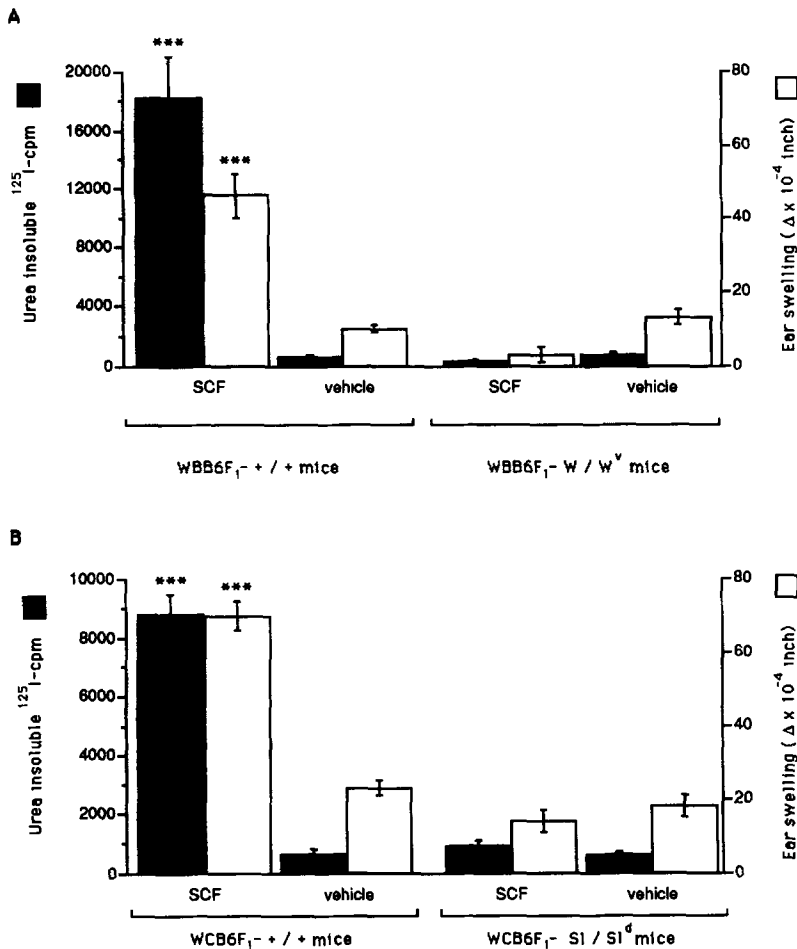
A

B

Figure 3. Crosslinked ^{125}I-fibrin–associated cpm (urea-insoluble ^{125}I-cpm) (*filled bars*) and ear swelling (*open bars*) determined 2 h after injection of *E. coli*-rrSCF[164] (30 μg/kg/site, left ears) or vehicle alone (right ears) in (*A*) genetically mast cell–deficient *W/W^v* vs. congenic normal (+/+) mice and (*B*) genetically mast cell–deficient *Sl/Sl^d* vs. congenic normal (+/+) mice. ***p < 0.001 vs. values for contralateral vehicle-injected ears by paired Student's *t* test (two-tailed).

Discussion

rrSCF[164] induced dermal mast cell activation and a mast cell–dependent acute inflammatory response when injected intradermally at doses as low as 140 fmols/site. On a molar basis, rrSCF[164] thus is at least 10-fold more potent as an activator of mouse dermal mast cells than is the classical CTMC agonist substance P (24). Our results indicate that the ability of rrSCF[164] to induce augmented cutaneous vascular permeability, local fibrin deposition, and tissue swelling were primarily, and perhaps entirely, mast cell dependent. Injection of rrSCF[164] into either *W/W^v* or *Sl/Sl^d* mast cell–deficient mice was without significant effect, even when the cytokine was injected at a dose 1,000 times that which induced significant mast cell activation and acute inflammation in +/+ mice. By contrast, rrSCF[164] was able to induce an acute inflammatory response when injected into the skin of *W/W^v* mice repaired of their cutaneous mast cell deficiency by the adoptive transfer of bone marrow cells or BMCMC derived from the congenic normal (+/+) mice.

These observations posed at least two questions. First, by what mechanism does intradermal injection of rrSCF[164] induce mast cell activation? The cytokine could either activate mast cells directly or indirectly, via effects on other cell populations or mediators. And direct effects on the mast cell ei-

ther could be mediated by an interaction between rrSCF[164] and the c-*kit* receptor or could reflect c-*kit* receptor-independent mechanisms. Our experiments with *W/W^v* mice strongly suggest that the ability of rrSCF[164] to induce mast cell activation is a direct effect of the cytokine and requires a functionally active c-*kit* receptor. Thus, rrSCF[164] induced extensive activation of dermal mast cells, as well as the rapid development of fibrin deposition and tissue swelling, in *W/W^v* mice whose skin contained adoptively transferred populations of mast cells derived from the congenic +/+ mice. by contrast, injection of rrSCF[164] into the skin of *W/W^v* mice that contained mast cells of *W/W^v* origin induced neither mast cell activation nor an inflammatory response. Both our previous studies (33, 34) and the present experiments showed that the dermal mast cells of *W/W^v* origin that develop as a result of chronic PMA treatment of the skin of *W/W^v* mice are fully competent to orchestrate IgE-dependent passive cutaneous anaphylaxis responses. The failure of *W/W^v* dermal mast cells to respond to challenge with rrSCF[164], therefore, does not reflect a general inability of these cells to degranulate and release mediators upon appropriate stimulation. Most probably, the unresponsiveness of *W/W^v* dermal mast cells to rrSCF[164] reflects the surface
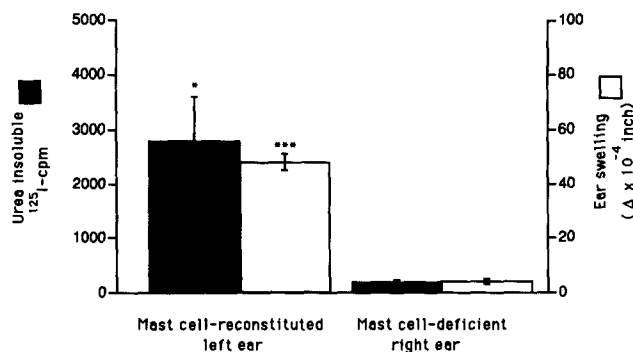
Figure 4. Crosslinked ^{125}I-fibrin–associated cpm (urea-insoluble ^{125}I-cpm) (*filled bars*) and ear swelling (*open bars*) determined 2 h after injection of *E. coli*–rrSCF^{164} into both the mast cell–reconstituted left ears and the mast cell–deficient right ears of W/W^v mice that underwent selective local reconstitution of skin mast cells by injection of BMCMC of congenic +/+ origin into the left ear 10 wk prior to the injection of rrSCF^{164}. *$p <$ 0.05 and ***$p <$ 0.001 vs. values for the contralateral mast cell–deficient ears by the paired Student's t test (two-tailed).

expression by these cells of c-*kit* receptors with markedly impaired tyrosine kinase activity (36).

In contrast to its potency as an activator of dermal mast cells in vivo, rrSCF^{164} is a relatively weak agonist of mast cell activation in vitro (21). Certain populations of in vitro–derived mast cells exhibited modest activation in response to challenge with rrSCF^{164}, but these effects were observed only at concentrations of rrSCF^{164} of 50–500 ng/ml, levels 10- to 100-fold higher than those necessary to induce proliferation of these cells (21). Moreover, rrSCF^{164} did not induce significant [^{3}H]5HT release from freshly purified mouse or rat peritoneal mast cells, even when tested at concentrations of up to 5.0 μg/ml (21). Although the results of in vitro and in vivo experiments should be compared cautiously, 5.0 μg/ml represents an ~30-fold higher concentration of rrSCF^{164} than the lowest concentration necessary to induce significant dermal mast cell degranulation and inflammation when injected intradermally in vivo. There are at least two possible explanations for the apparent differences in the responsiveness of mast cells to challenge with rrSCF^{164} in vitro or in vivo. First, optimal responsiveness to rrSCF^{164} may require a cofactor that is present in the skin in vivo but not in the conditions tested in vitro. Second, responsiveness to rrSCF^{164} as a stimulus of mast cell mediator release may vary according to mast cell phenotype. Although dermal mast cells and peritoneal mast cells are both regarded as examples of CTMC in murine rodents (reviewed in reference 22), these populations differ in size and morphometric characteristics of cytoplasmic granules (37). They may also vary in their responsiveness to rrSCF^{164}, whether because of differences in the amounts of c-*kit* ligand expressed endogenously in the skin as opposed to the peritoneal cavity or because of other factors.

The second major question prompted by our observations concerns the biological relevance of the findings. We have reported that rrSCF^{164} can induce c-*kit*–dependent mast cell proliferation in vitro (3) and in vivo (2), and that rrSCF^{164} also can promote maturation of immature mast cells, as judged
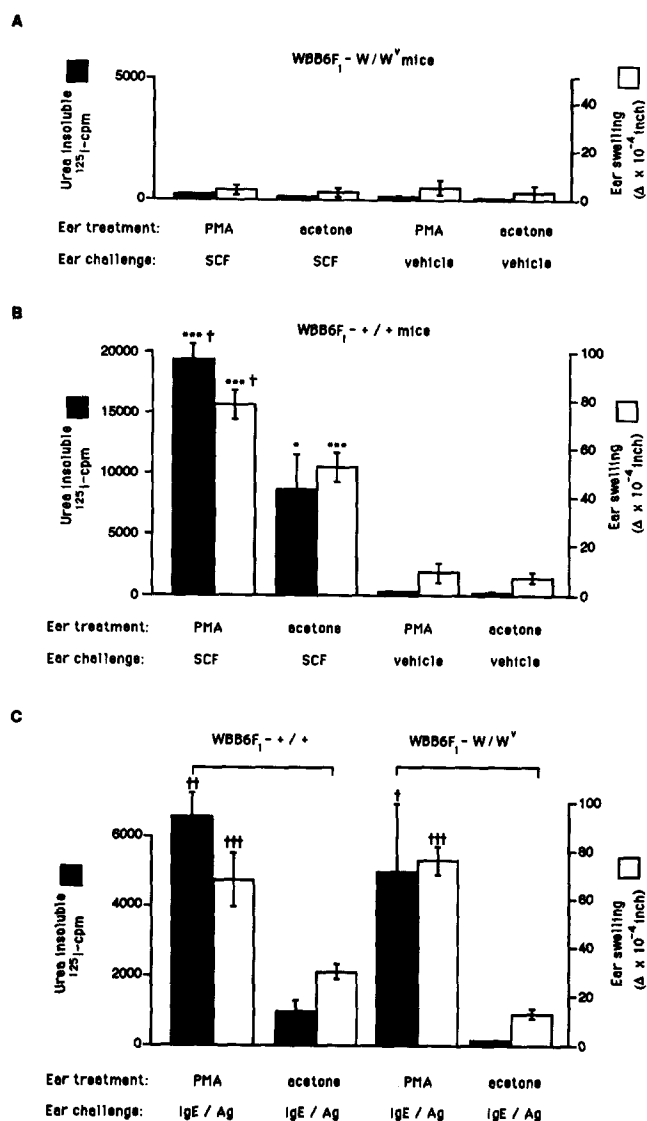
Figure 5. Crosslinked ^{125}I-fibrin–associated cpm (urea-insoluble ^{125}I-cpm) (*filled bars*) and ear swelling (*open bars*) determined 2 h after injection of *E. coli*–rrSCF^{164} (10 μg/kg/site) or vehicle alone (*A* and *B*), or 2 h after challenge for induction of a passive cutaneous anaphylaxis reaction (*C*). In each experiment (*A–C*), the left ears had been treated repeatedly with PMA in acetone, and the right ears with acetone alone (as a control), to induce local development of W/W^v dermal mast cells in the skin of PMA-treated W/W^v mice (*A* and *C*), and to increase numbers of +/+ mast cells locally at PMA-treated sites in the skin of congenic +/+ mice (*B* and *C*) (see Materials and Methods). 16 d after cessation of PMA/acetone treatment, some mice were challenged in both ears by intradermal injection of rrSCF^{164} (*A* and *B*), other mice were challenged in both ears by injection of vehicle alone (*A* and *B*), and other mice were challenged in both ears by injection of a monoclonal anti-DNP IgE, followed, 24 h later, by intravenous injection of specific antigen (DNP$_{30–40}$HSA). (*A*) W/W^v mice do not respond to challenge with rrSCF^{164}, either in PMA-treated ears or acetone-treated ears. (*B*) +/+ mice develop inflammatory reactions in response to rrSCF^{164} (*$p <$ 0.05, or ***$p <$ 0.001 vs. values for identically pretreated, but vehicle-challenged ears) and these reactions are significantly more intense in PMA-treated ears than in acetone-treated skin ($^†p <$ 0.05 vs. values for rrSCF^{164}-injected, acetone-treated ears). (*C*) +/+ mice develop passive cutaneous anaphylaxis reactions that are significantly more intense in PMA-treated ears than in acetone-treated ears; W/W^v mice develop intense passive cutaneous anaphylaxis reactions in PMA-treated ears, but not in acetone-treated ears ($^†p <$ 0.05, $^{††}p <$ 0.01, or $^{†††}p <$ 0.001, respectively, vs. values in identically challenged contralateral acetone-treated ears).

Table 2. *Numbers of Dermal Mast Cells, and Extent of Dermal Mast Cell Activation, in the Skin of Mice Containing* W/W^v *or Congenic* +/+ *Mast Cells 2 h After Intradermal Injection of* E. coli–SCF *(10 μg/kg/site) or Vehicle Alone, or 2 h After Challenge to Induce a Passive Cutaneous Anaphylaxis Reaction*

Mouse genotype	Ear	Treatment	Mast cell genotype	Challenge	Mast cells	Mast cell degranulation		
						None	Moderate	Extensive
					n/mm² dermis	%	%	%
WBB6F₁-W/W^v	L	PMA	WBB6F₁-W/W^v	SCF	36 ± 10	82 ± 2	16 ± 3	1 ± 1
	R	Acetone	None	SCF	0.2 ± 0.2	–	–	–
	L	PMA	WBB6F₁-W/W^v	Vehicle	43 ± 5	85 ± 5	12 ± 3	3 ± 2
	R	Acetone	None	Vehicle	0	–	–	–
	L	PMA	WBB6F₁-W/W^v	IgE/DNP-HSA	24 ± 3	0	6 ± 3	94 ± 3
	R	Acetone	None	IgE/DNP-HSA	0	–	–	–
WBB6F₁-+/+	L	PMA	WBB6F₁-+/+	SCF	111 ± 15	4 ± 1	24 ± 4	72 ± 4 (NS)
	R	Acetone	WBB6F₁-+/+	SCF	78 ± 12	8 ± 2	25 ± 3	65 ± 3
	L	PMA	WBB6F₁-+/+	Vehicle	216 ± 21	89 ± 2	8 ± 1	3 ± 1 (NS)
	R	Acetone	WBB6F₁-+/+	Vehicle	107 ± 15	82 ± 3	13 ± 3	4 ± 2
	L	PMA	WBB6F₁-+/+	IgE/DNP-HSA	116 ± 15	10 ± 3	16 ± 2	74 ± 2 (NS)
	R	Acetone	WBB6F₁-+/+	IgE/DNP-HSA	63 ± 7	22 ± 6	28 ± 3	50 ± 6

These data are from the same experiments depicted in Fig. 5.
NS, not significant ($p > 0.05$) when compared to values for contralateral acetone-treated ears by the χ^2 test.

by several criteria including histamine content and pattern of proteoglycan synthesis in vitro (3) and histochemistry and pattern of protease synthesis in vivo (2). Taken together with the profound mast cell deficiency expressed by both W/W^v and Sl/Sl^d mice (1, 12, 26, 27), these findings leave little doubt that interactions between the c-*kit* receptor and its ligand are essential for normal mast cell development.

But it is more difficult to form a firm conclusion about the physiological importance of SCF as a stimulus of mast cell mediator release. Using different approaches, two groups recently showed that activation of the c-*kit* receptor kinase by ligand binding resulted in a pattern of intracellular signal transduction that included binding and complex formation between the c-*kit* receptor and phosphatidylinositol 3′-kinase (38, 39). However, these reports did not determine whether such c-*kit* receptor–dependent signal transduction resulted in mast cell mediator release. The data presented here indicate that rrSCF^{164} can induce c-*kit* receptor–dependent mast cell activation and mediator release. In addition to demonstrating that mast cell activation via the c-*kit* receptor results in the development of a mast cell–dependent inflammatory response, our findings represent, to our knowledge, the first evidence that administration of the c-*kit* ligand in vivo can stimulate the functional activation of any cell type that expresses the c-*kit* receptor.

However, normal tissues do not ordinarily exhibit significant mast cell degranulation, or express inflammatory responses, without some sort of provocation. If interactions between

the c-*kit* receptor and its ligand importantly contribute to the maintenance of mast cell populations in normal tissues, as seems likely, then such interactions ordinarily must not result in sufficient mast cell activation to induce a perceptible inflammatory response. Several factors may account for the lack of detectable inflammation due to c-*kit* receptor–dependent mast cell activation in normal tissues. C-*kit* ligands exist in cell membrane–associated forms (9, 40, 41) and in soluble forms, such as that represented by rrSCF^{164}, which consist of the first 164 or 165 amino acids of the extracellular domain (4–6, 9, 10, 41). Although both forms of the ligand are biologically active with respect to effects on proliferation (9, 41), it is possible that soluble and membrane-associated forms of SCF may differ in their ability to induce mast cell degranulation. Moreover, no information is available concerning the levels of any forms of the c-*kit* ligand that are present in normal tissues. Nevertheless, the lack of significant mast cell degranulation in normal tissues would indicate that physiologic interactions between c-*kit* receptors expressed by mast cells and endogenous forms of the c-*kit* ligand do not generally result in extensive mast cell degranulation.

On the other hand, the possibility cannot be ruled out that low levels of mast cell mediator release induced by c-*kit* receptor-dependent mast cell activation may have a role in both the development and function of this cell type. Activation of mouse mast cells generated in vitro, via the FcₑRI or other mechanisms, induces the cells to develop increased levels of mRNA for several cytokines and/or to secrete the

products (42–45). Some of these cytokines, such as IL-3 and IL-4, can promote or augment mast cell proliferation, whereas others, such as granulocyte/macrophage CSF and IFN-γ, can suppress mast cell proliferation (reviewed in references 1, 22, 46, and 47). Recently, Razin et al. (48) reported evidence that both BMCMC and mouse peritoneal mast cells developed increased levels of mRNA for IL-3 after establishing contact with 3T3 fibroblasts in vitro. Several lines of evidence indicate that the ability of 3T3 fibroblasts to induce mouse mast cell proliferation and maturation requires an interaction between a c-kit receptor ligand expressed by the 3T3 cells and functionally active c-kit receptors expressed by the mast cells (reviewed in references 1–3, and 12). Taken together with the findings presented here, these observations raise the possibility that one of the mechanisms by which SCF influences mast cell development is to induce mast cells to produce other cytokines with autocrine effects on mast cell proliferation and/or maturation.

In addition, there has been a long history of speculation that one "physiological" role of the mast cell is to release constitutively very low levels of the same mediators which, when released rapidly in larger quantities, result in the development of an inflammatory response (reviewed in reference 49). Although our data by no means prove that physiological interactions between the c-kit receptor and its endogenous ligands actually result in low levels of mast cell mediator release in normal tissues, they nevertheless can be considered compatible with this hypothesis. Furthermore, our findings suggest that conditions that result in high local concentrations of soluble c-kit ligand, whether of endogenous or exogenous origin, may be associated with significant mast cell activation in murine rodents. It will be important to determine whether the same is true for human mast cells, since this information will clearly influence the development of plans for any clinical applications of this cytokine.

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