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IN VIVO ADMINISTRATION OF STEM CELL FACTOR ENHANCES BOTH PROLIFERATION AND MATURATION OF MURINE MEGAKARYOCYTES

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ABSTRACT

Background. **Stem cell factor (SCF) has already been shown to participate in the regulation of erythro- and granulopoiesis. The aim of this study was to define the possible role of SCF in the regulation of megakaryocytopoiesis.**

Methods. **Stem cell factor activity has been assessed in an *in vivo* murine model, in which different doses of the factor were either given alone or in association with recombinant human erythropoietin (rhEpo). Mice were sacrified after a six-day treatment to evaluate the effect of SCF on the number of bone marrow and spleen colony-forming units-megakaryocyte (CFU-Mk), and after a two-day treatment for evaluation of thrombopoietin-like activity.**

Results. **We found that SCF induces a dose-related increase in the number of CFU-Mk in both the bone marrow and spleen of the treated mice, and that in the range of the doses used (from 25 to 200 mg/kg/day) the greatest activity was observed when a dose of 200 mg/kg/day was injected. The effect was enhanced by adding rhEpo to optimal SCF concentrations.**

SCF also stimulated megakaryocyte maturation as assessed by the megakaryocyte number, the size of acetylcholinesterase-positive cells, ^{35}Sulphur (^{35}S) incorporation into the newly formed platelets. All these parameters were only minimally affected by the addition of rhEpo.

Conclusions. **These data suggest that SCF participates in the regulation of megakaryocytopoiesis and that its administration might have a role in the treament of disorders of platelet production.**

Key words: SCF, rhEpo, megakaryocytopoiesis

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SCF or mast cell growth factor, the ligand for the tyrosine kinase-associated receptor encoded by the c-kit locus, has been shown to have direct hematopoietic colony-stimulating activity,[1] and to synergize with other cytokines to stimulate erythropoiesis and granulopoiesis in in vitro studies with human bone marrow cells,[2] and in vivo murine models.[3-5]

Nevertheless, only limited data are available on the effect of SCF on the proliferation and differentiation of cells of the megakaryocyte lineage. The receptor for SCF is expressed in murine bone marrow megakaryocyctes[6] and this factor stimulates the proliferation of immortalized megakaryocyte cell lines.[7] In an *in vivo* study in non human primates both the number of bone marrow megakaryocyets and the platelet number were increased by SCF administration.[8]

In this study experiments were carried out in order to evaluate the influence of SCF, either alone or in combination with erythropoietin (Epo), on megakaryocytopoiesis in an *in vivo* murine model.

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Materials and Methods

Male Swiss mice weighing 25-30 g were used in all the experiments.

Factors

Rat PEG-SCF, generously provided by Amgen (Thousand Oaks, CA), was stored at 4°C and used after dilution with phosphate buffered saline (PBS) plus 0.1% bovine serum albumin (BSA) (Bayer Diagnostici, Italy).

PEG-SCF was found to be at least as active as the unmodified molecule when injected in primates,[4] sometimes giving an even more rapid increase in the peripheral blood cells. Mice were injected intraperitoneally twice a day with freshly prepared solutions of SCF to reach total daily doses of 25, 50, 100, 200 μg/kg; these doses were chosen according to the data published by Molineux et al.[4] regarding the effect of SCF on granulocytopoiesis in mice, and those of Andrews et al. who evaluated the activity of SCF on hematopoiesis in primates.[8,9]

RhEpo (Amersham International, U.K.) was administered at a dose of 8 U/mouse/day. This amount had been found to produce the maximal increase in the number of splenic megakaryocytes in a previous study.[10]

Doses were fractioned in two separate administrations per day, and in no case did the total volume exceed 250 μL per injection. Control mice (3 to 5/group depending on the number of experimental mice) were injected with PBS-BSA using the same schedule as that for the SCF-treated animals.

CFU-Mk cultures

For these experiments groups of three mice were treated with SCF daily for six days. Bone marrow CFU-Mk were grown as described previously.[10,11] Briefly, 2×10^5/mL mononuclear cells were cultured in McCoy's 5A medium containing 15% fetal calf serum and 0.3% Bacto agar. Cultures were fed with 10% pokeweed-mitogen (Gibco U.K.) spleen conditioned medium (PWM-SCM) as a source of megakaryocyte colony-stimulating activity.

Splenic cells were cultured at 5×10^5/mL in serum-containing medium.[4] Triplicate cultures were incubated for 7 days at 37°C in a 5% CO_2 fully humidified atmosphere. Megakaryocyte colonies were recognized by acetylcholinesterase staining (Ache) of the agar preparation,[11] and aggregates of three or more cells were recorded as colonies.

Spleen cell conditioned medium was obtained by incubating 10^7/mL spleen cells in α-MEM with 5% heat-inactivated plasma and 2.5 mg/mL pokeweed mitogen.

Evaluation of thrombopoiesis in vivo

Thrombopoiesis was monitored by injecting five mice/group with the chosen amounts of SCF and rhEpo (see above) in two daily doses for two days. Next 30 μCi/mouse of ^{35}S were injected in the tail vein according to McDonald[12] and incorporation of the radioactive element was calculated as previously described.[13] PBS plus BSA was used in control mice.

The size of the megakaryocytes was studied by flushing bone marrow cells with CATCH medium (calcium- and magnesium-free Hank's balanced salt solution containing 2×10^{-3}M theophylline, 10^{-3}M adenosine, 3.8% sodium citrate, 3.5% bovine serum albumin, 25 mM HEPES buffer, 115 U/mL of DNAase I, pH 7.1, 295±5 mOsm, 3.5% poly-vinyl-pyrrolidone); 2×10^4 cells were cytocentrifuged and stained for Ache.

This staining allows identification of the smallest morphologically non recognizable megakaryocytes. At least 200 megakaryocytes from each femur were counted.

Splenic and bone marrow megakaryocytes were enumerated in hematoxylin-eosin-stained paraffin-embedded sections prepared from mice treated as for colony growth, and the number of recognizable megakaryocytes was determined using an eye-piece reticulum at 400× by counting five randomly chosen reticulum areas.[14]

Platelets were enumerated by phase contrast microscopy.

Statistical analysis

Values represent the mean and standard deviation of three experiments for each test. The Student-t test was employed for statistical analysis, and values of $p<0.05$ were considered to be statistically significant.

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Table 1. Effect of SCF on the number of bone marrow and spleen colony forming units-megakaryocyte.

	pure Mk		p	mixed MK		p
Bone marrow colonies (2x10^5 cells)						
controls	4.7	2.2		7.8	3.5	
25	4.6	2.1	n.s.	8.0	4.1	n.s.
50	6.1	2.6	n.s.	11.0	2.3	n.s.
100	7.5	2.3	<0.05	13.0	1.9	<0.01
200	7.8	1.5	<0.01	13.3	2.7	<0.01
Spleen colonies (5x10^5 cells)						
controls	7.5	2.2		10.0	1.0	
25	6.0	2.7	n.s.	12.0	3.5	n.s.
50	6.2	3.5	n.s.	13.6	5.0	n.s.
100	10.0	2.2	<0.01	17.8	3.7	<0.01
200	13.6	2.8	<0.01	18.5	3.0	<0.01

Mice were treated with SCF (25, 50, 100, 200 mg/Kg/day) divided in two daily doses for 6 days. Pure Mk: colonies containing only megakaryocytic cells. Mixed Mk: colonies containing also non megakaryocytic cells.

Results

Effects of SCF on ex vivo CFU-Mk growth

Groups of three mice were treated for six days with 25, 50, 100 and 200 μg/kg/day SCF. A significant increase with respect to controls was observed in the number of bone marrow pure megakaryocyte colonies in mice injected with 100 ($p<0.05$) and 200 μg/kg/day ($p<0.01$). The same amounts of SCF also induced a significant growth of mixed colonies containing some non megakaryocytic cells (Table 1).

Results obtained in splenic cells were similar to those in bone marrow for both pure megakaryocyte- and mixed colonies (Table 1).

In other experiments 200 μg/kg/day of SCF were injected together with rhEpo (8 U/mouse per day): with respect to controls, the treatment augmented the number of bone marrow ($p<0.01$) and spleen ($p<0.05$) CFU-Mk, and in this setting the combination of the two cytokines had more than an additive effect when compared to SCF alone ($p<0.01$) (Table 2).

Effects on thrombopoiesis in vivo

Groups of five mice were given SCF for two days, using the same range of doses as for colony growth. As shown in Figure 1, a significant increase over control values in platelet ^{35}S incorporation was obtained only in mice injected with 200 μg/kg/day ($p<0.01$). RhEpo (8 U/day) failed to stimulate the incorporation of ^{35}S significantly with respect to controls, and even the increase observed in mice treated with a combination of SCF (200 μg/kg/day) plus rhEpo was not significantly different from the value obtained with SCF alone.

The number of bone marrow and spleen megakaryocytes rose significantly when 100 and 200 μg/kg/day of SCF were injected for six days (Table 3), while a dose as low as 50 μg/kg/day was sufficient to augment the size of bone marrow megakaryocytes (Figure 2a). Finally, the number of platelets was not increased by SCF after two days of treatment (Figure 3), and the addition of rhEpo caused only an insignificant marginal rise with respect to control mice. Response in mice treated with rhEpo alone was no different from that observed in controls (Table 3; Figure 2b; Figure 3).

In comparison with SCF alone, the addition of

Table 2. Effect of treatment with SCF and/or rhEpo on colony forming units-megakaryocyte.

	pure Mk		p	mixed MK		p
Bone marrow colonies (2x10^5 cells)						
controls	3.0	1.2		3.5	2.0	
rhEpo	4.0	1.5	n.s.	4.2	2.1	n.s.
SCF	7.0	1.1	<0.01	11.5	2.5	<0.01
SCF+rhEpo	18.0	2.7	<0.001*	31.0	3.0	<0.001*
Spleen (colonies/5x10^5 cells)						
controls	4.0	2.1		4.5	1.5	
rhEpo	4.2	1.7	n.s.	3.9	2.0	n.s.
SCF	8.0	1.8	n.s.	11.0	3.1	<0.05
SCF+rhEpo	20.0	2.05	<0.001*	35.0	4.7	<0.001*

Mice were treated with SCF (200 μg/Kg/day) and/or rhEpo (8 U/day).
*The number of both bone marrow and spleen CFU-Mk was significantly higher in mice treated with SCF and rhEpo than in those treated with SCF alone ($p<0.01$).

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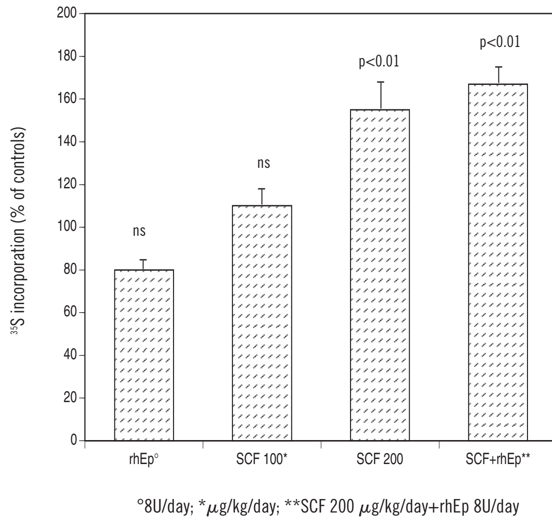

Figure 1. Effect of SCF alone or combined with rhEpo on ^{35}S incorporation in platelets. Mice were sacrified after a two-day treatment with the indicated doses of SCF and/or rhEpo. Results are expressed as percent of the control value.

suboptimal doses of SCF to Epo did not significantly affect CFU-Mk proliferation or thrombopoiesis (data not shown).

Discussion

Recombinant DNA technology has provided large amounts of SCF that are being used to elucidate its role in the regulation of hematopoiesis. The murine and rat molecules have proved their ability to stimulate hematopoiesis in rodents; the human molecule has shown activity in *in vitro* studies, and its *in vivo* administration in non human primates has resulted in a stimulation of bone marrow activity.

SCF is necessary for the development of BFU-E, and IL-3 and rEpo potentiate this biological activity in normal murine and human hemopoietic progenitors.[15-18] Moreover, the addition of SCF to *in vitro* cultures of bone marrow cells from patients with Diamond-Blackfan anemia,[19,20] aplastic anemia[21] and low-risk myelodysplastic syndromes[22] improves both erythropoiesis and granulopoiesis.

Very few *in vitro* studies have focused on the relationship between SCF and megakaryocytopoiesis. Avraham et al.[7] found that SCF alone is

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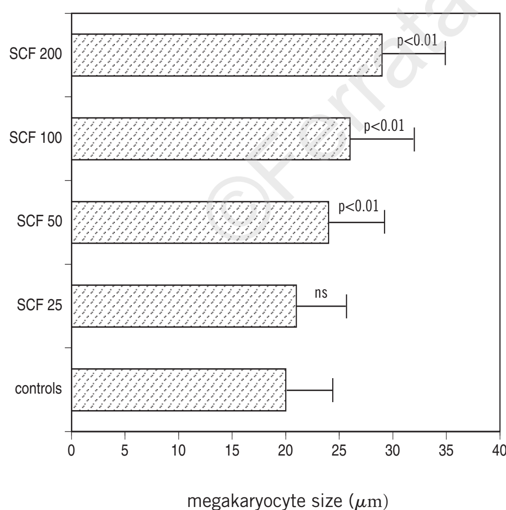

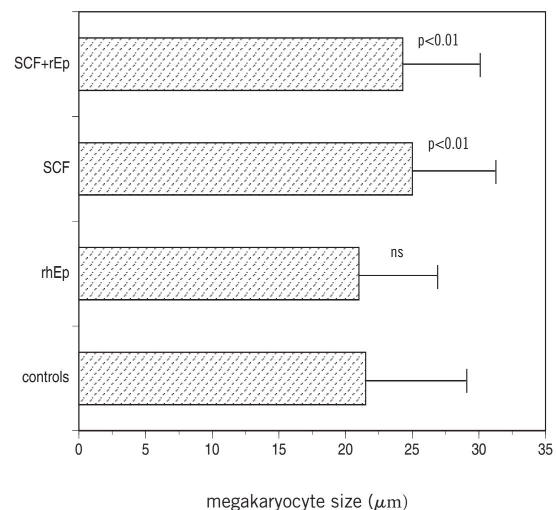

Figure 2. Effect of different doses of SCF alone (μg/kg/day) (A) or SCF (200 μg/kg/day) combined with rhEpo (B) on megakaryocyte size. Mice were sacrified after two days of treatment, and cells were detected by Ache staining in order to include morphologically non recognizable megakaryocytes.

doses tested can effectively stimulate megakaryocytopoiesis, both in the proliferation of CFU-Mk and in the maturation of megakaryocytes. The number of pure and mixed CFU-Mk was found to be significantly higher in the SCF-treated mice than in the controls, and the effect was similar in the bone marrow and the spleen. Combined treatment with rhEpo further increased the number of colonies, indicating a potentiating role for Epo. The importance of Epo in the regulation of megakaryocytopoiesis is still unclear; however, our study confirms that Epo is probably devoid of Mk-CSA activity, while it does possess a potentiating activity we had previously observed *in vitro*.[10]

SCF also seems to enhance megakaryocyte maturation and thrombopoiesis, as shown by the dose-dependent increase in the number of megakaryocytes, in their size, and in ^{35}S incorporation in platelets. However, our study indicates that SCF alone is not a sufficient stimulus to determine an increase in platelet number, a result that is not attributable to the treatment schedule (mice were injected for two instead of the six days used for megakaryocyte proliferation), which is appropriate for studying the early events following activation of megakaryocyte maturation.[25] In this setting the combination of rhEpo with SCF was of little additional benefit because platelet counts were not significantly different from those of controls or those of mice treated with SCF alone. The thrombopoietin-like activity of Epo is still uncertain: the studies published so far have shown contrasting results, perhaps due to the delivery schedules. In fact, acute high doses have been found to stimulate[26] and chronic large doses to inhibit[27] thrombopoiesis. However, with the same schedule utilized in this paper we previously reported that Epo did not show any thrombopoiesis stimulating activity.[10]

The results of our study add new insights into the role of SCF on the regulation of hematopoiesis. Its activity on erythropoiesis and granulopoiesis has already been shown and found to be boosted by the presence of other cytokines.[2,4,5,8] We demonstrate herein that SCF participates in the regulation of megakaryocytopoiesis, and its effect on the proliferation of

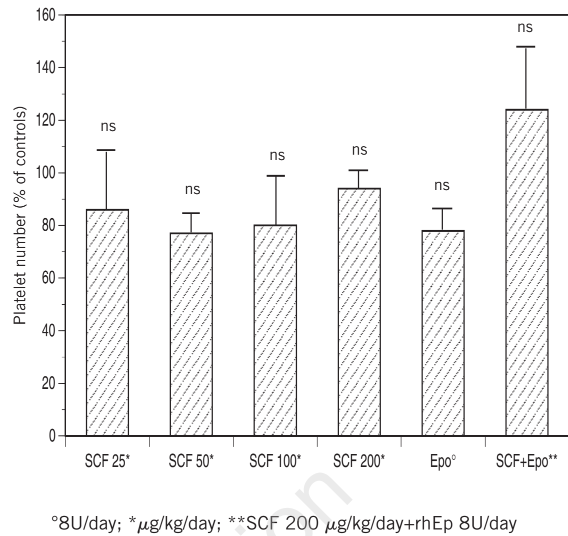

Figure 3. Platelet number expressed as percent of control value in mice treated as indicated.

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megakaryocyte progenitors is synergistically increased by Epo. However, this might not be the only interaction in the cytokine network because, given the experimental design of our study, it cannot be ruled out that SCF may act via the release by ancillary cells of other regulators of megakaryocytopoiesis, such as IL-3, GM-CSF or IL-6. In this respect it should be noted that SCF has been found to be capable of inducing the expression of IL-3 in cell lines,[7] and a role, together with other cytokines, in the regulation of megakaryocytopoiesis is also suggested by a recent report by Tanaka et al.[28] who observed that *in vitro* SCF enhanced the proliferation of CFU-Mk, while the maturation of cells was regulated by the presence of IL-6. The results of this study seem to confirm that the same cytokines do not interact in the control of CFU-Mk proliferation and thrombopoiesis. However, the very recent purification of the c-mpl ligand that possesses both meg-CSF and thrombopoietin-like activity and whose DNA has also been cloned,[29-33] and the role played by the oncogene c-mpl (a member of the receptor superfamily with sequence similarity to Epo and G-CSF receptors) in the regulation of platelet production[34,35] seem to question the model of separate

Table 3.

Number of megakaryocytes (I)						
	bone marrow		*p*	*bone marrow*		*p*
controls	33.0	5.0		27.0	5.0	
25	32.0	4.8	n.s.	28.2	6.0	n.s.
50	33.4	7.3	n.s.	29.2	8.2	n.s.
100	48.5	10.0	<0.05	42.0	11.3	<0.05
200	62.2	9.2	<0.001	50.5	10.4	<0.01

Five mice/group were treated with stem cell factor (25, 50, 100, 200 μg/kg/day) for 6 days.

Number of megakaryocytes (II)						
	bone marrow		*p*	*bone marrow*		*p*
controls	34.2	4.3		29.3	2.8	
rhEpo	20.0	5.0	n.s.	20.0	6.3	n.s.
SCF	54.8	5.7	<0.01	45.0	7.2	<0.01
SCF+rhEpo	69.7	7.1	<0.01	40.0	9.4	<0.01*

Mice treated with stem cell factor (200 μg/kg/day) and/or recombinant human erythropoietin (8 U/day).
**The number of bone marrow megakaryocytes was significantly higher in mice treated wth SCF+rhEpo than in those treated with SCF alone (p<0.05).*

regulation of CFU-Mk growth and megakaryocyte maturation. Hopefully, in the near future these new acquisitions will lead to a further definition of the growth factor interactions and suggest new strategies for the treatment of disorders of platelet production.

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