Induction of Colony-Stimulating Factor Receptor Expression on Hematopoietic Progenitor Cells: Proposed Mechanism for Growth Factor Synergism

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In many cell systems, the cellular interaction between two or more humoral factors leads to a synergistic response in terms of cellular growth and function. In particular, the growth and differentiation of hematopoietic progenitor cells involves numerous synergistic interactions between colony-stimulating factors (CSFs) that individually stimulate hematopoiesis (granulocyte-CSF, granulocyte-macrophage-CSF, and interleukin-3 [IL-3]), as well as between these factors and other cytokines that individually have a proliferative effect on progenitor cell growth (IL-1 and IL-6). The present study investigated whether hematopoietic growth factor (HGF) synergy could be mediated by upregulation of CSF receptors. Synergistic effects on bone marrow (BM) progenitor cell colony formation, regardless of the combination of factors used, were consistently preceded by increased CSF receptor expression on highly enriched BM progenitor cells, but not on unfractionated BM cells. Induction of CSF receptors preceded detectable differentiation and did not require cell division because nocodazole, an inhibitor of mitosis, blocked CSF-mediated cell proliferation, but not receptor upregulation. Furthermore, combinations of cytokines that did not synergize also failed to affect the level of CSF receptors on BM progenitors. These results have led us to propose a model for HGF synergy whereby one mechanism of action the investigated synergistic cytokines might be the ability to induce increased expression of CSF receptors. This is a US government work. There are no restrictions on its use.

THE PROLIFERATION and differentiation of hematopoietic progenitor cells is dependent on the presence of a family of glycoprotein hormones known as the colony-stimulating factors (CSFs),1 which include interleukin-3 (IL-3), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and macrophage-CSF (M-CSF).1-4 Synergistic responses in the growth of hematopoietic progenitor cells have been shown to occur both between different CSFs,5-6 as well as between the CSFs and other cytokines.7-10 However, growth factor synergism is not restricted to myelopoiesis, and the importance of this regulatory mechanism has also been shown in other cell types, including fibroblasts,11-15 T cells,16 and melanoma cells.17

Each CSF binds to and downmodulates its own unique high-affinity receptor.3 In addition, previous studies on unfractionated bone marrow (BM) cells have shown that the binding of a CSF to its own receptor can also decrease the number of unoccupied receptors for other CSFs.18 This rapid transdownmodulation occurs in a unidirectional hierarchical pattern, in that the multipotent CSFs (IL-3 and GM-CSF) can transdownmodulate the expression of the

lineage-specific CSFs (G-CSF and M-CSF), but not vice versa. Because all four CSFs induce granulocyte and macrophage production, a model was proposed in which IL-3 and GM-CSF, by transdownmodulating and thus activating the receptors for G-CSF and M-CSF, would induce granulocyte and macrophage proliferation and differentiation.18 Furthermore, this hierarchical model proposed that growth factor synergism could occur as a consequence of transdownmodulation of growth factor receptors by synergistic factors.

Previous studies of CSF receptor regulation on BM progenitor cells were performed on unfractionated BM cells,19-22 where progenitor cells are present in low frequency. Therefore, hematopoietic progenitor cells were enriched from normal BM by a newly developed technique for progenitor cell enrichment23 to investigate the role of CSF receptor modulation in synergy between hematopoietic growth factors (HGFs).

This study provides evidence supporting a new model for HGF synergism in which synergistic factors might partially act by inducing an increase in CSF receptor expression on hematopoietic progenitor cells. This increase can only be detected on purified BM progenitors, and not on unfractionated BM cells.

MATERIALS AND METHODS

HGFs. Purified recombinant murine (rMu) GM-CSF and recombinant human (rHu) G-CSF was supplied by Ian K. McNiece and Tom Boone (Amgen Corporation, Thousand Oaks, CA). Purified rMuIL-3 for radiolabeling was purchased from R and D Systems (Minneapolis, MN), while rMuIL-3 used for cold competition and biologic assays was obtained from the supernatants of Cos-7 cells transfected with a plasmid containing the IL-3 cDNA as described.21 rHuIL-1 was supplied by Hoffmann-LaRoche (Nutley, NJ), and rHuIL-6 by Interpharm (Ness-Ziona, Israel).

Cells. Normal murine BM cells used in radioligand binding experiments were obtained by aspiration of BALB/c mouse femurs. Enriched lineage negative (Lin-) BM progenitor cells were obtained as previously described.20 Briefly, unfractionated BM cells were layered over lymphocyte separation medium (Organon Teknika Corp, Durham, NC). The light-density cells were suspended in Hanks' Balanced Salt Solution (HBSS) and incubated for 30 minutes at 4°C with a cocktail of antibodies: RA3-6B2 (B220 antigen) and RB6-8C5 (GR-1 antigen) from Robert Coffman...
(DNAX Corp, Palo Alto, CA); MAC-1 (Boehringer-Mannheim, Indianapolis, IN); Lyt-2 (CD8) and L3T4 (CD4) (Becton Dickinson, Rochelle Park, NJ). Cells were washed twice and resuspended in HBSS containing immunomagnetic beads (Dynal, Oslo, Norway) at a ratio of 40:1 (beadscells) and then incubated for 30 minutes at 4°C. Labeled lineage positive (Lin+) cells were removed by a magnetic particle concentrator (Dynal), and Lin- cells were recovered.

**Soft agar colony formation.** A modification of the method described by Stanley et al was used to measure colony formation of Lin- progenitor cells in vitro. Briefly, 1 x 10^4 Lin- cells were resuspended in 1 mL Iscove’s modified Dulbecco’s medium (IMDM), 10% fetal calf serum (FCS; Innovar, Gaithersburg, MD), 2 mmol/L L-glutamine, 15 mg/L gentamicin (complete IMDM), and 0.3% seaplaque agarose and incubated in 35-mm Lux Petri dishes at 37°C and 5% CO2 for 7 days before being scored for colony growth (> 50 cells).

**3H-Thymidine incorporation assays.** Lin- cells were incubated with HGFs at 37°C and 5% CO2 in 96-well microtiter plates at a density of 5 x 10^3 cells in 100 µL complete IMDM. DNA synthesis was assessed with a pulse of 1 µCi [3H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) for the last 6 hours of the incubation period. Radioactivity was determined by liquid scintillation counting. In some experiments, nocodazole (Sigma Chemical Co, St Louis, MO) was used to block mitosis.

**Single cell proliferation assay.** Lin- cells were seeded in Terasaki plates (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well in 20 µL complete IMDM. Wells were scored for proliferation (> 10 cells) after 6 to 8 days of incubation at 37°C, 5% CO2.

**Radioiodination of CSFs.** Radioiodination of rMuIL-3 was performed by the chloramine-T method as previously described. rMuGM-CSF and rHUG-CSF were labeled by a modification of the chloramine-T method. Briefly, 7.5 x 10^4 rMuGM-CSF or rHUG-CSF, 20 µL 0.1 mol/L sodium phosphate buffer (pH 7.0) with 10% dimethyl sulfoxide and 100 µg/mL polyethylene glycol, 1 µCi 125Iodine (125I; Amersham, Arlington Heights, IL) and 10 µL 0.1 mg/mL chloramine-T were mixed and incubated at 4°C for 5 minutes. Then, 10 µL 0.3 mg/mL sodium metabisulfite and 10 µL 0.1 mol/L potassium iodine was added. Iodinated CSFs were separated on a Sephadex G-25 column. The specific activities were 9.2 to 13.5 x 10^6 cpm/pmol, 1.0 to 1.9 x 10^6 cpm/pmol, and 1.1 to 2.4 x 10^6 cpm/pmol for GM-CSF, G-CSF, and IL-3, respectively. The biologic activity of all CSFs was retained after radiolabeling, as determined by their ability to induce colony formation.

**Radioligand binding experiments.** CSF receptor binding studies were performed by a modified phthalate oil separation method. Unfractionated BM cells or Lin- cells (2.0 to 4.0 x 10^5 cells/sample) were pretreated in 1 mL 50 mmol/L glycine-HCl (pH 3.0) for 1 minute to release bound ligands. Cells were then washed twice in RPMI 1640 containing 2% bovine serum albumin (BSA; Sigma), 20 mmol/L HEPES, and 0.1% sodium azide (binding medium). Specific binding of radiolabeled CSFs (20 to 250 pmol/L) was determined as the difference in CSF binding in the absence and presence of a 50-fold excess of unlabeled CSF after 90 minutes of incubation at 22°C (GM-CSF) or 37°C (G-CSF and IL-3). Cell-bound radioactivity was separated from free radioligands by centrifugation through a mixture of dibutyl phthalate and 1 bis (2 ethylhexyl) phthalate oil (ratio 1.5:1) (Eastman Kodak, Rochester, NY), and radioactivity was determined in a Beckman Biogamma 2 counter (Beckman Instruments, Irvine, CA).

**Cell autoradiography.** Autoradiography of cytokine-treated and untreated Lin- cells was performed as described after equilibrium binding with 300,000 to 500,000 cpm 125I-GM-CSF as described above. Briefly, 1 x 10^5 cells resuspended in phosphate-buffered saline (PBS)/FCS (1:1) were centrifuged onto microscope slides, fixed in methanol for 10 minutes, coated with Kodak NTB2 photographic emulsion (diluted 1:1 with water), air-dried, placed in a light-proof box with drierite, and exposed at 4°C for 4 to 6 weeks. Slides were developed in Kodak D-19 developer, fixed in Kodak fixer, and stained with 5% Giemsa for 10 minutes. The number of specific and unspecific grains were determined for 150 to 200 blast cells on each slide under oil at 1,000 x magnification.

**RESULTS**

**Synergy between HGFs.** In the present study, HGF synergy was examined on a highly enriched progenitor cell population. A modification of a previously published protocol was used to obtain progenitor cells designated Lin-, due to the absence of cell surface markers characteristic of B and T lymphocytes, monocytes, and granulocytes. The Lin- cells represent 2% to 3% of the total BM and their high proliferative potential is underscored by the finding that up to 1 in 5 Lin- cells proliferate under optimal conditions in single cell cultures supplemented with FCS and multiple HGFs (Keller et al, unpublished observations).

As previously shown, GM-CSF, G-CSF, and IL-3 alone promoted colony formation of Lin- cells in vitro (Fig 1A). A synergistic response, 77% above additive, was observed with the combination of GM-CSF and IL-3 (Fig 1A). Similarly, a marked synergy was observed between G-CSF and GM-CSF as well as between G-CSF and IL-3, increasing the number of colony-forming units granulocyte-macrophage (CFU-GM) by 86% and 42%, above additive, respectively (Fig 1A). A second class of synergistic factors is represented by IL-1 and IL-6, which alone did not support colony growth, but synergized with GM-CSF to increase colony formation 66% and 58%, respectively (Fig 1B). IL-6 also increased IL-3-stimulated CFU-GM 61%, but did not synergize with G-CSF (Fig 1B). IL-1 did not affect G-CSF- or IL-3-induced colony formation (Fig 1B). IL-2, IL-4, IL-5, IL-7, and leukemia inhibitory factor (LIF) at saturating concentrations had no effect on CFU-GM formation alone or in combination with any of the three CSFs (data not shown). Finally, Lin- cells in single-cell cultures followed the same pattern of synergy with IL-1 and IL-6, indicating that the observed effects were direct (Table 1).

**CSF receptor modulation by synergistic HGFs.** To determine whether CSF receptor transmodulation might account for the observed synergistic effects on Lin- cells, equilibrium binding studies with radiolabeled CSFs were performed on unfractionated BM cells as well as purified Lin- cells after treatment with HGFs.

As expected, GM-CSF rapidly downmodulated the binding of radiolabeled GM-CSF to unfractionated BM by 2 hours (80%) and 6 hours (62%) (Fig 2A). In agreement with the hierarchical model, IL-3 transdownmodulated GM-CSF-specific binding 50% to 60% after 2 and 6 hours of treatment, whereas G-CSF did not affect GM-CSF binding at either time point (Fig 2A). Furthermore, for the same time period, IL-1 and IL-6 did not modulate GM-CSF binding to unfractionated BM (Fig 2A). The same modulation pattern of GM-CSF binding to purified Lin- cells was observed as on unfractionated BM (Fig 2B), suggesting that at least the synergistic effects of IL-1 and IL-6 on GM-CSF-
stimulated colony formation is mediated through a different mechanism than transdownmodulation of CSF receptors. Differential regulation of CSF receptors on hematopoietic cells. Because hematopoietic progenitor cells require prolonged cytokine exposure to induce colony formation,27 we next examined GM-CSF binding to unfraccionated BM and Lin− cells after 24 hours of cytokine treatment. Similar to the observation made after 2 and 6 hours, IL-3 and GM-CSF downmodulated GM-CSF-specific binding to unfracionated BM by 41% and 52%, respectively (Fig 3). Furthermore, G-CSF induced a 52% increase in GM-CSF binding, while IL-1 and IL-6 had no effect (Fig 3). In contrast, using purified Lin− cells, IL-3, G-CSF, IL-1, and IL-6 induced a 347%, 316%, 156%, and 138% increase, respectively, in GM-CSF binding after 24 hours of treatment (Fig 3). This GM-CSF receptor upregulation occurred in the absence of increased cell numbers or reduced cell viability (Table 2). Surprisingly, GM-CSF itself induced a modest 67% increase in GM-CSF-specific binding to Lin− cells (Fig 3). Although significantly lower than the increase induced by the synergistic factors, the GM-CSF-mediated GM-CSF receptor upregulation might play a role in enhancing and prolonging the effect of GM-CSF on responding progenitor cells. Finally, GM-CSF binding to Lin− cells was not altered after 24 hours of incubation in medium alone (data not shown).

The upregulation of GM-CSF-specific binding by synergistic factors was time and dose dependent. For example, G-CSF increased GM-CSF binding in a dose-dependent manner with maximum stimulation at 2 to 20 ng/mL and an ED50 of 0.2 ng/mL (data not shown). An increase in GM-CSF binding in response to G-CSF and IL-1 was not observed by 12 hours; however, a significant upregulation was detected by 18 hours with maximum stimulation by 24 hours (Table 3), and the increased GM-CSF binding was prolonged for at least 48 hours (data not shown). In response to IL-3, GM-CSF-specific binding was initially downmodulated at 6 hours, and later increased after 12 hours. Finally, GM-CSF–specific binding to unfraccionated BM and Lin− cells was not affected by IL-2, IL-4, IL-5, IL-7, or LIF after 2, 6, or 24 hours, in agreement with their inability to synergize with GM-CSF in the biologic assay.

In agreement with previous reports,18,28 equilibrium binding and subsequent Scatchard analysis of GM-CSF binding to unfraccionated BM showed two classes of GM-CSF binding sites, one of high affinity (kd = 58 pmol/L; 60 receptors/cell) and one of low affinity (kd = 1.6 nmol/L; 215 receptors/cell). Similarly, Lin− cells showed both high-affinity (kd = 30 pmol/L; 66 receptors/cell) and low-affinity (kd = 0.69 nmol/L; 306 receptors/cell) receptors (Fig 4), and both IL-1 and G-CSF treatment for 24 hours increased the number of high- and low-affinity GM-CSF receptors on Lin− cells without significantly affecting the receptor affinities (Fig 4).

Lin− cells were preincubated for 24 hours in the absence

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**Fig 1.** Synergistic effects between HGFs on CFU-GM formation of Lin− BM cell. Lin− cells, separated as described in Materials and Methods were plated at 1 × 10^6 cells/mL in IMDM with 10% FCS and 0.35% seaplague agarose, supplemented as indicated with predetermined optimal concentrations of purified rMuGM-CSF (20 ng/mL), rHuG-CSF (20 ng/mL), rHuL-1 (20 ng/mL), rHuL-6 (500 U/mL), and rHuL-3 (200 U/mL) and incubated at 37°C, 5% CO2 for 7 days before being scored for colony growth (>50 cells). IL-1 and IL-6 alone did not induce any colony formation. Results are presented as the mean of duplicate determinations ± SD and are representative of four separate experiments.

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**Table 1. Direct Effects of Synergistic Factors on CSF-Induced Proliferation of Lin− Progenitors**

<table>
<thead>
<tr>
<th>CSF</th>
<th>Synergistic Factor</th>
<th>Frequency</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>—</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>IL-1</td>
<td>12</td>
<td>67</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>IL-6</td>
<td>14</td>
<td>43</td>
</tr>
<tr>
<td>G-CSF</td>
<td>—</td>
<td>70</td>
<td>—</td>
</tr>
<tr>
<td>G-CSF</td>
<td>IL-1</td>
<td>75</td>
<td>7</td>
</tr>
<tr>
<td>G-CSF</td>
<td>IL-6</td>
<td>65</td>
<td>8</td>
</tr>
<tr>
<td>IL-3</td>
<td>—</td>
<td>22</td>
<td>—</td>
</tr>
<tr>
<td>IL-3</td>
<td>IL-1</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>IL-3</td>
<td>IL-6</td>
<td>16</td>
<td>47</td>
</tr>
<tr>
<td>—</td>
<td>IL-1</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>IL-6</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Lin− cells separated according to Materials and Methods were seeded as single cells in Terasaki plates as described. Cultures were incubated at 37°C, 5% CO2, with predetermined optimal concentrations of CSF, IL-1, and IL-6 (as described in Fig 1). The frequency of responding progenitors (>10 cells/well) was determined after 6 to 7 days of incubation. Results presented are the mean of four separate experiments, with a minimum of 1,200 wells scored per group.
or presence of growth factors synergizing with GM-CSF, replated in soft agar, supplemented with GM-CSF, and scored for colony formation after 7 days of incubation. The numbers of colonies formed (Fig 5) correlated closely to the synergistic factors abilities to upregulate GM-CSF receptor expression on Lin− cells (Fig 3).

Equilibrium binding studies were next performed to investigate whether other CSF receptors, such as those for G-CSF and IL-3, could be induced on Lin− progenitors. In agreement with data on unfractionated BM cells, G-CSF, GM-CSF, and IL-3 all rapidly (by 2 hours) downmodulated G-CSF-specific binding on Lin− cells, while only IL-3 reduced IL-3-specific binding (data not shown). In contrast, IL-3 induced a 66% increase in G-CSF binding to purified Lin− cells by 24 hours, while no increase was observed on unfractionated BM (Fig 6A). While G-CSF potently upregulates GM-CSF receptor expression on Lin− cells, GM-CSF downmodulates G-CSF receptor expression 28% by 24 hours. In addition, IL-1 and IL-6 did not affect G-CSF binding to Lin− cells by 24 hours (Fig 6A), in agreement with their inability to synergize with G-CSF (Fig 1). G-CSF and IL-6 increased IL-3 binding 49% and 147%, respectively, to enriched progenitors after 24 hours, but not to unfractionated BM (Fig 6B). Furthermore, IL-1 had no effect on IL-3 binding to Lin− cells after 24 hours, which again was correlated with an absence of biologic synergy (Fig 1).

Therefore, HGFs that synergize with CSFs upregulate the number of CSF receptors on purified hematopoietic progenitor cells by 24 hours, while HGFs that do not synergize fail to do so. The magnitude of synergism exhib-

Table 2. Effects of HGFs on Viability of Lin− Progenitor Cells

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Cells/mL (× 10^9)</th>
<th>Viable Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.90</td>
<td>88</td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.90</td>
<td>92</td>
</tr>
<tr>
<td>IL-1</td>
<td>0.85</td>
<td>86</td>
</tr>
<tr>
<td>IL-3</td>
<td>0.95</td>
<td>91</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.95</td>
<td>85</td>
</tr>
</tbody>
</table>

Lin− cells were incubated at 1.0 × 10^6 cells/mL in the presence or absence of HGFs at optimal concentrations (see Fig 1). Cell number and viability was determined by trypan blue staining and hemocytometer after 24 hours of incubation. Results were obtained by counting at least 200 cells per group. Results presented are representative of four separate experiments.
Table 3. Kinetics of GM-CSF Receptor Induction on Lin- Cells

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>98</td>
<td>105</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>IL-1</td>
<td>ND</td>
<td>115</td>
<td>176</td>
<td>258</td>
</tr>
<tr>
<td>G-CSF</td>
<td>ND</td>
<td>118</td>
<td>209</td>
<td>324</td>
</tr>
<tr>
<td>IL-3</td>
<td>62</td>
<td>123</td>
<td>247</td>
<td>348</td>
</tr>
</tbody>
</table>

Lin- cells (1.0 x 10^6 cells/mL) were incubated at 37°C in the presence of 20 ng/mL of IL-1, G-CSF, or IL-3, or in the absence of growth factors. Equilibrium binding with 150,000 cpm ¹²⁵I-GM-CSF was determined as described in Materials and Methods after 6, 12, 18, or 24 hours of incubation as indicated. Results are the mean of duplicate determinations and are representative of two separate experiments.

Abbreviation: ND, not determined.

Induction of CSF receptors precedes HGF synergy. If upregulation of CSF receptor expression is essential for HGF synergy, one would predict that increased CSF receptor levels would precede biologic synergy. Therefore, proliferative effects of HGFs on Lin- cells were determined by tritiated thymidine incorporation assays. The results showed that CSFs alone induced proliferation, but that no synergistic effects occurred by 12 hours (Fig 7A) and 18 hours (Fig 7B), while synergy was observed by 72 hours (Fig 7C) in a pattern similar to the synergistic effects seen on colony formation (Fig 1). Thus, CSF receptor upregulation by synergistic factors precedes the synergistic biologic responses.

CSF receptor induction by synergistic HGFs precedes cell division and differentiation. Because IL-1 (Fig 6) or IL-6 (data not shown) alone did not induce the proliferation of Lin- progenitors, their ability to upregulate GM-CSF receptor expression was not a consequence of cell division. However, because both G-CSF and IL-3 induced tritiated thymidine incorporation within 12 hours (Fig 7A), we could

![Figure 4](image-url)  
Fig 4. Induction of GM-CSF receptor expression on Lin- progenitor cells. Lin- cells were incubated for 24 hours at 37°C in the presence of IL-1 (A) (20 ng/mL), G-CSF (20 ng/mL), or (B) in the absence of growth factors. Increasing concentrations of unlabeled GM-CSF were added to 2.0 x 10^6 cells in 200 L binding medium in the presence of 15 to 25 pmol/L ¹²⁵I-GM-CSF and incubated at 22°C for 75 minutes. Equilibrium binding data were analyzed according to the Scatchard method. Results are the mean of duplicate determinations and are representative of three separate experiments.

![Figure 5](image-url)  
Fig 5. The effect of growth factor preincubation on GM-CSF-stimulated colony formation by Lin- cells. Lin- cells were preincubated for 24 hours in complete IMDM (control) or in the presence of optimal concentrations of cytokines (as in Fig 1). Cells were then washed twice in IMDM and replated at 1 x 10^6 cells/mL in complete IMDM supplemented with GM-CSF 20 ng/mL. Colonies were scored after 7 days of incubation at 37°C, 5% CO₂. Results presented are the mean of duplicate determinations ± SD and are representative of three separate experiments.
HGF SYNERGY AND CSF RECEPTOR INDUCTION

Fig 6. The effect of HGFs on G-CSF- and IL-3-specific binding to hematopoietic progenitor cells. (A) Unfractionated BM or Lin- cells were incubated at 37°C for 24 hours in the presence or absence of optimal concentrations of cytokines (as in Fig 1). (A) G-CSF- and (B) IL-3- specific binding was determined as outlined in Materials and Methods. Results are presented as the mean of duplicate determinations ± SD and are representative of four separate experiments.

not rule out that upregulation of GM-CSF receptor levels was a consequence of cell division. Therefore, we investigated the effect of nocodazole, a mitotic inhibitor, on G-CSF-induced proliferation and upregulation of GM-CSF receptor expression on Lin- progenitors (Table 4). Nocodazole (0.2 μg/mL) inhibited G-CSF-induced proliferation by 24 hours (Table 4) without significantly affecting cell viability (data not shown). In contrast, G-CSF-induced upregulation of GM-CSF binding was not significantly affected under the same conditions. Thus, upregulation of CSF receptors is not a consequence of cell division.

To determine whether the ability of HGFs to upregulate CSF receptor expression preceded their effects on differentiation, we examined the expression of MAC-1 and RB6-
8CS5 differentiation antigens that can be induced by growth factors on Lin- cells after 48 to 72 hours of incubation (Keller et al., unpublished observations). However, none of the investigated cytokines (IL-1, IL-3, G-CSF, and GM-CSF) induced the expression of these antigens on Lin- cells by 24 hours, and no change in cell morphology was observed after 24 hours of cytokine treatment (data not shown).

Even though the observation that synergistic factors induce CSF receptors on Lin- cells but not on un fractionated BM indicated otherwise, it was still possible that the increased binding of CSFs to the enriched populations of progenitors occurred on contaminated mature cells rather than immature progenitors. Lin- cells were therefore cultured for 24 hours in the presence or absence of IL-1, IL-3, and G-CSF, and GM-CSF binding was determined by autoradiography. Untreated Lin- cells, morphologically identified as blast cells, had an average of 5.1 specific grains per cell, and this did not significantly change after 24 hours of incubation in medium alone (Table 5). IL-1, IL-3, and G-CSF treatment for 24 hours increased the mean specific grain count over blast cells to 12.6, 18.9, and 16.5, respectively (Table 5). These data were in agreement with the results obtained by equilibrium binding experiments, with IL-3 > G-CSF > IL-1 with regard to their ability to induce increased GM-CSF receptor expression on Lin- cells (Fig 4). Mor phologically more mature cells (approximately 5%) within the Lin- cell population had GM-CSF-specific grains as well, but the number of grains were not significantly altered by cytokine treatment after 24 hours (data not shown). Therefore, the upregulation of GM-CSF receptors after treatment with synergistic factors was due to increased binding to undifferentiated blast cells.

The analysis of autoradiographs further showed a profound heterogeneity with regard to GM-CSF receptor expression on Lin- cells (Table 5). Specifically, 27% of untreated blast cells had no specific gains. IL-1, IL-3, and G-CSF dramatically reduced the number of GM-CSF receptor negative cells to 9%, 4%, and 5%, respectively, while a corresponding increase was observed in the number of high expressing cells (> 10 grains/cell; Table 5).

**DISCUSSION**

Although upregulation of CSF receptors has been proposed previously as a mechanism for synergistic interactions between HGFs, the present studies provide the first experimental evidence for such a mechanism on a population of highly enriched murine BM progenitor cells. This upregulation occurred in a dose- and time-dependent manner, and preceded the enhanced proliferative response, cell morphology changes, and induction of differentiation antigens on the progenitor cell surface. The maximum upregulation of CSF receptor expression observed by 24 hours was seen in the absence of increased cell numbers. However, the significant 3H-TdR incorporation induced by both G-CSF and IL-3 alone after 12 hours suggested that proliferation and cell division could account for the upregulation of CSF receptors. However, this seems unlikely because IL-1 or IL-6 did not induce proliferation, but were potent inducers of CSF receptors. Furthermore, nocodazole, an inhibitor of mitosis, blocked G-CSF-induced proliferation of Lin- cells without significantly affecting G-CSF-induced upregulation of GM-CSF receptor expression.

The modulation of CSF receptors showed a different pattern on un fractionated BM than on Lin- cells. While IL-3 and GM-CSF induced a significant upregulation of CSF receptor expression on Lin- cells by 18 to 24 hours, a downmodulation could still be observed on the un fractionated BM. The autoradiography suggested that this difference was due to the synergistic cytokines only upregulating CSF receptor expression on the more immature fraction of the BM, while receptors could not be induced on the mature blood cells of the BM. In contrast, the rapid downmodulation of GM-CSF receptors by GM-CSF and IL-3 occurred on both populations. Thus, un fractionated BM represents an incomplete model for studying CSF receptor modulation on hematopoietic progenitor cells.

Although we were unable to detect any significant effect...
of the synergistic cytokines on Lin− cell viability after 24 hours, as determined by trypan blue staining, it was still possible that selective death or inactivation of unstimulated progenitor cells was responsible for increased CSF-specific binding. However, this does seem unlikely because GM-CSF binding to Lin− cells was not altered significantly after 24 hours of incubation in medium alone. Furthermore, we have recently found that IL-1 administration in vivo also results in increased GM-CSF receptor expression on Lin− progenitor cells (Hestdal et al, Blood, in press). Although this in vivo upregulation of GM-CSF receptor expression could be indirect through production of other cytokines, such as G-CSF or IL-6, it supports the conclusion that it is not a consequence of cell death.

The present study proposes one mechanism for how synergism between HGFs can occur through the upregulation of CSF receptor expression on hematopoietic progenitor cells. In this regard, synergistic effects on colony formation of Lin− cells were consistently preceded by increased CSF receptor expression, involving either one or both CSF receptors (summarized in Table 6). Furthermore, the magnitude of synergy was correlated to the magnitude of increase in CSF receptor expression. In the case of synergy between two CSFs, a correlation between receptor modulation and biologic synergy has to be based on the modulation of receptors by both CSFs. With regard to the synergy observed between G-CSF and GM-CSF, the stimulatory effect of G-CSF on GM-CSF receptor expression was much more dramatic (300%) than the ability of GM-CSF to downmodulate G-CSF receptor expression (28%). Also, combinations of growth factors that were not synergistic failed to increase CSF receptor expression. Single-cell cloning experiments showed that growth factor synergy was a direct effect and that there was a strict correlation between the ability to synergize at the single-cell level and the ability to upregulate CSF receptor expression. These data would predict that 24 hours of preincubation of progenitor cells with a synergistic factor would result in increased receptor expression. In agreement with this, we found that IL-1, IL-6, IL-3, and G-CSF preincubation, for example, increased GM-CSF-induced colony formation by 55% to 218% when compared with preincubation in medium alone.

The importance of receptor upregulation as a mechanism for enhanced growth factor responsiveness has been questioned due to the spare receptor concept which proposes that only a low percentage of receptors need a ligand bound to achieve a maximum biologic response. However, a more recent study suggests that as many as 50% of the CSF receptors on hematopoietic progenitor cell lines need to be occupied to elicit a maximum response. Furthermore, the spare receptor concept was established using cells with high expression of growth factor receptors, while BM progenitor cells express only 100 to 200 CSF receptors per cell. On Lin− cells examined in the present study, the half-maximum receptor occupancy occurs at approximately 30 pmol/L (0.6 ng/mL) GM-CSF, which is similar to the concentration of GM-CSF needed for half-maximum proliferation (0.1 to 0.5 ng/mL; Keller et al, unpublished observation). These findings, combined with the present observation showing profound heterogeneity with regard to CSF receptor expression on BM progenitors (Table 5), suggest that induction of CSF receptor expression on progenitors lacking detectable receptors might be a mechanism for HGF synergy. The ability of synergistic factors to increase the number of CSF receptors on progenitor cells with no or very low receptor levels has the most biologic significance, because this could increase the number of progenitor cells with a threshold level of CSF receptors necessary to generate a biologic response. In support of this, a previous study showed that on a T-cell population with a heterogeneous distribution of IL-2 receptors, the proliferative response was dependent on IL-2 receptor density. Similarly, we believe that upregulation of CSF receptors on cells already expressing high levels of receptors probably has less biologic significance, because these cells might already have spare receptors.

Because the increase in CSF receptor expression on progenitor cells after 24 hours of treatment with synergistic factors often followed rapid transdownmodulation, it is possible that the hierarchical transmodulation model of CSF receptors might represent another mechanism for growth factor synergism. However, the inability of IL-1 and IL-6 to transdownmodulate the receptors for the CSF with which they synergized indicate that rapid transdownmodulation of growth factor receptors could not be a general, but rather a restricted mechanism, for growth factor synergism only applicable among the CSFs themselves. In addition, we have recently shown that another HGF, tumor necrosis factor-α (TNF-α), rapidly and dramatically transdownmodulates the expression of all CSF receptors on Lin− cells, and that this transdownmodulation is strictly correlated to an inhibitory effect on CSF-stimulated proliferation of these cells, suggesting that transdownmodulation of CSF receptors results in inhibitory rather than stimulatory responses.

In further support of our model for HGF synergy are recent studies from our laboratory showing that transforming growth factor-β (TGF-β) induces increased GM-CSF receptor expression on hematopoietic progenitor cells and that this is correlated with enhanced GM-CSF-induced proliferation, while TGF-β-induced inhibition of IL-3–stimulated proliferation is seen in the presence of a rapid and prolonged downmodulation of IL-3 receptor expression.
The cellular mechanism(s) by which synergistic factors induce increased CSF receptor expression remains to be determined. Potential mechanisms include regulation at the transcriptional and translational level, as well as effects on receptor protein processing and recycling.

In conclusion, the investigated HGFs synergizing with CSFs are potent inducers of CSF receptor expression on hematopoietic progenitor cells resulting in enhanced biologic response. Such induction can only be definitively detected by the use of purified progenitors. It is likely that also yet unidentified mechanisms are involved in growth factor synergism. Although certain studies suggest that upregulation of growth factor receptors could be a pathway for growth factor regulation applicable to hematopoietic\(^2\) as well as nonhematopoietic cell types,\(^3,^4\) the general applicability of this model, particularly in other developmental stem cell systems, remains to be investigated.

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Induction of colony-stimulating factor receptor expression on hematopoietic progenitor cells: proposed mechanism for growth factor synergism

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