

# Ultrastructural analysis of human skin biopsy specimens from patients receiving recombinant human stem cell factor: Subcutaneous injection of rhSCF induces dermal mast cell degranulation and granulocyte recruitment at the injection site

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We performed an ultrastructural analysis of 10 skin biopsy specimens that had been obtained from three women who were undergoing daily subcutaneous dosing with recombinant methionyl-human stem cell factor (rhSCF) as part of a phase I clinical trial. The biopsy specimens were obtained at sites of subcutaneous administration of rhSCF, within approximately 1 to 2 hours of rhSCF injection, and, at the same time, at contralateral control sites that had not been directly injected with rhSCF. We previously reported that subcutaneous dosing with rhSCF in these subjects induced the local development of a wheal and flare response, which was associated with evidence of mast cell degranulation, as well as a systemic increase in numbers of cutaneous mast cells. The present electron microscopic analysis revealed that all biopsies of swollen, erythematous rhSCF-injected sites exhibited anaphylactic degranulation of both mature and immature mast cells, an acute inflammatory response characterized by the migration of neutrophils, basophils (some of which exhibited evidence of piecemeal degranulation), and eosinophils through blood vessel walls into the perivascular and extravascular spaces, and edema and fibrin deposition within the interstitium. By contrast, the control biopsies contained no evidence of mast cell degranulation or acute inflammation. However, both control and rhSCF-injected sites exhibited mast cells that were undergoing granule building and maturation. Thus at the doses tested in these subjects, subcutaneous injection of rhSCF induced anaphylactic-type degranulation of dermal mast cells at the injection site, with an acute inflammatory response that was associated with the recruitment of granulocytes. By contrast, mast cells at sites distant from those directly injected with rhSCF exhibited no evidence of enhanced secretion. (*J Allergy Clin Immunol* 1998;101:793-806.)

**Key words:** Anaphylactic degranulation, basophil, piecemeal degranulation, microvessels, eosinophil, neutrophil, secretion, granulogenesis

Stem cell factor (SCF), also known as *kit* ligand (KL) or mast cell growth factor (MGF), the ligand of the *c-kit* receptor, is a pleiotrophic growth factor that represents a major mast cell survival and developmental factor, both in murine rodents and in humans.<sup>1,2</sup> SCF has many effects in mouse mast cell development: It can suppress apoptosis both in vitro<sup>3-5</sup> and in vivo,<sup>5</sup> promote the proliferation of cells in the mast cell lineage at multiple stages of their maturation,<sup>2,6</sup> and promote certain aspects of mast cell maturation.<sup>7-9</sup> Recombinant human SCF (rhSCF) can also promote the development of immature human mast cells in vitro.<sup>10-13</sup>

However, SCF can also induce mast cells to express secretory function. SCF can promote *c-kit*-dependent mast cell degranulation and mast cell-dependent local cutaneous inflammation in mice in vivo.<sup>14</sup> In vitro studies indicate that SCF can directly induce purified human skin mast cells to release histamine and prostaglandin D<sub>2</sub> (PGD<sub>2</sub>),<sup>15</sup> can enhance Fc<sub>ε</sub>RI-dependent mediator secretion by purified human lung<sup>16</sup> or skin<sup>15</sup> mast cells, and can induce mouse mast cells to release the cytokines IL-6 and, to a lesser extent, TNF- $\alpha$ , in the absence of significant release of preformed mediators such as histamine or serotonin.<sup>17</sup>

In addition to its effects on mast cells, SCF can have synergistic effects with many other hematopoietic growth factors, which in turn promote the development of multiple hematopoietic lineages.<sup>1,2,18</sup> Accordingly, it is envisioned that SCF may be useful clinically, both in settings in which bone marrow function is inadequate and in approaches to enhance the mobilization of hematopoietic precursor cells into the peripheral circulation.<sup>18</sup>

Although the effects of SCF on human mast cell populations have been studied extensively in vitro, only one report has analyzed the effects of dosing with recombinant methionyl-human SCF (rhSCF) on human mast cell populations in vivo.<sup>19</sup> Our previous study<sup>19</sup> as well as this report are based on findings that were obtained in a phase I study of rhSCF in patients with

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*Abbreviations used*

|                     |                                    |
|---------------------|------------------------------------|
| AND:                | Anaphylactic degranulation         |
| EM:                 | Electron microscopy                |
| Fc <sub>ε</sub> RI: | Fc <sub>ε</sub> receptor I         |
| KL:                 | Kit ligand                         |
| MGF:                | Mast cell growth factor            |
| PGD <sub>2</sub> :  | Prostaglandin D <sub>2</sub>       |
| PMD:                | Piecemeal degranulation            |
| rhSCF:              | Recombinant human stem cell factor |
| SCF:                | Stem cell factor                   |
| TNF-α:              | Tumor necrosis factor-alpha        |

advanced breast carcinoma. We previously reported, on the basis of light microscopic evidence, that a 14-day course of daily subcutaneous dosing with rhSCF (at 5 to 50 µg/kg per day) resulted in a modest systemic hyperplasia of dermal mast cells, as reflected by an approximately 67% increase in the number of mast cells/mm<sup>2</sup> of dermis in sites that had not been directly injected with rhSCF.<sup>19</sup> In addition, each subject developed a pruritic cutaneous wheal and flare response at each rhSCF injection site, typically within approximately 60 minutes of injection of the cytokine. By transmission electron microscopy, we found that many mast cells at rhSCF injection sites exhibited evidence of anaphylactic-type degranulation.<sup>19</sup> However, our previous report did not include an analysis of the inflammation that was induced locally at sites injected with rhSCF.

In this article we report in detail the ultrastructural features of (1) the secretory responses induced in mast cells in response to the local injection of rhSCF, (2) the acute cutaneous inflammatory responses that developed at sites of subcutaneous injection of rhSCF, and (3) the dermal mast cells at sites distant from those directly injected with rhSCF. This study indicates that in these subjects, rhSCF induced anaphylactic degranulation of mast cells (and associated acute inflammatory responses that included recruitment of granulocytes) at sites that were directly injected with rhSCF but did not induce detectable mast cell secretory responses, or acute inflammation, at sites that had not been injected with rhSCF.

## METHODS

The skin biopsy specimens were obtained (after receiving signed informed consent forms that had been approved by the Institutional Review Board for Human Studies) from three women with advanced breast carcinoma (stage IIIB or IV) who had volunteered to participate in a phase I trial of rhSCF.<sup>19</sup> The selection criteria for the subjects and a description of the protocol for daily subcutaneous dosing with rhSCF are described in detail in Reference 19. Briefly, in the subjects studied in this report, rhSCF was administered before the initiation of chemotherapy. Two patients (Nos. 1 and 2) received daily subcutaneous injections of rhSCF (5 µg/kg per day) in the anterior thigh over a period of 13 days; One patient (No. 3 in this study, No. 185 in Reference 19) received only five daily subcutaneous injections of 25 µg/kg of rhSCF in the abdominal

wall; she then was withdrawn from the study because of the development of a systemic adverse event.<sup>19</sup> After local intradermal injection (approximately 0.5 ml) of a 1.0% solution of lidocaine within approximately 1-2 cm of the planned biopsy site, punch biopsies (4 to 6 mm) of skin were obtained from the indurated wheal and flare lesion that developed within approximately 60 minutes of injection of rhSCF at each injection site; the biopsies were obtained between 1 hour, 40 minutes and 2 hours, 30 minutes after administering the rhSCF into the site. Identically prepared, lidocaine-injected, contralateral control sites (i.e., sites not directly injected with rhSCF) on the opposite thigh or side of the abdomen of the same patients were also studied. The control biopsy specimens for patients 1 and 2 were obtained at the same time as the biopsies of the rhSCF injection sites (i.e., on rhSCF dosing days 1 and 13 or 6 and 13, respectively), whereas for patient 3, the rhSCF injection site was examined on day 1 of dosing (i.e., the first rhSCF injection site in this subject was examined) and a control biopsy specimen was obtained 11 days after the cessation of dosing with rhSCF. Biopsy specimens were immediately immersed in a pool of freshly prepared fixative (2.0% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl<sub>2</sub> in 0.1 mol/L sodium cacodylate buffer, pH 7.4) at room temperature, trimmed into small blocks while immersed, then fixed and processed for transmission electron microscopy (EM) as previously described.<sup>20</sup>

## RESULTS

A total of 10 biopsy specimens were obtained from three subjects. Control biopsy specimens were obtained either 11 days after cessation of rhSCF dosing (in patient 3) or on days 1, 6, or 13 of rhSCF dosing (in patients 1 and 2); these specimens were examined by EM as controls for the biopsy specimens of rhSCF injection sites. The rhSCF injection site specimens were obtained from the same three patients, at intervals between 1 hour, 40 minutes and 2 hours, 30 minutes after subcutaneous injection of rhSCF on days 1, 6, or 13 of rhSCF dosing (Table I). The latter biopsy specimens all demonstrated prominent wheal and flare reactions, which developed within 60 minutes of subcutaneous injections of rhSCF. By contrast, the control biopsy sites appeared clinically normal.

### Control biopsies specimens

*Day 1.* The control biopsy specimen that was obtained 1 day after initiation of rhSCF therapy appeared normal. There were no inflammatory cells or edema, nor were there any fibrin deposits amid the extracellular collagen bundles of the dermis. There were no mast cells present in this EM sample, and the sampled blood vessels appeared normal.

*Day 6.* The control biopsy specimen of another patient, which was obtained 6 days after initiation of rhSCF therapy, appeared similar to the 1-day biopsy specimen described above. However, some mast cells were present in this specimen. None of the mast cells exhibited morphologic criteria of anaphylactic degranulation (AND), but some of these mast cells appeared immature. These small mast cells exhibited numerous cytoplasmic polyribosomes and often had enlarged nucleoli; several of such cells were located in the perivascular

**TABLE I.** Ultrastructural findings in skin biopsy specimens from rhSCF injection sites or control sites in subjects undergoing dosing with rhSCF in vivo

|                       | Day of<br>rhSCF<br>dosing on<br>which<br>biopsy<br>specimen<br>was<br>obtained | Time after<br>rhSCF<br>injection<br>at which<br>biopsy<br>specimen<br>was<br>obtained† | Mast cells    |       |                            |      |                                              |           |     |             |          |             |          |
|-----------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|---------------|-------|----------------------------|------|----------------------------------------------|-----------|-----|-------------|----------|-------------|----------|
|                       |                                                                                |                                                                                        | Extracellular |       | Mature<br>cells<br>present | AND§ | Granulogenesis/<br>Immature<br>cells present | Basophils |     | Eosinophils |          | Neutrophils |          |
|                       |                                                                                |                                                                                        | Fibrin        | Edema |                            |      |                                              | Present   | PMD | Present     | Necrosis | Present     | Necrosis |
|                       |                                                                                |                                                                                        |               |       |                            |      |                                              |           |     |             |          |             |          |
| Control sites         |                                                                                |                                                                                        |               |       |                            |      |                                              |           |     |             |          |             |          |
| (Patient No.)         |                                                                                |                                                                                        |               |       |                            |      |                                              |           |     |             |          |             |          |
| 1                     | 1                                                                              |                                                                                        | 0             | 0     | 0                          | NA‡  | NA‡                                          | 0         | NA‡ | 0           | NA‡      | 0           | NA‡      |
| 2                     | 6                                                                              |                                                                                        | 0             | 0     | +                          | 0    | +                                            | 0         | NA‡ | 0           | NA‡      | 0           | NA‡      |
| 1                     | 13                                                                             |                                                                                        | 0             | 0     | +                          | 0    | +                                            | 0         | NA‡ | 0           | NA‡      | 0           | NA‡      |
| 2                     | 13                                                                             |                                                                                        | 0             | 0     | +                          | 0    | +                                            | 0         | NA‡ | 0           | NA‡      | 0           | NA‡      |
| 3*                    | 11 days<br>after<br>cessation<br>of rhSCF                                      |                                                                                        | 0             | 0     | +                          | 0    | +                                            | 0         | NA‡ | 0           | NA‡      | 0           | NA‡      |
| rhSCF injection sites |                                                                                |                                                                                        |               |       |                            |      |                                              |           |     |             |          |             |          |
| (Patient No.)         |                                                                                |                                                                                        |               |       |                            |      |                                              |           |     |             |          |             |          |
| 3                     | 1                                                                              | 1 hr, 40 min                                                                           | +             | +     | +                          | +    | +                                            | 0         | NA‡ | 0           | NA‡      | +           | +        |
| 1                     | 1                                                                              | 2 hrs                                                                                  | +             | +     | +                          | +    | +                                            | 0         | NA‡ | 0           | NA‡      | +           | +        |
| 2                     | 6                                                                              | 2 hrs, 30 min                                                                          | +             | +     | +                          | +    | +                                            | +         | +   | +           | +        | +           | +        |
| 1                     | 13                                                                             | 2 hrs                                                                                  | +             | +     | +                          | +    | +                                            | 0         | NA‡ | +           | +        | +¶          | +        |
| 2                     | 13                                                                             | 1 hr, 45 min                                                                           | +             | +     | +                          | +    | +                                            | +         | +   | +           | +        | +           | +        |

\*Patient 3 received five daily injections of rhSCF at 25 µg/kg, developed an adverse systemic reaction (see Reference 19), and was discontinued from the trial 11 days before this biopsy.

†Time elapsed between subcutaneous injection of rhSCF at the site and subsequent skin biopsy for electron microscopy. All rhSCF injection sites had developed wheal and flare reactions by approximately 60 minutes after injection of rhSCF.

‡Not applicable, that is, that cell type was not present in the EM sample.

§AND of some mast cells.

||PMD of some basophils.

¶Neutrophilic myelocytes also present.

area, and many were closely contacted by dermal fibroblasts, macrophages, or dermal dendrocytes. The surfaces of these mast cells had narrow, elongated folds, and the cytoplasm displayed prominent mitochondria. Granulogenesis was evident, characterized by the presence of condensing nucleoids located centrally within granules that also contained less dense granule matrix materials. Some granules contained well-ordered crystals of several periodicities; others had focal scrolls, and many granules contained mixtures of crystals and scrolls. Some granules that had less evident condensation patterns displayed particulate matrix material.

**Day 13.** The control biopsy specimens obtained 13 days after the initiation of rhSCF therapy, which were derived from two different patients, showed no extracellular edema or fibrin and no infiltrating granulocytes, and the sampled blood vessels were undamaged. Mast cells were plentiful in these specimens; however, they exhibited no morphologic evidence of secretory activity. As in the control biopsy specimen obtained on day 6 of rhSCF dosing, some mast cells exhibited morphologic evidence of immaturity and granulogenesis; these cells were more elongated and contained more granules that were filled with dense materials than the mast cells in the

sample obtained on day 6 of rhSCF dosing. Mast cells were located in the perivascular spaces as well as in the relatively avascular, collagen-rich dermis. Some mast cells were closely applied to the elongated, narrow processes of dermal dendrocytes; contacts between mast cells and fibroblasts were also noted.

**Eleven days after cessation of rhSCF dosing.** A control biopsy specimen was obtained from one patient 11 days after the cessation of rhSCF dosing. The biopsy specimen showed neither damage of resident cells or blood vessels nor inflammation. No fibrin deposits or edema were observed between dermal collagen bundles. Mast cells were present but without evidence of secretion.

**Control biopsy specimens: Summary.** The five control biopsy specimens had no evidence of inflammatory infiltrates, nor did they display extracellular edema, fibrin deposition, or vascular damage (Fig. 1, A). Mast cells were present in four of five EM samples of the control biopsy specimens. These cells exhibited no morphologic criteria of AND or piecemeal degranulation (PMD), but some of these cells were actively building new granules. Many mast cells were in contact with dermal dendrocytes and fibroblasts.

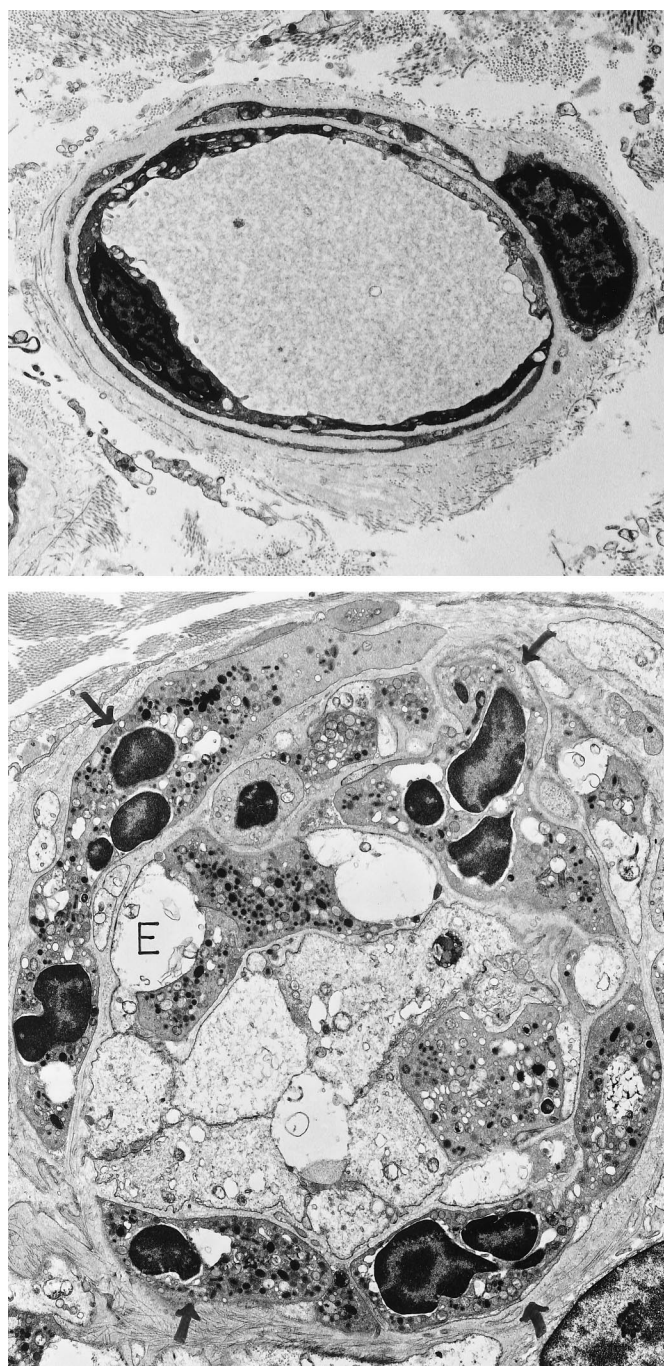


**FIG. 1.** **A**, Normal skin blood vessel in a control biopsy site obtained 6 days after the initiation of rhSCF dosing. Thin endothelial cells appear intact. **B**, Vessel in rhSCF injection site obtained on first day of rhSCF dosing. Vessel exhibits swollen, electron-lucent, necrotic endothelial cells (*E*). Lumen is packed with portions of cytoplasm from damaged cells and remnants of neutrophils. Numerous polymorphonuclear neutrophilic granulocytes (*arrows*) have migrated through vessel and reside beneath vascular basal lamina. **A**, magnification  $\times 6000$ ; **B**,  $\times 4500$ .

### Biopsies of rhSCF-injected sites

*Day 1.* Two biopsy specimens that were obtained on the first day of rhSCF dosing were examined by EM. The biopsy specimens from patients 1 and 3 (Table I) were

obtained 1 hour, 40 minutes or 2 hours after the local subcutaneous injection of rhSCF. Both patients had developed a wheal and flare reaction by approximately 60 minutes after injection. Both of the biopsy sites

exhibited extensive interstitial edema as well as fibrin deposits in perivascular areas and in association with interstitial collagen. The biopsy specimen from patient 3, the only patient studied who received rhSCF at 25  $\mu\text{g/kg}$ , also had focal necrosis of endothelial cells (Fig. 1, *B*). Neutrophils were present in both specimens but were more numerous in the specimen from patient 3 than in that from patient 1. Some of these polymorphonuclear cells were packed within blood vessels, some were migrating through them, and some were located in the perivascular and interstitial spaces (Fig. 1, *B*). In all of these locations, some of the neutrophils were undergoing necrosis, with release of large numbers of membrane-bound granules.

Both biopsy specimens exhibited mast cells that were undergoing AND. AND was characterized by the extrusion of membrane-free mast cell granules into the extracellular space. Some mast cells also exhibited the formation of intracytoplasmic degranulation chambers, resulting from the fusion of granule membranes, which contained altered granules. Extruded, membrane-free mast cell granules rested between the closely apposed surfaces of mast cells and fibroblasts or dermal dendrocytes. Some of the released mast cell granules were focally attached to the elongated, narrow surface processes of dermal dendrocytes, structures that sometimes contained coated and uncoated cytoplasmic vesicles. Extruded granules were also evident at some distance from the apparent mast cells of origin and were often located close to interstitial collagen bundles. Some mast cells undergoing AND also showed extensive shedding of cytoplasmic and surface processes. These cells were viable, with a nucleus containing heavily condensed chromatin and with only a narrow rim of residual cytoplasm. Some of the mast cells undergoing AND were immature, as identified by their content of numerous immature granules with condensing nucleoids.

**Day 6.** An rhSCF injection site biopsy from patient 2 was obtained on day 6 of rhSCF dosing. This site was biopsied at 2 hours, 30 minutes after injection of rhSCF, after the development of a wheal and flare reaction. Extensive interstitial edema, as well as deposits of fibrin, were present in the dermis (Fig. 2, *A*). Necrosis was not evident in blood vessels. Numerous neutrophils and some eosinophils were identified, some of them within vascular lumens, migrating through blood vessel walls, or within perivascular and interstitial spaces; some of these cells exhibited extensive necrosis, with release of membrane-bound secretory granules (Fig. 2, *B*).

Basophilic leukocytes were also observed en route through vessel walls or within interstitial spaces. These basophils exhibited neither evidence of necrosis nor evidence of granule extrusion (i.e., extruded granules or diminished numbers of cytoplasmic granules). Instead, they displayed the ultrastructural morphology of PMD, characterized by partially or completely electron-lucent, nonfused granule chambers within their cytoplasm. Some granules that retained an apparently complete

complement of their particulate materials were also observed.

Some of the dermal fibroblasts in this biopsy specimen had extensively dilated cisterns of rough endoplasmic reticulum, and some fibroblasts and macrophages exhibited numerous focal contacts with fibrin strands.

Mast cells were plentiful in this biopsy specimen. Many mast cells were elongated and contained numerous mature granules. Others were immature, and the cytoplasmic granules featured prominent condensation of dense, central nucleoids, as well as less dense matrix materials. Many mast cells exhibited extensive AND (Fig. 3), characterized by intracytoplasmic degranulation channels (formed by fusion of granule membranes), extrusion of membrane-free granules (Fig. 3), and shedding of surface folds. In this biopsy specimen, as in control biopsies and in biopsies of rhSCF injection sites obtained on day 1 of rhSCF therapy, some mast cells were in contact with dermal fibroblasts (Fig. 3) or dermal dendrocytes. Released mast cell granules were entrapped in narrow interstitial spaces between dermal dendrocytes (or fibroblasts) and mast cells. Several dendritic processes of dermal dendrocytes contained internalized mast cell granules, a finding that we did not observe in fibroblasts. Some of the mast cells undergoing AND had large nucleoli and condensing immature granules, thus illustrating the ability of morphologically immature mast cells to undergo AND. Small immature mast cells with diminished cytoplasm and few cytoplasmic granules were surrounded by extruded, membrane-free mast cell granules. These cells, which had numerous cytoplasmic polyribosomes, resembled the small immature mast cells that have been observed in populations of human lung mast cells that were recovering, in short-term cultures, from IgE-mediated AND.<sup>21</sup>

**Day 13.** Biopsy specimens from patients 1 and 2 were obtained on day 13 of rhSCF dosing. Each rhSCF-injected site (at biopsy 2 hours or 1 hour, 45 minutes after subcutaneous injection of rhSCF, respectively) clinically exhibited a wheal and flare reaction, which, at the ultrastructural level, was accompanied by extensive edema and extravascular deposits of fibrin. Blood vessels were undamaged. Neutrophils and eosinophils were present in both biopsy specimens (Fig. 4). Both neutrophils and eosinophils were present in interstitial tissues, and some of these granulocytes exhibited necrosis and release of membrane-bound granules. Some of the eosinophils also showed focal losses of secondary granule matrix or core materials (Fig. 4, *B*). Some of the viable extravascular eosinophils had few cytoplasmic granules and exhibited increased amounts of cytoplasmic tubulovesicular structures and lipid bodies (Fig. 4, *A*).

As in the biopsy of the rhSCF injection site that was obtained 6 days after the beginning of rhSCF dosing, the biopsy specimen that was obtained from the same patient (patient 2) on day 13 of rhSCF dosing contained many basophilic leukocytes that were migrating through vessels or were present in the adjacent edematous

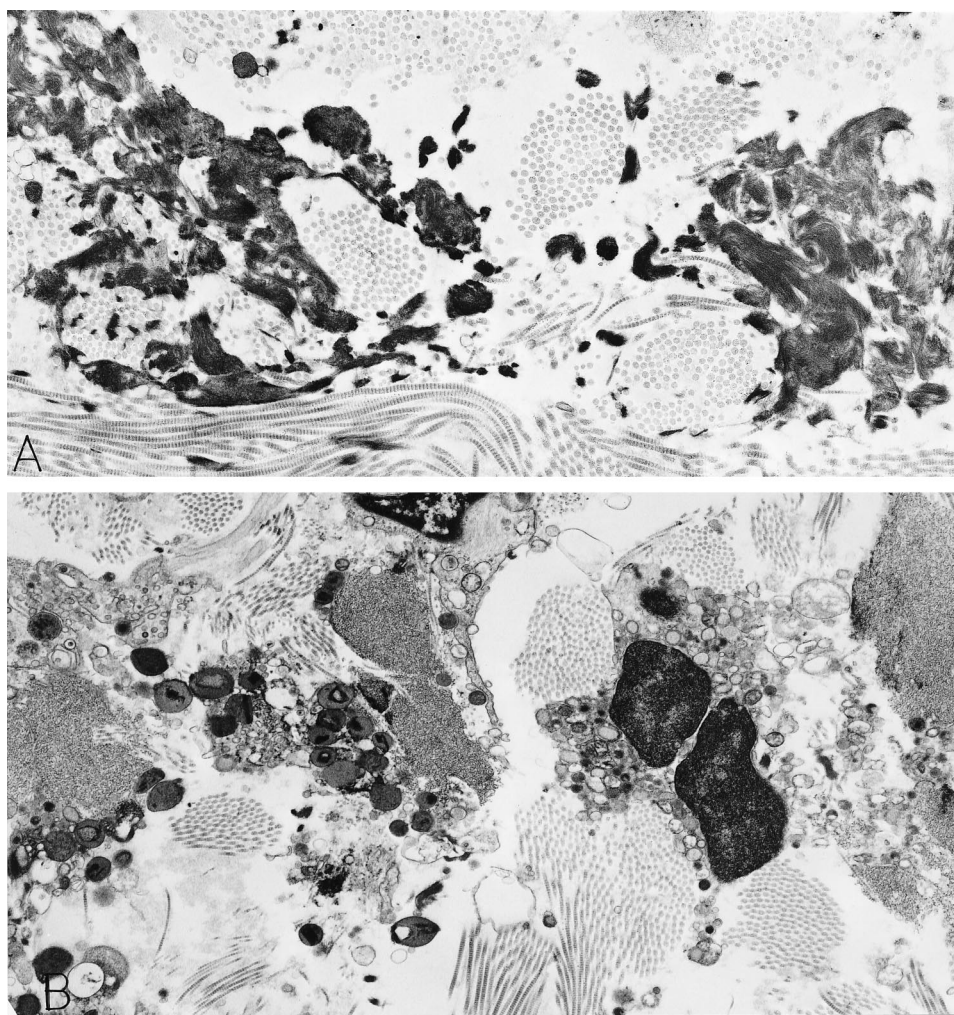


**FIG. 2.** Biopsy specimen of rhSCF injection site obtained on day 6 of rhSCF dosing. Papillary dermis shows dense aggregates of fibrin, some of them bound to collagen (in **A**) and necrotic eosinophils and neutrophils among collagen bundles (in **B**). Extracellular eosinophil and neutrophil granules retain their granule membranes, indicating their origin from cells undergoing necrosis rather than degranulation. **A**, magnification  $\times 14,000$ ; **B**,  $\times 9500$ .

interstitium (Fig. 4). These cells were viable, according to morphologic criteria, exhibiting no evidence of injury or necrosis, but they did display extensive PMD (Fig. 4). These basophils contained increased numbers of cytoplasmic vesicles, which either contained electron-dense particles or were largely electron-lucent. Increased stores of cytoplasmic glycogen were also present. Neither of the control biopsies from patient 2 (which were also obtained on days 6 and 13 of rhSCF dosing) contained any basophils. Thus the subcutaneous injection of rhSCF can be rapidly followed (by 2 hours, 30 minutes in the day 6 biopsy, and by 1 hour, 45 minutes in the day 13 biopsy [Fig. 4]) by the migration of basophils from the blood to the interstitial tissues at the site of rhSCF injection.

Both of the biopsy specimens obtained on day 13 of rhSCF dosing exhibited numerous mast cells (Figs. 5 and 6). These cells were undamaged and consisted of a

mixture of mature and immature cells (Fig. 5). The mature or immature cells that were not undergoing secretion contained cytoplasmic granules that were larger in size and increased in number compared with those in the mast cells in biopsy specimens that were obtained earlier in the course of rhSCF dosing. The substructural patterns of the more mature granules in these cells (Fig. 6) consisted primarily of crystals (Fig. 6, *B*), scrolls (Fig. 6, *C*), or mixtures of these materials. Some granules containing particles (Fig. 6, *A*), which generally were admixed with other materials, were also noted; these typically were relatively small granules that appeared to be especially frequent in immature cells. Many mast cells contained several immature granules with focal condensations of electron-dense material in areas of less dense granule matrix (Fig. 5). Granules with these distinctive condensing foci (Fig. 6, *D*) were easily discriminated from the small number of granules that



**FIG. 3.** Mast cell (*M*) in rhSCF injection site (obtained on day 6 of rhSCF dosing) has extruded most of its granules (*arrows*). The mast cell is in close contact with a fibroblast (*F*), which has greatly expanded cisterns of rough endoplasmic reticulum (*arrowhead*). Magnification  $\times 14,000$ .

exhibited irregular and focal piecemeal losses of electron-dense matrix associated with PMD (Fig. 6, *E*). The images of PMD (Fig. 6, *E*) were also quite different from those illustrating the relatively decreased electron density of newly crystallized foci of granule matrix with regular parallel arrays that were numerous in the cytoplasmic granules of nondegranulating mast cells. These arrays were apparent at low magnifications (*closed arrowhead* in Fig. 5, *B*) and were even more readily appreciated at higher magnifications (Fig. 6, *B*).

Many mast cells in these biopsy specimens exhibited extensive AND (Figs. 7 and 8); rarely, single granules in mast cells without AND showed evidence of piecemeal losses (Fig. 6, *E*). The major secretory process—AND—resembled that which was present in the biopsies of the rhSCF-injected sites which were obtained at earlier intervals of rhSCF dosing. That is, such mast cells had prominent intracytoplasmic degranulation channels and were accompanied by extruded granules (Fig. 7). Indeed, extrusion of granules, which resembled that seen in association with IgE-mediated degranulation of isolated human skin mast cells *in vitro*,<sup>22</sup> was a more common observation in these biopsies than was evidence of intracytoplasmic degranulation channel formation in mast cells. Rounded, membrane-free, altered granule matrices, representing many extruded mast cell granules, were identified in several locations, including immediately adjacent to the surfaces of the cells from which they were released, between mast cells and dermal dendrocyte processes (Fig. 8) or between mast cells and activated fibroblasts, widely dispersed in edema-rich interstitial fluid that also contained fibrin deposits (Fig. 7), and among dermal collagen fibers and bundles (Fig. 8). As in the other biopsies of rhSCF-injected sites, deposits

of fibrin were closely associated with the surfaces of activated fibroblasts. In the biopsy specimens obtained on day 13 of rhSCF dosing, we also noted that fibrin deposits were focally attached to the surfaces of some of the mast cells that were undergoing AND (Fig. 7).

**Biopsies of rhSCF-injected sites: Summary.** The dermis at each rhSCF injection site exhibited an extensive inflammatory response, with extracellular edema and fibrin deposits, regardless of the number of days that the individual patient had been receiving dosing with rhSCF. The most extensive inflammatory response, which occurred on day 1 of rhSCF dosing in one patient, was accompanied by focal necrosis of vascular endothelial cells. Mast cells were present in all of the biopsy specimens. In addition to findings that were also observed in the control biopsy specimens, such as the presence of immature mast cells with evidence of granule development, and contacts between mast cells and dermal dendrocytes or fibroblasts, many of the mast cells in each rhSCF injection site exhibited extensive AND. By contrast, PMD of mast cells in these biopsies was a minor finding and involved single granules only in a small number of cells.

The inflammatory infiltrate in the rhSCF-injected sites was composed of neutrophils, eosinophils, and basophils, but neutrophils outnumbered eosinophils and basophils by approximately 100:1:1. The primary morphologic finding in some of the neutrophils and eosinophils was cell necrosis, whereas basophils displayed evidence of extensive PMD, both during their migration through vascular walls and after they had migrated into interstitial spaces of the papillary dermis. The basophils in the rhSCF injection site biopsies did not exhibit necrosis.

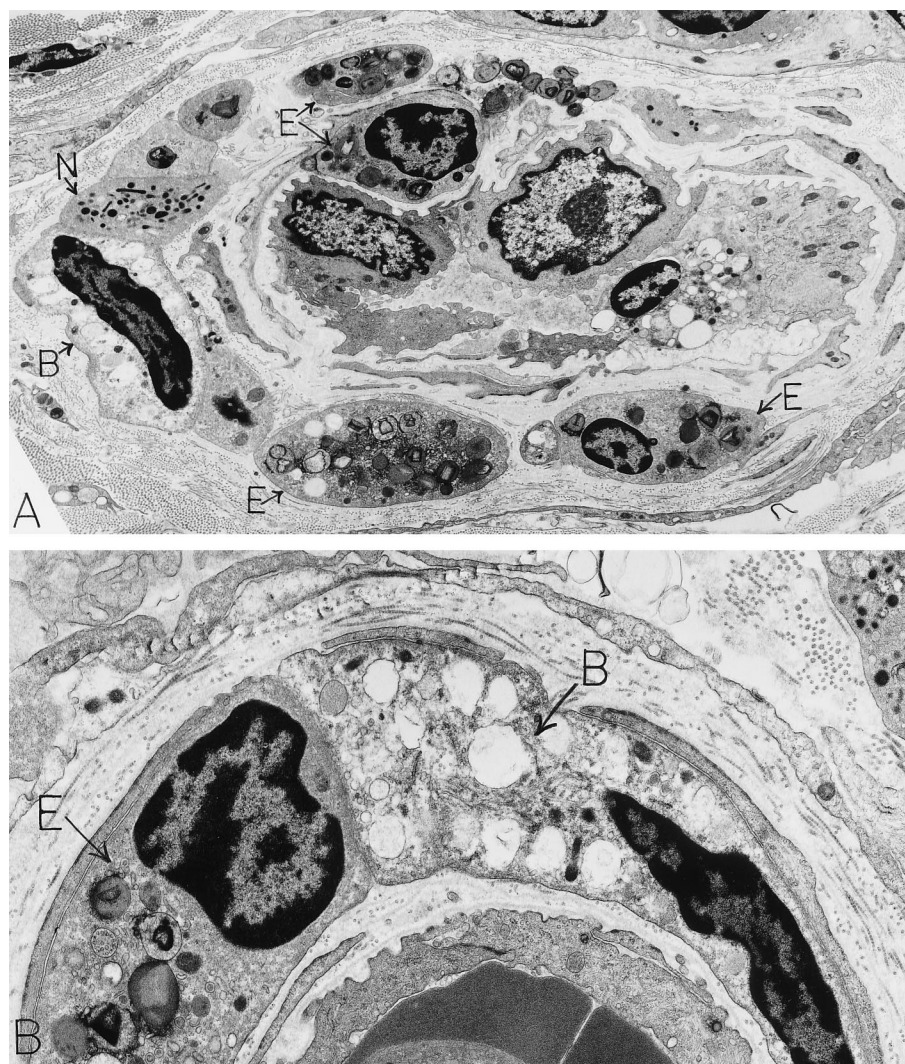


**FIG. 4.** Blood vessels and granulocytes in rhSCF injection site biopsy specimen obtained on day 13 of rhSCF dosing. Blood vessels exhibit perivascular neutrophils (*N*), eosinophils (*E*), and basophils (*B*). Basophils (e.g., *B* in **A** and **B**) exhibit extensive PMD, characterized by presence of numerous empty granules in their cytoplasm. Some eosinophils exhibit alterations in their secondary granules, involving both matrix and core compartments. **A**, magnification  $\times 7000$ ; **B**,  $\times 13,500$ .

## DISCUSSION

The ultrastructural analysis of skin biopsy specimens from subjects who were undergoing subcutaneous dosing with rhSCF established three major findings: (1) at sites of subcutaneous injection, rhSCF induced extensive anaphylactic-type degranulation (AND) of dermal mast cells; (2) such rhSCF-induced mast cell AND was associated with the local development of an acute inflammatory response, which typically included recruitment of granulocytes and which, in some cases, was associated with the recruitment of basophils that expressed piecemeal degranulation (PMD); and (3) at sites distant from those directly injected with rhSCF, some dermal mast cells exhibited evidence of maturation and/or granulogenesis, but no mast cells exhibited AND or PMD, nor was there any associated inflammatory response.

## rhSCF-induced AND of dermal mast cells in vivo

Skin biopsies of sites that had been injected with rhSCF subcutaneously were obtained from 1 hour, 40 minutes to 2 hours, 30 minutes after rhSCF injection, by which time clinically obvious inflammation, with features of the wheal and flare responses that are observed at sites of injection of other agents that can induce mast cell AND, had already developed.<sup>19</sup> Many of the mast cells at such sites exhibited evidence of AND, which, in its specific ultrastructural features, was remarkably similar to that previously reported for isolated human dermal mast cells that had been activated by IgE-dependent mechanisms *in vitro*.<sup>22</sup> Such mast cells had released membrane-free granules directly to the extracellular milieu or, less frequently, granule matrices were exteriorized by their release into intracytoplasmic de-

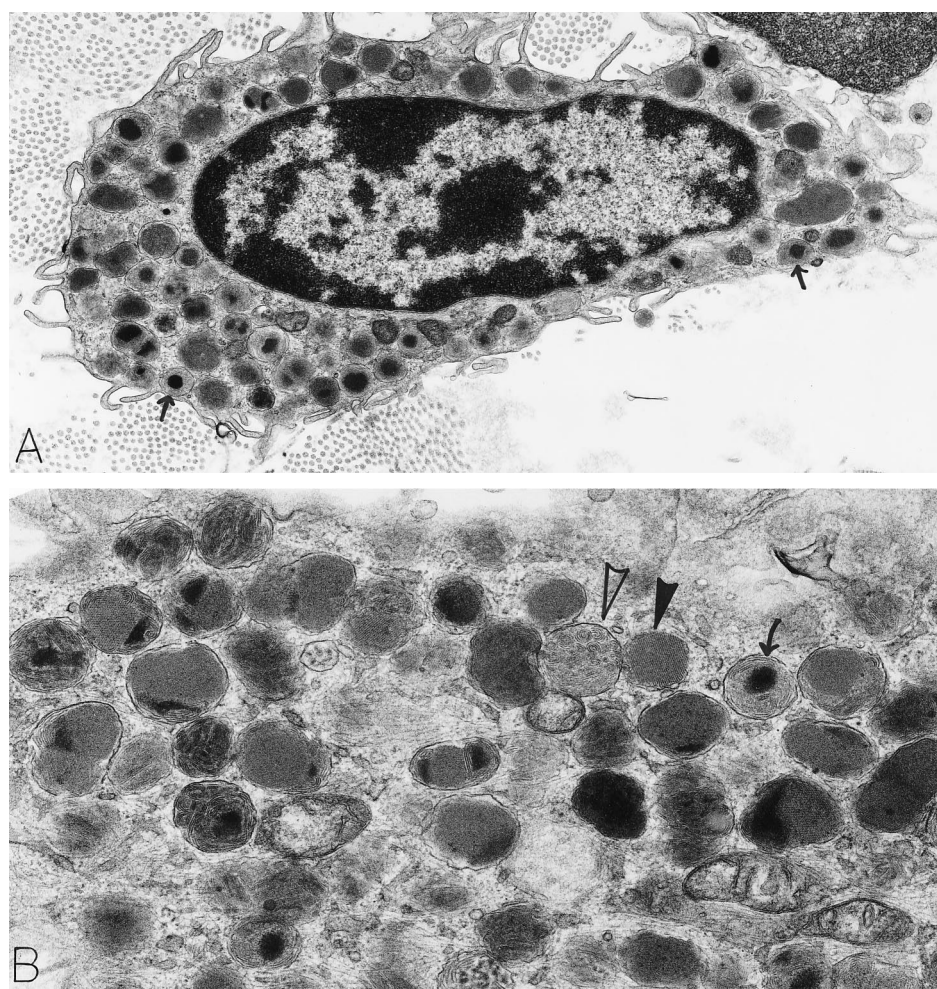


**FIG. 5.** Biopsy specimen of rhSCF injection site obtained on day 13 of rhSCF dosing contains some mast cells that have many secretory granules that exhibit no evidence of AND. Many developing granules show central dense foci of condensation (arrows) superimposed on underlying granule patterns. These patterns include crystals (closed arrowhead in B) and scrolls (open arrowhead in B). A, magnification  $\times 16,000$ ; B,  $\times 36,000$ .

granulation channels formed by fusion of the membranes of adjacent granules. Shedding of cytoplasmic processes accompanied the massive release of granules in some cells, which, as a result, appeared as small cells with few cytoplasmic granules; some of these cells exhibited morphologic evidence of early synthetic recovery.<sup>23</sup> Extruded, membrane-free mast cell granules were readily identified in the rhSCF-injected test sites remote from mast cells; they could be observed bound to interstitial collagen bundles, attached to mast cell surfaces, or sandwiched between mast cells and adjacent activated fibroblasts or the elongated, narrow processes of dermal dendrocytes. Extruded mast cell granules have also been observed near the processes of dermal dendrocytes when skin organ cultures were exposed to either substance P or morphine, known secretagogues for human skin mast cells.<sup>24</sup> Extruded mast cell granules persisted in the extracellular space for up to 2.5 hours after rhSCF injection—the latest interval we were able

to study. AND was by far the predominant form of secretion that was exhibited by dermal mast cells at sites of rhSCF injection; only rare cells had an occasional cytoplasmic granule with ultrastructural changes typical of mast cell PMD (for example, see Fig. 6, E).

#### **Subcutaneous injection of rhSCF induced the local development of an acute inflammatory response**

It is well known that IgE-dependent AND of dermal mast cells typically is followed by the development of a local acute inflammatory response (i.e., a “wheal and flare” response) and that this can be followed, typically 4 to 8 hours later, by the development of a “late-phase reaction” that is characterized by the local infiltration of the site with neutrophils, eosinophils, basophils, and other leukocytes.<sup>25-27</sup> Although it has been shown that recombinant rat SCF can induce dermal mast cell AND and increased vascular permeability in mice,<sup>14</sup> there has

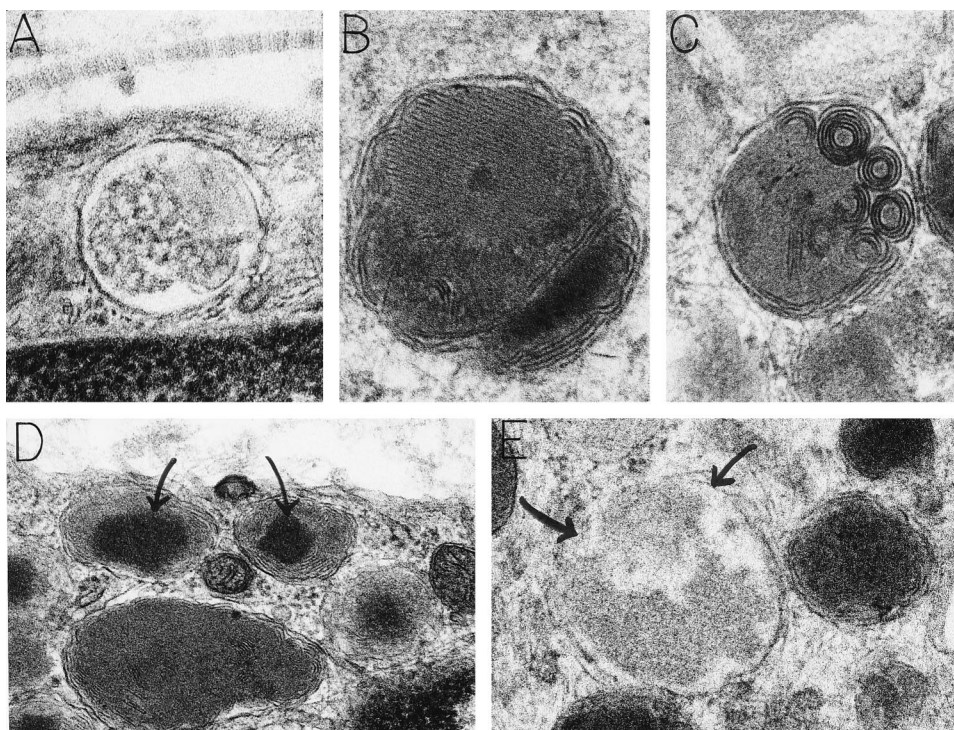


**FIG. 6.** Montage of cytoplasmic granules from mast cells in rhSCF injection site biopsy specimen (obtained on day 13 of rhSCF dosing) illustrating different ultrastructural patterns and processes. In **A**, granule contains dense particles. In **B**, regular crystalline array fills large portion of this granule, which exhibits mixed structural pattern. Mixed granule in **C** contains prominent scrolls at periphery of homogeneously dense material. **D**, Two granules with central condensing nucleoids (*arrows*), finding typical of mast cell granule building. **E**, Granule that exhibits focal, irregular electron-lucent areas within otherwise electron-dense granule matrix is characteristic of PMD (*arrows*). **A**, magnification  $\times 84,000$ ; **B**,  $\times 90,000$ ; **C**,  $\times 78,000$ ; **D**,  $\times 52,000$ ; **E**,  $\times 61,000$ .

been no previous report characterizing the leukocyte infiltration, or other ultrastructural features, of the inflammation induced by rhSCF *in vivo*, either in experimental animals or human beings.

In this study, we provide evidence of a brisk acute inflammatory response in the dermis in each site of subcutaneous injection of rhSCF. The intensity of this inflammatory response may have been dose related in that the most severe exudate, which was accompanied by focal necrosis of vascular endothelial cells in small blood vessels, was evident only in the biopsy of the single subject who received rhSCF at 25  $\mu\text{g}/\text{kg}$ . We did not observe vascular endothelial cell injury in biopsy specimens of subjects who received the lower dose of rhSCF (i.e., 5  $\mu\text{g}/\text{kg}$ ). Focal injury or necrosis of vascular endothelial cells can often be observed at sites of intense cutaneous inflammatory responses, such as strong T cell-dependent reactions to intradermal antigens.<sup>28</sup> However, in the case of subjects who received subcutaneous injections of rhSCF, none of the local inflammatory responses, including that at the biopsy site, which exhibited focal vascular endothelial cell necrosis by transmission electron microscopy, developed clinical evidence of cutaneous damage. Furthermore, except for the development of increased epidermal melanization at

some sites of rhSCF injection,<sup>19</sup> all of the lesions appeared to resolve completely.

All of the inflammatory responses that we analyzed exhibited extensive interstitial edema and the local deposition of interstitial fibrin, evidence of markedly enhanced vascular permeability.<sup>28</sup> It has long been thought that the development of interendothelial cell gaps represents the mechanism by which histamine induces enhanced vascular permeability.<sup>29</sup> Histamine may also increase extravascular edema formation by enhanced permeability through newly defined endothelial cell structures, an interconnected system of vesicles and vacuoles, termed the vesiculo-vacuolar organelle.<sup>30</sup> At the time that our biopsy specimens were obtained (1 hour, 40 minutes to 2 hours, 30 minutes after rhSCF injection), we identified no interendothelial cell gaps. The subjects in this study had been premedicated with H<sub>1</sub> (diphenhydramine 50 mg, orally, each 6 hours) and H<sub>2</sub> (ranitidine 150 mg, orally, each 12 hours) antihistamines to minimize the pruritus associated with the reactions to subcutaneous rhSCF,<sup>19</sup> and this may have influenced the nature of the vascular responses in these sites.

Extravasation of fibrinogen, and deposition of cross-linked fibrin, occurs at most, if not all, sites of enhanced

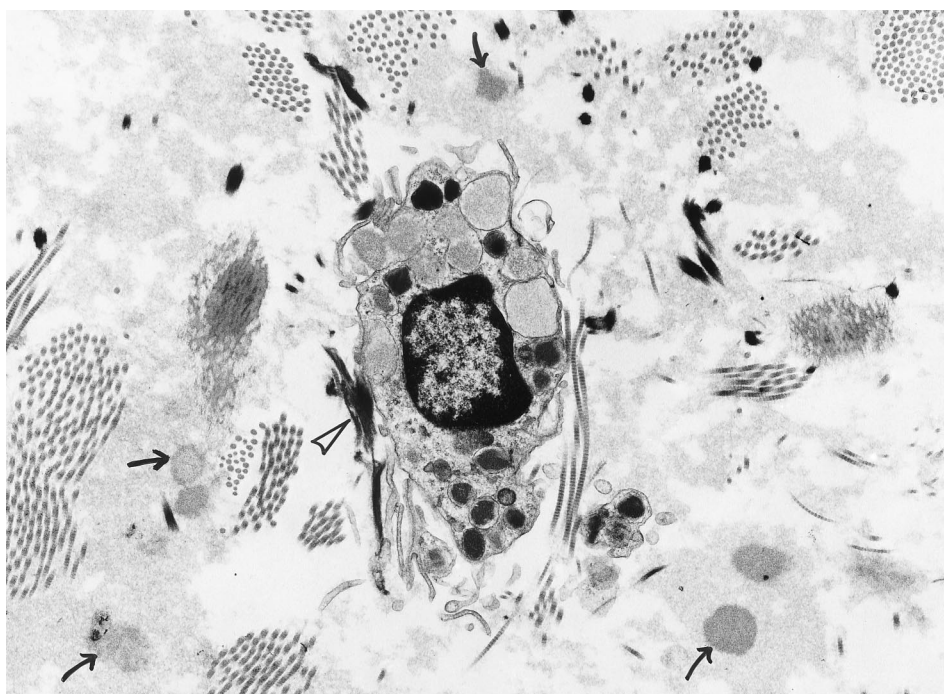


**FIG. 7.** Mast cell in papillary dermis of rhSCF injection site biopsy specimen (obtained on day 13 of rhSCF dosing) reveals morphology typical of AND. Altered, swollen granules are present in intracytoplasmic degranulation chambers that open to cell surface, and swollen, membrane-free, extruded granules (arrows) are present in interstitium at distance from probable mast cell of origin. These extruded granules rest in amorphous, slightly dense pools of interstitial edema that separate adjacent bands of collagen. Electron-dense fibrin fragments are also visible, some of which (open arrowhead) are attached to mast cell surface. Magnification  $\times 13,000$ .

vascular permeability.<sup>31</sup> In the rhSCF-injected sites, extravascular deposits of fibrin were observed bound to collagen, concentrated around activated fibroblasts,<sup>32</sup> and attached to the surfaces of degranulating mast cells. It is likely that such fibrin deposits significantly contributed to the palpable induration of the rhSCF injection sites.<sup>19, 28, 31, 33</sup>

The cellular infiltrate in the dermis at the SCF injection sites consisted primarily of neutrophils, which appeared to outnumber basophils and eosinophils by approximately 100:1:1. These mature granulocytes were observed en route through blood vessel walls, as well as within and adjacent to blood vessels. Some of these granulocytes had migrated well into the papillary dermis, but none were present in the epidermis. Some of the infiltrating neutrophils exhibited necrosis, with release of membrane-bound granules and cellular debris. Some eosinophils also exhibited necrosis and release of membrane-bound granules and cellular debris, whereas some eosinophils in blood vessel walls exhibited loss of granule materials characteristic of PMD<sup>34, 35</sup> as well as increased lipid bodies.<sup>36, 37</sup> Basophils showed neither injury nor necrosis but often exhibited evidence of secretion, typically PMD. Notably, many of the basophils that were present in rhSCF-containing suspension cultures of human cord blood mononuclear cells also exhibited PMD.<sup>38</sup> However, the mechanism responsible

for PMD of basophils, whether in cutaneous sites injected with rhSCF or in cultures containing rhSCF *in vitro*, remains to be determined. Indirect mechanisms may be involved because it has been shown that human basophils express only low (or undetectable) levels of *c-kit* on their surfaces and that short-term exposure to even high concentrations (up to 3  $\mu\text{g/ml}$ ) of rhSCF fails to induce detectable histamine release from purified human basophils *in vitro*.<sup>15</sup>

#### **Dosing with rhSCF did not induce systemic effects on dermal mast cell secretion**

In contrast to dermal mast cells at sites that had been directly injected with rhSCF, none of the mast cells at control biopsy sites exhibited evidence of either AND or PMD, nor did these sites exhibit any ultrastructural evidence of acute inflammation. However, many mast cells at these sites exhibited evidence of immaturity, particularly granulogenesis. In our previous study, we found that patients who were dosed with rhSCF subcutaneously daily for 14 days exhibited a mean increase in mast cells/ $\text{mm}^2$  dermis (at sites that had not been directly injected with rhSCF) of 67%.<sup>19</sup> In mice that had been injected with 30 or 100  $\mu\text{g/kg}$  per day of recombinant rat SCF subcutaneously for 21 days, the ensuing rhSCF-induced dermal mast cell hyperplasia reflected both the proliferation of differentiated dermal mast cells

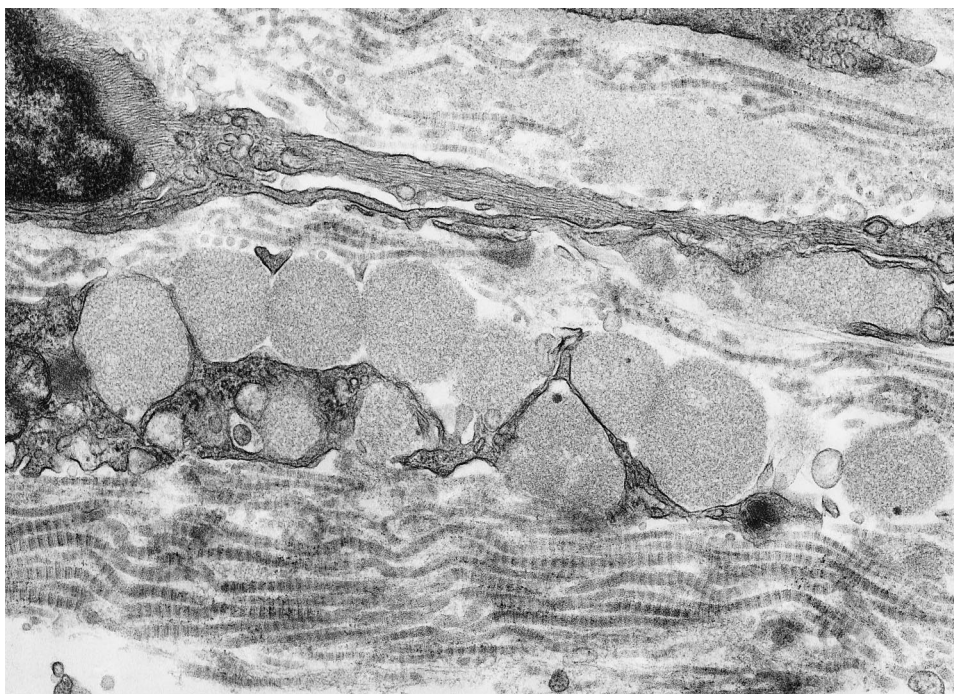


**FIG. 8.** High-magnification micrograph of rhSCF injection site biopsy specimen, which was obtained on day 13 of rhSCF dosing, illustrating extracellular, membrane-free mast cell granules in close proximity to surfaces of elongated, narrow cell processes of dermal dendrocytes. Dermal dendrocyte at top illustrates intermediate filament-packed process of dermal dendrocyte in continuity with dendrocyte cell body. Banded interstitial collagen fibers are plentiful. Magnification  $\times 37,000$ .

and the local recruitment/retention and maturation of mast cell precursors.<sup>7</sup> In the present ultrastructural study, we found no mast cells in mitosis. Instead, over the period of rhSCF dosing analyzed (6 or 13 days), we identified small mast cells with relatively few cytoplasmic granules (especially at day 6) and larger, but still immature, mast cells (especially at day 13) that contained increased numbers of granules, many of them still in the process of maturation. On the basis of these findings, we believe that the modest systemic increase in dermal mast cell numbers observed in human subjects dosed with rhSCF may have reflected to a greater extent the enhanced maturation of mast cells from less-differentiated precursors rather than the division of mature dermal mast cells.

It is important to emphasize that the mast cells in the control biopsy specimens from subjects who were dosed with rhSCF often were in close proximity to dermal fibroblasts or dendrocytes, were frequent in the perivascular region of the papillary dermis, and never occurred in the epidermis. Thus these mast cells occupied the normal anatomic locations of mast cells in the skin.<sup>24, 39, 40</sup> Moreover, the ultrastructural features of granulogenesis in mast cells in subjects who were dosed with rhSCF, that is, mast cells with many immature granules with prominent central condensation foci of dense granule materials, were essentially identical to those observed in mast cells that were undergoing maturation from agranular umbilical cord blood mono-

nuclear cells that were cocultured with fibroblasts in vitro.<sup>41-44</sup> Several lines of evidence indicate that mast cell maturation in such fibroblast coculture systems reflects the production, by these fibroblasts, of membrane-associated and/or soluble SCF.<sup>1, 2, 40, 44-46</sup> Finally, the mature granules in the mast cells in the control biopsy sites displayed the usual substructural patterns that are encountered in human mast cells; notably, crystal patterns, a characteristic feature of human dermal mast cells,<sup>22, 39, 40</sup> were especially prominent.

However, we have insufficient evidence to provide an explanation for the presence of immature mast cells in the control biopsy specimens. This finding is not common. Indeed, one of the authors has examined by transmission EM more than 90 biopsies of essentially normal skin from adult human subjects. Immature mast cells undergoing granulogenesis were rarely seen in just a few of these biopsy specimens and were absent from most of them. It is possible that the systemic distribution of rhSCF contributed to some of the findings in the mast cells present in the control biopsies, particularly those obtained after several days of dosing with rhSCF. On the other hand, some mast cells with features of immaturity, including granulogenesis, were present even in the two control biopsy specimens that were obtained on day 1 of rhSCF, within 1 hour, 40 minutes or 2 hours of rhSCF injection at the contralateral site. This interval would seem quite short for the development of a systemic effect of rhSCF on mast cell granule formation. Given the

limited number of specimens available for analysis and the fact that we were not able to obtain biopsy specimens from these patients for ultrastructural analysis before the beginning of dosing with rhSCF, we are unable to ascertain whether or not the presence of immature mast cells in the control biopsy specimens had any relation to the rhSCF dosing.

## Conclusions

In summary, control skin biopsies of sites not directly injected with rhSCF but obtained from patients who were receiving daily dosing of rhSCF at other subcutaneous sites revealed increased numbers of mast cells. Although some of these mast cells exhibited ultrastructural evidence of ongoing maturation, none of them exhibited ultrastructural evidence of secretion. Control biopsy specimens also exhibited no evidence of acute inflammation. By contrast, the wheal and flare reaction sites observed clinically at sites that had recently received subcutaneous injections of rhSCF exhibited extensive AND of mast cells as well as acute inflammatory responses. These sites contained cellular infiltrates consisting primarily of neutrophils but including occasional eosinophils and basophils.

There have been few ultrastructural studies of classic wheal and flare reaction sites, such as those produced by the injection of allergen into subjects with sensitivity to those agents, or in classic dermatographism. However, such studies have not documented prominent infiltration of granulocytes.<sup>47, 48</sup> Indeed, leukocyte recruitment is regarded as a much more characteristic feature of cutaneous late-phase reactions than acute wheal and flare responses.<sup>49</sup> Notably, the wheal and flare reactions to subcutaneous injection of rhSCF in our patients developed more slowly than the typical acute reactions that are provoked in response to allergen in sensitized subjects. While the reason for this difference in the kinetics of the reactions in these two settings is uncertain, our results indicate that the interval of 1 hour, 40 minutes to 2 hours, 30 minutes between the time of rhSCF injection and biopsy of the injection site was sufficient for the elicitation of granulocyte recruitment to these sites, presumably by mast cell-dependent mechanisms.

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