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Stem Cell Factor Is Localized in, Released from, and Cleaved by Human Mast Cells

Amato de Paulis,* Giuseppina Minopoli, † Eloisa Arbustini, § Gennaro de Crescenzo,* Fabrizio Dal Piaz, ‡ Piero Pucci, ‡ Tommaso Russo, § and Gianni Marone 2 *

Stem cell factor (SCF) is the most important cytokine regulating human mast cell growth and functions. The immunogold technique showed SCF in the secretory granules of skin mast cells and in lung parenchymal mast cells (HLMC). Immunoreactive SCF (iSCF) was detected in cell lysates of HLMC, but not in basophils; iSCF and histamine were detected in supernatants of HLMC 3 min after challenge with anti-FcεRI or anti-IgE, and iSCF in supernatants rapidly declined after 30 min, whereas histamine remained unchanged for 120 min. HPLC and electrospray mass spectrometry (ES/MS) analysis of recombinant human SCF1–166 (18,656.9 ± 0.9 Da) treated with chymase showed a polypeptide of 17,977.1 ± 0.6 Da and a minor component of 697.4 ± 0.1 Da generated by specific cleavage at Phe159. SCF1–166 and SCF1–159 similarly activated HLMC, potentiated anti-IgE-induced activation of these cells, and stimulated HLMC chemotaxis. SCF1–159–166 had no effect on mast cells. Western blot analysis of supernatants of anti-IgE-activated HLMC incubated with recombinant human SCF1–166 showed that SCF1–166 was rapidly cleaved to SCF1–159 and SCF1–144. Experiments with supernatants of anti-IgE-activated HLMC incubated with SCF1–166 yielded similar results. In conclusion, SCF is stored in mast cell secretory granules and is immunologically released by human mast cells. SCF1–166 is rapidly and specifically cleaved to SCF1–159 by chymase, which retains its biological effect on mast cells. SCF is also cleaved by other proteases to several SCF species whose possible biological activities remain to be established. The Journal of Immunology, 1999, 163: 2799–2808.

The proto-oncogene c-kit encodes a transmembrane tyrosine kinase receptor that is a member of the receptor family for platelet-derived growth factor and M-CSF receptor (1, 2). The ligand for c-kit has been cloned and variously designated stem cell factor (SCF), mast cell growth factor, kit ligand, or steel factor, and exists in both membrane-bound and soluble forms (3–5). The gene encoding SCF resides at the steel locus (Sl) on human chromosome 12 (6). The Sl gene encodes a primary translation product of 248 aa with a leader sequence and extracellular, transmembrane, and cytoplasmic domains (7, 8). The protein contains a proteolytic cleavage site encoded by the exon 6 sequence, and post-translational processing at this site leads to the secretion of a 165-aa, biologically active protein (9). An alternatively spliced cDNA form encodes a smaller 220-aa polypeptide that lacks exon 6 sequence, including the proteolytic cleavage sites, and hence results in a membrane-bound protein (7).

SCF is produced by fibroblasts (7, 10), stromal cells (11, 12), keratinocytes (13, 14), endothelial cells (12, 14–16), neuroblastoma cells (17), and tumor cell lines (18). SCF binds the c-kit receptor (c-kitR), activating its tyrosine kinase, leading to autophosphorylation of c-kitR on tyrosine and to association of c-kitR with substrates such as phosphatidylinositol 3-kinase (19). The c-kit product is selectively expressed on rodent (9, 20–25) and human mast cells (26–30), on melanocytes (19), and on a small progenitor cell fraction derived from bone marrow (27, 31), fetal liver (29), and cord blood mononuclear cells (30). SCF acts synergistically with other hemopoietic growth factors to stimulate the growth and differentiation of a variety of progenitor cells, including human mast cell progenitors (27, 29, 32).

Using immunogold staining of human heart tissue, we provided the first evidence that the secretory granules of human heart mast cells store SCF (33). Longley et al. demonstrated that human mast cell chymase, a chymotrypsin-like protease (34) also present in the secretory granules of human mast cells (34–37), cleaves SCF at the peptide bond between Phe158 and Met159 (38), which are encoded by exon 6 of the SCF gene (9). Recently, constitutive synthesis of SCF mRNA was demonstrated in human mast cells by RT-PCR (39).

This study was undertaken to investigate 1) whether SCF is present in mast cell granules from tissues other than the heart and in patients with diseases other than cardiomyopathies, 2) whether SCF can be immunologically released by isolated and purified mast cells, and 3) whether cleavage of SCF by chymase results in products active on human mast cells.

Materials and Methods

Reagents

The following were purchased: 60% HClO4 (Baker Chemical Co., Deer-\textvisiblespace}v\textvisiblespace}ter, The Netherlands); human serum albumin (HSA), α-chymotrypsin, PIPE\textvisiblespace}s, hyalurondi\textvisiblespace}s, chymopapain, collagenase, elastase type I, PMSF (Sigma, St. Louis, MO); HBSS, FCS (Life Technologies, Grand Island, NY); DNase I and pronase (Calbiochem, La Jolla, CA); RPMI 1640 with...
Buffers

The PIPES buffer used in these experiments was made up of 25 mM PIPES (pH 7.37), 110 mM NaCl, and 5 mM KCl. The mixture is referred to as P. P(25 mM PIPES, 110 mM NaCl, and 1 g/L dextrose; pH 7.4); PGMD contains 0.25 g/L MgCl2 gelatin in addition to P, pH 7.37.

Patients with mastocytosis

The skin samples used in this study were obtained from four patients (27–45 yr old) with mastocytosis, undergoing skin biopsy for diagnostic purposes. Two (a 31-yr-old man and a 26-yr-old woman) presented with multiple pigmented diffuse hyperpigmented and thickened skin that had remained unchanged for at least 10 yr. Hepatomegalic and systemic signs were present, although the results of routine blood tests were normal. The other two patients (an 18-yr-old man and a 45-yr-old woman) presented with multiple pigmented macules approximately 3 cm in diameter that developed wheals when stroked. In the first two patients the results of skin and bone marrow biopsies indicated systemic mastocytosis; in the other two patients cutaneous mastocytosis was diagnosed according to previously established criteria (42). Skin tissue was also obtained from two individuals (43 and 52 yr old) undergoing cosmetic surgery.

Isolation and purification of human lung and skin mast cells

Lung tissue was obtained from patients undergoing thoracotomy and lung resection, mostly for lung cancer. These patients were anesthetized with the following drugs: droperidol plus fentanyl and atropine (premedication); and thiopenthal, thiopental, succinylcholine, and pancuronium (anesthesia). Macroscopically normal lung parenchyma was dissected free from pleura, bronchi, and blood vessels and minced into a single-cell suspension as previously described (43). Yields with this technique ranged from 3 × 10^6 to 18 × 10^6 mast cells, and purities were between 1–8%. HLMC were purified by countercurrent elutriation (J2-21, Beckman). Several fractions were collected, and fractions containing HLMC were identified by discontinuous Percoll gradient (46). Yields ranged from 3 to 10 × 10^6 basophils with a purity of 74–98%, as assessed by basophil staining with Alcian blue and counting in a Spiers-Levy eosinophil counter (46).

Histamine release

Cells (∼3 × 10^6 mast cells/tube) were resuspended in P2CG, and 0.3 ml of the cell suspensions were placed in 12 × 75-mm polyethylene tubes (Sarstedt, Princeton, NJ) and warmed to 37°C; 0.2 ml of each prewarmed releasing stimulus was added, and incubation was continued at 37°C (37). At the end of this step, the reaction was stopped by centrifugation (1000 × g, 22 °C, 1 min), and the cell-free supernatants were stored at −20 °C for subsequent assay of histamine content, using an automated fluorometric technique (47). Total histamine content was assessed by lysis induced by incubating the cells with 2% HCO3 before centrifugation. To calculate histamine release as a percentage of total cellular histamine, the spontaneous release of histamine from mast cells (2–14% of the total cellular histamine) was subtracted from both numerator and denominator (48). All values are based on means of duplicate or triplicate determinations. Replicates differed in histamine content by <10%.

Chromatin assay

Mast cell chemotaxis was performed with a modified Boyden chamber technique as previously described (49). Briefly, 25 µl of PACGM buffer or various concentrations of the stimuli in the same buffer were placed in triplicate in the lower compartment of a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). The lower compartments were covered with a polycarbonate filter sandwich. The media were replaced every 24 h. The harvested supernatants and total SCF contents of HLMC were assessed for iSCF content using the SCF Quantikine kit (R&D Systems). The minimum detectable concentration was 10 pg/ml. All experiments were performed at least three times with duplicate samples.

Ultrastructural study

Samples for ultrastructural study were fixed in Karnowsky solution at 4°C for 2 h, rinsed in sodium cacodylate buffer (pH 7.3, 0.2 M), postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°C, dehydrated in ethanol and propylene-oxide, and embedded in Epon-Araldite. Ultrathin sections were stained with uranyl acetate and Reynolds’s lead citrate. The stained sections were examined with a Zeiss EM10 electron microscope (Zeiss, New York, NY) (50).

Electron immunocytochemistry

Ultrathin sections were deosmicated in aqueous saturated solution of 5% sodium metaperiodate for 10 min, rinsed in 1% OVA in 0.01 M Tris buffer/0.5 M NaCl, pH 7.6-Triton buffer, and washed for 1 h in TBSB/0.5 M NaCl, pH 7.6-Triton-lysin buffer; the sections were then preincubated with a 1% heat-inactivated normal goat serum and subsequently incubated overnight with the anti-SCF antiserum (7H6) diluted 1/100 in TBSB/1% BSA/0.5% sodium azide buffer. The sections were washed three times in TBSB/1% BSA/0.5% sodium azide buffer for 10 min each. They were incubated for 1 h with protein A/gold complex diluted 1/30 with TBSB/1% BSA/0.5% sodium azide buffer (33). After a 2-h wash in TBSB/1% BSA/0.5% sodium azide buffer, the grids were dried and stained for 15 min with aqueous uranyl acetate (5%) and for 10 min with Reynolds’s lead citrate (36). Ultrathin sections were incubated with another anti-SCF mAb (hkl-12) or a rabbit or sheep polyclonal anti-SCF Ab. The following controls were run: omission of the Ab layer, replacement of specific Ab with isotype-matched irrelevant Ab at the same concentration,
and neutralization of specific Ab with rhSCF (3 μg/ml): equal amounts were mixed and allowed to stand for 1 h at 22°C until used for immunolabeling. The control procedures excluded any nonspecific reactivity.

**Western blot analysis**

Proteins were denatured in a buffer containing 50 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM DTT, and 0.01% bromophenol blue; resolved by SDS-PAGE; and transferred to Immobilon-P membranes (Millipore, Bedford, MA) according to the manufacturer’s instructions. For the Western blot experiments, the filters were blocked in 5% dried nonfat milk in TBS solution and incubated with appropriate dilutions of the mAb against SCF H66 for 2 h at 22°C. The excess Ab was removed by sequentially washing the membranes in TBS-T, then a 1/5000 dilution of HRP-conjugated anti-mouse Ab (Amersham, Aylesbury, U.K.) was added to the filters for 1 h at 22°C. Filters were washed, and the signals were detected by chemiluminescence using the enhanced chemiluminescence system (Amersham) (51).

**Characterization of rhSCF**

rhSCF1–166 was characterized by ES/MS to verify its amino acid sequence and the homogeneity of the protein preparation (52). An aliquot of the protein was analyzed by HPLC on a Vydac C18 (Phase Separation Group, Hesperia, CA) column, giving a single symmetric peak. The molecular mass of the HPLC fraction was directly determined by electrospray mass spectrometry (ES/MS), showing a molecular mass of 18,656.9 ± 0.9 Da, in agreement with the expected value (18,656.5 Da) (38).

**Limited proteolysis**

In limited proteolysis experiments rhSCF1–166 was treated separately with α-chymotrypsin and chymase in 50 mM ammonium bicarbonate, pH 7.5, at 37°C using enzyme:substrate ratios of 1:1000 and 1:500 (w/w) respectively. Proteolysis was monitored on a time-course basis by sampling the reaction mixture at different times. Proteolytically digested SCF samples were fractionated by RP-HPLC on a Vydac C18 column; peptides were eluted using a linear gradient of 10–80% acetonitrile in 0.1% trifluoroacetic acid over 40 min; elution was monitored at 220 nm. Individual fractions were collected and identified by ES/MS (53).

**Mass spectrometry**

SCF and its proteolytic fragments were analyzed by ES/MS using a Bio-Q triple quadruple mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray ion source. Samples were injected directly into the ion source by loop injection at a flow rate of 2–10 μL/min. Data were acquired and elaborated using the MASS-LYNX program (Micromass, Manchester, U.K.) (53). Mass calibration was performed using the multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass, 16,950.5 Da); all masses are reported as the average.

**Statistical analysis**

The results are the mean ± SEM. Values from groups were compared using Student’s t test (54). Significance was defined as p < 0.05.

**Results**

**SCF in skin mast cells of patients with systemic mastocytosis and ultrastructural evidence of exocytosis of granule-associated SCF**

Skin tissue from patients with mastocytosis (n = 4) and from individuals undergoing cosmetic surgery (n = 2) was studied by electron microscopy and the immunogold technique. About 8% of HSMC from mastocytosis patients showed a degranulation pattern. The ultrastructural localization of SCF in HSMC was examined by the immunogold technique using mAb 7H6 against region 79–97 of human SCF (40). After immunogold staining, gold particles were present throughout the secretory granules of skin mast cells, but not in the perigranular cytoplasm (Fig. 1A). Gold particles were also found throughout the secretory granules of mast cells from the skin tissue of individuals undergoing cosmetic surgery (data not shown). Similar results were obtained with rabbit or sheep polyclonal Abs against multiple epitopes of SCF and another mAb (hkl-12) against region 150–154 of SCF (14) (M. Brockhaus, unpublished observation). HSMC incubated with a murine myeloma against an irrelevant Ag at concentrations similar to those of the anti-SCF Ab lacked particles (Fig. 1B). Results were similar when HSMC were incubated with IgG from nonimmunized rabbit or nonimmunized sheep or when the anti-SCF Abs were neutralized with rhSCF (3 μg/ml) (data not shown).

Different phases of degranulation can be seen in the skin mast cell Fig. 1A, from extrusion of altered, membrane-free granules to the cell’s exterior to the opening of intracytoplasmic degranulation channels. Inset A shows a higher magnification of the early phase of granule extrusion in which granules are still in the cell, but the cell membrane is partly lost at the external surface of the cell itself. Inset B, at high magnification, shows that extruded secretory granules retain the specific immunocytochemical localization of SCF. This suggests that SCF in situ in secretory granules of HSMC may be released into the extracellular milieu in the process of exocytosis. Similar results were obtained in eight preparations of human lung parenchyma from patients undergoing thoracotomy and lung resection, examined for SCF expression in lung mast cells (data not shown).

**Measurement of iSCF protein in human lung mast cells**

Using ELISA, iSCF protein was measured in cell lysates of highly purified (>95%) preparations of HLMC and basophils purified (>98%) from peripheral blood. In a series of seven experiments the concentration of iSCF in lysates of HLMC was 50.2 ± 10.9 pg/10⁶ cells. The iSCF protein was undetectable in lysates of five preparations of purified basophils (Fig. 2).

**Kinetics of iSCF and histamine release from immunologically activated HLMC**

Mast cells isolated and purified (>96%) from lung parenchyma (HLMC) were challenged in vitro with an optimal concentration (1 μg/ml) of anti-IgE to evaluate the release of histamine and iSCF. Fig. 3 compares the kinetics of iSCF and histamine release from immunologically challenged HLMC. The release of histamine and iSCF induced by maximal stimulation with anti-IgE was complete within 1–15 min. The kinetics of histamine release reached a plateau after 1 min and remained unchanged for 60–120 min. In contrast, a peak of iSCF, detected after 3–15 min, progressively declined between 30–120 min. Similar results were obtained in two experiments in which purified HLMC (>96%) were challenged in vitro with anti-IgE (data not shown).

These data indicate that iSCF can be immunologically released by HLMC, but the immunoreactivity in the cell supernatants is rapidly lost, suggesting that the immunological activation of human mast cells also releases enzymatic activity capable of degrading iSCF.

**Selective conversion of rhSCF**

Nonglycosylated rhSCF1–166 purified from transfected Escherichia coli cells as previously described (38) was submitted to limited proteolysis by pancreatic α-chymotrypsin or recombinant human chymase. To identify protease-sensitive sites within the rhSCF1–166 molecule, enzymatic hydrolysis was performed under strictly controlled conditions to ensure maximal stability of the protein conformation and to address protease action toward specific sites as previously reported (53). rhSCF1–166 was incubated with each protease using an appropriate enzyme to substrate ratio (E/S), and the process was monitored on a time-course basis by sampling the incubation mixture at intervals, followed by HPLC fractionation. Fragments released from the recombinant protein were identified by ES/MS, leading to the assignment of cleavage sites.
Fig. 4A shows the HPLC profile of the sample at 2 h of incubation of rhSCF1–166 with α-chymotrypsin using an E/S of 1/1000 (w/w). Peptides were analyzed by ES/MS and located within the rhSCF1–166 sequence on the basis of molecular mass. The major component yielded a molecular mass of 17,977.1 ± 0.6 Da and was identified as fragment 1–159 (theoretical mass value, 17,976.6 Da), originating from a single proteolytic event at Phe159. This was confirmed by identification of the smaller fraction containing the complementary peptide 160–166 (molecular mass, 697.4 ± 0.1 Da).

These two fragments were observed at very early stages of hydrolysis; they were present in the HPLC profile at 15 min of incubation, when intact rhSCF1–166 was not yet detectable. At later stages, two other species were identified (Fig. 4A) whose mass values were 17,977.1 ± 0.6 Da and 17,976.6 Da, originating from a single proteolytic event at Phe159. This was confirmed by identification of the smaller fraction containing the complementary peptide 160–166 (molecular mass, 697.4 ± 0.1 Da).

Hydrolysis of rhSCF1–166 with chymase was performed under the same experimental conditions using an E/S of 1/500 (w/w). Fig. 4B shows the HPLC profile of the sample withdrawn after a 3-h incubation. Chymase displayed greater cleavage selectivity; only two fragments were generated by proteolysis. Mass spectral analysis of the major fraction 2 showed two components, whose mass values were 17,977.1 ± 0.6 and 18,656.9 ± 0.9 Da (Fig. 5).

The major component was identified as peptide 1–159, already observed in the α-chymotrypsin experiment, and the minor species corresponded to the intact rhSCF1–166 still present in the incubation mixture. Analysis of the minor fraction (Fig. 4B) showed the complementary peptide 160–166 (mass value, 697.4 ± 0.1 Da). No further cleavages were observed at later stages even when proteolysis was continued for 24 h (data not shown), indicating that chymase very specifically cleaves rhSCF1–166 at Phe159.

Rapid conversion of SCF1–166 to SCF1–159 by human chymase

We next determined whether recombinant human chymase cleaves rhSCF1–166 by Western blot, using the mAb 7H6. Cleavage of rhSCF1–166 by chymase was extremely rapid and was essentially complete within 24 h (Fig. 6). The 3 h sample had a higher percentage of the cleaved molecule, in agreement with the results of the ES/MS experiment. At the end of the assay, a homogeneous immunoreactive band was present at about 18 kDa (lane 5), corresponding to SCF1–159.

Biological effects of SCF1–166 and SCF1–159 on HLMC and HSMC

The results reported above indicated that chymase rapidly and specifically cleaves SCF1–166 at a site encoded within exon 6 of the SCF gene, leading to the formation of SCF1–159 and a C-terminal...
septapeptide (9, 38). To investigate the possible biological roles of these three peptides, we evaluated the effects of rhSCF1–166, SCF1–159, and the septapeptide on HLMC and HSMC. Fig. 7A shows the results of a series of eight experiments, in which SCF1–166 and SCF1–159 induced the release of histamine from HLMC in a similar manner, whereas the small cleavage product, the C-terminal septapeptide, had no activating effect whatsoever. SCF1–166 and SCF1–159 also enhanced the release of histamine from HLMC induced by anti-IgE (Fig. 7B). The septapeptide had no such effect. Results were similar when rhSCF1–166, SCF1–159, and the septapeptide were examined on HSMC alone or in combination with anti-IgE (data not shown).

In a series of six experiments we evaluated the effects of SCF1–166, SCF1–159, and the septapeptide SCF160–166 on HLMC chemotaxis. Fig. 8 shows the results of a typical experiment in which SCF1–166 and SCF1–159 concentration-dependently induced the chemotaxis of HLMC. The C-terminal septapeptide had no effect.

**Kinetics of the interaction between SCF1–166 and HLMC**

In a first group of experiments purified (>97%) HLMC challenged with anti-IgE (1 μg/ml) for 10 min at 37°C were incubated with rhSCF1–166 for periods between 30 s and 30 min at 37°C. At different times, HLMC were centrifuged, and supernatants were analyzed by Western blot using the 7H6 mAb. After 30-min incubation at 37°C, three bands appeared, one corresponding to SCF1–166, a band of 15 kDa, and an intermediate band slightly lower than 18 kDa. HLMC incubated with buffer for 10 min at 37°C and then incubated with rhSCF1–166 for periods between 30 s and 30 min did not affect SCF1–159 (Fig. 9). These data indicate that rhSCF1–166 in the presence of activated HLMC is rapidly converted to at least two different forms, one of which has a molecular mass corresponding to that of SCF1–159. The second form was identified by HPLC and ES/MS analysis of the immunoprecipitate as another, shortened form of SCF cleaved at Thr144.
To identify the 15-kDa band, the HLMC supernatant sample withdrawn after 15 min of incubation was fractionated by HPLC, and the individual fraction was analyzed by ES/MS. Besides a small percentage of SCF1–159, mass spectral analysis showed a major component with a molecular mass of 16,309.0 ± 0.6 Da, which was identified as SCF1–144 originating from proteolytic cleavage at Thr144. Minor species corresponding to SCF158 and SCF154 were also detected.

These results do not exclude the possibility that at least part of exogenous SCF1–166 is internalized by HLMC (55). To assess this we conducted a similar experiment using supernatants of anti-IgE-stimulated HLMC (Fig. 10). Incubation of SCF1–166 with supernatants of anti-IgE-challenged HLMC induced rapid (~3 min) cleavage of SCF1–166 to a faster migrating form with a molecular mass of approximately 15 kDa. After 30 min of incubation at 37°C, the approximately 15-kDa band increased in intensity, and an approximately 18-kDa band appeared, corresponding by ES/MS analysis to SCF1–159. These results indicate that the interaction between SCF and products released by human mast cells leads to the formation of several SCF species, which suggests that several putative cleavage sites occur within SCF1–166. Supernatants of HLMC incubated with buffer for 10 min at 37°C and then incubated with rhSCF1–166 for periods between 30 s and 30 min did not affect SCF1–166.

To demonstrate cleavage specificity, we added PMSF (3 mM) to the supernatants of anti-IgE-stimulated HLMC before adding SCF1–166. Under these conditions chymase activity is virtually completely inhibited (38). Supernatants of stimulated HLMC incubated with PMSF (3 mM) and then incubated with rhSCF1–166 for periods between 3 and 30 min did not induce a band of about 18 kDa corresponding to SCF1–159 (Fig. 11). As a negative control, we performed similar experiment with purified (98%) basophils challenged with an optimal concentration of anti-IgE (0.3 µg/ml) or buffer for 20 min at 37°C and then incubated with rhSCF1–166 for periods between 30 s and 30 min at 37°C. Basophils were centrifuged for various lengths of time, and supernatants were analyzed by Western blot using the 7H6 mAb. The results show that neither anti-IgE-activated nor nonactivated basophils affected rhSCF1–166 (Fig. 12).

**FIGURE 5.** Mass spectrometric analysis of the major product of digestion of SCF1–166 by human chymase (see Fig. 4A). The figure shows the multiple charged ions (right panels) and the transformed (left panel) mass spectra. The major species (A) has a molecular mass of 17,977.1 ± 0.6 Da, corresponding to fragment 1–159 (theoretical mass, 17,976.6 Da) originating from a single proteolytic event at Phe159. The smaller component (B) represents the residual undigested SCF1–166.

**FIGURE 6.** Western blot analysis of cleavage products of 18.6-kDa SCF1–166 generated by mast cell chymase. Lane 1, SCF1–166; lane 2, HPLC purified SCF1–159; lanes 3–5, time course of mast cell chymase hydrolysis of SCF1–166. SCF1–166 (0.1 µg) and mast cell chymase (0.1 µM) were incubated at 37°C for the times indicated. The reaction was stopped by addition of PMSF at a final concentration of 3 mM. Western blot analysis was performed as described in Materials and Methods, using mAb 7H6 anti-SCF. Samples were electrophoresed on a 15% SDS-PAGE gel transferred to Immobilon-P membrane using a Transblot cell.

**FIGURE 6.** Western blot analysis of cleavage products of 18.6-kDa SCF1–166 generated by mast cell chymase. Lane 1, SCF1–166; lane 2, HPLC purified SCF1–159; lanes 3–5, time course of mast cell chymase hydrolysis of SCF1–166. SCF1–166 (0.1 µg) and mast cell chymase (0.1 µM) were incubated at 37°C for the times indicated. The reaction was stopped by addition of PMSF at a final concentration of 3 mM. Western blot analysis was performed as described in Materials and Methods, using mAb 7H6 anti-SCF. Samples were electrophoresed on a 15% SDS-PAGE gel transferred to Immobilon-P membrane using a Transblot cell.

**Discussion**

We demonstrate that SCF, the most important cytokine for human mast cell growth and function, is localized in, immunologically released, and cleaved by human mast cells. Mast cell chymase rapidly and specifically cleaves SCF1–166 to SCF1–159 and a C-terminal septapeptide. Although SCF1–166 and SCF1–159 activate mast cells similarly, other proteases released by these cells cleave SCF to additional cleavage products (SCF1–144 and others). Thus,
the interaction between SCF and mast cell products leads to the formation of several SCF species, illustrating the complexity of these biological interactions in vivo.

In an earlier study we reported the presence of SCF in the secretory granules of human heart mast cells from patients with idiopathic and ischemic cardiomyopathy, detected using the immunogold technique (33). Roche and his collaborators, using RT-PCR, have recently demonstrated that human mast cells constitutively express SCF mRNA (39). Our study extends the previous observations, showing that SCF can be found in several tissues (skin and lung) from different patients. The specificity of this observation was demonstrated by the fact that gold particles were seen throughout the secretory granules of skin and lung mast cells, but not in the perigranular cytoplasm. Similar results were obtained with two different mAb anti-SCF (7H6 and hkl-12) and two polyclonal (rabbit and sheep) Abs that recognize different epitopes of SCF (14, 40). Dilated cardiomyopathies (33) and systemic mastocytosis (13, 14, 42) are characterized by mast cell hyperplasia, respectively local or systemic. SCF was found in secretory granules of mast cells from patients with dilated cardiomyopathy (33) and mastocytosis and also in the secretory granules of HLMC.

This is the first study to demonstrate that iSCF is present in human mast cells and that it can be rapidly released by immunologically challenged HLMC, in parallel with the preformed mediator histamine. However, whereas histamine is stable in the supernatants of anti-IgE-activated HLMC, iSCF declines rapidly. This might be due to prompt internalization of SCF bound to its cognate receptor, c-kit (55) or to rapid hydrolysis by proteolytic enzymes. Longley et al. demonstrated that rhSCF 1–166 is rapidly cleaved to SCF 1–159 and a septapeptide by human mast cell chymase (38). We confirmed their findings by showing that chymase, unlike α-chymotrypsin, selectively and rapidly cleaves SCF 1–166 into two components. The major component identified by ES/MS yielded a molecular mass of 17,977.1 ± 0.6 Da and was identified as the fragment SCF 1–159 originating from a single proteolytic event at Phe 159. The smaller fraction contained the complementary peptide 160–166 (molecular mass, 697.4 ± 0.1 Da). Both polypeptides,
the native molecule SCF$^{1-166}$ and the cleavage product of chymase, SCF$^{1-159}$, apparently had biological effect on HLMC and HSMC, because they induce their activation and chemotaxis. The septapeptide had no such effect.

The complexity of the in vivo interactions between proteases and SCF, localized in and released from secretory granules of human mast cells, can be easily envisioned, since they contain several proteases (56–58) in addition to chymase (34, 35).

Tissue mast cells and circulating basophils, the only two cells known to express FcεRI and synthesizing histamine (20, 21, 59), differ with respect to SCF. iSCF was undetectable in cell lysates of basophils and could not be identified by the immunogold technique. Interestingly, SCF is the principal cytokine regulating human mast cell growth and proliferation (27, 29–32) and functions in vitro (26, 28, 33, 36, 37) and in vivo (60). In contrast, SCF has marginal effects on human basophils (28), and rhSCF$^{1-166}$ incubated with anti-IgE-activated purified basophils is not modified. Thus, these findings confirm and extend the many immunological and biochemical differences between human basophils and mast cells (59) also in terms of cytokines synthesized and released (61–66).

Human mast cells contain not only chymase (34, 35) but several proteases, such as tryptase (35–37), carboxypeptidase (56, 58), and cathepsin G (57). The experiment with α-chymotrypsin and chymase indicated that although chymase very specifically cleaves rhSCF$^{1-166}$ at Phe$^{159}$, there are further cleavage sites within SCF$^{1-166}$. This was suggested by the experiments in which rh-SCF$^{1-166}$ was incubated for different intervals with activated HLMC. After 30 min at 37°C, SCF$^{1-166}$ was converted to at least two different forms, with molecular masses corresponding to those of SCF$^{1-159}$ and SCF$^{1-144}$, respectively.

To exclude the possibility that the disappearance of SCF$^{1-166}$ is due to its internalization by HLMC (55), we incubated SCF$^{1-166}$ with supernatants of anti-IgE-activated HLMC. SCF$^{1-166}$ was again rapidly cleaved to at least two forms, one with a molecular mass compatible with that of SCF$^{1-159}$. These results indicate that the immunological secretion of proteases from HLMC leads to the formation of several SCF species, suggesting there may be several cleavage sites within SCF$^{1-166}$. As a control we found that anti-IgE-activated basophils, which contain extremely low concentrations of proteases (35), did not cleave rhSCF$^{1-166}$.

These results indicate that besides the native form of SCF$^{1-166}$, at least two others, SCF$^{1-159}$ and SCF$^{1-144}$, may be formed in vivo. Thus, chymase might exert specific enzymatic activity that selectively acts at Phe$^{159}$. Other as yet unknown protease(s), released from mast cells and not inhibited by PMSF may cleave SCF, leading to the formation of several SCF species. SCF$^{1-166}$ and SCF$^{1-159}$ are equally active on human mast cells. The biological activities of the third form of SCF$^{1-144}$ generated by incubation of SCF$^{1-166}$ with HLMC or their supernatants remain to be determined. Whatever the findings, it appears that SCF, chymase, and other proteolytic enzymes present in human mast cells participate in a complex biochemical system similar to the angiotensin I/angiotensin-converting enzyme/angiotensin II/angiotensin III system. Interestingly, chymase in human skin (34, 35) and heart mast cells (36) exerts angiotensin-converting enzyme activity, cleaving angiotensin I to angiotensin II (69–71).

A novel finding of this study is that mast cell proteases can degrade mast cell-derived cytokine. Previous studies have provided contrasting results on the production of cytokines by human mast cells. Several cytokines have been detected by immunocytochemical techniques (64–66) and in mRNA (39, 62, 63), but there are still technical difficulties in measuring cytokine release from isolated and purified human mast cells at the protein level (70).
example, constitutive synthesis of SCF mRNA was demonstrated in human lung and skin mast cells by RT-PCR, but ISCF was undetectable in supernatants of anti-IgE-activated HLMC (39). Our results showing that chymase and probably other mast cell-associated proteases can rapidly and efficiently cleave SCF explain the latter findings. Thus, the immunological activation of human mast cells could lead to the concomitant release of cytokines and cytokine-digesting proteases.

Although the in vivo significance of the low amounts of SCF immunologically released from human mast cells remains to be established, it is not inconceivable that they exert local autocrine and paracrine functions. In fact, our results also highlight the complexity of the autocrine loops and negative feedbacks involving human mast cells. These cells not only synthesize but also release the autocrine factor SCF acting on c-kit receptor, and display high levels of c-kit receptors and produce a soluble form of the c-kit receptor. Blood 83:2145.


