Brief Definitive Report

Recombinant Human Stem Cell Factor (Kit Ligand) Promotes Human Mast Cell and Melanocyte Hyperplasia and Functional Activation In Vivo

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Summary

Stem cell factor (SCF), also known as mast cell growth factor, kit ligand, and *Steel* factor, is the ligand for the tyrosine kinase receptor (SCFR) that is encoded by the c-*kit* proto-oncogene. We analyzed the effects of recombinant human SCF (r-hSCF, 5-50 μg/kg/day, injected subcutaneously) on mast cells and melanocytes in a phase I study of 10 patients with advanced breast carcinoma. A wheal and flare reaction developed at each r-hSCF injection site; by electron microscopy, most dermal mast cells at these sites exhibited extensive, anaphylactic-type degranulation. A 14-d course of r-hSCF significantly increased dermal mast cell density at sites distant to those injected with the cytokine and also increased both urinary levels of the major histamine metabolite, methyl-histamine, and serum levels of mast cell α-tryptase. Five subjects developed areas of persistent hyperpigmentation at r-hSCF injection sites; by light microscopy, these sites exhibited markedly increased epidermal melanization and increased numbers of melanocytes. The demonstration that r-hSCF can promote both the hyperplasia and the functional activation of human mast cells and melanocytes in vivo has implications for our understanding of the role of endogenous SCF in health and disease. These findings also indicate that the interaction between SCF and its receptor represents a potential therapeutic target for regulating the numbers and functional activity of both mast cells and cutaneous melanocytes.

The stem cell factor receptor (SCFR) is expressed on immature hematopoietic progenitor cells, mast cells, melanocytes, and germ cells, and both in vitro studies and analyses of experimental animals indicate that stem cell factor (SCF) is critical for the survival and normal development of these cell types (1–3). Apart from its effects on mast cells, the major actions of SCF in hematopoiesis in vitro are to promote the survival of immature hematopoietic progenitor cells and to synergize with other hematopoietic growth factors in promoting the proliferation and differentiation of committed progenitor cells (1–3). Accordingly, *Escherichia coli*–derived recombinant methionyl human SCF (r-hSCF), which represents essentially the entire extracellular ligand domain of the native transmembrane molecule, as well as the most prevalent native soluble molecule, has been introduced into phase I clinical trials; preliminary results indicate that r-hSCF may indeed be useful in mobilizing hematopoietic progenitors into the circulation and in promoting hematopoiesis (4).

However, SCF has an exceedingly complex biology; r-hSCF certainly represents the most pleiotropic of the "hematopoietic" growth factors that have been introduced into the clinic. For example, recombinant SCF can promote mast cell hyperplasia in mice (5), rats (5), and nonhuman primates (6) in vivo; human mast cell development in vitro (7–10); degranulation and mediator release from mouse dermal mast cells in vivo (11) or human cutaneous mast cells in vitro (12); and, in synergy with phorbol esters, the proliferation of human melanocytes in vitro (13).

In this study, we investigated whether r-hSCF can influence human mast cell or melanocyte development or function in vivo. Portions of this work have been reported in abstract form (14, 15).

Materials and Methods

Patients and r-hSCF Dosing Protocol. 10 women with stage IIIB or IV breast carcinoma who had volunteered to participate in an

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/06/2681/06 $2.00
Volume 183 June 1996 2681–2686

open-label phase I trial of r-hSCF administered before and after up to five cycles of chemotherapy with cyclophosphamide and doxorubicin were enrolled after providing informed consent and meeting the following major eligibility requirements: no prior chemotherapy for metastatic disease, no adjuvant chemotherapy within the preceeding 6 mo, <250 mg/m^2 prior anthracyclines, age >18 yr, Karnofsky performance status ≥70%, absolute neutrophil count ≥2,000/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥9 g/dl, adequate organ function, and no active asthma or other significant immediate hypersensitivity disorders. The protocol, which was approved by the Institutional Review Board for Human Studies of the Dana–Farber Cancer Institute, called for patients to receive an initial 14-d course of r-hSCF (5, 10, 25, or 50 μg/kg/d, with each patient receiving only one of these doses) via daily subcutaneous injection in the upper arm, anterior thigh, or abdominal wall (cycle 0), followed by a 7-d period of observation before initiation of chemotherapy.

Histology. Written informed consent was obtained from each patient to procure 4-mm punch biopsies of skin after induction of local anaesthesia (0.5–1.0 ml of 1% or 2% lidocaine, injected intradermally at least 1 cm away from the site to be biopsied). To assess the systemic effects of r-hSCF on dermal mast cells, biopsies (separated by at least 3 cm) were obtained from the skin over the posterior iliac crest (i.e., at a site not injected with r-hSCF) before (day -1 or 0) and on the last day (day 14) of the first period of r-hSCF dosing. Half of each biopsy was immediately placed in 10% neutral buffered formalin (NBF) and the other half into freshly prepared Carnoy's fixative. All sections were coded, to conceal each specimen's identity, before microscopic analysis. Carnoy's fixed, paraffin-embedded sections (4 μm thick) were stained for 10–15 min with 0.5% alcian blue (Rowley Biochemical Institutes, Inc., Rowley, MA) in 0.7 M HCl, rinsed, and counterstained with 0.25% safranin (Rowley Biochemicals) in 0.125 M HCl for 2 h (5), and the numbers of mast cells per square millimeter of dermis were quantified at 400 × by use of the Bioquant Morphometric System (R & M Biometrics, Inc., Nashville, TN) (6). Biopsies of hyperpigmented lesions were fixed in NBF, embedded in paraffin, and stained with the Fontana–Masson technique to identify melanin (16) or with hematoxylin and eosin (H and E) to quantify melanocytes as number of basal clear cells per square millimeter of skin (17).

Electron Microscopy. Skin biopsies (performed as above) of the wheal-like lesions that developed 90–120 min after r-hSCF injection, and control biopsies of identically prepared contralateral sites (not injected with r-hSCF), were immediately immersed in a pool of freshly prepared fixative (2.0% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4) at room temperature, trimmed, and then fixed and processed for transmission electron microscopy (18).

Quantification of Mast Cell Mediators. Serum specimens obtained before r-hSCF dosing (day −1) and on day 14, just before the last r-hSCF injection, and urine specimens obtained on day −1 and on day 14 or 15 (before or ~1 d after the last r-hSCF injection), were stored as 0.5-ml aliquots at −20°C. Because of histamine's short half-life in the circulation (~1 min), urinary levels of the major histamine metabolite, methyl-histamine, are used to assess systemic changes in histamine levels (19). Urine methyl-histamine was measured with the methyl-histamine RIA kit, exactly as specified by the manufacturer (Pharmacia Inc. Diagnostics, Columbus, OH; detection range, 0.2–10.0 μg/l). To account for individual variation in urinary concentration, values were expressed as normalized methyl-histamine, i.e., micrograms of methyl-histamine per milligram of creatinine in the same aliquot of urine. In all patients, the serum blood urea nitrogen and creatinine levels were stable over the 2-wk period of r-hSCF dosing.

Tryptase (stored in the cytoplasmic granules of most, if not all, human mast cells) is a more specific marker of mast cells than is histamine (20, 21). At least two tryptase genes occur in the human genome, encoding α-tryptase and the ~93% identical β-tryptase (22). Serum tryptase was measured with the Tryptase RIACT kit (exactly as specified by the manufacturer [Pharmacia Diagnostics]); the capture antibody used in this assay (G5) recognizes primarily β-tryptase, which is not detectable (<1.0 ng/ml) in serum obtained from healthy control subjects (20). Most samples were also analyzed by using the new B12 capture assay that measures both α- and β-tryptase and can detect α-tryptase in blood from normal individuals (mean levels of ~5 ng/ml, all values <20 ng/ml) (20, 21).

Statistics. Serial observations for each individual were compared by use of the 1-tailed paired Student's *t* test (after first establishing that the differences between the baseline and post–r-hSCF dosing values conformed to a normal distribution). Data are expressed as mean ± SD.

Results and Discussion

r-hSCF Induces Dermal Mast Cell Degranulation. All 10 patients developed a raised, pruritic wheal with surrounding erythema at r-hSCF injection sites (Fig. 1). The lesions were clinically most prominent ~90–120 min after r-SCF injection, they were induced by every dose of r-hSCF, and, at any given dose of r-hSCF, the intensity of the lesions that developed after each of the injections in individual subjects did not appear to change over the 14-d treatment period. Oral premedication with H_1 (diphenhydramine 50 mg, orally, each 6 h) and H_2 (ranitidine 150 mg, orally, each 12 h) antihistamines minimized the pruritis associated with these reactions but appeared to have little or no effect on the wheal and flare component of the responses.

Biopsies of the wheals at r-hSCF injection sites, as well as control biopsies of contralateral normal skin, were obtained twice from two individuals and once from a third subject, providing five r-hSCF injection sites and five control sites for electron microscopy. As illustrated in Fig. 2, many of the dermal mast cells at all five r-hSCF injection sites exhibited extensive degranulation, including cytoplasmic granule extrusion, a characteristic feature of anaphylactic-type degranulation in human skin mast cells (18). By

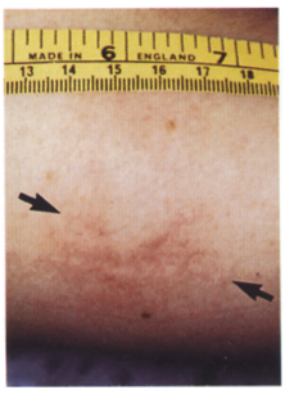

Figure 1. A wheal (area of central pallor) with surrounding erythema (*arrows*) that developed ~90 min after the subcutaneous injection of r-hSCF (10 μg/kg).

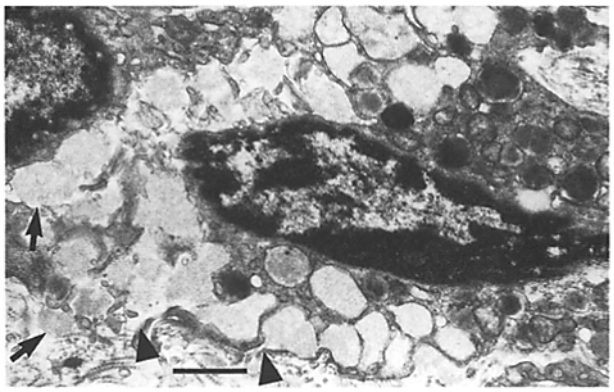

Figure 2. Electron micrograph demonstrating extensive anaphylactic-type degranulation of a dermal mast cell in an r-hSCF injection site biopsy obtained 2 h after subcutaneous administration of the agent. Many structures that represent fused cytoplasmic granule membranes, some of them in continuity with the plasma membrane (*arrowheads*), are present. There is also evidence of extrusion of cytoplasmic granule contents at the cell surface (*arrows*). Bar, 1.0 μm.

contrast, none of the mast cells in any of the control biopsies demonstrated anaphylactic degranulation (not shown). These findings are the first to demonstrate that any cytokine can induce human mast cell degranulation in vivo.

Three subjects were withdrawn from the study because they exhibited distant adverse clinical effects that may have reflected mast cell activation in response to injection of r-hSCF, including moderate to severe (grade 3 in severity according to the World Health Organization toxicity scale [22]) urticarial reactions and upper airway symptoms (cough, hoarseness, or throat tightness). A complete description of the results of the phase I trial of r-hSCF, including both the hematological findings and a more detailed description of the adverse events, will be reported separately (our unpublished data).

r-hSCF Induces Dermal Mast Cell Hyperplasia. The mean total dermal mast cell density (number per square millimeter) in the seven subjects who completed the first course of r-hSCF treatment increased by 67% (75 ± 31 [range 49–120] at day 14 vs. 45 ± 22 [range 28–85] at baseline, $P = 0.011$), with significant increases in the mean density of mast cells in both the papillary dermis (254 ± 87 on day 14 vs. 173 ± 89 at baseline, $P < 0.001$) and reticular dermis (51 ± 19 at day 14 vs. 34 ± 16 at baseline, $P \cong 0.043$). This is the first demonstration that any cytokine can induce human mast cell hyperplasia in vivo. Moreover, human subjects appear to be more sensitive to the systemic effects of r-hSCF on dermal mast cell development than are cynomolgus monkeys (*Macaca fascicularis*), in which administration of r-hSCF subcutaneously at 100 μg/kg/d for 21 d, did not result in significantly increased numbers of dermal mast cells in skin distant to sites of r-hSCF injection (6).

r-hSCF Treatment Results in Increased Systemic Levels of Mast Cell Mediators. Each of the five patients for whom complete data are available (numbers 103, 125, 128, 138, and 162) exhibited an increase in normalized urine methylhistamine over the course of r-hSCF dosing. Mean (± SD) values for micrograms of methyl-histamine per milligram of creatinine increased ~44%, from 0.126 (± 0.042) at baseline to 0.182 (± 0.031) at day 14 or 15, $P = 0.006$.

The G5 assay, which detects primarily β-tryptase, detected no serum tryptase (<1.0 ng/ml) at baseline or on day 14 in all but one of the seven patients who completed 14 d of r-hSCF dosing without exhibiting any symptoms suggestive of systemic mast cell activation; this subject (number 128) had <1.0, 1.0, and 1.8 ng of tryptase/ml on days 0, 7, and 14, respectively. By contrast, the B12 assay, which detects both α- and β-tryptase, demonstrated a two-fold or greater increase in tryptase levels after 14 d of r-hSCF dosing in each of the five patients for whom complete data are available (Table 1); the magnitude of the increase in percentage of serum tryptase between day 0 and day 14 was strongly correlated with the dose of r-hSCF ($R = 0.97$, $P \cong 0.006$), and marked increases were observed even in patients who exhibited no systemic clinical adverse events during the 14-d dosing period. In three patients, the B12 assay also documented increases in serum tryptase (of 68–166%) in specimens obtained 4–20 h after systemic clinical adverse events that were suggestive of widespread mast cell degranulation; the G5 assay detected

Table 1. *Administration of r-hSCF Results in Increased Levels of Serum Tryptase*

Patient	r-hSCF dose	Study day	Serum tryptase
	μg/kg/d		*ng/ml*
161	5	0	4
		14	10
103	10	0	12
		14	29
106	10	0	11
		‡7	19
		§7	32
		14	22
*185	25	0	35
		‖5	93
*123	50	0	2
		¶0	5
128	50	0	19
		7	200
		14	251
138	50	0	14
		14	140

*Withdrawn from study after development of a systemic reaction.
‡,§Specimens obtained on day 7 before (‡) and 5 h after (§) one 50 μg/kg dose of r-hSCF that was associated with a systemic reaction; this subject had no subsequent systemic reactions to 10 μg/kg doses of r-hSCF.
‖,¶Specimens obtained 20 h (‖) or 4 h (¶) after onset of systemic reactions.

Figure 3. Areas of persistent cutaneous hyperpigmentation (*arrows*) at 3 sites (on the upper anterior thigh) that had been injected subcutaneously with r-hSCF (50 μg/kg/site) more than 6 mo earlier.

an increase in serum tryptase (to 2.6 ng/ml) in one of these three subjects (number 106). These findings are consistent with the hypothesis that β-typtase is released primarily in association with extensive, systemic, anaphylactic-type mast cell degranulation (21), whereas α-tryptase appears in the circulation as a result of a distinct pathway of nonanaphylactic release that reflects total body levels of mast cells (20).

Notably, the increases in serum tryptase levels detected over the 14-d course of r-hSCF dosing (range = 100 – 1220% increase) greatly exceeded the corresponding increases in total dermal mast cell numbers (range = 4.5 – 100% increase) in the same five subjects; in four of these patients, the day 14 serum tryptase values were in the range observed in naturally occurring systemic mastocytosis (i.e., >20 ng/ml) (20). *M. fascicularis* that had been dosed for 21 d with 100 μg of r-hSCF/kg/d exhibited no significant increase in noninjection-site skin mast cells, but developed marked elevations of mast cell numbers (4- to 128-fold increases) in organs with a relatively porous vasculature, such as the mesenteric lymph nodes, bone marrow, liver, and spleen (6). Taken together, these findings suggest that the quantification of the systemic effects of r-hSCF on dermal mast cell numbers in our patients may have underestimated the effects of the course of r-hSCF dosing on total body mast cell levels.

SCF can maintain mast cell survival by suppressing apoptosis (23–25). Our protocol did not permit us to obtain additional biopsies of clinically normal skin in our patients, but serum tryptase levels in other subjects dosed with r-hSCF fell to approximately baseline levels within 2 wk of cessation of r-hSCF dosing (L.B. Schwartz et al., unpublished data). This finding suggests that in humans, as in mice (25) and *M. fascicularis* (6), rSCF-induced mast cell hyperplasia is reversible upon cessation of cytokine dosing.

r-hSCF Promotes Epidermal Hyperpigmentation and Enhanced Melaninization as Well as Melanocyte Hyperplasia. Five of the 10 subjects treated with r-hSCF developed areas of striking cutaneous hyperpigmentation, 3–5 cm in diameter, at one or more r-hSCF injection sites (Fig. 3). These lesions were first noted during the initial 14 d of r-hSCF administration before chemotherapy (cycle 0) in three subjects and after chemotherapy, during cycle 3 or 4 of dosing, in two other subjects. In most instances, the lesions were first noted 3 or 4 d after r-hSCF injection at those sites. When hyperpig-

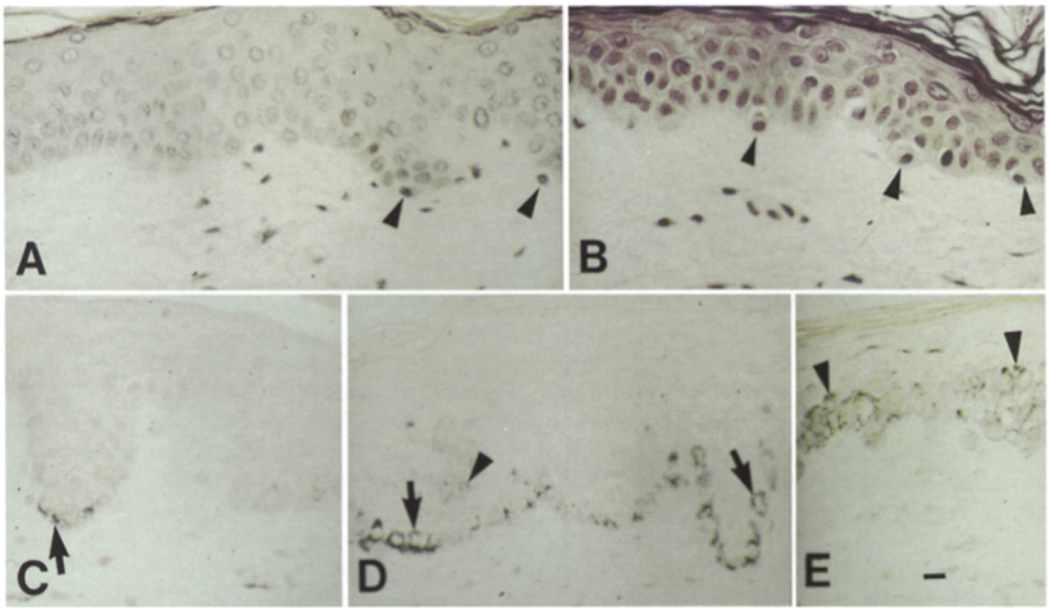

Figure 4. (*A* and *B*) H and E–stained sections of skin biopsies of (*A*), a site not injected with r-hSCF, obtained at the end (day 14) of the first course of r-hSCF administration, or of (*B*), an area of hyperpigmentation at a site that had received a single injection of r-hSCF 2 mo earlier. There are increased numbers of melanocytes (*arrowheads*), which appear as basal clear cells with separation from surrounding keratinocytes, in biopsy *B* compared with biopsy *A*. (*C* and *D*) Fontana–Masson–stained sections, in which melanin is stained purple-black. (*C*) In a biopsy obtained before the start of r-hSCF injections, melanin is present focally in basal keratinocytes (*arrow*). (*D*) The same biopsy shown in *A*, demonstrating prominent melanin in virtually all basal keratinocytes (*arrows*) and in occasional keratinocytes (*arrowheads*) above the basal layer. (*E*) The same biopsy as in *B*, showing markedly increased melanization, with prominent melanin in all basal keratinocytes and in many keratinocytes (*arrowheads*) above the basal layer. All biopsies are from the same patient. Bar in *E*, 25 μm.

mentation developed during cycle 0, this response was observed at each r-hSCF injection site over the entire course of the study. The hyperpigmented skin lesions persisted for variable but generally long periods of time (2–12 mo), during which they gradually faded.

In comparison with the skin biopsies obtained from the same subjects before the initiation of r-hSCF administration (Fig. 4 *C*), the hyperpigmented lesions exhibited markedly enhanced epidermal melanization (Fig. 4 *E*). While some of the sites also exhibited focal perivascular infiltrates of lymphocytes and eosinophils and occasional basophils, few melanophages were present, indicating that the enhanced melanization at these sites is unlikely to be of a postinflammatory etiology (26). Moreover, modest increases in epidermal melanization were also detected in some skin biopsies obtained at the end (day 14) of the first cycle of r-hSCF dosing at sites that had not been injected with r-hSCF and that clinically did not appear to be hyperpigmented (Fig. 4 *D*). Taken together, these findings indicate that r-hSCF can promote the functional activation of human melanocytes, as well as human mast cells.

Biopsies of both pretreatment skin and sites of persistent (2–12-mo-old) r-hSCF-induced hyperpigmentation were available in three subjects (numbers 161, 128, and 138, treated with 5, 50, or 50 μg of r-hSCF/kg/d, respectively). The mean number of melanocytes in the hyperpigmented lesions was increased by >300% compared with that in the pretreatment biopsies (38 ± 8 [range 29–44] vs. 9 ± 2 [range 7–11] melanocytes per linear millimeter of epidermis, respectively; $P \cong 0.015$) (Figs. 4, *A* and *B*). None of the melanocytes appeared dysplastic. Moreover, melanocyte numbers were not increased in hyperpigmented areas that were biopsied within 2 wk of r-hSCF injection (n = 2), indicating that r-hSCF–induced hypermelanosis can occur before the onset of melanocytic hyperplasia. Nor were melanocyte numbers significantly increased after 14 d of r-hSCF dosing in skin biopsies from the iliac crest, a site distant from those directly injected with the cytokine (n = 7) (data not shown). This finding suggests that human melanocytes may be less sensitive to the systemic effects of r-hSCF on proliferation than are human mast cells.

It is not clear why some subjects injected with r-hSCF did not develop clinically detectable areas of hyperpigmentation at the injection sites, or whether this represented a qualitative or quantitative difference in the responses of the different patients. Notably, r-hSCF does not induce human melanocyte proliferation in vitro unless a second agent (e.g., PMA) is also present (13), raising the possibility that individual subjects may vary in the levels of other factors that contribute to the expression of r-hSCF–induced effects on melanocyte development or function.

Conclusions. Our observations provide direct evidence that r-hSCF can promote the hyperplasia and the functional activation of two distinct SCFR$^+$ populations in humans in vivo, mast cells and melanocytes. These findings thus not only strengthen the evidence that endogenous SCF represents a critical regulator of the development and function of mast cells and melanocytes in humans, as well as in experimental animals, but also support the view that the increased production or bioavailability of endogenous SCF may contribute to certain diseases associated with mast cell and/or melanocyte hyperplasia or hyperpigmentation. For example, our observations support the concept that changes in levels of endogenous SCF may account for two of the cardinal features of urticaria pigmentosa (27), and may also contribute to mast cell hyperplasia and/or epidermal hyperpigmentation in other settings.

Our findings also suggest that enhancing or suppressing local levels of SCF (or other approaches for manipulating SCFR-dependent signaling) may be useful for increasing or reducing mast cell numbers or for promoting or reducing cutaneous melanization, in settings in which these effects are clinically desirable. Finally, since blood levels of α-tryptase are elevated in both naturally occurring (20) and r-hSCF–induced iatrogenic systemic mastocytosis, α-tryptase may prove to be a practical and sensitive index for estimating total body mast cell numbers.

We thank Dr. Zhen-sheng Wang for assistance with the histological analysis, Rita A. Monahan-Earley, and Patricia Fox for assistance with the electron microscopy, and Bernard J. Ransil, Ph.D., M.D. for assistance with the statistical analysis.

This work was supported by United States Public Health Science grants CA/AI-72074, AI/GM-23990 and AI-31982 (to S.J. Galli), AI-33372 (to A.M. Dvorak), and AI-20487 (to L.B. Schwartz), by AMGEN, Inc., and by the Beth Israel Hospital Pathology Foundation. J.J. Costa, G.D. Demetri, and S.J. Galli have performed research funded by AMGEN, Inc. and consult for AMGEN, Inc. under terms that are in accord with Beth Israel Hospital (J.J. Costa, S.J. Galli), Dana–Farber Cancer Institute (G.D. Demetri) and Harvard Medical School conflict-of-interest guidelines; L.B. Schwartz performs research funded by Pharmacia under terms that are in accord with Virginia Commonwealth University's conflict-of-interest guidelines.

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Received for publication 29 January 1996 and in revised form 5 March 1996.

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