Lack of significant skin inflammation during elimination by apoptosis of large numbers of mouse cutaneous mast cells after cessation of treatment with stem cell factor

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We previously reported that subcutaneous (s.c.) administration of stem cell factor (SCF), the ligand for the c-Kit receptor, to the back skin of mice promotes marked local increases in the numbers of cutaneous mast cells (MCs), and that cessation of SCF treatment results in the rapid reduction of cutaneous MC populations by apoptosis. In the present study, we used the ¹²⁵I-fibrin deposition assay, a very sensitive method for quantifying increased vascular permeability, to assess whether the clearance of large numbers of apoptotic MCs is associated with significant cutaneous inflammation. The s.c. injection of rrSCF¹⁶⁴ (30 or 100 µg/kg/day) or rrSCF¹⁶⁴-peg (polyethylene glycol-treated SCF, 30 or 100 µg/kg/day) for 23 days increased the numbers of dermal MCs at skin injection sites from 5.1 ± 0.7 MCs/mm² to 36.4 ± 4.1 , 34.7 ± 9.7 , 52.5 ± 5.8 , and 545 ± 97 MCs/mm², respectively. In contrast, MC numbers were markedly lower in mice that had been treated with SCF for 21 days, followed by 2 days of injection with the vehicle alone. Notably, when tested during the period of rapid reduction of skin MCs, ¹²⁵I-fibrin deposition in the skin was very similar to that in mice receiving continuous treatment with SCF or vehicle. We conclude that the rapid elimination of even very large populations of MCs by apoptosis, which also results in the clearance of the considerable quantities of proinflammatory products stored by these cells, does not lead to significant local cutaneous inflammatory responses.

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It is widely believed that apoptosis, or programmed cell death, can result in the elimination of cell populations without inducing an associated inflammatory response.^{1–3} Yet few studies have actually tested this hypothesis by using sensitive methods to search for, and to quantify, evidence of local inflammation in tissues that include large populations of cells that are undergoing apoptosis.

Therefore, we searched for evidence of inflammation at cutaneous sites that contained greatly expanded populations of dermal mast cells (MCs) that were undergoing apoptosis after the withdrawal of treatment with recombinant rat stem cell factor (rrSCF).⁴ We selected this model for three reasons. First, MCs are arguably among the most ‘proinflam-

matory’ of the cell types that might be examined to assess the potential effects of apoptosis on the induction of tissue inflammation. In addition to being able to synthesize potent proinflammatory lipid mediators and cytokines upon appropriate activation, MCs contain preformed stores of biogenic amines (histamine, and, in mice and rats, serotonin) and other inflammatory mediators in their prominent cytoplasmic granules.^{5,6} Accordingly, it is thought that agents that induce MC injury or necrosis can cause the local development of an inflammatory response in parallel with the release of MC cytoplasmic granule contents to the interstitial space.^{5,6} Indeed, inflammation can also be induced by the intracutaneous injection of cytoplasmic granules that have been purified from MCs.⁷

Second, we previously reported that the repetitive subcutaneous (s.c.) injection of mice with polyethylene glycol-modified rrSCF resulted in a marked expansion of tissue MC populations,^{4,8} and that the numbers of dermal MCs in cutaneous sites injected with rrSCF rapidly diminished after cessation of

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rrSCF treatment.^{4,8} Both studies of the effects of stem cell factor (SCF) on mouse MC apoptosis *in vitro*,⁴ which assessed MC apoptosis both by morphology and by detection of the characteristic pattern of DNA fragmentation associated with apoptosis, and our histological examination of the sites of repetitive s.c. rrSCF injection *in vivo*,⁴ which documented the presence of apoptotic MCs (as well as occasional adjacent eosinophils or macrophages) in sections obtained 24 or 36 h after stopping SCF treatment, indicated that the rapid fall in dermal MC numbers, which occurred upon cessation of rrSCF treatment *in vivo*, reflected the apoptosis of such MCs. These studies thus established an *in vivo* model system that can be used to examine the extent to which the massive loss of dermal MC populations via apoptosis might result in the induction of a cutaneous inflammatory response.

Third, several studies indicate that the extravasation of intravenously administered ¹²⁵I-fibrinogen, as well as the local interstitial deposition of cross-linked ¹²⁵I-fibrin, represents an extremely sensitive method for detecting and quantifying the enhanced vascular permeability that is associated with inflammatory responses elicited in mouse skin.^{9–13} Such responses include cutaneous contact hypersensitivity reactions,⁹ IgE-dependent passive cutaneous anaphylaxis responses¹⁰ and reactions to the intradermal injection of substance P¹¹ or SCF¹² or the epicutaneous application of PMA.¹³ For example, in tests of cutaneous contact hypersensitivity reactions, the ¹²⁵I-fibrin deposition assay was more sensitive than standard measurements of tissue swelling in detecting weak responses.⁹

In the present study, we assessed the local extravasation of ¹²⁵I-fibrinogen and deposition of crosslinked ¹²⁵I-fibrin, and performed histological analysis, to search for evidence of cutaneous inflammation at sites in which dermal MC numbers were dropping dramatically after cessation of treatment with rrSCF. The results strongly support the conclusion that even greatly expanded populations of mouse MCs can be eliminated by apoptosis in the virtual absence of any induction of a significant local inflammatory response.

Materials and methods

Animals

C57BL/6 mice (male, 6–8 weeks old at the beginning of the experiments) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were kept in community cages at the Beth Israel Deaconess Medical Center Animal Care Facilities at light periods of 12 h and were fed water and mouse chow *ad libitum*. All animal experiments were conducted in accordance with the guidelines of the NIH and the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Elicitation of MC Apoptosis

To induce increased numbers of cutaneous MC populations, mice received for either 3 weeks or 3 months daily s.c. injections (always to the same flank site) of vehicle alone, *Escherichia coli*-derived rrSCF¹⁶⁴ or rrSCF¹⁶⁴, which had been modified by the covalent attachment of polyethylene glycol (ie, rrSCF¹⁶⁴-peg), to increase the biological half-life of the cytokine, at 30 or 100 µg/kg in 150–250 µl of vehicle (vehicle = sterile saline containing 0.1% bovine serum albumin (fraction V, fatty acid free: ICN Immunobiologicals, Lisle, IL, USA)). To assess the effects of a rapid reduction of MC numbers, in some mice (the 'SCF withdrawal' group), the injections with rrSCF¹⁶⁴ or rrSCF¹⁶⁴-peg were then stopped and these mice received subsequent s.c. injections of vehicle (into the same sites used for rrSCF or rrSCF-peg treatment) at ~6 and 30 h before the mice were killed (see below). Other mice continued to receive injections of rrSCF or rrSCF-peg (the 'SCF continued' group) or of vehicle alone. In some experiments, skin histamine content was determined using a Histamine Enzyme Immunoassay Kit (Immunotech, Miami, FL, USA) according to the manufacturer's instructions.

Induction of Cutaneous Reactions to Histamine or IgE and Antigen

In some experiments, cutaneous sites in mice in the SCF or vehicle treatment groups were injected intradermally with histamine (2 µg in 20 µl of vehicle) or vehicle (sterile saline, 20 µl) alone. In other studies, cutaneous sites in mice in the SCF or vehicle treatment groups were injected intradermally with a mouse monoclonal IgE with specificity for DNP¹⁴ at 100 ng in 20 µl of vehicle or 20 µl vehicle alone, and the mice were injected approximately 24 h later intravenously with 200 µg of DNP-HSA in 100 µl of vehicle to induce IgE-dependent passive cutaneous anaphylaxis reactions.

Assessment of Skin Inflammation by Measurement of ¹²⁵I-fibrinogen Extravasation and ¹²⁵I-fibrin Deposition

At 4 h before they were killed (eg, in the SCF withdrawal group, 50 hours after the cessation of SCF treatment), mice were injected i.v. with 3×10^6 counts per minute (cpm) of ¹²⁵I-guinea-pig fibrinogen. After the mice were killed by cervical dislocation, ¹²⁵I-fibrinogen extravasation and ¹²⁵I-fibrin deposition were assessed as previously described.⁹ Briefly, mice received i.v. injections with a mixture of antifibrinolytic agents and anticoagulants to impede additional clotting or fibrinolysis during specimen preparation. Each mouse received 0.2 ml of a mixture containing 80 U heparin, 20 mg D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (P-PAC), 2 mg ε-aminocaproic acid (EACA),

and 56U Trasylol, all in 0.15 M sterile NaCl. Immediately thereafter, mice were anesthetized with ether, and a sample of retro-orbital 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 plasma for radioactive counting. One 6-mm punch biopsy from the skin injection site and a skin control site and half the spleen were 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), P-PAC (5 mg/ml), EDTA (2 mg/ml), EACA (0.1 M), Trasylol (10 U/ml), 2 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetate and 2 mM *N*-ethylmaleimide. The minced tissues were then extracted in the buffer by keeping the tubes for 18 h at 4°C. Each tube was centrifuged (1000 *g* for 20 min at 20°C), and the supernatant decanted. The pellet containing the tissue fragments was resuspended vigorously in 2 ml of the same buffer and was centrifuged again as described before. The supernatant was aspirated and was pooled with the earlier extract for radioactive counting as the aqueous extract. The centrifuged tissue fragments were then resuspended in 2 ml freshly prepared 3 M urea, extracted for 2 h at 37°C, centrifuged (1000 *g* for 20 min at 20°C), and the urea-soluble supernatant and the urea-insoluble pellet were counted for radioactivity in a gamma counter.

Histologic Studies

Specimens from the biopsies taken from the skin injection sites and from the contralateral control sites (which had received no injections), and specimens from the spleens, were transferred to Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.025% CaCl_2 in 0.1 M cacodylate buffer, pH 7.4, for 12 h at 4°C) or Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid, for 4 h at 4°C). The presence of tissue MCs was assessed in 1 μm , Epon-embedded, Giemsa-stained sections as previously described.¹³ Briefly, sections were coded so that the observer was not aware of the identity of individual specimens and examined at $\times 1000$ by light microscopy to quantify MCs/mm² of dermis or spleen parenchyma.

Statistical Analysis

The data were analyzed for statistical significance using the unpaired two-tailed Student's *t*-test to compare values derived from mice from the various treatment groups, and using the paired two-tailed Student's *t*-test to compare values from skin injection and contralateral control sites of the individual mice.

Results

S.c. Injections with rrSCF¹⁶⁴ or rrSCF¹⁶⁴-peg Result in Increased Numbers of Cutaneous and Spleen MCs and Cessation of Treatment with rrSCF¹⁶⁴-peg Induces Rapid Reductions of Skin MC Populations

In confirmation of our previous studies employing WCB6F₁ mice,^{4,8} we found that the chronic treatment of C57BL/6 mice with daily s.c. injections of polyethylene glycol-treated rrSCF¹⁶⁴ (rrSCF¹⁶⁴-peg) for 23 days resulted in increased numbers of MCs at the site of injection, in untreated control skin and the spleen (Figures 1c, f and 2).

We also assessed whether rrSCF¹⁶⁴ that had not been treated with polyethylene glycol also can induce increased MC numbers and whether prolonged treatment with rrSCF¹⁶⁴-peg for 92 days can further enhance the increase in MC numbers over that observed after a 23-day treatment period. Figure 2 shows that injections of rrSCF¹⁶⁴ at 30 or 100 $\mu\text{g}/\text{kg}/\text{day}$ also resulted in increased MCs at the site of injection and a contralateral skin control site as compared to continued injections with vehicle. When rrSCF¹⁶⁴-peg (100 $\mu\text{g}/\text{kg}/\text{day}$) was injected daily for 92 days, MC numbers increased markedly as compared to that observed with continued injection of vehicle. However, only the increase of MC numbers in the spleen was enhanced as compared to that observed after a 23-day treatment period with the same dose of rrSCF¹⁶⁴-peg (Figure 2).

When injections of rrSCF¹⁶⁴ or rrSCF¹⁶⁴-peg were discontinued for 54 h, MC numbers in the skin at the injection sites were significantly lower (by >50%) in mice that had been treated with rrSCF¹⁶⁴-peg at 100 $\mu\text{g}/\text{kg}/\text{day}$ for 21 or 90 days (reductions of ~ 356 or ~ 205 MC/mm², respectively) and in mice that had been treated with rrSCF¹⁶⁴ at 30 $\mu\text{g}/\text{kg}/\text{day}$ for 21 days (a reduction of ~ 23 MC/mm²), as compared to mice that received continued injections with SCF (ie, for a total of 23 or 92 days) (Figures 1b, e and 2). In these groups, reductions of MC numbers upon cessation of rrSCF¹⁶⁴-peg treatment also occurred in the skin control sites that had not been directly injected with rrSCF¹⁶⁴-peg (reductions of ~ 32 or 20 MC/mm² for mice treated with 100 $\mu\text{g}/\text{kg}/\text{day}$ for 21 or 90 days, respectively) or rrSCF¹⁶⁴ (a reduction of ~ 11 MC/mm² for mice treated with 30 $\mu\text{g}/\text{kg}/\text{day}$) but not in the spleens. By contrast, a 54 h cessation of SCF treatment did not result in significant reductions in MC numbers in the skin of mice treated with rrSCF¹⁶⁴ at 100 $\mu\text{g}/\text{kg}/\text{day}$ or rrSCF¹⁶⁴-peg at 30 $\mu\text{g}/\text{kg}/\text{day}$ for 21 days, compared to the corresponding values in mice that received continued treatment with SCF for 23 days (Figure 2). Accordingly, we used mice treated with rrSCF¹⁶⁴-peg at 100 $\mu\text{g}/\text{kg}/\text{day}$ for the majority of the other studies reported herein.

We also determined whether the increase of skin MCs after chronic treatment with SCF was associated with an increase of tissue histamine content

Figure 1 S.c. injection with rrSCF¹⁶⁴-peg at 100 μ g/kg/day for 23 days results in increased numbers of cutaneous MCs and cessation of treatment on day 21 induces rapid reductions of skin mast cell populations. Alcian Blue staining of skin sections at injection sites of rrSCF¹⁶⁴-peg (100 μ g/kg/day) or vehicle demonstrates markedly increased numbers of dermal MCs after 23 days of injection with rrSCF¹⁶⁴-peg (c + f; 'SCF-continued') as compared to skin of mice treated only with vehicle (a + d; 'vehicle-continued'). When SCF-injections were discontinued after 21 days, MC numbers dropped rapidly within the next 54 h (b + e; 'SCF withdrawal'). a–c = $\times$ 100 magnification, d–f = $\times$ 400 magnification; 4- μ m paraffin-embedded, Alcian blue-stained sections with eosin counterstain.

and whether the reduction of MCs after cessation of SCF injections was paralleled by a decrease of histamine levels. We examined skin histamine content at the site of daily injections with rrSCF¹⁶⁴-peg (100 μ g/kg/day) for 21 days at 54 h after discontinuation of SCF injections ('SCF withdrawal' group) or after continued injections with SCF, for a total of 23 days ('SCF continued' group) (Figure 3). In mice that received rrSCF¹⁶⁴-peg for a total of 23

days, skin histamine levels at the site of injection and untreated control skin were markedly increased as compared to values in vehicle-treated mice. When SCF injections were stopped after 21 days, the skin histamine content at the site of injection and at the control site dropped rapidly and significantly (by >50%) within the next 54 h (Figure 3). The reductions in skin histamine content and numbers of cutaneous MCs upon cessation of

Figure 2 Numbers of cutaneous and spleen MCs in mice treated with various amounts of rrSCF¹⁶⁴ or rrSCF¹⁶⁴-peg. Numbers of MCs in the dermis at the skin injection site or contralateral control skin site or spleen, of C57BL/6 mice that had been injected with vehicle, rrSCF¹⁶⁴ (30 or 100 µg/kg/day), or rrSCF¹⁶⁴-peg (30 or 100 µg/kg/day, s.c., *n* = 6 per group). After 21 or 90 days of treatment, injections of SCF were discontinued ('SCF withdrawal') to induce MC apoptosis or continued ('SCF continued') and mice were killed after 54 h for morphometric quantification of MCs. Bars represent mean ± s.e.m. of MC numbers per mm² of tissue (alkaline Giemsa, × 1000); for low values, only mean numbers of MCs are shown. Significant differences vs control ('vehicle continued') mice are shown as **P* < 0.05, ***P* < 0.005, ****P* < 0.001 (Student's *t*-test for unpaired samples, two-tailed). Significant differences between 'SCF withdrawal' and 'SCF continued' mice in the same SCF dose groups are shown as **P* < 0.05, ***P* < 0.005, ****P* < 0.001 (Student's *t*-test for unpaired samples, two-tailed), as indicated by the comparison bars.

SCF treatment were quite similar in magnitude (cf. results in Figures 2 and 3). These findings confirm that reductions in cutaneous MC numbers by

apoptosis indeed result in parallel reductions of an inflammatory mediator that is stored in the MC granules.

Figure 3 Clearance of apoptotic skin MCs after cessation of chronic treatment with SCF-peg at 100 µg/kg/day is associated with a reduction of skin histamine content. Changes in histamine content in the skin at SCF or vehicle injection sites and contralateral control skin sites (no injections) of C57BL/6 mice after cessation of chronic treatment with vehicle or rrSCF¹⁶⁴-peg (100 µg/kg/day) for 21 days (s.c., $n = 4$ per group, to induce MC apoptosis, = 'SCF withdrawal') or after continued injections of vehicle ('vehicle continued') or after daily injections with SCF for 23 days ('SCF continued'). At 24 h and 48 h after the last (21st) injection, mice in the SCF withdrawal and Vehicle groups received an injection with vehicle and mice in the SCF continued group received an injection with SCF. At 54 h after the 21st injection, all mice were killed and a 6-mm punch biopsy was taken from the site of injection and a contralateral control (no injection) site. Biopsies were weighed and histamine was extracted and measured by EIA. Bars represent means $\pm$ s.e.m. Statistical significance of control (no injection) vs injection sites of animals within individual treatment groups: paired Student's *t*-test, two-tailed ($^+P < 0.05$, $^{+++}P < 0.005$). Statistical significance of differences between injection or control sites in different treatment groups: unpaired Student's *t*-test, two-tailed ($^*P < 0.05$, $^{**}P < 0.01$ for SCF continued vs SCF withdrawal; $^{***}P < 0.005$ for SCF continued or SCF withdrawal vs vehicle continued).

Figure 4 Induction of MC apoptosis by cessation of chronic treatment with SCF is not associated with increased vascular permeability. (a) ¹²⁵I-Fibrinogen-derived CPM in biopsies of skin injection sites, contralateral skin control sites (no injections) and spleens of C57BL/6 mice after cessation of chronic treatment with rrSCF¹⁶⁴ (30 or 100 µg/kg/day, in two different experiments, for 21 days, s.c., $n = 5$) to induce MC-apoptosis ('SCF withdrawal') or after continued injections of SCF ('SCF continued'). At 24 and 48 h after the last (21st) injection with SCF, mice in the SCF withdrawal group received an injection with vehicle and mice in the SCF continued group received an injection with SCF (ie, for a total of 23 days of treatment with SCF). At 50 h after the 21st injection with SCF, mice in both groups received ¹²⁵I-fibrinogen i.v. and the amount and pattern of extractability of ¹²⁵I-CPM were determined 4 h later. Numbers in parentheses over the bars = mean value for skin injection site divided by the corresponding value for the control skin site. At the time of killing, the ¹²⁵I-CPM in 50 µl of serum in the two experiments were as follows: SCF withdrawal (30 µg/kg), 35 597 $\pm$ 4622; SCF continued (30 µg/kg), 29 752 $\pm$ 2375; SCF withdrawal (100 µg/kg), 19 598 $\pm$ 2621; SCF continued (100 µg/kg), 21 715 $\pm$ 5157. (b) ¹²⁵I-Fibrinogen-derived CPM in biopsies of skin injection sites, contralateral skin control sites (no injections) and spleens of C57BL/6 mice after cessation of chronic treatment with rrSCF¹⁶⁴-peg (30 µg/kg/day for 21 days, s.c., $n = 5$) to induce MC-apoptosis ('SCF withdrawal') or after continued injections of SCF ('SCF continued') or after daily injections of vehicle ('vehicle continued'). At 24 and 48 h after the last (21st) injection with SCF or vehicle, mice in the SCF withdrawal and vehicle continued groups received an injection with vehicle and mice in the SCF continued group received an injection with SCF (ie, for a total of 23 days of treatment with SCF). At 50 h after the 21st injection with SCF or vehicle, mice in all groups received ¹²⁵I-fibrinogen i.v. and the amount and pattern of extractability of ¹²⁵I-CPM were determined 4 h later. Numbers in parentheses over the bars = mean value for skin injection site divided by the corresponding value for the control skin site. Statistical significance of differences of skin injection sites vs control sites in the same treatment groups: paired Student's *t*-test, two-tailed ($^{**}P < 0.01$, $^{***}P < 0.005$). At the time of killing, the ¹²⁵I-CPM in 50 µl of serum were as follows: SCF withdrawal, 2317 $\pm$ 173; SCF continued, 2377 $\pm$ 206; vehicle continued, 2556 $\pm$ 207. (c) ¹²⁵I-species in skin injection sites, contralateral skin control sites (no injection) and the spleens of C57BL/6-mice after cessation of chronic treatment with rrSCF¹⁶⁴-peg (100 µg/kg/day for 21 or 90 days, s.c., $n = 5$) to induce MC apoptosis ('SCF withdrawal') or after continued injections of SCF ('SCF continued'). At 24 and 48 h after the last (21st or 90th) injection with SCF, mice in the SCF withdrawal and SCF continued groups received an injection with vehicle or SCF (ie, for a total of 23 or 92 days of treatment with SCF), respectively. At 50 h after the 21st or 90th injection with SCF, mice in both groups received ¹²⁵I-fibrinogen i.v. and the amount and pattern of extractability of ¹²⁵I-CPM were determined 4 h later. Numbers in parentheses over the bars = mean value for skin injection site divided by the corresponding value for the control skin site. Statistical significance of differences of skin injection sites vs skin control sites in the same treatment groups: paired Student's *t*-test, two-tailed ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). For skin injection sites in mice treated with rrSCF¹⁶⁴-peg at 100 µg/kg/day for 21 days, all comparisons for values in the SCF continued vs SCF withdrawal groups are $P \sim 0.07$ – 0.36 . At the time of killing, the ¹²⁵I-CPM in 50 µl of serum were as follows: SCF withdrawal (21 days of treatment), 2216 $\pm$ 304; SCF continued (21 days of treatment), 2162 $\pm$ 294; SCF withdrawal (90 days of treatment), 904 $\pm$ 40; SCF continued (90 days of treatment), 800 $\pm$ 51.

Induction of MC apoptosis by Cessation of Chronic Treatment with SCF is not Associated with Increased Vascular Permeability

To assess whether the rapid clearance of markedly increased MC populations after cessation of chronic injections with SCF is associated with increased vascular permeability, the extravasation

of radio-labeled fibrinogen was measured at the skin injection site, a skin control site that had not been subject to injection, and the spleen in mice treated with rrSCF¹⁶⁴ (30 or 100 µg/kg/day for 21 or 23 days) and with rrSCF¹⁶⁴-peg (30 or 100 µg/kg/day for 23 or 92 days) (Figure 4a–c).

In all treatment groups except rrSCF¹⁶⁴ at 30 $\mu\text{g/kg/day}$ for 21 days, values for crosslinked (urea insoluble) ¹²⁵I-fibrin, and in many instances other ¹²⁵I-fibrinogen-derived species, were higher in the skin sites that had been injected with SCF (skin injection sites) than in skin sites in the same mice that had not been directly injected with SCF (ie, skin control sites) (Figure 4a–c). In some instances, these differences achieved statistical significance. However, there were no statistically significant differences in the values for mice in the SCF continued vs the SCF withdrawal groups, whether one considers skin sites directly injected with SCF, control skin sites not injected with SCF, or the spleen (Figure 4a–c). This was true even for the groups in which the most striking changes in skin MC numbers occurred upon administration and then withdrawal of SCF, that is, those mice treated with rrSCF¹⁶⁴-peg at 100 $\mu\text{g/kg/day}$ for 21 or 90 days (Figure 4c).

In one set of experiments, we included a group of mice that was treated with vehicle alone ('vehicle continued' group in Figure 4b) instead of rrSCF¹⁶⁴-peg at 30 $\mu\text{g/kg/day}$ for 21 ('SCF withdrawal' group) or 23 ('SCF continued' group) days (Figure 4b). Such vehicle-injected mice exhibited values for ¹²⁵I-fibrin and other ¹²⁵I-fibrinogen-derived species, in skin injection sites, control skin sites and spleen, that were statistically indistinguishable from those in the SCF continued or SCF withdrawal groups. In the SCF continued group, values for ¹²⁵I-fibrin and other ¹²⁵I-fibrinogen-derived species in skin injection sites were significantly higher than those in the corresponding control skin sites (Figure 4b). However, none of the values in the SCF continued group was significant when compared to the corresponding data for the SCF withdrawal or vehicle-treated groups.

We think that the simplest interpretation of all of our results is that the presence of a greatly expanded population of cutaneous MCs in SCF-treated mice may result in slightly enhanced local cutaneous vascular permeability, but that the rapid reduction of such populations of cutaneous MCs by apoptosis upon the withdrawal of SCF treatment results in no significant further enhancement of this effect.

Effect of Chronic Treatment with SCF, or SCF Withdrawal, on Cutaneous Responses to Histamine or IgE and Antigen

In one experiment, we attempted to elicit IgE-dependent passive cutaneous anaphylaxis reactions in mice that had been treated with rrSCF¹⁶⁴-peg at 30 $\mu\text{g/kg/day}$ or with vehicle alone for 21 days, and in mice that were left untreated for 21 days. IgE anti-DNP was injected into the skin at SCF injection sites 6 h after the injection of SCF or vehicle on the day before the end of the experiment (ie, day 20 of SCF or vehicle injection) and DNP-HSA was injected i.v.

the next day, 24 h after the mice received their last injection of SCF or vehicle. Although injection of IgE and antigen resulted in a significant enhancement of vascular permeability in the SCF injection sites compared to sites in control mice injected with vehicle rather than IgE before antigen challenge, the values were significantly lower than those in mice that had been injected repeatedly at these sites with vehicle rather than SCF (total cpm: 2112 ± 376 vs 4734 ± 537 per biopsy, urea insoluble cpm: 714 ± 121 vs 1612 ± 241 , respectively, $P < 0.05$). This finding is consistent with our previous observation that mice treated chronically with rrSCF¹⁶⁴ can exhibit both markedly increased numbers of tissue MCs and, paradoxically, *reduced* pathological consequences of systemic IgE-dependent reactions.¹⁴

rrSCF¹⁶⁴-peg can induce MC degranulation upon acute injection into the skin of normal mice.¹² Therefore, it is possible that repeated injections of rrSCF¹⁶⁴-peg can result in such sites exhibiting tachyphylaxis to the effects of mast cell mediators. To test this hypothesis, we injected histamine or vehicle alone intradermally into the skin injection sites or skin control sites of mice that had been injected with rrSCF¹⁶⁴-peg at 100 $\mu\text{g/kg/day}$ for 21 days (in the 'SCF withdrawal' group) or 23 days, (in the 'SCF continued' group), or with vehicle alone. As shown in Figure 5, we found that histamine induced significantly more enhancement of vascular permeability, as assessed by quantification of ¹²⁵I-fibrinogen-derived species, than did vehicle, whether one evaluated the responsiveness of skin injection sites or skin control sites in the SCF continued, SCF withdrawal or vehicle continued groups. However, many of the ¹²⁵I-cpm values in the histamine injections sites were slightly lower in the SCF continued or the SCF withdrawal groups than in the vehicle continued group. These results are consistent with the possibility that some tachyphylaxis to histamine can occur at cutaneous sites of mice that have been chronically injected with rrSCF¹⁶⁴-peg.

Discussion

The dermis of the mouse ordinarily is extremely sensitive to the proinflammatory effects of MC activation. In a previous report, we found that the induction of minimal degranulation of skin MCs (10 and 28% of MCs exhibiting morphological evidence of extensive or moderate degranulation, respectively) was sufficient to induce near-maximal levels of tissue swelling, as well as significant deposition of ¹²⁵I-fibrin.¹² Increasing levels of dermal MC activation resulted in progressively increasing amounts of local ¹²⁵I-fibrin deposition.¹² In the prior study, MC degranulation was induced in otherwise normal mice by the acute intradermal injection of rrSCF; thus, these mice had normal, or 'baseline', levels of dermal MCs.¹²

Figure 5 Reduced histamine-induced enhancement of vascular permeability at SCF cutaneous injection sites and control skin sites in mice that have been chronically treated with SCF. ^{125}I -species in skin injection (a) and skin control (non-injected) sites (b) of C57BL/6-mice ($n = 5/\text{group}$) injected with rrSCF 164 -peg at $100 \mu\text{g}/\text{kg}/\text{day}$ for 23 days ('SCF continued') or for 21 days followed by a 2-day treatment with vehicle ('SCF withdrawal') to induce MC apoptosis, or of mice injected with vehicle for 23 days ('vehicle continued'). On day 23, mice from all treatment groups as well as untreated control mice (Control, c) received intradermal injections of histamine ($2 \mu\text{g}$ in $20 \mu\text{l}$) or vehicle alone and the amount and pattern of extractability of ^{125}I -cpm were determined 2 h later. Unpaired two-tailed Student's t -test: $^{\#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.005$ for histamine vs vehicle; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.005$ for SCF withdrawal or SCF continued vs vehicle continued. At the time of killing, the ^{125}I -CPM in $50 \mu\text{l}$ of serum of histamine-injected mice as compared to vehicle-injected mice were $26\,730 \pm 5579$ vs $40\,216 \pm 1962$ (SCF withdrawal), $40\,247 \pm 1456$ vs $48\,734 \pm 1966$ (SCF continued) and $41\,979 \pm 2794$ vs $55\,575 \pm 1980$ (vehicle continued), respectively.

In the present study, withdrawal of chronic treatment with rrSCF resulted in the loss, by apoptosis, of a population of dermal MCs that was, depending on the rrSCF treatment protocol examined, from 7 to 65 times greater than that present normally in the dermis. Yet this marked reduction in dermal MC populations after cessation of rrSCF treatment in rrSCF-treated mice occurred with little or no local enhancement of dermal vascular permeability over that detected in corresponding sites in mice that remained on treatment with rrSCF, as assessed by the ^{125}I -fibrin deposition assay, nor did it induce significant local infiltration of circulating leukocytes (Iemura *et al.*⁴ and histological observations from this study, not shown).

In contrast to the results obtained at sites of massive MC apoptosis, the IgE-dependent activation of the expanded populations of dermal MCs at the sites of chronic injection of rrSCF induced significant local augmentation of ^{125}I -fibrin deposition. In addition to demonstrating that MCs in mice chronically treated with rrSCF are capable of producing

proinflammatory mediators upon appropriate IgE- and antigen-dependent activation, this finding also showed that the local vasculature at sites of chronic treatment with rrSCF retained responsiveness to MC-derived mediators of enhanced vascular permeability. On the other hand, the induction of IgE-dependent passive cutaneous anaphylaxis induced significantly greater levels of ^{125}I -fibrin deposition in mice chronically injected with vehicle than in those chronically treated with rrSCF, even though the latter mice contained much higher numbers of dermal MCs. Moreover, responsiveness to histamine, as judged by the ^{125}I -fibrin deposition assay, was also slightly reduced in rrSCF-treated as opposed to vehicle-treated mice.

We cannot rule out the possibility that tachyphylaxis, induced by chronic, presumably low level, extracellular release of biogenic amines from MCs, may have contributed to the low levels of vascular permeability at sites of rrSCF treatment. At least two mechanisms theoretically could have contributed to the occurrence of low levels of histamine release at

such sites. In the 'SCF withdrawal' group, some histamine and serotonin may have been released extracellularly from MCs undergoing apoptosis upon withdrawal of SCF. In the 'SCF continued' group, injections of SCF might have induced c-kit-dependent degranulation and release of biogenic amines from *viable* MCs. However, it bears emphasis that mechanisms other than (or, in addition to) tachyphylaxis to biogenic amines certainly may have contributed to the relatively low levels of vascular permeability and/or reduced responsiveness to injected histamine that were detected at sites of chronic injection with SCF.

Using a very different model system, Tokita and Yamamoto¹⁵ recently reported that, upon intradermal injection into the skin of guinea-pigs, necrotic HL-60 cells enhanced local extravasation of plasma whereas apoptotic HL-60 cells had little or no effect. In the present study, we investigated the effects, on local vascular permeability (as assessed by ¹²⁵I-fibrinogen extravasation and ¹²⁵I-fibrin deposition), of the induction of apoptosis in *native* dermal MC populations whose numbers had been greatly expanded by the repetitive injection of the MC growth/survival factor, SCF. Our findings clearly show that the very large population of dermal MCs induced to develop by repetitive treatment with rrSCF can be eliminated by apoptosis under conditions that result in little or no detectable local enhancement of local vascular permeability. Our results thus provide dramatic evidence that apoptosis can indeed result in the removal of even extremely large endogenous populations of highly 'proinflammatory' cells without the concomitant induction of a significant inflammatory response.

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