Cascade Transactivation of Growth Factor Receptors in Early Human Hematopoiesis

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Highly purified progenitors (including erythroid [BFU-E], granulo-monocytic [CFU-GM], multipotent [CFU-GEMM] progenitors, as well as multipotent progenitors with self-renewal capacity [CFU-B]) express high-affinity growth factor receptors (GFRs), with prevalent interleukin-3 receptors (IL-3Rs) (2,700/cell), a 2·10-fold lower number of IL-6Rs (145/cell) and granulocyte-macrophage colony-stimulating factor receptors (GM-CSFRs) (300/cell), and a barely detectable level of erythropoietin (Ep) receptors (75/cell). Hematopoietic growth factor (HGF) dosages inducing peak clonogenetic effects are associated with partial/subtotal occupancy of the homologous HGF receptor (HGFR). Cross-reactivity between GFRs and heterologous GFSs (including IL-6, IL-3, GM-CSF, Ep, and the kit ligand [KL]) was explored by competition experiments on purified progenitors with radiolabeled and excess cold HGFs at +4°C. No cross-reaction was observed between IL-6, IL-3, Ep, and the heterologous GFSs, whereas the GM-CSFR showed cross-reactivity with IL-3 and, to a lesser extent, KL. Modulation of GFRs was examined after 18 or 40 hours of incubation with GFSs at 37°C, followed by ligand-binding assay at 20°C. IL-6, IL-3, GM-CSF, and Ep induce a marked down-modulation of their own receptors. Interestingly, each GF induces the transactivation of the R(s) for the "distal" GF(s): (1) IL-6 induces transactivation of IL-3R, but not of GM-CSFR/EpR; (2) IL-3 causes a rapid upmodulation of GM-CSFR/EpR ("pure" progenitors treated with IL-3 show upmodulation of GM-CSFR a-chain mRNA by reverse transcriptase-polymerase chain reaction); whereas (3) GM-CSF induces the transactivation of the EpR. This chain upmodulation of HGFRs may underlie the synergistic interactions between the HGFs in clonogenetic culture. It is emphasized that KL does not induce upmodulation of the other GFRs. Finally, Ep, GM-CSF, and IL-3 do not modulate the expression of the "proximal" HGFs (ie, GM-CSFR/IL-3R/IL-6R, IL-3R/IL-6R, and IL-6R, respectively). These results allow insight into the cellular basis of hematopoiesis, ie, the complex and coordinate interactions between HGFs and their receptors. They are compatible with a model of cascade transactivation via upmodulation of GFRs in the initial key steps of hematopoietic differentiation, whereby the action of each GF enhances the effect of the distal GF(s) by a multistep chain-potentiation mechanism.

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Hematopoiesis is a multistep cell proliferation/differentiation process that is sustained by a pool of stem cells. The stem elements can self-renew and differentiate into progenitors. These progenitors are committed to specific lineages and are functionally defined as colony- or burst-forming units (CFUs or BFUs), ie, progenitors of the erythroid series (BFU-E or CFU-E), the granulocyte-monocytic lineage (CFU-GM), and multipotent CFU for the GM, erythroid, and megakaryocytic series (CFU-GEMM). The progenitors in turn differentiate into morphologically recognizable precursors that mature to terminal elements circulating in peripheral blood.

A group of glycoprotein hematopoietic growth factors (HGFs), termed colony stimulating factors (CSFs) or interleukins (ILs), control the survival, proliferation, and differentiation of stem and/or progenitor cells. In addition, they affect a variety of functional activities of mature/terminal cells.

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HGFRs exert either a multilineage or unilineage stimulus. IL-3 acts on the early progenitors pool, ie, multipotent (CFU-GEMM), early erythroid (BFU-E), GM (CFU-GM), and megakaryocytic progenitors. GM-CSF exerts similar effects, but possibly stimulates progenitors at a more distal differentiation stage. Erythropoietin (Ep), G-CSF, IL-5, and M-CSF are largely specific for end-stage progenitors of the erythroid (CFU-E), granulocytic (CFU-G), eosinophilic (CFU-Eo), and monocytic (CFU-M) lineage, respectively.

Recent studies indicate an important role for a group of "permissive" GFSs (particularly kit ligand [KL], but also IL-6 and bFGF). Under stringent culture conditions, these GFSs themselves exert little activity, but potentiate the stimulatory activity of other HGFRs, particularly the multilineage ones. These permissive GFSs exert their effect at a very early stage of hematopoiesis: indeed, a defect of kit proto-oncogene or KL underlies the stem cell defect in W/W<sup>v</sup> or Sib/Sib mice, respectively, thereby suggesting that the KL represents a stem cell factor (SCF).

Other polypeptide GFSs modulate the growth of hematopoietic progenitors when added together with the above mentioned ILs/CSFs. Thus, IL-1 acts in synergy with HGFRs to stimulate proliferation and differentiation of primitive multipotent hematopoietic cells. Similarly, in combination with different HGFRs, IL-4 may either enhance or suppress colony formation by hematopoietic progenitors. It has been suggested, however, that these cytokines exert their effect by modulation of HGFRs released by accessory cells.

The availability of recombinant ILs/CSFs favored not only the analysis of their biologic activities, but also studies on their specific cell surface receptors (Rs). HGFRs have been recently cloned and classified into two different categories. (1) Rs belonging to the Ig superfamily, eg, IL-1R platelet-derived growth factor receptor (PDGFR), and the M-CSFR;
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Analysis of step III and IV purified human hematopoietic progenitors from normal adult blood. The cells, purified as outlined in Materials and Methods, were labeled with either a non-reactive FITC-labeled mouse Ig (control) or with FITC-labeled 8G12 anti-CD34 MoAb and analyzed with a FACScan flow cytometer. A representative experiment is shown here. (B) Clonogenetic analysis of step III and IV purified progenitors grown in semisolid medium with different GFs (same experiment as above). (i) CFU-GEMM + BFU-E; (ii) CFU-GM.

(2) Rs belonging to the peculiar "cytokine receptor family," which includes IL-2R β chain, EpR, IL-6R, IL-4R, GM-CSFR, and IL-3R.

Elucidation of HGFs/HGFRs interactions is obviously crucial in unveiling the cellular and molecular basis of hematopoiesis, particularly at the level of progenitor cells, which represent the main target of ILs/CSFs. Murine bone marrow cells incubated with HGFs at 37°C (but not at 4°C) show downmodulation within minutes of the expression of both their own Rs and other HGFRs. Thus, IL-3 downmodulates all CSFRs (GM-CSF, both G-CSFRs and M-CSFRs) and G-CSF at high concentrations downmodulates M-CSFRs. The hypothesis that regulation of the murine HGFRs follows a hierarchical pattern, whereby multilineage GFs (IL-3/GM-CSF) induce downmodulation and hence activation of the Rs for lineage-specific GFs was considered. In contrast, usurpation by IL-3 of GM-CSFR at 37°C and 0°C has been observed on both leukemic cell lines and terminal hematopoietic cells.

The expression and modulation of GFRs on early hematopoietic progenitors remains to be clarified. We have recently developed new methodology that enables stringent purification and abundant recovery of early hematopoietic progenitors from normal adult peripheral blood. The final, "pure" population of progenitors comprises a majority of BFU-E/CFU-GM and a minority of progenitors with multipotent differentiation (CFU-GEMM) and self-renewal capacity (CFU-B, giving rise to primary blast cell colonies, which in turn generate secondary colonies of all types). The availability of a large number of highly purified progenitors allowed us to explore the expression of selected HGFRs and the regulation thereof in early hematopoietic differentiation.

MATERIALS AND METHODS

Materials

**HGFs**

Recombinant human IL-3 (specific activity, $4 \times 10^6$ U/mg), GM-CSF ($1.7 \times 10^7$ U/mg), and IL-6 (specific activity, $2 \times 10^6$ U/mg) were supplied by Dr S. Clark (Genetics Institute, Pilot Development Laboratory, Cambridge, MA). Recombinant human Ep ($1.1 \times 10^5$ U/mg) was kindly provided by Amgen (Thousand Oaks, CA). Recombinant human c-kit ligand was supplied by Immunex (Seattle, WA).

**Other Materials**

Isotonic Percoll ($d = 1.124$) was obtained from Seromed-Biochrom (Berlin, Germany). Different monoclonal antibodies (MoAbs) were used for both the purification of the cells and the analysis of their interactions with the HGFRs.

Fig 1. (A) Flow cytometry analysis of step III and IV purified human hematopoietic progenitors from normal adult blood. The cells, purified as outlined in Materials and Methods, were labeled with either a non-reactive FITC-labeled mouse Ig (control) or with FITC-labeled 8G12 anti-CD34 MoAb and analyzed with a FACScan flow cytometer. A representative experiment is shown here. (B) Clonogenetic analysis of step III and IV purified progenitors grown in semisolid medium with different GFs (same experiment as above). (i) CFU-GEMM + BFU-E; (ii) CFU-GM.
membrane phenotype: OKT3, OKT4, OKT8, OKT11, OKT16, OKM1, and OKM5 (Ortho Diagnostic Systems, Raritan, NJ); Leu7, Leu9, Leu11, Leu12, Leu19, LeuM1, LeuM3, LeuM9, HPCA-I, and anti–HLA-DR (Becton Dickinson, Mountain View, CA). Immunomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG and IgM (Dynabeads M450 diameter 4.5 µm, 1.3 × 10^7 particles/mg) were obtained from Dynal Laboratories (Oslo, Norway). Purified bovine serum albumin (BSA), human low-density lipoproteins (LDL), and ferric ammonium citrate were purchased from Sigma (St Louis, MO).

Progenitor Purification

Cells

Adult peripheral blood was obtained from 20- to 40-year-old healthy male donors after informed consent. Blood (450 mL ± 10%) was collected in preservative-free CPDA-I anticoagulant (citrate-phosphate-dextrose-adenine). A buffy coat was obtained by centrifugation (Beckman J6M/E 1,400 rpm/20 min at room temperature; Beckman, Palo Alto, CA).

Purification Methodology

Step IA. The buffy coat samples were separated over a Ficoll-Hypaque density gradient (d = 1.077) (Pharmacia Fine Chemicals, Piscataway, NJ) at 400g for 40 minutes at 20°C. The interface mononuclear cells were collected, washed twice, and resuspended in Iscove’s modified Dulbecco’s medium (IMDM). Cell vitality was assessed by trypan blue exclusion test; cell mortality never exceeded 5%.

Step IB. Peripheral blood mononuclear cells (PBMC) were resuspended in IMDM containing 20% heat-inactivated fetal calf serum (FCS; Flow Laboratories, Glasgow, UK) at a cellular density of 3 × 10^6 cells/mL and then treated with two 60-minute plastic adherence cycles.

Step II. Cells were washed three times and resuspended in IMDM containing 10% FCS (5 × 10^6 cells/mL) and then separated by density centrifugation (600g for 30 minutes at 20°C) on a four-step discontinuous isotonic Percoll gradient (Biochrom KG, Berlin, Germany) (3 mL fractions; densities: 1.052, 1.056, 1.062, and 1.072).

Step III. Lower density cells from fraction I (d = 1.056), containing the majority of hematopoietic progenitors, were collected, washed three times in IMDM, resuspended in 3 to 6 mL of the same medium, and incubated for 60 minutes at 4°C with appropriate amounts of the following MoAbs: OKT3, OKT4, OKT8, OKT11,
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OKT16, OKM1, OKM5 (Ortho), Leu7, Leu9, Leu11, Leu12, Leu14, Leu19, LeuM1, and LeuM3 (Becton Dickinson, Oxnard, CA). Fifty microliters of each antibody was incubated with $3 \times 10^7$ cells. After three washes with cold IMDM, cells were incubated for 40 minutes at 4°C with a fivefold excess of immunomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG and IgM (Dynabeads M450; Dynal). The immunomagnetic beads were first washed three times with cold IMDM to remove preservatives and then three times with the same medium containing BSA (2 mg/mL) to decrease their nonspecific binding to the cells. The beads, together with rosetting cells, were then retained along the tube wall with a magnet for 60 seconds before the supernatant fluid containing negative cells was recovered. The negative cells were then incubated again for 30 minutes at 4°C with immunomagnetic microspheres and processed as described. The negative cells contained in the supernatant were washed twice in cold IMDM, resuspended in 0.5 mL IMDM, counted, and then cultured in semisolid medium. The remaining cells required for the last steps of purification were incubated overnight at 1 x 10^6 cells/mL in IMDM containing 20% FCS.

Step IV Cells were incubated for 60 minutes at 4°C in the presence of an appropriate amount of two anti-CD34 MoAbs, HPCA-1 (Becton Dickinson; 40 μL/L x 10^6 cells) and BI3C5 (Sera Lab, London, UK; 20 μL/L x 10^6 cells), washed three times in cold Iscove’s medium containing 2 mg/mL BSA, and then incubated for 60 minutes at 4°C in the same medium containing a fivefold excess of immunomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG and IgM (Dynabeads M450; Dynal). The beads, together with rosetting cells (CD34^+), were then retained along the tube wall with a magnet for 60 seconds, the supernatant fluid containing negative cells recovered, and the beads washed twice with 1 mL of Iscove’s medium containing BSA. The CD34^+ cells rosetting with the beads were then resuspended in a small volume of medium, counted, and plated in semisolid medium for clonogenic assays as indicated below.

In some experiments, the progenitors purified from the four normal donors were pooled at the end of the purification procedure (step III or IV) and then assayed for their HGF binding capacity.

Clonogenetic Culture

**Standard Serum-Supplemented (FCS^+) Cultures**

PBMC were cultured at a concentration of 3 x 10^6 cells/mL, and step III or IV purified progenitors were cultured at a concentration of 2 or 1 x 10^6 cells/mL, respectively (two plates per point), in 0.9% methylcellulose, 40% FCS (Flow), and 3.0 IU/mL Ep in IMDM supplemented with α-thioglycerol (10^-4 mol/L; Sigma) at 37°C in a 5% CO2/O2, humidified atmosphere.

** Serum-Free (FCS^-) Cultures**

In these cultures, FCS was replaced by a mixture of: (1) BSA (10 mg/mL; each stock solution was prepared according to McLeod et al, deionized, adjusted to pH 7.3, and filtered); (2) human LDL (Sigma) (40 μg/mL); (3) pure human transferrin (Behring Institute) (0.7 to 1.0 mg/mL) fully iron-saturated with FeCl3 as described; and (4) a mixture of pancreatic bovine insulin (10 μg/mL), nucleotides (10 μg/mL each), rare inorganic elements as in Eliaisons supplemented with iron sulphate (4 x 10^-8 mol/L) (GIBCO, Glasgow, UK).

The dishes (at least two plates per point) were incubated as for FCS^+ cultures. Colony maturation is slightly delayed in FCS^- conditions.

**HGF Stimulus**

When total PBMC were plated for clonogenic assays, 3 U/mL human recombinant Ep or 10 ng/mL human recombinant GM-CSF (specific activity, 1.7 x 10^7 U/mg) was added to sustain the formation of BFU-E and CFU-GM colonies, respectively. When step III or IV purified hematopoietic progenitors were grown in vitro for clonogenic assays, Ep (3 U/mL), GM-CSF (10 ng/mL) and IL-3 (100 U/mL);
specific activity, $4 \times 10^4$ U/mg), or GM-CSF (10 ng/mL) and IL-3 (100 U/mL) were added to stimulate the formation of BFU-E and CFU-GM colonies, respectively.

Receptor Binding Studies

**Incubation of Step III Hematopoietic Progenitors With HGFs Before Binding Analysis**

In experiments on GFR modulation, step III hematopoietic progenitors (containing routinely 25% to 35% CD34+ progenitor cells, see Gabbianelli et al18) were incubated at 37°C (5 $\times$ 10^5 cells/mL) in IMDM containing 10% FCS in absence (control) or presence of HGFs, ie, KL (10 ng/mL), IL-6 (10 ng), IL-3 (10 or 100 U), GM-CSF (10 ng), or Ep (3 U). After 16 or 40 hours of incubation, a cell aliquot was harvested, counted, and processed for binding with iodinated HGFs as outlined below.

In some experiments, cells were preincubated for 2 hours at 37°C in the presence of either IL-3 or GM-CSF, harvested, counted, and processed for binding with iodinated HGFs as outlined below. Step III progenitors incubated for 16 hours at 37°C without exogenous GFs (control) did not show a significant decrease of their cloning efficiency (data not shown).

**Binding of Iodinated IL-6, IL-3, GM-CSF, and Ep to Step III Purified Progenitors**

$^{125}$I-GM-CSF (specific activity, 101 $\mu$Ci/µg) was obtained from New England Nuclear (Boston, MA). $^{125}$I-Ep (specific activity, 118 $\mu$Ci/µg), $^{125}$I-IL-3 (specific activity, 22.5 $\mu$Ci/µg), and $^{125}$I-IL-6 (specific activity, 58 $\mu$Ci/µg) were purchased from Amersham (Buckinghamshire, UK).

Cells used for binding were washed three times and then resuspended in a buffer consisting of IMDM containing 25 mmol/L HEPES and BSA (2 mg/mL, pH adjusted to 7.4). Cells preincubated for 16 or 40 hours with cold HGF before the binding reaction with an iodinated ligand were washed using a stringent procedure to ensure the removal of HGF still bound to the cell membrane. Thus, cells were first washed with neutral phosphate-buffered saline (PBS) (pH = 7.4), then with acid PBS (pH = 5.0), 2 minutes of incubation at 4°C), and finally with neutral PBS.

Cells preincubated for 2 hours at 37°C in the presence of either IL-3 or GM-CSF were washed twice with IMDM, treated with 1 mL cold PBS (titrated to pH = 3.0 with HCl) for 1 minute at 4°C to remove the large majority of surface bound GM-CSF or IL-3 molecules, and finally incubated with iodinated HGFs.

Additional studies were performed to evaluate the efficacy of the acid wash in removing surface-bound IL-3 or GM-CSF. Thus, cells were first incubated for 2 hours at 4°C in medium containing 0.02% sodium azide and either $^{125}$I-GM-CSF or $^{125}$I-IL-3; cells were then carefully washed and incubated with acid PBS. Under these experimental conditions the acid wash removed greater than 90% of the cell-associated radioactivity.

Preliminary experiments were performed to define the optimal number of cells for binding studies. Using $1 \times 10^4$ to $2 \times 10^6$ cells per point, a linear relationship through the origin was observed between the amount of ligand bound to the cells and the cell number. On the basis of this preliminary observation we used $4 \times 10^6$ cells for each binding point. This cell number provides a low, but clearly measurable level of binding of the various ligands and is compatible with the availability of purified progenitors. Thus, $4 \times 10^6$ cells in 200 µL of binding buffer containing various concentrations of $^{125}$I-GM-CSF, $^1^2$I-IL-6, $^1^2$I-IL-3, or $^1^2$I-Ep were incubated at 23°C for 40 minutes (binding at 23°C was rapidly saturated, and reached equilibrium within 30 minutes). Binding at 4°C was much slower and required at least 2 hours to reach equilibrium. After incubation, cells were resuspended and carefully transferred onto 200 µL of a phthalate oil mixture.
Figure 5. Downmodulation of IL-3R and GM-CSFR by incubation of step III cells in the presence of the autologous ligand. Step III cells were grown in the absence of exogenous GFs (control) or the presence of (C, top) IL-3 (10 ng/mL), (□) IL-3 (10² ng/mL) + IL-6 (10 ng/mL), or (C, bottom) GM-CSF (10 ng/mL) for 16 or 40 hours at 37°C. Mean values ± SD from three experiments are shown. P < .01 when comparing IL-3, IL-3 + IL-6, and GM-CSF–treated and corresponding control groups.

Statistical Analysis

Significance of differences between groups was determined by the two-sided Student's t-test for paired samples.

Assay of Cytokines in Culture Supernatant

Step III progenitors were incubated in liquid suspension culture in the absence or presence of IL-6 (10 ng/mL), IL-3 (100 U/mL), or GM-CSF (10 ng/mL). At each day of culture cells were harvested, centrifuged (5 min/8,000 rpm), and the supernatants stored at −80°C until assay. IL-6, IL-3, GM-CSF, G-CSF, tumor necrosis factor-α (TNF-α), and IL-1β concentrations in the supernatants were evaluated by sensitive and specific immunoenzymatic assays (R&D System, Minneapolis, MN).

Analysis of α-Chain GM-CSFR mRNA in "Pure" Step IV Progenitors

GM-CSFR α-chain mRNA expression was assayed in step IV CD34⁺ cells by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Step IV cells (2 × 10⁶) were collected and total RNA was purified by a modification of the guanidine thiocyanate CsCl gradient technique⁶⁷ using a microcentrifuge (TL-100; Becton Instruments, Palo Alto, CA). Total RNA from the same number of cells was quantitated by RNA dot-blot hybridization with a ribosomal RNA probe. After densitometric analysis, normalized RNA was reverse transcribed into cDNA (MMLV-RT; Bethesda Research Laboratories, Gaithersburg, MD) using 0.2 µg of oligo-dT as primer. An aliquot of each RT reaction was incubated in the presence of a ³²P dCTP tracer and the reverse-transcribed RNA/DNA was normalized accordingly. The amplification procedure involved denaturation at 95°C for 1.5 minutes, annealing at 53°C for 1.5 minutes, and extension at 72°C for 1.5 minutes during 20 PCR cycles, ie, within the range of linear amplification. The 5' and 3' 18-bp primers corresponding to a 330-bp sequence of GM-CSF α-chain mRNA were: 5'GCCAGGCGCGGTGGCTCA-3' and 5'CCAAGTAGCTGGGATGATY, respectively. PCR was performed in a total volume of 100 µL. Ten microliters of each sample were amplified; the dose/response curves showed linearity for all points. The relative intensities of the bands were quantified by scanning with a laser densitometer (LKB).
Purified human hematopoietic progenitors purified from normal adult peripheral blood contain, respectively, 25% to 35% (occasionally down to 20% and up to 50%) and 75% to 95% CD34+ progenitors (CFU-GEMM, BFU-E, and CFU-GM) (Fig 1; see also Gabbianelli et al). These highly undifferentiated progenitors are largely quiescent, give rise to large colonies in clonogenetic culture, and exhibit an approximately

Expression of HGFRs on Step III Purified Human Hematopoietic Progenitors

In a first set of experiments, we evaluated the saturation binding of [125I]-IL-6, [125I]-IL-3, [125I]-GM-CSF, and [125I]-Ep to the purified progenitors (Fig 2 and Table 1). Scatchard analysis of the data indicated the presence of a single class of high-affinity binding sites for the ligands (Table 1). It is emphasized that the affinity of the receptors for their specific ligands was similar using either labeled (saturation binding experiments) or unlabeled (competition binding experiments) ligands (data not shown), as indicated by statistical analysis from three separate experiments (data not shown).

As mentioned above, step III cells were composed of 20% to 50% CD34+ hematopoietic progenitors and 50% to 80% contaminating CD34- cells. Thus, control studies were performed to verify whether CD34- cells express membrane receptors for IL-6, IL-3, GM-CSF, and Ep. The results show that step IV CD34- cells possess IL-6Rs and GM-CSFRs, but they are unable to bind IL-3 or Ep (Table 1). Thus, in the step III cells, the IL-3 and Ep binding capacity is selectively exhibited by CD34+ progenitors, whereas IL-6Rs and GM-CSFRs are expressed on both CD34+ progenitors and CD34- accessory cells. Taking into account the expression of these receptors on both total step III cells and the CD34- fraction, it was possible to evaluate the number of IL-6Rs, IL-3Rs, GM-CSFRs, and EpRs on CD34+ step III progenitors (Table
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1. These cells possess a high number of IL-3Rs (1,300 binding sites/cell), an approximately 10-fold lower level of IL-6Rs and GM-CSFRs (180 and 85 binding sites/cell, respectively), and an even lower number of EpRs (75 binding sites/cell).

It was of interest to correlate the R binding curve with the clonogenetic activity of the homologous HGF. The number of hematopoietic colonies (BFU-E + CFU-GEMM and CFU-GM) generated in methylcellulose cultures of step III progenitors (2 x 10^5 cells/plate) stimulated by graded amounts of IL-6, IL-3, GM-CSF, or Ep in the presence of saturating doses of the other GFs was measured (data not shown). The clonogenetic dose-response curve of each HGF fits with the binding curve of the homologous R; particularly, the plateau level of each GF in clonogenetic culture is associated with partial/subtotal R occupancy.

Cross-Reactivity Between HGFs and Rs

Recent reports described cross-modulation of human IL-3Rs and GM-CSFRs at both 37°C and 0°C on hematopoietic terminal cells (ie, macrophages and eosinophils). We evaluated whether this phenomenon could also be observed at 4°C in the presence of 125I-IL-3 or 125I-GM-CSF alone, and a >100-fold excess of unlabeled KL, IL-6, IL-3, GM-CSF, or Ep. These studies showed that (1) an excess cold ligand inhibits the binding of the homologous iodinated GF; (2) cold GM-CSF did not inhibit the binding of 125I-IL-3; (3) cold IL-3 induced a marked decrease in the binding of 125I-GM-CSF, less pronounced than that caused by cold GM-CSF; (4) cold KL caused a moderate, but significant, decrease of 125I-GM-CSF binding; and (5) finally, the addition of cold IL-6 or Ep had no effect on the binding of either 125I-IL-3 or 125I-GM-CSF (Fig 3).

The inhibition of 125I-GM-CSF binding by unlabeled IL-3 is particularly relevant. Increasing amounts of IL-3 displace virtually all 125I-GM-CSF bound to purified progenitors at either 4°C or 37°C (ie, 30 and 40 ng/mL are required for a 50% reduction of the binding at 37°C and 4°C, respectively) (Fig 4). Furthermore, this phenomenon is observed on both total and CD34+ step III cells (Fig 4).

Altogether, these data suggest that the high-affinity GM-CSFRs present on peripheral blood progenitors exhibit cross-reactivity with IL-3 and, to a lesser extent, with KL.

Similar experiments performed to explore the competition of HGFs for the binding of either 125I-IL-6 or 125I-Ep indicated that IL-6R and EpR do not cross-react with nonhomologous GFs (Fig 3 and data not shown).

Each HGF Downmodulates its own Receptor

Modulation of GFR(s) was explored after 18 or 40 hours of incubation with GF(s), followed by a ligand binding assay. IL-6, IL-3, GM-CSF, and Ep induce a marked downmodulation of homologous receptors. Figure 5 shows that step III progenitors treated with either IL-3 or GM-CSF for 16 or 40 hours exhibit a pronounced decline of the IL-3 or GM-CSF binding capacity, respectively (see below).

Each HGF Upmodulates the Receptors for Distal HGF(s)

To evaluate the effects of IL-6, IL-3, or GM-CSF on the "distal" GFR (ie, IL-6 on IL-3R/GM-CSFR/EpR, IL-3 on GM-CSFR/EpR, GM-CSF on EpR). Step III cells were first grown for 16 or 40 hours in the presence of either IL-6, IL-3, or GM-CSF and then assayed for their ability to bind iodinated IL-6, IL-3, GM-CSF, and Ep. Control experiments involved (1) 16 or 40 hours of incubation of step III progenitors in the absence of HGFs, and (2) evaluation of the effect of HGFs on the expression of "proximal" HGFRs on step III cells (ie, Ep on GM-CSFR/IL-3R/IL-6R; GM-CSF on IL-3R/IL-6R; IL-3 on IL-6R).

Control experiments were performed to evaluate whether 16 hours of incubation of step III purified progenitors in the absence (control) or presence of these HGFs modifies their clonogenetic capacity, cycling status, and/or membrane phenotype. The results indicate that, after 16 hours of culture in the absence or presence of saturating levels of IL-6, IL-3, GM-CSF, or Ep, step III progenitors do not show differential characteristics from the corresponding control (time 0) progenitors, in terms of: (1) clonogenetic capacity (colony number, type, and size) under optimal culture conditions (ie, in the presence of KL, IL-3, GM-CSF, and Ep); (2) membrane antigen pattern (CD33, CD15, and CD45RO expression); and (3) level of [3H]-thymidine incorporation (<100 cpm/2 X 10^4 cells even after incubation with IL-3 or GM-CSF) (results not presented).

As shown in Fig 6A, incubation of step III progenitors in the presence of IL-6 elicited a twofold upmodulation of IL-3 binding capacity, whereas the level of iodinated GM-CSF and Ep binding was virtually unmodified. Scatchard analysis of [125I] IL-3 binding data showed that the stimulatory effect of IL-6 on IL-3R expression is due to an approximately twofold increase of the number of IL-3Rs (representative results in Fig 6B) without significant modifications of receptor affinity. This analysis also showed that IL-3 induced a marked downmodulation of the number of IL-3Rs (Fig 6B). KL, GM-CSF, and Ep were unable to significantly modify the expression of IL-3Rs (see below).

Incubation of step III progenitors in the presence of IL-3 elicited a pronounced upmodulation of both GM-CSFRs and EpRs (Fig 7A); after 40 hours of incubation in the presence of IL-3, the cells showed a 2.5-fold and fivefold increase of their GM-CSF and Ep binding capacity, respectively. Scatchard analysis indicated a 2.2-fold and 8.2-fold increase of GM-CSFRs and EpRs number, respectively, without significant changes in their affinity (Fig 7B and C). Dose-response experiments showed that the stimulatory effect of IL-3 on both GM-CSFR and EpR expression is observed at low IL-3 concentrations (0.25 ng/mL), but is markedly increased at higher concentrations (25 ng/mL) (results not shown).

Because GM-CSFRs are expressed on both CD34+ and CD34- cells, experiments were performed to differentially evaluate the effect of IL-3 on GM-CSFR on these two populations. As expected, 16 or 40 hours of incubation of CD34+ cells in the presence of IL-3 did not modify their GM-CSF binding capacity (Fig 7A). It is concluded that IL-3 selectively upmodulates GM-CSFRs expressed on CD34+ progenitor cells, as predicted by virtual absence of IL-3Rs on CD34- cells.

Furthermore, we evaluated GM-CSFR expression after a short 2-hour incubation of step III progenitors in the presence
of IL-3 (1 to 10 ng/mL) or GM-CSF (10 ng/mL) at 37°C. The cells were then washed twice with IMDM, treated with 1 mL of PBS (pH ~3.0) for 1 minute at 4°C to remove surface-bound GM-CSF or IL-3, and then incubated at 23°C for 40 minutes in the presence of radiolabeled GM-CSF. The GM-CSF binding capacity is not significantly modified upon incubation with GM-CSF, whereas it is slightly increased in cells preincubated with IL-3 when compared with control values (Fig 8). The absence of GM-CSFR downmodulation after 2 hours of incubation in the presence of cold GM-CSF could be due to the relatively low concentration of GM-CSF (10 ng/mL) used for the preincubation.

Finally, incubation of step III progenitors in the presence of IL-3 did not modify the IL-6 binding capacity, even 48 hours after IL-3 addition (data not shown).

Additional experiments were performed to evaluate whether combined addition of IL-6 and IL-3 may induce a synergistic upmodulation of GM-CSFRs and EpRs, as compared with IL-3 alone. IL-3 + IL-6 addition induced: (1) an enhancement of GM-CSFR expression, over the level observed after IL-3 treatment alone; and (2) a stimulation of EpR expression comparable to that observed after incubation with IL-3 alone (Fig 7A).

Finally, we investigated the effect of GM-CSF on the expression of different GFRs. After 16 or 40 hours of incubation, GM-CSF induced a threefold increase in Ep binding capacity, as compared with control values (Fig 9). It is emphasized that the stimulatory effect of GM-CSF on EpR expression is lower than that of IL-3. On the other hand, 16 or 40 hours of incubation of step III progenitors with GM-CSF did not significantly modify IL-3 or IL-6 binding capacity (data not shown).

Additional experiments were performed to evaluate the effect of KL on IL-6, IL-3, GM-CSF, and Ep R expression. Sixteen or 40 hours of incubation of step III progenitors in the presence of KL did not significantly alter the expression of these R (Fig 10).

Further experiments were performed to determine the release of secondary cytokines, including IL-6, IL-3, GM-CSF, G-CSF, and IL-1, by step III progenitors incubated in FCS+/IMDM suspension culture supplemented or not with IL-3 or IL-6. Analysis by sensitive and specific immunoenzymatic assays, indicated that (1) IL-6 is present at similar levels in both control and IL-3-treated cells; (2) IL-3, GM-CSF, and G-CSF are undetectable; and (3) very low levels of IL-1β (40 to 60 pg/mL) are observed, with no significant differences among control, IL-3-, or IL-6-treated cells (Table 2).

IL-3 Upmodulates the GM-CSFR α-Chain mRNA in “Pure” Step IV Hematopoietic Progenitors

In additional experiments on ~90% “pure” step IV progenitors, we evaluated the effect of IL-3 on the expression of GM-CSFR α-chain mRNA by RT-PCR analysis. This mRNA species is present in step IV progenitors and its level is apparently increased in cells grown for 16 or 40 hours in the presence of IL-3 (Fig 11). Because the analysis of GM-CSFR α-chain mRNA was performed on “pure” hematopoietic progenitors, these results indicate that IL-3 upmodulates GM-CSFR gene expression on hematopoietic progenitors through transcriptional or posttranscriptional mechanisms.

DISCUSSION

Hematopoiesis is a complex cell proliferation/differentiation system that is dependent on regulation of the proliferative/differentiative activity of stem/progenitor cells. Analysis of the key control mechanisms of early hematopoiesis has been hindered so far by the lack of a sufficient number of highly purified stem/progenitor cells. Thus, although the action of HGFs on early hematopoietic cells has been extensively investigated, little is known on the expression of HGF receptors on stem/progenitor cells and the regulation thereof. In this study we have analyzed for the first time the expression and HGF-induced modulation of HGF receptors on highly purified early hematopoietic progenitors (CFU-GEMM, BFU-E, and CFU-GM).

Previous studies reported the expression of EpRs on late erythroid progenitors (CFU-E) generated after 7 days of culture by partially purified BFU-E.51-53 Our results indicate that early CD34+33+ progenitors express a variety of high-affinity HGF receptors, with prevalent IL-3Rs (2,700 Rs/cell), an approximately 10-fold lower number of IL-6R/GM-CSFRs (145 and 300 Rs, respectively), and a barely detectable level of EpRs (75 Rs). The GFR binding and GF dose/response curves are strictly correlated, ie, GF dosages required for partial/subtotal R saturation induce peak clonogenic effects.

Incubation of purified progenitors with HGFs showed three different phenomena: (1) downmodulation of each HGF receptor by the homologous GF; (2) cross-reactivity between IL-3 and KL and GM-CSFRs; (3) transactivation by HGFs of the receptor(s) for “distal” but not “proximal” HGF(s). The two latter phenomena are drastically different in that (1) cross-modulation indicates receptor usurpation by a heterologous ligand; and (2) transactivation reflects modulation of receptor...
The cross-reactivity is conceivably related to the structure of the GM-CSFR. Two distinct polypeptide chains (α and β) form the GM-CSFR. (1) The 80-Kd protein (α-chain) constitutes a low-affinity receptor (kd, ~1 to 10 nmol/L) that is apparently expressed on all cells binding to GM-CSF. (2) The 135-Kd protein (β-chain) forms the high-affinity receptor (kd, 10 to 40 pmol/L) together with the α-chain, but does not bind GM-CSF by itself; its expression is restricted on hemopoietic cells that respond to GM-CSF. Human GM-CSFRs and IL-3Rs share the 130-Kd β subunit. This seemingly underlies the competition of IL-3 with GM-CSF for binding to the high-affinity GM-CSFR. Further observations suggest that cross-inhibition of GM-CSF binding by IL-3 may be mediated by interaction of IL-3 with the β-chain of the high-affinity GM-CSFR. Indeed, (1) IL-3 inhibits GM-CSF binding to high-affinity, but not low-affinity GM-CSFR (this study); and (2) in COS cells transfected with the α-chain GM-CSFR cDNA, cross-linking studies have shown that IL-3 selectively inhibits the interaction of 125I-GM-CSF with the β-chain, but not the α-chain of GM-CSFR. It is of interest that inhibition by IL-3 of GM-CSF binding to its own R was observed in human leukemic cell lines that bore the Rs for both GM-CSF and IL-3, but not in those with only GM-CSFRs.

We observed that IL-3 is able to compete with GM-CSF for R binding, but not vice versa. The latter finding is in line with previous studies on human leukemic lines. However, studies on mature hemopoietic cells, eg, monocytes and eosinophils, showed that cross-modulation occurs in both directions.
Effect of KL on the expression of IL-6, IL-3, GM-CSF, and Ep Rs on Step III cells. Step III purified progenitors were incubated for 16 or 40 hours at 37°C in the absence (control) or presence of KL (10 ng/mL) and then analyzed for IL-6, IL-3, GM-CSF, and Ep binding capacity. Mean ± SD from three experiments are shown. No significant difference was found between control and corresponding KL-treated groups.

The finding that KL is able to interact, although with low affinity, with the GM-CSFR is novel. The biochemical basis of this phenomenon is not yet understood. In this regard, genetic and structural homology between KL and M-CSF has been reported. Cross-reaction of KL with the GM-CSFR may underlie the generation of a few GM colonies in semi-solid cultures of purified progenitors supplemented with KL alone.

The transactivating effect of HGFs on "distal" HGFRs deserves extensive discussion. IL-6 upmodulates IL-3R expression, without affecting the expression of other HGFRs; IL-3 upmodulates the expression of both GM-CSFRs and EpRs; and GM-CSF upmodulates EpR. This pattern of cascade upmodulation of HGFRs is novel and in line with the effects of HGFs on the clonogenetic activity of purified step III progenitors.

Conversely, these HGFs do not modulate the expression of the Rs for "proximal" HGF (ie, Ep, GM-CSF, and IL-3 did not modify the expression of the receptors for IL-6/IL-3/GM-CSF, IL-6/IL-3, and IL-6, respectively). Finally, as expected, each HGF induces a marked downmodulation of its own receptor.

It is of interest that modulation of Rs number is already observed at 16 hours after HGF stimulus. Conversely, the clonogenetic capacity (colony number, type, and size), cycling status, and membrane phenotype of the progenitors were unmodified after 16 hours of incubation in both control and GF-treated culture, as compared with the corresponding population of progenitors at time 0. These findings suggest that R modulation occurs at a very early stage after the GF stimulus, before significant modification of the phenotypic characteristics of the progenitors (ie, before demonstrable differentiation of the progenitors at cellular level).

The cascade transactivation of GFRs described here is only apparently at variance with a previous report indicating that murine HGFRs are downmodulated according to a hierarchical pattern, ie, lineage-specific HGFRs are activated via downregulation by IL-3 and/or GM-CSF. However, (1) these studies analyzed total murine bone marrow cells, composed by ≥99% mature hemopoietic precursors/terminal cells, whereas we used highly purified human hemopoietic progenitors; and (2) the above downmodulation was observed within minutes after addition of HGFs; the upmodulation reported here occurs 16 to 40 hours after HGF(s) addition.

Little is known on the expression and modulation of IL-6R and IL-3R on hematopoietic progenitors. We observed that incubation of the human progenitors with IL-6 induced an upmodulation of IL-3R expression. This phenomenon may underlie the synergistic effect of IL-6 with IL-3 in stimulating the formation of both blast and GM/erythroid colonies (present report).

Studies on the modulation of GM-CSFR expression in normal and leukemic human myeloid cells have shown that: (1) GM differentiation of human leukemic cell lines, induced by chemical agents, is coupled with an increase of the number of GM-CSFRs; (2) GM-CSF as well as TNF-α downmodulate the GM-CSFR on normal human myeloid cells; (3) TNF-α enhances the expression of GM-CSFR on human leukemic blasts; and (4) IL-1β upregulates the expression of GM-CSFR on a factor-dependent human hematopoietic cell line (TF-1).

The present study indicates that IL-3 (but not KL, IL-6, or Ep) is able to stimulate the expression of GM-CSFRs. This effect is specific for hematopoietic progenitors, in that...
Table 2. Evaluation of the Concentration of Several Cytokines in the Supernatant of Step III Cells Grown Without Exogenous HGFs (control) or in the Presence of IL-3 (100 U/mL), GM-CSF (100 ng/mL), or IL-6 (10 ng/mL)

<table>
<thead>
<tr>
<th>Culture Treatment</th>
<th>TNF-α (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-3 (pg/mL)</th>
<th>GM-CSF (pg/mL)</th>
<th>G-CSF (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>115</td>
<td>20</td>
<td>35</td>
<td>40</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>IL-3</td>
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<td>40</td>
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</tr>
<tr>
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<td>30</td>
<td>55</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>IL-6</td>
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<td>80</td>
<td>45</td>
<td>50</td>
<td>45</td>
<td>25</td>
</tr>
</tbody>
</table>

Step III hematopoietic progenitors (5 × 10⁶ cells/mL) were grown in IMDM containing 20% FCS under different experimental conditions. At each day of culture, one aliquot of the cell suspension was removed, centrifuged for 5 minutes at 8,000 rpm, and the supernatants stored at −80°C until assay. The concentrations of some cytokines, including TNF-α, IL-1β, IL-6, IL-3, GM-CSF, and G-CSF, were evaluated using the sensitive immunoenzymatic assays. Results represent the mean values observed in five separate experiments.

it is observed for CD34⁺, but not CD34⁻ cells. This finding was expected, in view of virtual lack of IL-3Rs on CD34⁺ cells. The stimulatory effect of IL-3 on GM-CSFR number is associated with a parallel increase of GM-CSFR mRNA level in "pure" progenitors and thus seems to be mediated through transcriptional and/or posttranscriptional mechanisms. The IL-3-induced upmodulation of GM-CSFRs seemingly underlies the synergistic effect of IL-3 and GM-CSF to stimulate the formation of GM and erythroid colonies.

 Autoradiography studies on partially purified human erythroid progenitors showed that only 20% of peripheral blood BFU-E bear EpRs.³¹ The number of the Rs was lower on BFU-E than on CFU-E.⁵² The Ep binding declined with further progression along the erythroid pathway.⁵³ In line with these findings, our observations indicate that the EpR is barely present on early progenitors, but is rapidly upmodulated by IL-3 and/or GM-CSF. We observed that IL-3 is more potent than GM-CSF to induce EpRs. This correlates with the differential burst generating activity exhibited by these GFs. Altogether, it is apparent that, after the IL-3/GM-CSF stimulus, BFU-E undergo differentiation and expansion of EpRs, thus becoming very sensitive to the action of Ep (this report) and strictly dependent on it for viability.⁵⁵

In conclusion, our studies provide a novel analysis of the expression and modulation of HGFRs on highly purified or "pure" early hematopoietic progenitors.

These observations are compatible with a model of cascade...
transactivation via upmodulation of GFs in early hematopoietic differentiation, whereby the action of each GF enhances the effect of the distal GF(s) in a multistep chain-potentiation mechanism. This model may explain the interactions between HGFs in semisolid or liquid culture; more importantly, it unveils key control mechanisms underlying early hematopoiesis.

It may be suggested that HGFs direct hematopoietic proliferation/differentiation in a step-wise fashion. Accordingly, IL-6 clearly amplifies the responsiveness of early progenitors to IL-3, which plays a pivotal role in stimulating early hematopoiesis.

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Cascade transactivation of growth factor receptors in early human hematopoiesis

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