Stem Cell Factor Induction of In Vitro Murine Hematopoietic Colony Formation by “Subliminal” Cytokine Combinations: The Role of “Anchor Factors”

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The high levels of hematopoietic growth factors required for in vitro and in vivo activity raise questions as to their role in normal hematopoietic maintenance. We hypothesize that the use of combinations of cytokines to stimulate hematopoietic progenitors might allow individual factors to exert their influence at lower, more physiologically relevant concentrations. Growth factor combinations were assessed by their ability to stimulate both total colonies and high proliferative potential colony-forming cells (HPP-CFC), an early murine hematopoietic progenitor, in double-layer agar cultures. Very-low-level combinations of colony-stimulating factor (CSF)-1, granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), interleukin (IL)-1α, and IL-3 had little or no clonogenic capacity. Plateau levels of rr stem cell factor (rrSCF), a c-kit ligand, used alone also had negligible clonogenic capacity, but when combined with the low-level combination of the other five factors produced total colony and HPP-CFC growth approaching that produced by all factors at plateau levels. Delayed addition experiments suggest that this effect may represent sequential activity of SCF and the other factors. We propose a model of the normal hematopoietic microenvironment in which SCF at locally high concentration on the stromal cell surface “anchors” the hematopoietic stem cell’s response to multiple other cytokines at physiologically relevant levels.

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THE IDENTIFICATION and purification of the hematopoietic growth factors has revolutionized the study of hematopoiesis. However, the levels of cytokines required for in vitro colony growth and in vivo response are far in excess of those observed circulating in normal marrow or in the supernatants of Dexter-type liquid marrow cultures, which raises questions as to their role in stromal-associated hematopoiesis.

It has been previously noted in our laboratory and others that early murine hematopoietic progenitors require multiple growth factor stimulation for optimal proliferation.2-5 The recently described ligand for the c-kit receptor, variously identified as Steel factor,6 stem cell factor (SCF),7 mast cell growth factor,8 and kit ligand9, has proved to be a particularly potent component of these combinations, with significant increases in colony number, size, and secondary proliferative capacity with its addition.3,10-12 Combinations of colony-stimulating factor (CSF)-1, granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), interleukin (IL)-1α, and IL-3 at plateau levels have consistent clonogenic potential both in terms of total colony production and the stimulation of the high proliferative potential colony-forming cell (HPP-CFC), an early murine progenitor closely related to marrow repopulating capacity.13 Log dilutions of the five-factor combination result in loss of colony-forming capacity. We now report that addition of SCF to these diluted multifactor combinations restores total colony and HPP-CFC generation to unfraccionated marrow populations and to populations enriched for early progenitors. Experiments with time delayed additions of factors other than SCF suggest that this effect may represent sequential activity of the factors.

SCF is active as a transmembrane molecule,7,8 suggesting a refinement of the hematopoietic microenvironment model based on a locally concentrated presentation of SCF at the adherent stromal cell surface priming the stem cell to respond to local or circulating combinations of other factors at physiologically relevant concentrations.

MATERIALS AND METHODS

Marrow. Bone marrow was obtained from C57 BL/Ka-Thy-1.1 mice maintained in our own breeding colony by flushing tibias and femurs with cold 1x Hank’s balanced salt solution (HBSS) with 5% fetal calf serum. Marrow was used unfraccionated or enriched for early progenitors by one of two methods. 5-Fluorouracil (5-FU) is a cell-cycle active agent. Its administration to mice removes actively cycling intermediate hematopoietic progenitors and enriches for early hematopoietic progenitors.13 Marrow taken after 5-FU administration is particularly enriched for HPP-CFC, with marrow taken 2 days after 5-FU primarily enriched for “early” HPP-CFC, while marrow taken 8 days after 5-FU has expanded populations of both early and intermediate HPP-CFC.6,14 Mice were injected with 150 mg/kg 5-FU via tail vein and marrow harvested after 2 days (FU2 marrow) or 8 days (FU8 marrow).

Early murine progenitors have also been isolated based on unique patterns of surface antigen expression.15,16 We isolated cells expressing the Ly-6A.2 antigen (stem cell antigen or SCA+) and lacking terminal lineage markers (Lin+) from normal marrow using methods described by Spangrude and Scollay.16 Initial immunomagnetic depletion of CD4 and CD8 cells was followed by staining with fluoresceinconjugated antibody to stem cell antigen and immunomagnetic enrichment for that population. Lineage markers (B220, Gr-1, Mac-1, and CD5) were stained with biotinylated antibody and secondarily labeled with a streptavidin-phycoerythron conjugate (Biomedica, Foster City, CA). SCA+Lin- cells were isolated by fluorescence-activated cell sorting and incorporated into standard assay systems.

 Colony assays. Colonies were grown in 35-mm Petri dishes using double-layer agar as described previously.18 Briefly, growth factors were incorporated into 1-mL underlays with 0.5% agar and a-medium with 20% fetal calf serum. Whole marrow was plated at 50,000 cells per plate, FU2 marrow at 100,000 cells per plate, FU8 marrow at 200,000 cells per plate, FU2 marrow at 100,000 cells per plate.

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marrow at 10,000 cells per plate, and SCA+Lin- cells at 50 to 400 cells per plate. Target cells were incorporated into a 0.5-mL 0.3% agar overlay with medium and fetal calf serum. Plates were incubated at 37°C for 14 days in holding boxes gassed for 20 minutes with 5% O₂, 10% CO₂, 85% N₂ and then sealed.

For serial addition experiments, plates were initially prepared as above. At 96 hours, plates were sterilely removed from their holding boxes and 0.3 mL of medium containing growth factors at appropriate concentration layered gently on top of the cell layer. Plates were regassed and incubation continued as before to complete 14 days.

Plate evaluation. Plates were initially scored on a dissecting microscope for HPP-CFC and total colony numbers as previously described. Agar preparations were then fixed with 10% formalin and transferred to glass slides. Slide preparations were stained for acetyl cholinesterase, counterstained with hematoxylin, and evaluated for cellular morphology.

Statistics. Group means were compared using the Wilcoxon test on unpaired samples.

Growth factors. Growth factors were the kind gift of Amgen (Thousand Oaks, CA; recombinant human [rh]G-CSF, recombinant mouse [rm]GM-CSF, Immunex (Seattle, WA; rmIL-3), Hoffman-LaRoche (Nutley, NJ; rhIL-1a), and Cetus (Emeryville, CA; rhCSF-1). rrSCF was kindly provided by Dr Krisztina M. Zsebo of Amgen.

Growth factor concentrations were chosen relative to previously established "plateau" levels of activity: CSF-1, 1,600 U (37.2 ng) per dish; G-CSF, 10,000 U (100 ng) per dish; GM-CSF, 50 U (5 ng) per dish; IL-1α, 500 U (0.2 ng) per dish; IL-3, 100 to 200 U (58.5 to 117 ng) per dish; and rrSCF, 100 ng per dish. These "plateau" levels were designated as "1x." Activity in assays using factors at 10-fold increased concentration ("10x"), or at 10-fold or 100-fold dilutions ("0.1x" and "0.01x", respectively) was then compared with standard plateau levels.

RESULTS

In two initial experiments with unfractionated marrow and three with FU8 marrow, we established that the five-factor combination of CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3, all at 1x concentration levels, produced consistent numbers of HPP-CFC and total colonies. SCF alone produced few colonies and no HPP-CFC in these populations. Reduction of the five-factor combination concentrations by 1 log resulted in significant reduction of colony-forming capacity, but addition of SCF at 1x concentration to these reduced-level factor combinations significantly restored HPP-CFC and total colony-forming capacity (data not shown).

FU2 marrow. Both whole marrow and FU8 marrow contain significant numbers of intermediate progenitors. As SCF may have particularly potent effects on the earliest hematopoietic progenitors, we next tested its effects on FU2 marrow, which is enriched for early HPP-CFC, in two experiments.

Figures 1 and 2 summarize those results. SCF alone had virtually no clonogenic capacity, while the five-factor combination of CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3 again produced consistent colony formation at 1x concentrations. SCF addition to this combination did not significantly change colony numbers, although previous work in our laboratory has suggested that average colony size and density are increased.

One- and two-log dilutions of the 5-factor combination led to a progressive loss of colony-forming capacity, which was significantly restored by readdiction of SCF at 1x concentrations.

SCA+Lin- cells. Although FU2 marrow is enriched for early progenitors, it is still "contaminated" by multiple accessory and other additional cells. Previous work in our laboratory and elsewhere has suggested that SCF acts primarily by direct stimulation of early progenitors. To confirm that the "rescue" of colony production of SCF from "subliminal" multifactor combinations was similarly due to
direct action, we performed factor concentration-reduction experiments with SCA+Lin- cells, isolated as described above, in a total of six experiments.

Figures 3 and 4 summarize the results with SCA+Lin- cells. Five-factor stimulation with CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3, all at 1x concentration, without or with SCF again produced good HPP-CFC and total colony production, while SCF alone produced minimal or no colonies. Reduction of the five-factor combination to 0.1x concentration essentially eliminated HPP-CFC production and markedly reduced total colony formation. These capabilities were significantly restored by the readdition of SCF at 1x. Reduction of the five-factor combination to 0.01x abrogated all colony-forming capacity. Addition of SCF at 1x to the 0.01x five-factor combination partially restored clonogenic capacity, although not to a level equivalent to all factors used at plateau concentration.

In additional experiments with SCA+Lin- cells, we investigated the effects of 1-log increases of all factors above plateau (10x) and found no significant additional effect. Two further experiments demonstrated that SCF at 1 log below plateau (0.1x) had intermediate ability to restore clonogenic capacity to “subliminal” five-factor combinations at reduced concentration. Experiments with normal and post-5-FU marrow yielded similar results (data not shown).

Ability of other factors to restore colony production to reduced-concentration five-factor combination-stimulated cells. CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3, used individu-
I. CSF-1
G-CSF
GM-CSF at 1x
IL1α
IL3

II. CSF-1
G-CSF
GM-CSF at 0.1x
IL1α
IL3

III. CSF-1
G-CSF
GM-CSF at 0.01x
IL1α
IL3

Plating Density 300 cells per plate
Serum Replete Medium

Fig 4. Macroscopic appearance of plates from a typical experiment with SCA+Lin- cells. Plates are shown without (left) and with (right) SCF readdition to indicated concentrations of the five-factor combination of factors shown.

ally at plateau concentration, could not restore colony production to reduced-concentration five-factor combination–stimulated cells with a potency equivalent to SCF. IL-1α, and to a lesser extent, GM-CSF and IL-3, do seem to have intermediate rescue capacity, which we are currently exploring (data not shown).

Serial addition of factors. Our basic experimental design involves addition of all growth factors in initial cultures, and therefore does not distinguish whether their action is simultaneous or sequential. To approach this question, we performed a series of four experiments with SCA+Lin- cells and three experiments with FU2 marrow with temporarily separated addition of SCF and other factors.

Figure 5 presents representative results with SCA+Lin- cells. If no factors were added initially, even the addition of all factors at plateau levels at 96 hours resulted in only marginal colony production. SCF addition on day 0 followed by medium addition at 96 hours produced minimal colonies, consistent with the results in standard culture. In contrast, SCF addition initially followed at 96 hours by addition of CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3 produced significant numbers of HPP-CFC and total colonies that approached those seen when all factors were added on day 0. Studies using FU2 marrow have had similar results (data not shown).

Colony morphology. Macroscopically, the morphology of HPP-CFC produced by reduced factor combinations anchored by SCF was no different than the colonies produced by all factors at plateau levels, although the average size did decrease somewhat when the five-factor combination was used at a 0.01x (see Figs 2 and 4). Colonies produced by serial addition experiments were essentially equivalent to those seen in standard culture.

Multifactor-stimulated HPP-CFC derived from enriched progenitor populations usually contain cells of at least two lineages: macrophage and granulocyte. Approximately 10% of colonies additionally contain megakaryocytes.

DISCUSSION

The hematopoietic growth factors have a well-established potential to stimulate in vitro and in vivo hematopoietic proliferation and are probably the prime mechanism of

[Graph]

Fig 5. Results pooled from two experiments with triplicate plates in each employing serial growth factor addition with SCA+Lin- cells. Additions on day 0 (at initiation of culture) and on day 4 (96 hours after initiation of culture) are indicated on the horizontal axis. “MED” indicates addition of medium, “SCF” indicates SCF addition, and “5” indicates the simultaneous addition of CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3. All factors are used at plateau levels and results are expressed with standard errors indicated. Addition of SCF on day 0 when followed by addition of the five factors on day 4 produced significantly more HPP-CFC and total colonies (P ≤ .01) compared with medium alone addition on day 0 followed by five-factor addition on day 4 (a). Five-factor addition on day 0 followed by either medium or SCF on day 4 produced equivalent numbers of HPP-CFC and total colonies as compared with SCF addition on day 0 with five-factor addition delayed until day 4, (b) and (c). Addition of SCF and the five-factor combination all on day 0 resulted only in a marginally significant increase in HPP-CFC (P > .01, but ≤ .05) and a nonsignificant increase in total colonies compared with delayed addition of the five-factor combination until day 4 (d). (■) HPP-CFC; (▲), total colonies.
inflammation, and infection. Their role in the normal modulation of the earliest steps of hematopoiesis has remained less clear, due in part to the lack of significantly detectable levels circulating in vivo and in vitro in Dexter-type culture systems. Early hematopoietic progenitors do require intimate contact with stromal cells for optimal proliferation, suggesting that the necessary signals for maintenance and proliferation are provided within the context of that close association.

The identification in our laboratory and others of multiple growth factor requirements for proliferation of early hematopoietic progenitors suggested a model wherein locally high concentrations of multiple growth factors presented at or near the stromal cell surface might modulate early commitment, proliferation, and differentiation steps. Although we have established the capability of early hematopoietic progenitors to produce all of these factors, the model is somewhat cumbersome in its requirement for coordinate expression of all of these factors at significant local concentration.

The identification of the ligand for the proto-oncogene c-Kit has dramatically expanded the understanding of early stimuli in hematopoietic proliferation. Although it has limited clonogenic capacity acting alone, SCF has potent effects in combination with other factors in stimulating in vitro hematopoietic colonies, with these effects occurring apparently via direct stimulation of very early progenitors.

In the current series of experiments, we have observed that SCF can “rescue” significant colony production from 1- and 2-log dilutions of the combination of CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3. This effect was observed in whole bone marrow and in populations partially purified for early hematopoietic progenitors by pretreatment with 5-FU or by direct isolation of populations expressing the Ly-6A.2 antigen (SCA+) and lacking terminal lineage markers (Lin-).

This suggests a model of local control of normal hematopoiesis by one or a few factors concentrated at intermediate to high levels at the surface of the stromal cell and directly stimulating intimately associated hematopoietic stem cells. This “juxacrine” stimulation of early hematopoietic cells, though insufficient in and of itself to induce proliferation and differentiation, could be modulated by physiologically relevant “subliminal” levels of other growth factors either similarly presented locally or freely diffusing (Fig 6).

We have designated this activity “anchor factor” stimulation, as the primary factor is not independently highly functional, but can provide the basic stimulation required to allow secondary response to very low levels of other factors.

SCF is a particularly good candidate for this activity, as it is synthesized in an active trans-membrane form, has potent effects on very early hematopoietic cells, but requires synergistic interaction with other factors for optimal activity. The recent demonstration that SCF may have a dual role as growth factor and adhesion molecule for early progenitors and the indication that the membrane-bound form, as opposed to the soluble species, may preferentially induce long-term hematopoiesis, further supports its role as a potential anchor factor.

Although other factors tested failed to show equivalent effects, the expression of factors such as IL-1α in an active membrane form and our preliminary finding of intermediate anchor factor-like activity with IL-1α suggests potential parallel but attenuated effects with other factors.

The current data clearly support the ability of SCF to synergize with other factors at much lower concentrations than previously demonstrated. We suggest that the “hematopoietic niche” may be functionally defined as a stromal microenvironment characterized by the local stromal cell surface presentation of a factor such as SCF at high concentration. The hematopoietic stem cell is locally complexed either directly through SCF-c-Kit interaction, or additionally with adhesion molecules such as fibronectin, and is thus exposed to SCF in concentrations functionally equivalent to those used in these experiments. “Normal” hematopoiesis proceeds under modulation by local or circulating low levels of multiple other factors.
Traditional culture designs have not distinguished between the simultaneous and sequential activity of synergistic factor combinations. Using subculturing techniques, Metcalf and Nicola have found indications that SCF has activity both early and late in the sequence of hematopoietic proliferation, and that there is a temporal hierarchy with G-CSF.11

We have developed a technique for investigating temporally separated additions of growth factors, which avoids the potential artifacts of disrupting colonies in initial culture and transfer to new culture conditions. Experiments using this technique indicate that SCF alone is largely sufficient to support the first 96 hours of proliferation and, when followed by addition of CSF-1, G-CSF, GM-CSF, IL-1α, and IL-3 at that point, has a clonogenic capacity approaching that seen when all factors are added at initiation of culture. At least in this assay system, all factors combined exclusive of SCF incorporated from day 0 had similar clonogenic capacity, suggesting that the effect of SCF was not exclusively required. However, we plan additional separation schemes and morphologic analysis to test whether this represents stimulation of the same or different starting progenitor populations. Interestingly, in contrast to the findings of Metcalf and Nicola, we found little effect of the delayed addition of SCF beyond 96 hours as compared with potent effects used alone or in synergy with other factors when added at day 0.

The findings of the delayed addition experiments are consistent with a temporal hierarchy of growth factor effect. The findings of relatively early action of SCF are consistent with the characterization of c-kit expression as being concentrated on early stem cells and progenitors,23 and suggest an expansion of the model to indicate a potential temporal sequence of stimulation and response.

The evolving model is particularly attractive, as it allows for the flexibility that characterizes hematopoiesis both normally and in response to stress. Various "niches" might be defined by a unique local anchor factor or factors, which in turn defines a differentiation preference for stem cells adherent in that niche. Tonic stimulation by low-level circulating or locally produced factors would allow hematopoiesis to proceed, producing differentiated daughter progeny in normal ratios. The stress of infection, inflammation, or cell loss would effect increased cell production and altered ratios of daughter progeny via modulation of the concentration of circulating factors. This could have its effect though selective stimulation of clones in specific, selected anchor factor niches. Alternatively, the high circulating growth factor levels might "override" the usual program determined by the local niche and induce all early progenitors along a common differentiation pathway. In either case, the model allows for both the divergent differentiation of normal hematopoiesis and a convergent differentiation in response to stress.

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Stem cell factor induction of in vitro murine hematopoietic colony formation by "subliminal" cytokine combinations: the role of "anchor factors"

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