Mammalian Granulocyte–Macrophage Colony-stimulating Factor Receptor Expressed in Primary Avian Hematopoietic Progenitors: Lineage-specific Regulation of Proliferation and Differentiation

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Abstract. The cytokine Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF) regulates proliferation, differentiation, and apoptosis during myelopoiesis and erythropoiesis. Structure–function relationships of GM-CSF interactions with its receptor (GM-R), the biochemistry of GM-R signal transduction, and GM-CSF action in vivo are relatively well understood. Much less is known, however, about GM-R function in primary hematopoietic cells. In this paper we show that expression of the human GM-R in a heterologous cell system (primary avian erythroid and myeloid cells) confirms respective results in murine or human cell lines, but also provides new insights how the GM-R regulates progenitor proliferation and differentiation. As expected, the hGM-CSF stimulated myeloid progenitor proliferation and differentiation and enhanced erythroid progenitor proliferation during terminal differentiation. In the latter cells, however, the hGM-R only partially substituted for the activities of the erythropoietin receptor (EpoR). It failed to replace the EpoR in its cooperation with c-Kit to induce long-term proliferation of erythroid progenitors. Furthermore, the hGM-R α chain specifically interfered with EpoR signaling, an activity neither seen for the β_c subunit of the receptor complex alone, nor for the α chain of the closely related Interleukin-3 receptor. These results point to a novel role of the GM-R α chain in defining cell type–specific functions of the GM-R.

THE tight regulation of proliferation, differentiation, and apoptosis required for homeostasis during hematopoiesis is to a large extent effected by numerous humoral factors (cytokines). These polypeptide factors induce and maintain the production of the correct amounts of immature and mature hematopoietic cells. Furthermore, cytokines are responsible for the fast responses of the hematopoietic system to specific needs arising from immune responses, blood loss, or during disease.

One of the best-studied cytokines is the Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF).[1] It represents a major regulator at different stages of hematopoiesis (Metcalf, 1980). GM-CSF induces a large variety of biological effects, such as proliferation induction in early progenitors, stimulation of differentiation along various myeloid lineages depending on factor concentration (Metcalf, 1989), cooperation with or even replacement of erythroid cytokines (Nishijima et al., 1995), as well as regulation of mature cell function (Metcalf, 1980). Finally, GM-CSF also stimulates the proliferation of multipotent CD34-positive progenitors (Sonoda et al., 1994).

GM-CSF binds to a heterodimeric receptor complex (GM-R) consisting of an α chain (GM-Rα) and a common β chain (β_c). Whereas the α chain confers specific binding to GM-CSF, β_c by itself does not bind the ligand, but alters the affinity of the receptor complex for GM-CSF from low

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1. *Abbreviations used in this paper*: AS, anemic serum; β_c, common β chain; BPA, burst-promoting activity; cMGF, chicken myelomonocytic growth factor; Epo, erythropoietin; EpoR, erythropoietin receptor; GM-CSF, Granulocyte–Macrophage Colony-Stimulating Factor; GM-R, GM-CSF receptor; GM-Rα, GM-R α chain; IL, interleukin; IRES, internal ribosome entry site; ts, temperature sensitive.

to high affinity. Furthermore, β_c associates with the intracellular tyrosine kinase Jak2 (Quelle et al., 1994). Since neither the GM-Rα, nor β_c have an intrinsic kinase activity, this association is thought to be a necessary step in triggering signal transduction. Interfering with the association between β_c and Jak2 renders respective GM-R mutant proteins unable to confer a mitogenic signal (Watanabe et al., 1996). After ligand activation of the GM-R Jak2 phosphorylates several tyrosine residues on β_c. These subsequently interact with the src homology region 2 (SH-2) domains of signaling intermediates like Stat5 (Ihle et al., 1994; Mui et al., 1995; Wakao et al., 1995), as well as phosphatidylinositol-3-kinase (PI-3-kinase), Grb2, Shc, and PTP-1D (Lanfrancone et al., 1995; Rao and Mufson, 1995; Pratt et al., 1996).

In contrast to β_c, the GM-R α chain has a very small cytoplasmic domain that lacks tyrosine residues phosphorylated by Jak-2 or other kinases. Nevertheless, the α chain contributes to the activity of the GM-R, since a receptor complex with an α chain lacking its cytoplasmic tail is unable to promote proliferation and differentiation (Sakamaki et al., 1992; Weiss et al., 1993; Matsuguchi et al., 1997). How the α chain contributes to receptor function is still obscure, but obviously important to understand receptor-specific signal transduction.

The β_c receptor subunit is not only used by the GM-R, but also by the interleukin (IL)-3 and IL-5 receptors. Like the GM-R, both of these receptors have a receptor-specific α chain, but they also require interaction with β_c for signal transduction and biological activity. Despite this shared use of β_c, all three receptor complexes have distinct biological activities when activated by their respective ligands. IL-5 induces differentiation of eosinophils (Dent et al., 1990), a specificity explained mainly by the restricted expression pattern of the IL-5 receptor α chain. In contrast, IL-3 functions on a similar subset of hematopoietic progenitors as GM-CSF. However, its activity is much less biased to myeloid progenitors as the activity of GM-CSF (Nimer and Uchida, 1995). Thus, receptor specificity may be dependent on signaling events mediated by the α chains of the receptors.

In contrast to this rather detailed knowledge on receptor structure and in vivo function, much less is known about how ligand activation of the GM-R affects cell proliferation, differentiation, and apoptosis in primary, differentiating hematopoietic progenitors. So far, mainly immortalized cell lines of human or murine origin were used in such studies (Spooncer et al., 1986; Jubinsky et al., 1993; Mui et al., 1995). However, many of these lines are altered in their response to cytokines and sometimes exhibit incomplete and/or aberrant differentiation (for review see Beug et al., 1995).

Clones of chicken erythroid and myelomonocytic progenitors capable of sustained proliferation offer an alternative. Long-term proliferation can be induced by transformation with temperature-sensitive (ts) oncoproteins (ts-v-sea, ts-gag-myb-ets; Beug et al., 1984; Knight et al., 1988) or—in the case of erythroid progenitors—by combinations of "self renewal factors," i.e., receptor tyrosine kinase ligands plus steroid hormones (Hayman et al., 1993; Beug et al., 1995). In both cases, the proliferating progenitors can be induced to terminal differentiation into erythrocytes or macrophages either by ts oncoprotein inactivation at 42°C or by replacement of "self-renewal factors" by cytokines required for differentiation.

In this paper, we sought to clarify the action of the GM-R and the cooperation between its α and β chains by expressing these proteins in the above-described heterologous cell systems. Since avian hematopoietic progenitors are unresponsive to mammalian GM-CSF or other cytokines, e.g., those present in serum (Steinlein et al., 1994; Wessely et al., 1997*b*; Beug, H., and E.M. Deiner, unpublished observations), this allowed to test for the specific contributions of the GM-R. The usefulness of such an approach to analyze mammalian hematopoietic regulators and oncoproteins has recently been demonstrated (Tran Quang et al., 1997). Here, we show that GM-CSF promotes differentiation of hGM-R–expressing myelomonocytic progenitors and cooperates with the EpoR in regulating erythroid differentiation. Interestingly, however, non-liganded exogenous hGM-R renders erythroid progenitors unresponsive to signaling via the endogenous aEpoR. This quenching of Epo-responsiveness is specifically caused by the hGM-R α chain, while neither β_c nor the hIL-3 receptor α chain induce this effect. Epo responsiveness of the GM-R–expressing cells can be restored by an exogenously expressed mEpoR. However, the hGM-R cannot substitute for all functions of the mEpoR, in particular it failed to cooperate with c-Kit to induce long-term proliferation in erythroblasts.

These results constitute the first demonstration that the α chain of the GM-R may contribute to lineage commitment by differential signal transduction in erythroid and myeloid hematopoietic cell types.

Materials and Methods

Viruses and Cells

The cDNAs encoding the hGM-Rα was a gift of Genentech Inc. (South San Francisco, CA), the hIL3-Rα and β_c were generously provided by DNAX (Palo Alto, CA). The construction of the pCRNCM/hGM-R retroviral vector will be described in detail elsewhere. In general, the strategy used was similar to that described in Tran Quang et al. (1997), namely separating the two cDNAs for GM-Rα and β_c by an internal ribosome entry site (IRES; Elroy-Stein et al., 1989). Additionally, to express the single chains of the receptor complex, β_c was cloned into the avian retroviral expression vector pOli (Wessely et al., 1997*b*) and the hGM-Rα, as well as the hIL3-Rα, were inserted behind the cytomegalovirus (CMV) promoter of pCRNCM (Steinlein et al., 1994). RCAS/EpoR was a kind gift of J. Ghysdael (Institute Curie, Orsay, France).

Chicken embryo fibroblasts expressing the retroviral constructs pCRNCM/hGM-R, pCRNCM/hGM-Rα, pCRNCM/hIL3-Rα and pOli/β_c were generated as described earlier (Zenke et al., 1990; Wessely et al., 1997*b*). All fibroblasts were grown in standard growth medium (Graf, 1973). Ts21-E26 transformed myeloblasts, SCF progenitors, and SCF/TGFα progenitors were grown from the bone marrow of 3–10-d-old Spafas chicks as described previously (Hayman et al., 1993; Schroeder et al., 1993; Woldmann et al., 1997). The HD3 erythroblast cell line HD3-EpoR-E22 has been described previously (Mellitzer et al., 1996). Steroids (estradiol, dexamethasone) were used at a final concentration of 10^{-6} M, cMGF at 10 ng/ml, aSCF at 100 ng/ml (Bartunek et al., 1996), TGFα (Promega, Heidelberg, Germany) at 5 ng/ml, IGF-1 (Sigma Chemical Co., St. Louis, MO) at 40 ng/ml, hGM-CSF (Promega) at 10 ng/ml, hEpo at 10 U/ml, insulin at 1.4 nM, and PD 153035 at 2.5 μM (Fry et al., 1994).

Infection of Primary Erythroblasts and Myeloblasts with Retroviruses

To infect erythroblasts, freshly prepared chicken bone marrow cells were

cocultivated with mitomycin C–treated CEF expressing the respective retroviral constructs for 2 d (Fuerstenberg et al., 1992). The cells were then either further propagated as a mass culture or seeded in CFU-E-methocel containing aSCF, TGFα, E2, and Dex. Outgrowing colonies were isolated and expanded in CFU-E medium plus aSCF, TGFα, E2, and Dex (Hayman et al., 1993) Myeloid progenitors were obtained by coculturing bone marrow cells with both mitomycin C–treated, ts21-E26–transformed myeloblasts and CEF expressing the respective retroviral constructs. Subsequently, the myeloid progenitors were maintained in the presence of cMGF (Beug et al., 1984).

Proliferation Kinetics

To determine the growth kinetics of erythroblast mass cultures, cells were subjected to daily partial medium changes plus re-addition of fresh factors. They were kept at densities of 2–4 $\times$ 10^6 cells/ml, and aliquots counted in an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, FRG). Cumulative cell numbers were determined as described in Fuerstenberg et al. (1992).

Differentiation of Myeloid Progenitors

Cells were washed twice in PBS and seeded at 1–2 $\times$ 10^6 cells/ml into 35-mm dishes containing 2 ml of differentiation medium (Woldmann et al., 1997) and were incubated at 42°C. When indicated, cMGF or hGM-CSF was added. The cells were maintained at densities of 2–4 $\times$ 10^6 cells/ml, counted daily, and aliquots were removed at days 3–5 for morphological analysis by cytocentrifugation onto slides and staining with histological dyes (Beug et al., 1984).

Differentiation of Erythroid Progenitors

Cells were washed twice in PBS and seeded at 1–2 $\times$ 10^6 cells/ml into 35-mm dishes containing 2 ml of differentiation medium (Dolznig et al., 1995). When indicated, 3–5% high titer anemic serum (as a source for aEpo), insulin, hGM-CSF, and/or hEpo were added. Cells were counted daily and maintained at densities of 2–4 $\times$ 10^6 cells/ml. Aliquots were removed for hemoglobin determination and morphological analysis at the days indicated.

Analysis of Differentiation by Cell Morphology and Staining

Cells were cytocentrifuged onto slides and subsequently stained with histological dyes and neutral benzidine for hemoglobin as described in (Beug et al., 1982). Images were taken using a CCD camera (Photometric, Tucson, AZ) and a blue filter (480 nm), so that mature cells (stained yellow to brownish) appear darkly stained. Images were processed with Photoshop (Adobe Systems Inc., Mountain View, CA).

Photometric Hemoglobin Assay

Three 50-μl aliquots of the cultures were removed and processed for photometric determination of hemoglobin as described (Kowenz et al., 1987). Values obtained were normalized to cell number.

Northern Blot Analysis

Cells were lysed in GITC buffer (Chomczynski and Sacchi, 1987) and NaAc, pH 4.0, was added to 25 mM. The solution was extracted with H_2O-saturated phenol, chloroform, and isoamylalcohol. After precipitation with isopropanol, the pellet was dissolved in 10 mM Tris/HCl, pH 7.0, 1 mM EDTA, 0.2% SDS, and proteinaseK was added to 200 μg/ml. After a 30-min incubation at 37°C, the solution was extracted with phenol/chloroform/isoamylalcohol (25:24:1), pH 7.0, and then precipitated with ethanol. 10–20 μg of RNA was run on a formaldehyde-containing agarose gel and transferred to nylon filters (Gene Screen, Dupont NEN, Boston, MA) using conventional procedures. Single-stranded DNA probes were radioactively labeled with ^{32}P by using an Oligolabeling Kit (Pharmacia Biotechnology Inc., Piscataway, NJ) and hybridized at 65°C in 7% SDS, 0.5 M Na phosphate, pH 7.0, 1 mM EDTA. Membranes were washed in 1% SDS, 40 mM Na phosphate at 65°C, and then autoradiographed.

Growth Factor Treatment and Cell Lysis

For growth factor stimulation primary myeloid and erythroid progenitors were washed once with PBS and then withdrawn from factors by cultivation for 12 h in differentiation medium (Dolznig et al., 1995). Afterwards the cells were washed twice with PBS, suspended in differentiation medium, and then treated as indicated with either 50 ng/ml TGFα, 50 U/ml hEpo, 100 ng/ml hGM-CSF, 100 ng/ml cMGF, or without factors for 15 min at 37°C. Stimulation of HD3-EpoR-E22 cells was performed similarly except that the cells were withdrawn from growth factors using serum-free differentiation medium using 2.5 μM PD153035 to inhibit v-ErbB activity (Mellitzer et al., 1996) and were shifted to 42° instead of 37°C.

After stimulation, 10^6 cells were lysed in 10 μl EMSA lysis buffer (20 mM Hepes, pH 7.9, 140 mM NaCl, 1.5 mM $MgCl_2$, 1.0% NP-40, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 2 μg/ml leupeptin), or in 20 μl immunoprecipitation buffer (1% Triton-X-100, 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 2 μg/ml leupeptin), respectively. Cell lysates were cleared by centrifugation for 15 min at 15,000 rpm before use.

Mobility Shift (EMSA) Assay

This assay was performed as described earlier (Mellitzer et al., 1996) using the IFP53-GUS oligonucleotide to detect complex formation with Stat5b.

Phosphotyrosine Blot

Lysates from growth factor–stimulated cells were subjected to Western blot analysis as described earlier (Hayman et al., 1993; Mellitzer et al., 1996). Samples were run on an 8% SDS-PAGE gel, transferred to nitrocellulose membranes (Dupont-NEN, Boston, MA), and then probed with an anti-phosphotyrosine antibody (4G10; Upstate Biotechnology Inc., Lake Placid, NY).

Results

hGM-R Functionally Expressed in ts21-E26–transformed Myeloid Progenitors

To demonstrate the functionality of the hGM-R in chicken cells, we introduced it into myelomonocytic progenitors transformed by the ts21 mutant of the E26 virus, expressing a thermolabile $p135^{gag\text{-}myb\text{-}ets}$ oncoprotein. These ts21-E26 myeloblasts proliferate at 37°C, if supplied with the avian cytokine cMGF (Leutz et al., 1988, 1989). After inactivation of the ts21-E26 oncoprotein at 42°C, the cells differentiate into macrophages, again requiring cMGF (Beug et al., 1984; Woldmann et al., 1997).

Stable expression of both chains of the hGM-R was achieved by constructing a retrovirus that coexpresses the α and the β_c chain (for a schematic drawing see Fig. 1 *A*). Both chains were inserted behind an internal CMV promoter, separated from each other by an IRES (Elroy-Stein et al., 1989). Chicken embryo fibroblasts producing this virus were cocultivated with a mixture of ts21-E26–transformed myeloblasts and freshly prepared chicken bone marrow cells. Methocel colonies growing in the presence of cMGF and G418 were expanded and analyzed for the expression of the hGM-R by Northern analysis. Fig. 1 *B* shows that these clones express mRNA for both the α and the β_c chain. Also, treatment with hGM-CSF caused only in hGM-R–expressing clones the phosphorylation of a specific 130-kD protein in phosphotyrosine blots, whereas cMGF treatment caused phosphorylation of a slightly smaller protein in both hGM-R expressing and control ts21-E26 cells (Fig. 1 *C*). Finally, recombinant hGM-CSF and cMGF evoked a similar, short-term proliferation response in hGM-R expressing ts21 cells (Fig. 2 *B*; see below). Control cells only responded to recombinant cMGF (Fig. 2 *A*). This clearly shows that the exogenous hGM-R is bioactive.

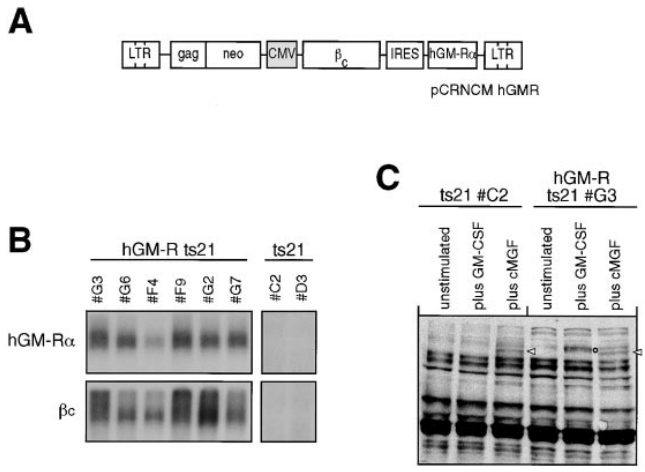

Figure 1. Expression of the hGM-R complex in ts21-E26 transformed myeloblasts. (*A*) Schematic drawing of the avian retroviral vector constructed to express both hGM-Rα and β_c. Besides avian AEV-ES4 LTRs, the virus contains a neomycin resistance gene fused to gag and an internal CMV promoter. The β_c and hGM-Rα cDNAs are separated by an IRES (Elroy-Stein et al., 1989). (*B*) Northern blot analysis of myeloblast clones expressing the hGM-R or infected with the control vector (*ts21#C2* and *#D3*). The blot was subsequently hybridized with both an α chain and a β_c-specific fragment. Blots from the GM-R–expressing and control clones were exposed for 5 and 20 d, respectively. Equal loading was verified by methylene blue staining of the blot before hybridization. (*C*) ts21-E26 transformed myeloblast clones harboring an empty vector (*#C2*) or the hGM-R expression vector (*#G3*) were withdrawn from factors overnight. They were then stimulated for 15 min with hGM-CSF, cMGF, or left untreated. Lysates were analyzed by phosphotyrosine blot as described in Materials and Methods. *Open circle*, the 130-kD β_c chain of the GM-R phosphorylated in response to hGM-CSF; *arrowheads*, the slightly smaller band phosphorylated in response to cMGF in both cell types. Note that some putative substrate proteins (e.g., ~75 kD) show enhanced phosphorylation upon treatment with hGM-CSF and cMGF.

Next, we analyzed whether hGM-CSF could replace cMGF in factor-dependent differentiation of ts21-E26 cells. Differentiation was analyzed by three parameters. First, daily cell counting shows that ts21-E26 myeloblasts differentiating at 42°C undergo a few cell divisions before becoming stationary after 2–3 d. Second, they become adherent and acquire a typical macrophage morphology in cytospin preparations stained with histological dyes. And thirdly, they express typical macrophage antigens (MC22/3, MC47/83) and lose myeloblast antigens (MC4M12/26; Beug et al., 1984). When ts21-E26 cells expressing the hGM-R were induced to differentiate at 42°C, hGM-CSF fully replaced cMGF. After initial transient proliferation (Fig. 2 *B*; somewhat prolonged in case of hGM-CSF treatment), hGM-R–expressing ts21-E26 cells differentiated into macrophage-like cells in both hGM-CSF and cMGF (Fig. 2 *C*, *right panels*). In contrast, control cells lacking the hGM-R showed the expected differentiation behavior in cMGF, but underwent apoptosis in hGM-CSF (Fig. 2 *C*, *left panels*). Similar to the cMGF-treated control cells, the hGM-R–expressing cells differentiating in the presence of hGM-CSF gained expression of macrophage surface markers detected by the monoclonal antibodies MC22-3 and MC47/83, while they lost the immature cell marker MC4M12/26 (data not shown; Beug et al., 1984).

Taken together, these results indicate that the hGM-R can be functionally expressed in avian myelomonocytic progenitors, inducing typical differentiation accompanied by transient proliferation.

Cooperation of the hGM-R with the EpoR during Erythroid Differentiation Requires hGM-CSF

In human or murine bone marrow, GM-CSF acts as a burst-promoting activity (BPA), supporting Epo-dependent erythroid differentiation (Metcalf, 1989). So far, we have been unable to find a similar activity for cMGF (Beug, H., and E.M. Deiner, unpublished observations). Sequence comparisons suggested cMGF to be homologous to IL-6 or G-CSF rather than to GM-CSF (Boulay and Paul, 1993). It was therefore of interest whether erythroid progenitors expressing the hGM-R would respond to hGM-CSF in a fashion analogous to early mammalian erythroid progenitors (BFU-E). To isolate normal erythroblast clones expressing the hGM-R, avian bone marrow cells growing in aSCF, TGFα, estradiol, and dexamethasone (Wessely et al., 1997*a*) were infected with the hGM-R retrovirus and seeded into methocel containing G418. Resistant colonies were picked and expanded. After confirming expression of both hGM-R chains by Northern and phosphotyrosine blot analysis (data not shown; see Fig. 1), hGM-R–expressing clones as well as empty vector control clones were induced to differentiate in insulin alone (control), hGM-CSF plus insulin, aEpo-containing anemic chicken serum (AS) plus insulin, and in both AS and hGM-CSF plus insulin. After 4 d, cells were subjected to cytocentrifugation followed by histochemical staining for hemoglobin. In addition, we quantitated hemoglobin (normalized to cell number) in these cell populations (see Materials and Methods).

As expected, control cells lacking the hGM-R differentiate in AS/insulin regardless of the presence or absence of hGM-CSF (Fig. 3, *A* and *B*, *left panels*). In the absence of AS (i.e., in insulin alone or hGM-CSF plus insulin) these cells undergo abortive differentiation (production of aberrant, partially mature cells with reduced hemoglobin levels, followed by apoptosis). This is evident both from hemoglobin accumulation (Fig. 3 *A*, *left*) and cytospin analysis (data not shown). The slight decrease in hemoglobin content caused by hGM-CSF in the presence of AS is most likely caused by endotoxin present in the hGM-CSF preparation used, since it could be avoided by using purer batches of hGM-CSF (data not shown).

Erythroblast clones expressing the hGM-R differentiated in the presence of insulin plus both hGM-CSF and AS, but showed abortive differentiation in hGM-CSF plus insulin alone (Fig. 3, *A* and *B*, *right panel*). This confirmed, that hGM-CSF cannot induce erythroid differentiation on its own, rather exhibiting a BPA-like activity in hGM-R–expressing chicken erythroblasts. Surprisingly however, the hGM-R–expressing cells no longer differentiated in the presence of AS plus insulin, showing only abortive differentiation as with insulin alone. They expressed only slightly elevated levels of hemoglobin (Fig. 3 *A*, *right panel*)

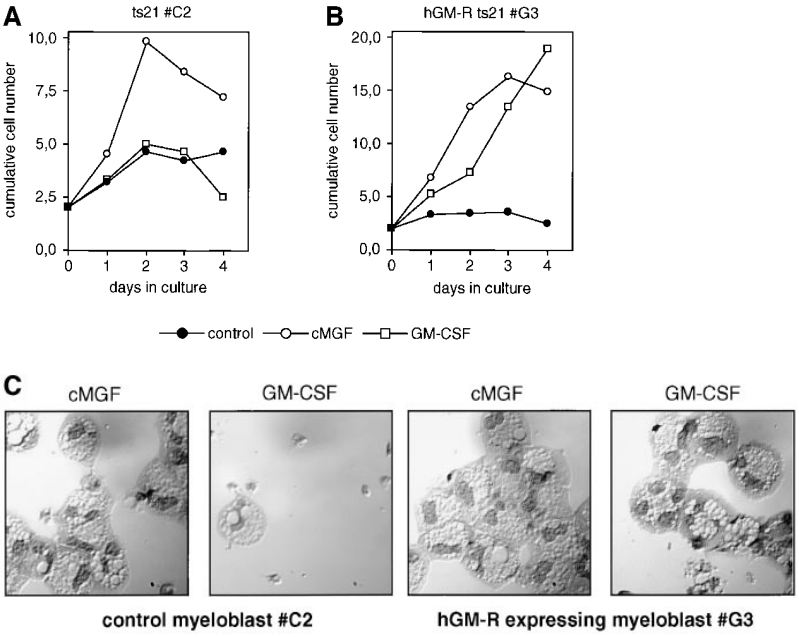

Figure 2. GM-CSF induces macrophage differentiation in ts21-E26 transformed myeloblasts expressing the hGM-R. (*A* and *B*) A ts21-E26 transformed myeloblast clone expressing the hGM-R (#*G3*; *B*) and a control clone (#*C2*; *A*) were seeded at 42°C into differentiation medium only (*closed circles*), or medium supplemented with either cMGF (*open circles*) or hGM-CSF (*open squares*). Cell numbers were determined daily and cumulative cell numbers calculated. (*C*) After 4 d at 42°C, aliquots of the cells grown in either cMGF or hGM-CSF were cytocentrifuged onto slides, stained with histological dyes, and then photographed using a CCD camera. The big, vacuolated cells with eccentric, sometimes fragmented nuclei are mature macrophages.

and appeared as either dead cells or partially mature cells expressing little or no hemoglobin in cytospins (Fig. 3 *B*, *right panels*).

These results show that the ligand-activated hGM-R supports AS-dependent erythroid differentiation and displays a BPA-like activity on erythroid progenitors. However, expression of the non-liganded receptor seems to interfere with erythroid differentiation, preventing a normal response to differentiation factors like aEpo.

The α Chain of the hGM-R Is Sufficient to Interfere with Erythroid Differentiation

The α chain of the GM-R is mainly responsible for ligand binding, whereas the β chain is necessary for interaction with JAK2 and other signal transduction intermediates (Quelle et al., 1994; Watanabe et al., 1996). Thus we sought to determine, whether the interference with AS-induced differentiation caused by the complete hGM-R receptor

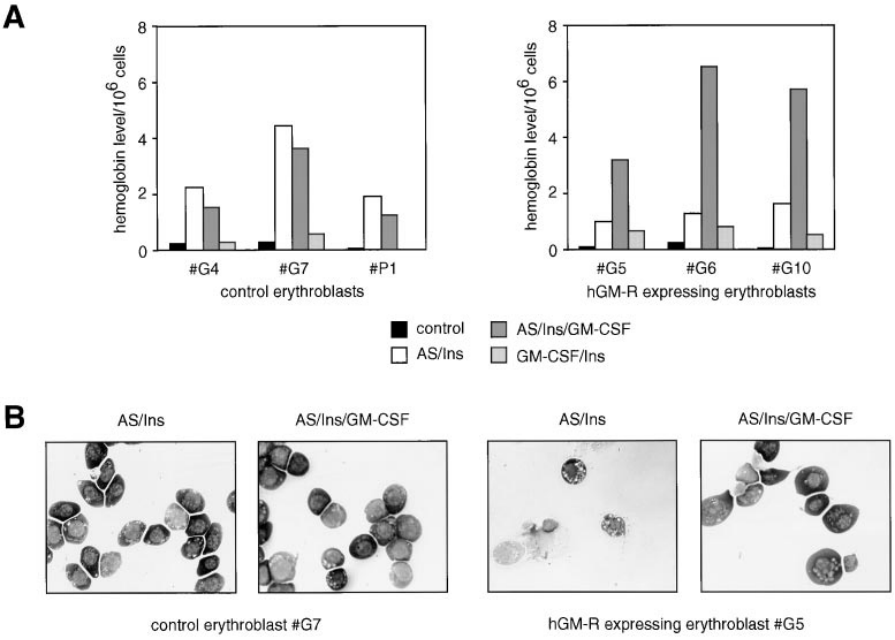

Figure 3. Differentiation of normal erythroid progenitors expressing exogenous hGM-R. (*A*) Three primary erythroblast clones expressing either the hGM-R or empty vector (*control erythroblasts*) were seeded into Epotest medium containing insulin only (*control*), anemic serum plus insulin (*AS/Ins*), anemic serum plus insulin and hGM-CSF (*AS/Ins/GM-CSF*), or hGM-CSF and insulin (*GM-CSF/Ins*). At day 3 of the experiment, hemoglobin was determined in aliquots of the cultures (Kowenz et al., 1987) and plotted after normalization to cell numbers. (*B*) 3 d after differentiation induction cytospins were prepared and stained with neutral benzidine plus histological dyes. Images were taken using a blue filter (480 nm) to reveal hemoglobin by dark staining (Beug et al., 1982). Shown are respective pictures from cultures grown in AS/Ins and AS/Ins/GM-CSF for one hGM-R expressing clone (#*G5*) as well as one control clone (#*G7*). Note that cells expressing the hGM-R fail to accumulate hemoglobin and undergo abortive differentiation in AS/Ins, i.e., in the absence of hGM-CSF.

heterodimer could also be induced by one of the two chains alone. For this, retroviral vectors were constructed which either expressed the α chain or the β_c chain of the hGM-R (see Fig. 4 *A* for schematic drawings).

Erythroblast clones infected with these constructs were generated and expression of the hGM-R proteins verified (data not shown). Well-expressing clones were then induced to differentiate in the presence of AS plus insulin or insulin alone and analyzed for erythroid differentiation parameters as above. Erythroblasts expressing the β_c chain behaved exactly as clones containing empty vector. In the presence of AS/insulin they expressed the same, high levels of hemoglobin (Fig. 4 *B*) and differentiated erythrocytes were visible in the stained cytospins (data not shown). In insulin alone, both cell types showed the expected abortive differentiation (see above; Fig. 3 *B*), characterized by much lower hemoglobin levels.

In contrast, the α chain of the hGM-R heavily interfered with erythroid differentiation. In both insulin alone and AS plus insulin, these erythroblasts accumulated only heavily reduced hemoglobin levels (Fig. 4 *B*) and failed to differentiate into mature erythrocytes, giving rise to malformed, partially mature cells instead (abortive differentiation; data not shown).

To test whether this suppression of AS-induced differentiation was specific for the hGM-R α chain, we also generated clones expressing the α chain of a related cytokine receptor, the hIL-3R (see Fig. 4 *A*). Surprisingly, the hIL-3R α chain did not affect the responsiveness of erythroblasts to AS/insulin. Rather, the hIL-3R α chain expressing clones differentiated normally into mature erythrocytes expressing high hemoglobin levels (Fig. 4 *B*; data not shown).

These data clearly demonstrate that the α chain of the hGM-R specifically interferes with erythroid differentiation. Neither β_c nor the related hIL-3R α chain could substitute for the hGM-R α chain in causing this effect. Thus, at least part of the lineage-specific effects of the GM-R are dependent on the α chain.

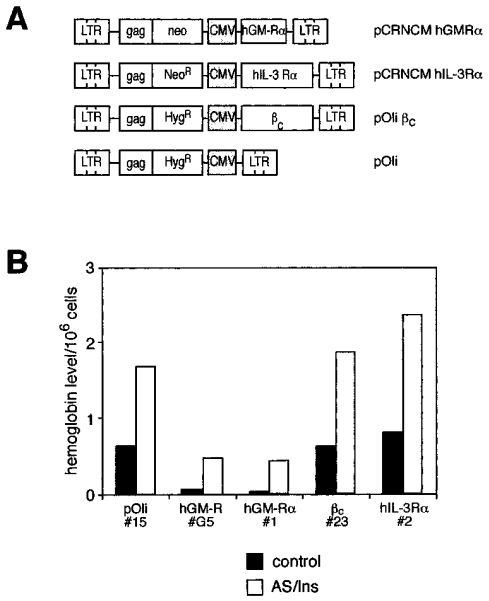

Figure 4. The hGM-R α chain is sufficient to interfere with erythroid differentiation induced by anemic serum. (*A*) schematic drawings of the retroviral vectors used to express the hGM-Rα, β_c, and the hIL3-Rα. *HygR*, hygromycine resistance gene; *hIL-3Rα*, cDNA of the human IL-3 receptor α chain; and *pOli*, empty control vector. (*B*) Primary erythroblast clones expressing the hGM-R (#*G5*), the hGM-R α chain (#*1*), the β_c subunit (#*23*), the hIl3-R α chain (#*2*), or the empty vector (#*15*) were seeded into Epotest medium containing either insulin (*control*) or anemic serum plus insulin (*AS/Ins*). 3 d after the onset of the experiment the hemoglobin content normalized to cell number was determined. Note that only cells expressing either the hGM-R complex (#*G5*) or the hGM-R α chain (#*1*) were impaired in hemoglobin accumulation resulting from abortive differentiation in the presence of anemic serum and insulin.

Exogenous Expression of the Murine EpoR Prevents Interference with AS-induced Differentiation by the Non-liganded hGM-R

The EpoR and the GM-R share several structural and functional characteristics, suggesting that overexpression of one receptor may interfere with signaling of the other. Unfortunately this question could not be addressed directly, since neither Epo nor the EpoR have been cloned in the chicken. However, aEpo shares most biochemical properties with mammalian Epo (Kowenz et al., 1987) and is neutralized by a polyclonal antibody to human Epo (Wessely, O., E.M. Deiner, and H. Beug, unpublished observations), even though aEpo fails to bind and activate the mammalian EpoR and vice versa (Kowenz et al., 1987; Beug, H., unpublished results). We therefore decided to ectopically express the murine EpoR in hGM-R–expressing erythroblasts. If the exogenously expressed hGM-R would interfere with the activity of the endogenous avian EpoR (presumably expressed at much lower levels) by quenching signals emanating from it, exogenous expression of both the mEpoR and the hGM-R at similar levels should neutralize this effect.

To allow simultaneous expression of three proteins (i.e., hGM-Rα, β_c, and mEpoR) in the same cell, we used an approach described previously (Tran Quang et al., 1997). Fibroblasts were co-transfected with the hGM-R retrovirus plus a replication competent retroviral vector expressing the mEpoR (RCAS-mEpoR; Fig. 5 *A*) and used to infect avian bone marrow cells. G418-resistant erythroblast clones were isolated from methocel, expanded, and then tested for expression of the three proteins (data not shown). Well expressing clones were analyzed for erythroid differentiation as above. 4 d after differentiation induction, aliquots of the cultures were processed for hemoglobin determination (Fig. 5 *B*) and histochemical staining for hemoglobin (Fig. 5 *C*). Similar to erythroblasts expressing the hGM-R only, mEpoR/hGM-R expressing cells terminally differentiated in the presence of hGM-CSF plus AS. In AS/insulin alone, the cells expressed only low levels of hemoglobin (Fig. 5 *B*) and developed into abortively differentiated, apoptotic cells (Fig. 5 *C*). Interestingly, and in clear contrast to AS, activation of the exogenous mEpoR by hEpo in the mEpoR/hGM-R–expressing cells induced their terminal differentiation into erythrocytes expressing high hemoglobin levels. Addition of hGM-CSF plus hEpo/insulin only slightly increased hemoglobin levels beyond those induced by the single ligands.

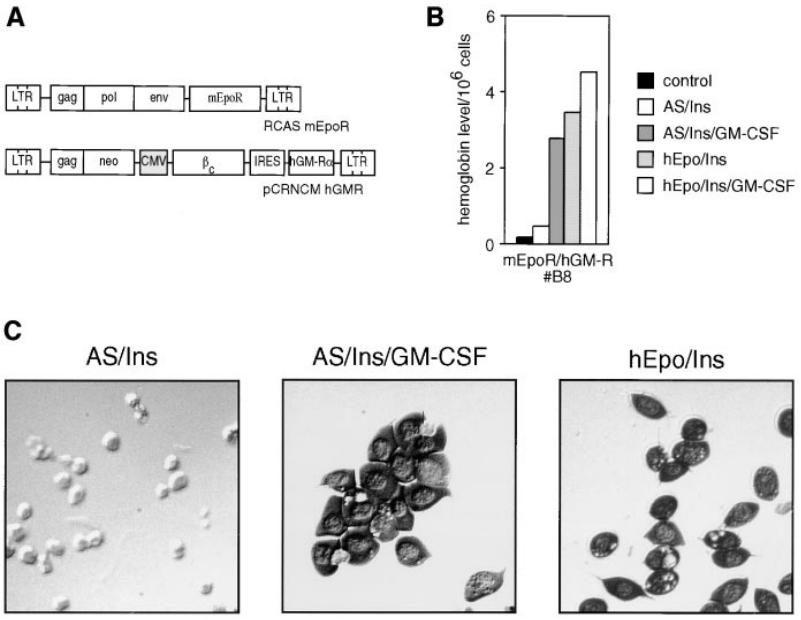

Figure 5. Ectopic expression of the mEpoR can restore Epo responsiveness in hGM-R–expressing erythroblasts. (*A*) Schematic drawings of the two retrovirus vectors used to co-express the hGM-R and the mEpoR (see text). (*B* and *C*) One representative erythroblast clone simultaneously expressing the hGM-R and the mEpoR (#*B8*) was seeded into Epotest medium containing insulin only (*control*), anemic serum plus insulin (*AS/Ins*), anemic serum plus insulin and hGM-CSF (*AS/Ins/GM-CSF*), hEpo plus insulin (*Epo/Ins*) or hEpo plus insulin and hGM-CSF (*hEpo/Ins/GM-CSF*). At day 3 of the experiment, hemoglobin accumulation (normalized to cell number) was determined (*B*). Simultaneously, aliquots of the culture were cytocentrifuged on slides, stained with neutral benzidine plus histological dyes, and then photographed under blue light (*C*). Dark color signifies hemoglobin expression.

These results show that the interference of the exogenous hGM-R with AS-induced differentiation could be overcome, if a mEpoR was expressed in an exogenous fashion as well, and therefore suggest that the GM-R interacts with the EpoR signaling pathway.

The Ectopic hGM-R Interferes with Signaling of the Endogenous aEpoR

The EpoR and the GM-R share several signal transduction pathways, for instance activation of the Ras/MapK pathway and the Stat5 pathway (Ihle et al., 1994; Mui et al., 1995; Wakao et al., 1995). Recently, activation of Stat5b by AS has also been reported for avian erythroid cells (Mellitzer et al., 1996). Therefore we used this signaling pathway to study the effects of exogenous hGM-R on EpoR signaling. However, primary erythroblasts do not tolerate complete withdrawal of growth factors before re-activation with ligand (Mellitzer et al., 1996, Wessely et al., 1997*b*). We therefore expressed the hGM-R and—as a control—empty vector in the HD3 erythroblast cell line HD3-EpoR-E22. These cells express low levels of a bioactive mEpoR (<1,000 receptors per cell; Mellitzer, G., unpublished observations) and undergo terminal differentiation in response to both avian and mammalian Epo, if exogenous (v-ErbB) and endogenous receptor tyrosine kinases (c-ErbB, c-Kit) causing sustained proliferation of these cells are inactivated by kinase inhibitors (PD 153.035) and ligand withdrawal (Mellitzer et al., 1996). Furthermore, these cells show the same interference of the hGM-R with aEpoR signaling as primary cells (data not shown). They are thus much more suitable for biochemical analysis of signal transduction pathways (Mellitzer et al., 1996).

HD3-EpoR-E22 cells were withdrawn from growth factors overnight and re-stimulated with hGM-CSF or hEpo. Extracts normalized to protein content were then analyzed by phosphotyrosine blot and EMSA (see Materials and Methods; Mellitzer et al., 1996). As a positive control, Stat5b activation was induced in the same cells by the c-ErbB ligand TGFα. Control HD3 cells lacking exogenous hGM-R showed specific tyrosine phosphorylation of the 80-kD EpoR after stimulation with hEpo (and of the 170-kD c-ErbB protein after TGFα stimulation), whereas no protein was specifically phosphorylated after stimulation with hGM-CSF (Fig. 6, *top panel*). Consequently, specific Stat5b DNA binding in EMSA assays was induced by hEpo and TGFα but not by hGM-CSF (Fig. 6, *bottom panel*). In contrast, the hGM-R–expressing HD3-EpoR cells showed the expected, hGM-CSF-induced phosphorylation of the 130-kD β_c protein, but surprisingly hEpo now failed both to induce EpoR autophosphorylation and to give rise to phosphorylated substrates. This impaired signal transduction could be confirmed with respect to activation of Stat5b; hGM-CSF (and TGFα) could induce Stat5b/DNA complex formation, but hEpo could not (Fig. 6).

Taken together, these results show that exogenous overexpression of the hGM-R actively interferes with the signaling from the EpoR.

The hGM-R Cannot Replace the EpoR in Its Cooperation with c-Kit and Steroid Hormone Receptors to Induce Sustained Erythroblast Self-Renewal

The ability of the hGM-R α chain to interfere with differentiation induction by the endogenous aEpoR prompted us to study whether the hGM-R would exhibit similar properties with respect to EpoR-dependent proliferation control. In mouse bone marrow cultures, erythroid colonies induced by Epo plus SCF are more numerous and bigger than those induced by Epo/Insulin, suggesting that a cooperation of c-Kit and the EpoR causes enhanced erythroid progenitor proliferation (Broxmeyer et al., 1991; McNiece et al., 1991; Muta et al., 1995). Furthermore, the EpoR is phosphorylated by ligand-activated c-Kit in certain cell lines (Wu et al., 1995*a*). These findings were recently extended by our finding that the EpoR cooperates with c-Kit and steroid hormone receptors in primary erythroid progenitors derived from chickens, humans and

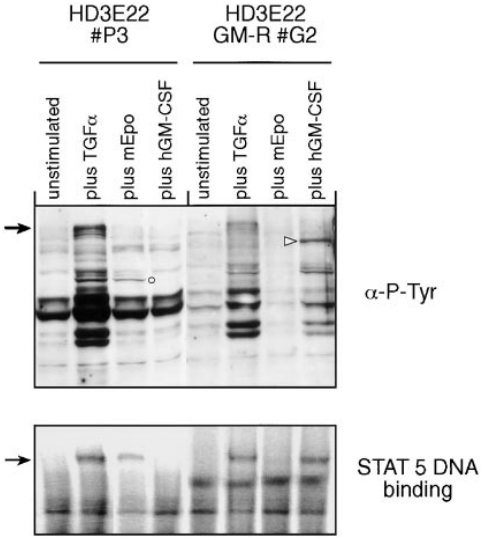

Figure 6. Non-liganded GM-R inhibits signal transduction from the EpoR. Cell extracts prepared from HD3-EpoR-E22 cells expressing the hGM-R (*#G2*) or, as a control, the empty vector (*#P3*) were withdrawn from factors. They were then stimulated for 15 min with TGFα, mEpo, hGM-CSF or left untreated. Lysates normalized to protein content were analyzed by phosphotyrosine blot (*top panel*) as described in Materials and Methods. Equal loading was verified by staining of the blot with Ponceau S before antibody incubation (not shown). *Open circle*, phosphorylated, 80-kD EpoR; *white arrowhead*, phosphorylated 130-kD β_c; *black arrow*, phosphorylated, 170-kD c-ErbB protein. In addition, lysates from the same cells were analyzed by EMSA (*bottom panel*), using a ^{32}P-labeled oligonucleotide specific for Stat5b (*IFP53-GAS*). Arrow indicates mobility shifted Stat5b/DNA complex. Identity of the shifted band with Stat5b was verified by supershift using Stat5b antibody (not shown; see Mellitzer et al., 1996).

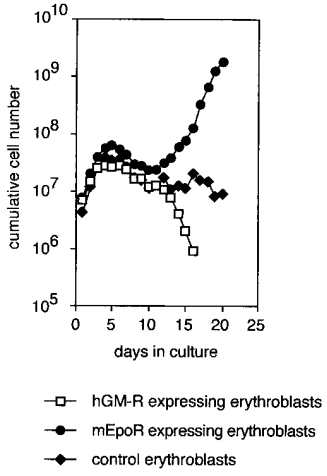

Figure 7. The hGM-R cannot cooperate with c-Kit to promote erythroblast outgrowth. Erythroid progenitors expressing the hGM-R (*open squares*), the mEpoR (*closed circles*) or the empty vector (*closed diamonds*) were grown in CFU-E medium containing hEpo, hGM-CSF, aSCF, IGF-1, E2, Dex, and the c-ErbB specific inhibitor PD153035 (Fry et al., 1994) to rule out proliferation stimulation by endogenously upregulated c-ErbB. Cells were counted daily and cumulative cell numbers calculated.

mice, causing their sustained proliferation (Wessely, O., E.M. Deiner, and H. Beug, unpublished observations; Beug, H., unpublished observations; Wessely, O., and H. Beug, unpublished observations). In the chicken system, this was shown by the fact that expression of the mEpoR (cooperating with c-Kit and the glucocorticoid receptor) allowed sustained proliferation of erythroid progenitors (see Fig. 7; Wessely, O., E.M. Deiner, and H. Beug, unpublished observations).

This system allowed us to test, if the activated hGM-R could replace the mEpoR in its contribution to self-renewal induction by c-Kit and steroid hormone receptors. Erythroid progenitors were infected as described above with retroviruses expressing the mEpoR or the hGM-R complex. Empty vector served as a control. Cells were cultivated in hEpo and hGM-CSF plus the other factors required to allow sustained proliferation (see Materials and Methods) and cell proliferation assayed by daily counting (Fig. 7). As expected, the cells expressing the mEpoR were capable of sustained proliferation, while control cells lacking the mEpoR ceased to proliferate around day 6–10. Interestingly, the cells expressing the hGM-R were completely unable to self renew. They disintegrated even faster than the control cells.

These results clearly show, that the cooperation between EpoR and c-Kit leading to erythroblast self-renewal is dependent on specific features of the EpoR and cannot be substituted by a related cytokine receptor mainly active in another hematopoietic lineage.

Discussion

In this paper we have analyzed the function of the hGM-R in a heterologous cell system. These are primary erythroid and myeloid progenitors of the chicken that can be induced at will to either proliferate or differentiate. The need for such experiments was obvious, since GM-CSF–dependent immortalized cell lines like TF-1 or FDCP-mix are extremely useful to analyze the biochemistry of GM-R action and contribution to proliferation control, but are less suitable to clarify how the GM-R controls the balance between proliferation and differentiation or how it causes lineage-specific effects. These effects of GM-CSF, however, are important in vivo. For instance, exogenously expressed GM-CSF in transgenic mice led to elevated numbers of macrophages, but did not cause leukemia (Elliott et al., 1991). This suggested that one of the major tasks of the GM-R is to control the balance between myeloid progenitor proliferation and differentiation (Metcalf, 1989).

The Ligand-activated hGM-R Promotes Myeloid Differentiation, but Does Not Contribute to Self-Renewal Induction

Expression of the hGM-R in ts21-E26–transformed myeloid progenitors was used to show that the GM-R can have similar functions in cultured primary cells as in vivo. These cells express a thermolabile p135$^{gag\text{-}myb\text{-}ets}$ oncoprotein and proliferate at 37°C, but are induced to differentiation into macrophages when shifted to 42°C (Beug et al., 1984). For both proliferation and differentiation the presence of the avian cytokine cMGF is absolutely required (Woldmann et al., 1997). Interestingly, in hGM-R–expressing ts21-E26 myeloblasts hGM-CSF induced the typical differentiation response of these progenitors i.e. terminally differentiating into macrophages accompanied by a limited number of cell divisions. However, hGM-CSF differed from cMGF in that it failed to induce long-term proliferation at 37°C, i.e., in the presence of a fully active p135$^{gag\text{-}myb\text{-}ets}$ oncoprotein (not shown). This is in clear

contrast to GM-CSF–dependent myeloid cell lines, in which GM-CSF is required for long-term proliferation, but has no clear effects on differentiation. Thus, the action of GM-CSF in suspension cultures of hGM-R–expressing primary avian myeloid progenitors closely corresponds to the established cytokine responses of mammalian bone marrow in colony assays, i.e., induction of differentiation in immature cells, stimulation of transient proliferation during differentiation and regulation of cell function in mature cells.

The fact that the ligand-activated hGM-R could not induce long-term proliferation at 37°C clearly sets GM-CSF apart from cMGF. This is important, since the relationship of cMGF to mammalian cytokines is still unclear. On the sequence and structural level, cMGF is most homologous to mammalian IL-6 with slightly less homology to G-CSF (Sterneck et al., 1992; Boulay and Paul, 1993). On the other hand, the in vivo activity of cMGF (i.e., a marked increase of functionally activated monocytes in the peripheral blood of chickens constitutively expressing high levels of cMGF) (York et al., 1996) is more easily comparable to the biological activities of M-CSF or GM-CSF. Our result that the ectopically expressed, ligand-activated GM-R was unable to substitute for all activities of cMGF now rules out the possibility that cMGF is the avian version of GM-CSF.

Function of the hGM-R in Erythroid Cells

The biological activity of GM-CSF is not restricted to the myeloid compartment. GM-CSF also supports erythroid differentiation (Metcalf, 1989; Nishijima et al., 1995) but in contrast to Epo is unable to induce or maintain it on its own. Such a BPA is also typical for other cytokines, e.g., IL-3. In the case of GM-CSF, it has been explained by the loss of ligand responsiveness resulting from downregulation of the GM-R during differentiation. However, extending these findings, we demonstrate here that the activated hGM-R is unable to promote terminal erythroid differentiation even when it is exogenously expressed in avian erythroblasts and thus cannot be downregulated. Rather, GM-CSF must cooperate with an activity present in anemic chicken serum. This activity is most likely Epo since it behaves like mammalian Epo during biochemical purification (Kowenz et al., 1987) and is neutralized by a polyclonal antibody to mammalian Epo (Wessely, O., unpublished observations). However, formal proof of this notion has to await molecular cloning of avian Epo and/or its receptor.

The cooperative action of the EpoR and the GM-R seems to be easily explainable in mechanistic terms, since both receptors mediate signaling via the intracellular tyrosine kinase JAK2 (Witthuhn et al., 1993; Quelle et al., 1994). They also activate a comparable subset of signaling molecules like STAT5, Ras, or PI-3-kinase (Mui et al., 1995; Pallard et al., 1995) and both receptors activate marker genes characteristic for mature erythrocytes like carbonic anhydrase II (CAII; Wessely, O., unpublished observations). But why does GM-CSF only act as a BPA even when its receptor is ectopically expressed? A possible answer to this question could be that the EpoR has multiple activities. In addition to inducing terminal erythroid differentiation, the EpoR physically associates with the tyrosine kinase receptor c-Kit and even to induce tyrosine phosphorylation of the latter receptor tyrosine kinase (Wu et al., 1995). As shown elsewhere (Beug, H., unpublished observation; Wessely, O., E.M. Deiner, and H. Beug, unpublished observations; Wessely, O., and H. Beug, unpublished observations) the combination of c-Kit and the EpoR also seem to be key players to induce long-term proliferation in human, murine, and avian erythroblasts. However when the hGM-R was tested in conjunction with ligand-activated c-Kit, it failed to induce long-term outgrowth. Thus the GM-R cannot substitute for the EpoR in its cooperation with c-Kit.

Our finding of a partial functional overlap between the EpoR and the GM-R agree with data obtained from cytokine/cytokine receptor knockout mice (Dent et al., 1990; Dranoff and Mulligan, 1994; Robb et al., 1995; Lin et al., 1996), showing that the various cytokine receptors exhibit a partial functional redundancy. Ablation of these genes usually does not lead to complete ablation of a given lineage, but shows much more subtle phenotypes. Only in case of specialized, cell type–specific receptors such as the EpoR or the IL-5 receptor (Wu et al., 1995*b*; Lin et al., 1996; Yoshida et al., 1996), the more generally expressed cytokine receptors such as the GM-R are unable to functionally substitute and thus hematopoiesis is more severely affected.

The α Chain of the hGM-R May Confer Myeloid Cell Specificity by Interfering with EpoR Signal Transduction

Many functions of the GM-R are similar to those of the IL-3 receptor. Both receptors can stimulate proliferation of immature myeloid and erythroid progenitors, support differentiation along these lineages, and act via similar signal transduction pathways (McNiece et al., 1991; Quelle et al., 1992; Sonoda et al., 1994; Mui et al., 1995). This is not surprising, since both receptors have to interact with β_c and Jak2 to initiate signal transduction. In this paper, however, we identified a biological activity specific for the GM-R and not shared by related cytokine receptors. In the absence of GM-CSF, the hGM-R was incompatible with erythroid differentiation. hGM-R–expressing erythroblasts disintegrated rather than differentiated in AS/Ins, whereas hIL-3R–expressing erythroblasts differentiate normally under the same conditions (Steinlein, P., E.M. Deiner, and H. Beug, unpublished observations). Furthermore, isolated expression of the α or β chains of the GM-R receptor complex showed that the α chain was sufficient to interfere with erythroid differentiation. In contrast, overexpression of neither β_c nor the IL-3R α chain exerted this effect. These biological observations were matched by biochemical data. When the hGM-R was expressed in the erythroblast cell line HD3, hGM-CSF caused both tyrosine phosphorylation and DNA binding of Stat5b. However, the non-liganded hGM-R inhibited both EpoR autophosphorylation and induction of Stat5b DNA binding in response to hEpo. In HD3-EpoR control cells lacking the exogenous hGM-R, hEpo induced both effects as expected. These results support the idea of a crucial role of the hGM-R α chain in lineage-specific signal transduction. Mutation analysis of the cytoplasmic tail of the GM-R α

chain recently revealed that this molecule is required together with the β_c chain for the activation of JAK2 (Matsuguchi et al., 1997). Therefore, interference by GM-R α with signal transduction intermediates such as Jak-2 or Stat5b in a fashion distinct from the β_c chain is one possible explanation for this cross talk. Finally, experiments by others show that overexpression of the EpoR in the murine cell line FDCP interfered specifically with signaling from the GM-R (Quelle and Wojchowski, 1991). This corresponded to the finding that a protein of 100 kD could no longer be tyrosine phosphorylated in response to hGM-CSF, when the EpoR was exogenously expressed as well (Quelle et al., 1992).

These results raise the interesting possibility that nonliganded cytokine receptors may contribute to lineage commitment by specific effects mediated via their lineage-specific α chains. Thus, cytokine receptors may use different mechanisms to regulate the balance between proliferation and apoptosis on the one hand and differentiation versus proliferation/apoptosis on the other. Whereas the former mechanism (the one easily studied in immortalized cell lines) is always ligand dependent, the latter mechanisms may be less ligand dependent, being mainly driven by tissue-specific expression of specific types of receptor α chains.

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