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A Human GM-CSF Receptor Expressed in Transgenic Mice Stimulates Proliferation and Differentiation of Hemopoietic Progenitors to All Lineages in Response to Human GM-CSF

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) mainly stimulates proliferation and maturation of myeloid progenitor cells. Although the signal transduction pathways triggered by GM-CSF receptor (GMR) have been extensively characterized, the roles of GMR signals in differentiation have remained to be elucidated. To examine the relationship between receptor expression and differentiation of hemopoietic cells, we used transgenic mice (Tg-mice) that constitutively express human (h) GMR at almost all stages of hemopoietic cell development. Proliferation and differentiation of hemopoietic progenitors in bone marrow cells from these Tg-mice were analyzed by methylcellulose colony formation assay. High affinity GMR interacts with GM-CSF in a species-specific manner, therefore one can analyze the effects of hGMR signals on differentiation of mouse hemopoietic progenitors using hGM-CSF. Although mouse (m) GM-CSF yielded only GM colonies, hGM-CSF supported various types of colonies including GM, eosinophil, mast cell, erythrocyte, megakaryocyte, blast cell, and mixed hemopoietic colonies. Thus, the effects of hGM-CSF on colony formation more closely resembled mIL-3 than those of mGM-CSF. In addition, hGM-CSF generated a much larger number of blast cell colonies and mixed cell colonies than did mIL-3. hGM-CSF also generated erythrocyte colonies in the absence of erythropoietin. Therefore, GM-CSF apparently has the capacity to promote growth of cells of almost all hemopoietic cell lineages, if functional hGMR is present.

INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates proliferation and maturation of myeloid progenitor cells, giving rise to granulocytes and monocytes, and enhances differentiated functions of these mature cells. Although the effects of GM-CSF are similar to those of IL-3, GM-CSF acts mainly on mature myeloid progenitor cells (Arai *et al.*, 1990; Gasson, 1991). These biological activities are mediated by cell surface receptors. The human GM-CSF receptor (hGMR) is composed of two distinct subunits designated as α and β (Miyajima *et al.*, 1993). GMR belongs to the cytokine receptor superfamily characterized by four well-conserved cysteine residues, the WSXWS motif in the extracellular domain, and the absence of

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distinct motifs in the cytoplasmic domain that may be linked to molecules capable of transducing signals. The α subunit is specific to GMR and binds to GM-CSF with low-affinity, whereas the β subunit in itself, which is shared by IL-3R and IL-5R, shows no detectable affinity with any ligand (Gearing *et al.*, 1989; Hayashida *et al.*, 1990). Both the α and β subunits are essential and sufficient to reconstitute a high-affinity receptor. In the mouse system, at least two types of β subunit (βIL-3 and βc) exist; βIL-3, also known as AIC2A, is the β subunit of mouse IL-3R, and βc, also known as AIC2B, is the common β subunit of mouse IL-3R, GMR, and IL-5R (Gorman *et al.*, 1990; Itoh *et al.*, 1990).

GM-CSF acts in a species-specific manner between mice and humans. The high-affinity hGMR, but neither α nor β subunit alone, reconstituted in mouse pro-B cell line BA/F3 can transduce growth-promoting signals in response to hGM-CSF (Kitamura *et al.*, 1991), thereby indicating that no species-specific molecule other than hGMR α and β subunits is required to mediate hGM-CSF function in mouse hemopoietic cells. Furthermore, NIH3T3 fibroblasts expressing high-affinity hGMR can also proliferate in response to hGM-CSF, thus, the molecule(s) specific to hemopoietic cells may not be essential to transduce hGM-CSF signals (Watanabe *et al.*, 1993).

All blood cells including lymphoid cells are probably derived from common progenitor cells termed pluripotent hemopoietic stem cells (Suda *et al.*, 1983b; Smith *et al.*, 1991). However, mechanisms that control self-renewal and differentiation of stem cells into cells of various lineages have not been clearly defined (Ogawa, 1993). In general, response of the cell to biological stimuli is regulated at two phases; i.e., the inducer phase, which controls the supply of external signals such as cytokines, growth factors, or cell adhesion molecules, and the effector phase, which is determined by the potential of the cell to respond to environmental signals (Arai *et al.*, 1990). Conceptually, the latter process is regulated by at least two elements; expression of receptors for external ligands and/or expression of intra-cellular signal transducing molecules linked to cell surface receptors. It has been suggested that expression of the cytokine receptor is regulated in a stage-specific manner (Broudy *et al.*, 1991). For example, GMR appears at a particular stage of hemopoietic cell development and is down-regulated at a later stage (Walker and Burgess, 1985; Walker *et al.*, 1985; Cannistra *et al.*, 1990). The effects of constitutive or ectopic supply of cytokine signals have been examined by generating transgenic (Tg) mice expressing cytokine genes (Lang *et al.*, 1987). In the present work, we directed attention to the effector phase by generating Tg-mice expressing high affinity hGMR at all stages of hemopoietic cell development. We asked whether the reconstituted hGMR in primary cells derived from Tg-mice is functional, as was noted in established cell lines. Our observations indicated that this is indeed the case and that hGM-CSF supports in vitro colony formation of bone marrow cells derived from Tg-mice. Based on these findings, we addressed the question of the role of GM-CSF in growth and differentiation of hemopoietic cells.

We attempted to determine whether GM-CSF plays an instructive role in generating cells of GM lineage through signals unique to GMR (instruction or deterministic model) or whether it simply permits the growth of cells expressing functional GMR (selection model). We manipulated the expression of GMR in early progenitors by constructing hGMR Tg-mice, and obtained evidence that hemopoietic progenitor cells expressing hGMR can develop into cells of a wide variety of lineages, including erythrocytes, in response to hGM-CSF. GM-CSF is apparently not acting in an instructive manner and simply permits the growth of cells, if functional GMR is present.

MATERIALS AND METHODS

Plasmid Construction and DNA Preparation

The 1.3-kb and 3.2-kb *Xho*I fragments of hGMRα and β cDNAs, respectively, were inserted into the *Sal*I site in the pLG1 expression vector (Suematsu *et al.*, 1992), which has the 1.2 kb of mouse major histocompatibility complex (MHC) L-locus gene (H2-Ld) promoter and the 0.8 kb of rabbit β globin intron upstream of the cloning sites. The 0.5 kb β globin polyadenylation sequence was placed downstream of these sites (Figure 1). The resulting pLd-GMRα and pLd-KH97 plasmids were 7.1 kb and 9.0 kb, respectively. pLd-GMRα and pLd-KH97 were digested with *Sph*I and *Xho*I and fragments were separated from the vector fragment by low-melting agarose gel (Sea Plaque, FMC Bio Products, ME). DNA fragments were purified using a QIAGEN tip 5 column (QIAGEN GmbH, Hilden, Germany) and were dissolved in 10 mM Tris-HCl (pH 7.5), 0.2 mM EDTA.

Generation and Maintenance of Tg-Mice

Tg-mice were produced using the C3H/HeN strain, by standard oocyte injection method (Hogan *et al.*, 1986). Six-week–old C3H/HeN mice were purchased from Nippon Clea (Tokyo, Japan) and grown in an environmentally controlled clean room with 12 h light-dark cycles, in an authorized animal facility at the laboratory animal research center at IMSUT. The mice were maintained under specific pathogen-free (SPF) conditions in micro-isolator cages; all the equipment and supplies, including cage, water bottles, wooden chips for bedding, and food pellets were sterilized.

DNA Blot Analysis

Mouse tail-tip DNA was prepared by the removal of 1 cm of tail and incubation in 0.7 ml of tail-tip buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.02 mg/ml proteinase K) at 55°C for 15 h. The lysate was extracted with an equal vol of phenol and chloroform. The DNA in aqueous phase was precipitated by 0.1 vol of 3 M sodium acetate and 2 vol of ethanol. Twenty micrograms of DNA was digested with *Bam*HI/*Eco*RI for pLd-GMRα and with *Bam*HI/*Bgl*II for pLd-KH97, and 10 μg of digested DNA was separated by agarose gel electrophoresis and then transferred to Hybond N+ nylon membrane (Amersham, Arlington Heights, IL) by capillary blotting. These membranes were hybrid-

ized with hGMR α or β cDNA full-length probe, as described by Sambrook *et al.* (1989).

RT-PCR Analysis

The expression of transgene RNA transcripts in different tissues was determined by reverse transcriptase polymerase chain reaction (RT-PCR), using oligonucleotides of hGMRα (sense, nucleotide positions 1042 to 1061; antisense, 1271 to 1251), β (sense, 2360 to 2379; antisense, 2655 to 2636), and β-actin as positive control (sense, 628 to 651; and antisense, 866 to 846) as primers. RNA was prepared from peripheral blood, bone marrow, brain, heart, kidney, liver, lung, muscle, skin, spleen, testis, and thymus of adult (8-wk old) mice, as described (Chomczynski and Sacchi, 1987). Total RNA (1 μg) was mixed with the following reagents in a final vol of 20 μl: 1× RT buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4], 2.5 mM $MgCl_2$, and 0.1 mg/ml bovine serum albumin [BSA]), 1 mM of each dNTP, 1 U/ml of RNase inhibitor, 100 pmol oligo-dT primer, and 30 U AMV reverse transcriptase. After incubation for 60 min at 42°C, 1 μl was removed for RT-PCR analysis, under standard conditions (30 cycles: 94°C for 40 s, 55°C for 50 s, and 72°C for 50 s (Innis *et al.*, 1990). Expression of hGMR mRNA of an individual colony was also determined, as described above.

Flow Cytometry

Bone marrow cells were harvested from 6- to 10-wk–old mice. Cells were washed twice with staining solution (phosphate-buffered saline containing 10% fetal calf serum [FCS] and 0.02% sodium azide). About 1.5×10^5 cells were incubated at 4°C for 1 h in 50 μl of staining solution containing 150 ng of mouse anti-hGMR α antibody (clone GMA1) or 150 ng of rat anti-β antibody (clone CRS-1) (Watanabe *et al.*, 1992). After washing twice with staining solution, samples were stained at 4°C for 30 min with 250 ng of fluorescein isothianate-conjugated goat anti-mouse IgG fragment or rabbit anti-rat IgG fragment in 50 μl of staining solution. Cells were washed twice and suspended in 100 μl of staining solution. Flow cytometry was performed with a Becton Dickinson FACScan (Mountain View, CA) using FACScan software, LYSIS II.

Hemopoietic Growth Factors

hGM-CSF produced in *Escherichia coli* was provided by Dr. R. Kastelein, DNAX Research Institute. mIL-3 produced by silkworm (*Bombyx mori*) was purified as described previously (Miyajima *et al.*, 1987). mGM-CSF produced in *E. coli* was a gift from Sumitomo Pharmaceutical (Hyogo, Japan). Mouse stem cell factor (mSCF) produced in *E. coli* was supplied by Amgen (Thousand Oaks, CA). rhEPO produced by Chinese hamster ovary cells was kindly provided by Kirin Brewery (Tokyo Japan).

Cell Preparation

Six- to ten-week–old Tg-mice and their normal littermates were used in this study. Bone marrow cells were prepared in α-MEM (Flow Laboratories, Rockville, MD) by repeated pipetting and were passed through a 50-μm Nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). 5-Fluoro-uracil (5-FU; Sigma Chemical, St. Louis, MO) was given through the tail vein of the mice in a dose of 150 mg/kg. Bone marrow cells were harvested on the second day after 5-FU injection.

Clonal Cell Culture

Methylcellulose culture was done using a modification of the technique described previously (Nakahata and Ogawa, 1982a). Usually, 1 ml of culture mixture containing 10^4 bone marrow cells from normal mice, 5×10^4 bone marrow cells from 5-FU–treated mice, αMEM, 1.2% (1500 centipoieses) methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% FCS (HyClone Laboratories, Logan, UT), 1% deionized fraction V BSA (Sigma Chemical), 10^{-4} M mercaptoethanol (Eastman Organic Chemicals, Rochester, NY), and hemopoietic growth factors were plated in 35-mm suspension culture dishes (#171099, Nunc, Naperville, IL). These dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Unless otherwise specified, concentrations of growth factors used in this study were as follows: hGM-CSF, 10 ng/ml; mGM-CSF, 10 ng/ml; mIL-3, 10 ng/ml; mSCF, 100 ng/ml; and hEPO, 2 U/ml, all of which are in the range of optimal concentrations for colony formation, as reported previously (Koike *et al.*, 1987, 1988; Tanaka *et al.*, 1992). Serum-free culture was carried out as described elsewhere (Koike, *et al.*, 1988). Culture mixture contained 1% crystallized globulin-free deionized BSA (Calbiochem-Behring, La Jolla, CA), 300 μg/ml fully iron-saturated human transferrin (Sigma Chemical), 96 μg/ml of cholesterol (Nacalai Tesque, Kyoto, Japan), and 10^{-7} M sodium selenite (Sigma Chemical), instead of FCS and fraction V BSA.

Determination of Colony Types

Colony types were determined on days 5–17 of incubation by in situ observation using an inverted microscope and according to the criteria described by Nakahata *et al.* (Nakahata and Ogawa, 1982b; Nakahata *et al.*, 1982). To assess the accuracy of in situ identification of the colonies, individual colonies were lifted with an Eppendorf micropipette, under direct microscopic visualization, spread on glass slides using a cytocentrifuge (Cytospin II; Shandon Southern, Sewickley, PA), then stained with May-Grünwald-Giemsa and acetylcholine esterase staining for megakaryocytes (Jackson, 1973; Tanaka *et al.*, 1992) and alcian blue-safranin staining for mast cells (Spicer, 1960; Nakahata *et al.*, 1986). Except for megakaryocyte colonies, cell aggregates consisting of more than 50 cells were scored as colonies, according to the criteria of Nakahata and Ogawa (1982a). Megakaryocyte colonies were scored as such when they had four or more megakaryocytes (Nakeff and McQueen, 1976; Tanaka *et al.*, 1992). Abbreviations for the colony types are as follows: G, granulocyte colonies; M, macrophage colonies; GM, granulocyte-macrophage colonies; Eo, eosinophil colonies; MK, megakaryocyte colonies; Mast, mast cell colonies; E, erythrocyte colonies; B, erythroid bursts; Mix, mixed hemopoietic colonies including GMM, granulocyte-macrophage-megakaryocyte colonies; GEMM, granulocyte-erythrocyte-macrophage-megakaryocyte colonies; and Blast, blast cell colonies.

Replating Experiment

To examine the specificity of action of hGM-CSF, we did replating experiments of individual Blast colonies developed in culture of bone marrow cells from 5-FU–treated Tg-mice, in the presence of hGM-CSF or mIL-3. On day 8 of primary cultures, Blast colonies consisting of fewer than 200 cells were identified, individually lifted from the methylcellulose medium using an Eppendorf micropipette on an inverted microscope, and suspended in 200 μl of αMEM. After gentle pipetting, samples were then divided into two parts; one half was added to the secondary culture medium with hGM-CSF and the other half was added to the culture medium with mIL-3. Cultures of the replated cells were incubated and colonies were scored in the same manner as for primary cultures.

RESULTS

Transgene Constructs

To achieve ubiquitous expression of cDNAs of both α and β subunits of hGMR in Tg-mice, we used the pLG1 vector that consists of 1.2 kb of the 5′-flanking sequence from the MHC class I H2-Ld gene, 0.8 kb of the intron, and 0.5 kb of polyadenylation site from the rabbit β globin gene. The H2-Ld promoter has been

used to ubiquitously express the transgene (Suematsu *et al.*, 1992). The α and β subunit cDNAs of hGMR were inserted into the *Sal*I site of the pLG1 expression vector to generate the pLd-hGMRα and pLd-KH97 constructs (Figure 1). To verify the expression of these α and β subunit constructs, NIH3T3 cells were co-transfected with the c-*fos*–luciferase gene, and the transiently expressed luciferase protein in response to hGM-CSF was examined using a luminometer (Watanabe *et al.*, 1993). The luciferase assay revealed the expression of protein products of α and β subunits from these pLd constructs.

Establishment of hGMR α and β Tg-Mice

To generate Tg-mice, the 3.8-kb and 5.7-kb *Sph*I-*Xho*I DNA fragments containing hGMR α and β cDNAs under the H2-Ld promoter, respectively, were coinjected into fertilized (C3H/HeN) eggs and the eggs were then transferred into the oviducts of pseudopregnant ICR females. 135 founder mice were generated and tested for the presence of transgenes, by analyzing tail DNA. Tail DNA was digested either with *Bam*HI and *Eco*RI for hGMRα, which generated an internal 1.8-kb GMRα transgene fragment or with *Bam*HI and *Bgl*II for KH97, which generated an internal 2.5-kb KH97 transgene fragment. The DNA blots were analyzed by Southern hybridization, using hGMRα and β probes. Consequently, 13 lines of Tg-mice were established. Among them, one contained only H2-Ld-GMRα sequence and five contained only H2-Ld-KH97 sequence whereas seven contained both H2-Ld-GMRα and H2-Ld-KH97 sequences, the copy number of these genes being between 1 and 20 (our unpublished observations).

Flow Cytometry Analysis of hGMRαβ Expression on the Hemopoietic Cell Surface

To confirm surface expression of transgenes in hemopoietic cells, we analyzed the cell surface expression of hGMR on bone marrow and spleen cells derived from Tg-mice by flow cytometry, using monoclonal antibodies against each subunit (Figure 2). Of seven lines of Tg-mice carrying integrated hGMRα and β cDNAs, two lines termed H2–71 and H2–81 were found to express both hGMRα and β subunits on the cell surface of bone marrow cells, spleen cells, and peripheral blood cells. A representative profile of the expression of both hGMRα and β subunits on bone marrow cells from line H2–71 is shown in Figure 2. One line of Tg-mice (H2–1) carrying only integrated hGMRα cDNA expressed the hGMRα subunit. Only one line (H2–31) of five lines of Tg-mice carrying integrated hGMRβ cDNA was found to express the hGMRβ subunit. The number of high affinity hGMR of H2–71 and H2–81 was comparable to the BA/Fαβ cell, which expresses about 10,000 molecules of hGMR on BA/F3 cell (our unpublished observations). In the following experiments, we used H2–71 for the methylcellulose colony formation assay.

Expression of Transgene RNA Products in Various Tissues of H2–71

The H2-Ld-hGMRα and H2-Ld-KH97 constructs were designed to achieve ubiquitous expression of the transgene products in Tg-mice. The expression of transgene RNA transcripts in various tissues was examined by RT-PCR, using hGMRα and β primers. There were high levels of 229-bp hGMRα and 295-bp β RT-PCR products in bone marrow, spleen, kidney,

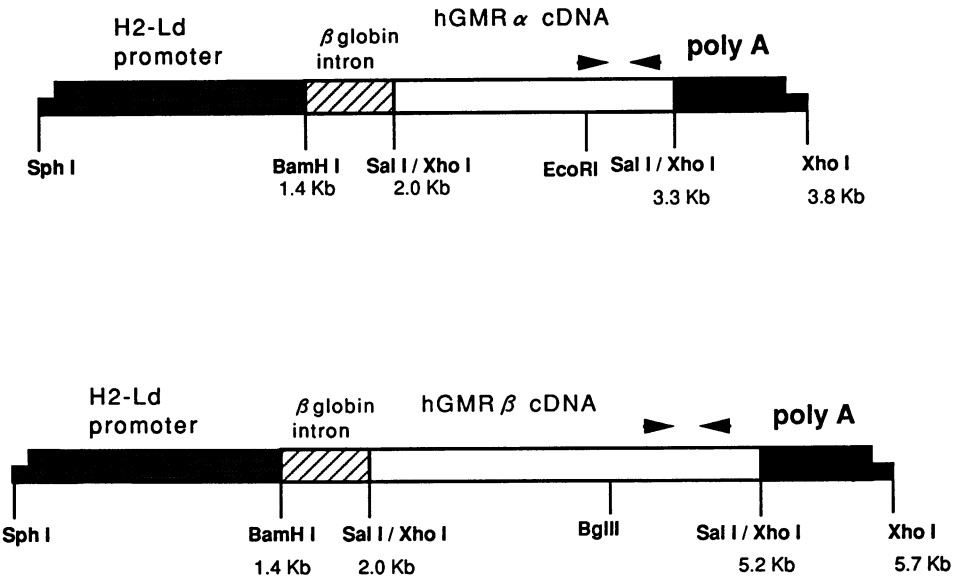

Figure 1. Restriction endonuclease cleavage maps of pLd-GMRα and pLd-KH97 constructs used to generate hGMR Tg-mice. Fragments derived from the H2-Ld promoter, rabbit β globin intron, the hGMR cDNA, and rabbit β globin polyadenylation sequence are shown. Restriction endonuclease cleavage sites used for plasmid construction (*Sal*I/*Xho*I) and for Southern blot analysis of mouse tail DNA (*Bam*HI and *Eco*RI for hGMRα; *Bam*HI and *Bgl*II for β) to detect insertion of the transgene are indicated. The arrow indicates position of the primer for RT-PCR.

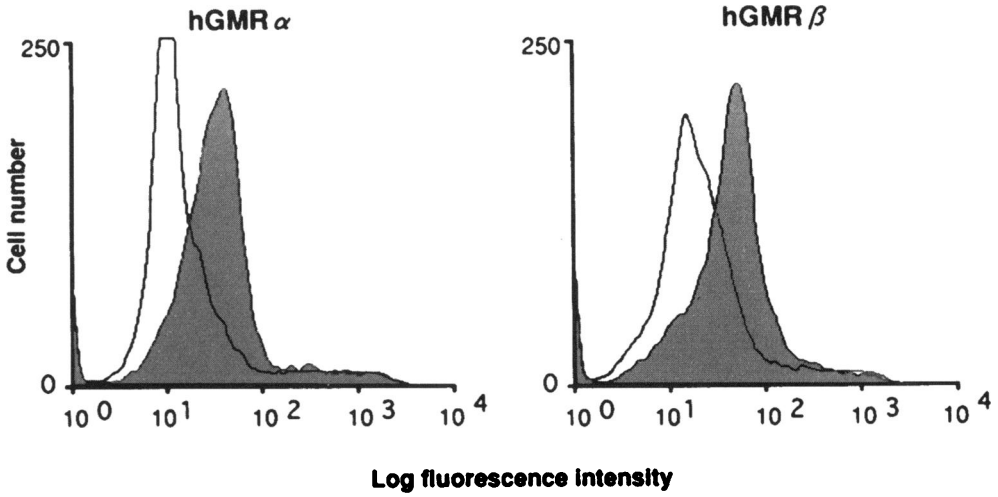

Figure 2. Cell surface expression of hGMRαβ on total bone marrow cells of Tg-mice analyzed by flow cytometry. Bone marrow cells of normal mice (white area) and the one line (H2–71) of Tg-mice (shaded area) were stained with antibodies recognizing hGMRα and β and with fluorescent-tagged antibodies, as described in MATERIALS AND METHODS. Fluorescence intensity of staining is plotted against relative cell number.

heart, liver, lung, and peripheral blood and low levels in brain, skin, testis, and skeletal muscle (Figure 3). Transgene expression was almost the same between H2–71 and H2–81 mice and between the sexes.

hGM-CSF Stimulates Colony Formation of Bone Marrow Cells Derived from hGMR Tg-Mice

To characterize the functional properties of cells expressing hGMR, the effects of hGM-CSF on colony formation in vitro were examined, using bone marrow cells derived from hGMR Tg-mice H2–71. We first examined the dose-dependent response of total colony formation to hGM-CSF. hGM-CSF stimulated colony formation in a dose-dependent manner and the number of total colonies was maximum at 10 ng/ml of hGM-CSF (Figure 4). Therefore, hGM-CSF at 10 ng/ml was used in subsequent studies. In contrast, hGM-CSF did not stimulate colony formation of bone marrow cells derived from either normal littermate mice or Tg-mice expressing only hGMRα or β subunit at concentrations of up to 100 ng/ml of hGM-CSF (our unpublished observations).

Next, we examined the effects of hGM-CSF on colony number and types of colonies derived from bone marrow cells of H2–71 Tg-mice (Table 1). hGM-CSF supported the formation of various types of colonies including GM, Eo, Mast, MK, B, E, Blast, and Mix colonies including GMM or GEMM, whereas mGM-CSF yielded only GM, G, and M colonies (Table 1). In Table 1, the number of GM, G, and M colonies stimulated by IL-3 is higher in the normal mice than in the transgenic mice; however, in other experiments, the number of GM, G, and M colonies stimulated by IL-3 is lower in the normal mice than in the transgenic mice. In situ appearance and May-Grünwald Giemsa staining of representative GEMM, MK, and Mast colonies induced from bone marrow cells of H2–71 Tg-mice in the presence of hGM-CSF alone are presented in Figure 5. The effect of hGM-CSF on types of colonies formed more closely resembled mIL-3 than mGM-CSF. It should be noted, however, that hGM-CSF stim-

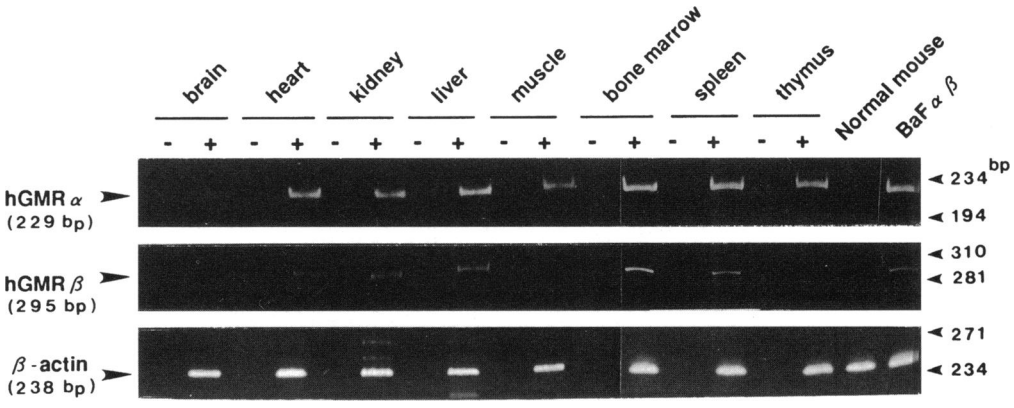

Figure 3. RT-PCR analysis of transgene expression in tissues of 8-wk-old Tg-mice. Each cDNA used was 0.05 μg (RNA equivalent). Lane marked (−) is the PCR product of a mock cDNA (no reverse transcriptase included in the cDNA synthesis reaction). Normal mouse indicates the PCR product of the bone marrow, and BaFαβ is the mouse Pro-B cell line expressing about 10,000 molecules of high affinity and functional hGMR.

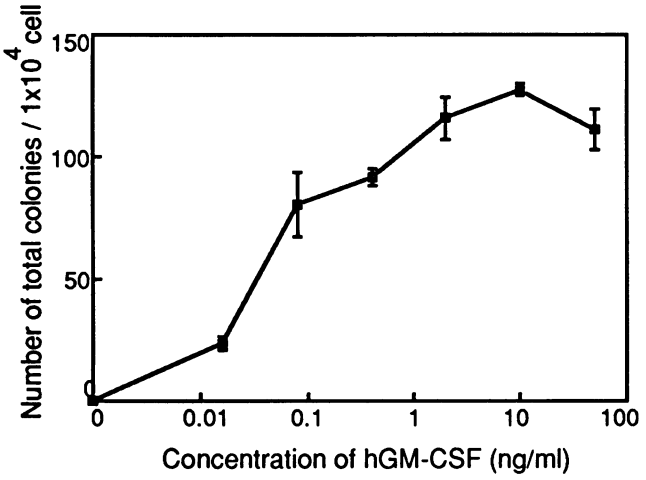

Figure 4. Dose-response curve for the effect of hGM-CSF on colony formation by bone marrow cells of Tg-mice. Data represent the mean of total colony numbers ± SD of triplicate cultures.

ulated a much larger number of colonies than did mIL-3. The addition of mIL-3 to culture containing hGM-CSF did not affect the total number of colonies or types of colonies (our unpublished observations), hence hemopoietic progenitors responsive to mIL-3 are likely to be already included in the population of cells responsive to hGM-CSF.

hGM-CSF Stimulates Erythrocyte Development in the Absence of Erythropoietin

In addition, as much as 60% of mixed colonies formed by hGM-CSF contained erythrocytes in the absence of EPO (our unpublished observations). In contrast, erythroid cells were rarely detected in mixed colonies formed by mIL-3, under the same conditions. Interestingly, hGM-CSF supported the proliferation of E colonies, which are derived from mature progenitors restricted to erythroid lineage, in the absence of EPO. Addition of anti-EPO antibody to the culture did not alter the number of E colonies produced by hGM-CSF, whereas the same antibody completely suppressed formation of E colonies induced by mIL-3 and EPO (our unpublished observations). This result rules out the possibility that hGM-CSF induces erythrocyte through stimulation of endogenous EPO production in the culture and strongly suggests that it directly activates the formation of erythrocyte from bone marrow cells.

Colony Formation in Serum-free Culture

The observed effects of hGM-CSF may be due to combined actions of hGM-CSF and unknown factor(s) contained in FCS rather than to hGM-CSF itself. To exclude this possibility, we carried out serum-free cultures of bone marrow cells of Tg-mouse, as described in MATERIALS AND METHODS. As shown in Table 2, the results were much the same as in the presence of 30% FCS. It should be noted that B and Mix colonies were also supported under serum-free conditions with hGM-CSF. This excluded the possibility that formation of erythroid cells observed without added EPO (Table 1) is due to contamination of EPO, if any, in serum used. Again, hGM-CSF supported more significantly a larger number of total colonies as

Table 1. Colony formation by bone marrow cells of hGMR Tg-mice

	No. of colonies[a]/10^4 bone marrow cells				
	G	M	GM	Eo	MK
Transgenic mice					
mIL-3	9.0 ± 1.0	3.3 ± 1.5	26.3 ± 3.1	0.6 ± 0.6	1.0 ± 1.0
mIL-3 + Epo	9.7 ± 4.5	4.0 ± 1.7	26.0 ± 2.0	1.0 ± 1.0	1.3 ± 0.6
mGM	10.3 ± 1.2	9.7 ± 5.5	17.3 ± 3.8	0	0
mGM + Epo	8.0 ± 4.4	8.0 ± 1.7	16.0 ± 4.4	0	0
hGM	10.0 ± 2.0	8.3 ± 1.2	25.7 ± 1.5	1.0 ± 1.0	4.7 ± 2.5
hGM + Epo	10.3 ± 2.5	4.6 ± 1.5	23.0 ± 5.6	1.0 ± 1.0	2.3 ± 0.6
Epo	0	0	0	0	0
Normal mice					
mIL-3	10.3 ± 1.5	9.3 ± 0.6	45.3 ± 9.3	1.0 ±1.0	1.0 ± 1.0
mIL-3 + Epo	14.7 ± 0.6	12.7 ± 3.5	46.3 ± 12.3	1.6 ± 0.6	2.7 ± 2.1
hGM	0	0	0	0	0

Values are mean ± SD of triplicate plates containing 10^4 bone marrow cells. hGM-CSF 10 ng/ml; mGM-CSF 10U/ml; mIL-3 10 ng/ml; mSCF 100 ng/ml.

[a]Abbreviations: G, granulocyte colonies; M, macrophage colonies; Eo, eosinophile colonies; GM, granulocyte-macrophage colonies; MK, megakaryocyte colonies; Mast, mast cell colonies; E, erythrocyte colonies; B, erythroid bursts; Mix, mixed hemopoietic colonies; Bl, blast cell colonies.

well as Mix colonies than was supported by mIL-3 in serum-free culture.

Colony Formation of Bone Marrow Cells from 5-FU-treated hGMR Tg-Mice

Our previous study, in which hGM-CSF generated a much larger number of immature colonies such as Mix and Blast colonies than mIL-3, suggested the possibility that early hemopoietic progenitors that normally do not respond to mIL-3 or mGM-CSF acquired the potential to respond to hGM-CSF in bone marrow cells of hGMR Tg-mice. To obtain support for this hypothesis, we examined the response of bone marrow cells derived from 5-FU–treated mice to hGM-CSF. 5-FU treatment enriched cell cycle–dormant immature progenitor cells (Suda *et al.*, 1983a). As shown in Table 3, mIL-3 stimulated the formation of a much smaller number of colonies than did hGM-CSF, hence, 5-FU treatment seemed to enrich progenitor cells with the potential to respond to hGM-CSF but not to mIL-3. This means that 5-FU treatment enriched immature progenitor cells expressing hGMR more significantly than it did those expressing mIL-3R. We then examined the effect of SCF, which in itself lacks the capacity to form colonies. SCF significantly enhanced colony formation by mIL-3 but not by hGM-CSF. The number of colonies formed by the combination of mIL-3 and SCF was much the same as that formed by hGM-CSF alone (Table 3). As expected, the stimulatory effect of SCF on mIL-3–dependent colony formation was pronounced in Mix and Blast colonies whereas SCF showed no stimulatory effect on hGM-CSF–dependent colony formation. These colonies are probably derived from cells at the early stage of differentiation.

Replating Experiments of Individual Blast Colonies Supported by hGM-CSF and mIL-3

The observed effects of hGM-CSF may be due to a mIL-3–like molecule produced by accessory cells, in response to stimulation with hGM-CSF, rather than to hGM-CSF itself. To eliminate the influence of accessory cells such as T cells and macrophages that secrete cytokines, we carried out replating experiments of individual Blast colonies developed in culture of bone marrow cells from 5-FU–treated Tg-mice in the presence of hGM-CSF or mIL-3. Mouse Blast colonies consist of various hemopoietic progenitors and do not contain detectable mature cells (Nakahata and Ogawa, 1982). One-half the primary Blast colonies were cultured with mIL-3, and another one-half were cultured with hGM-CSF. Out of 24 primary Blast colonies, one failed to produce secondary colonies, and four produced only secondary GM colonies; the remaining 19 formed various types of secondary colonies including MK, Mast, Blast, and Mix colonies in the presence of hGM-CSF, as shown in Table 4. These colonies were confirmed to express hGMR transgene RNA transcript (Figure 6).

DISCUSSION

We generated Tg-mice constitutively expressing hGMR and analyzed the hGM-CSF–dependent in vitro colony formation of hemopoietic cells. The unique feature of the approach used in this work is twofold, i.e., use of hGMR and establishment of Tg-mice. Our previous experiments indicated that reconstituted hGMR is functional in the mouse pro-B cell line BA/F3 and in NIH3T3 fibroblasts (Watanabe *et al.*, 1993). We confirmed that hGM-CSF also stimulates the proliferation of bone marrow cells of hGMR Tg-mice. The species-specificity of hGMR facilitates evaluation of activity, without perturbation from endogenous mGMR. Another advantage of using the Tg-mice is that it allows one to assess the developmental potential of cells, which is often difficult with established cell lines. We wanted to achieve the expression of hGMR throughout mouse development so that the effects of hGMR signal at various stages of hemopoi-

Table 1 continued

No. of colonies[a]/10^4 bone marrow cells					
Mast	E	B	Mix	Blast	Total
5.7 ± 1.5	4.3 ± 4.9	0	6.7 ± 1.2	1.3 ± 1.2	57.6 ± 10.0
8.0 ± 3.6	50.3 ± 6.7	0	11.3 ± 2.1	1.0 ± 0	112.6 ± 1.5
0	0	0	0	0	37.3 ± 7.5
0	33.6 ± 5.5	0	0	0	65.7 ± 6.4
7.0 ± 1.7	49.3 ± 15.4	1.3 ± 1.2	14.0 ± 3.7	2.6 ± 1.2	124.0 ± 17.0
4.7 ± 2.1	63.3 ± 9.0	2.0 ± 1.7	17.0 ± 5.3	4.0 ± 1.0	112.3 ± 6.1
0	53.3 ± 12.2	0	0	0	53.3 ± 12.2
8.7 ± 1.5	1.0 ± 1.7	0	12.0 ± 1.7	1.3 ± 1.5	90.0 ± 12.0
7.0 ± 1.0	46.7 ± 9.6	0	18.0 ± 4.6	0.6 ± 0.6	150.3 ± 20.6
0	0	0	0	0	0

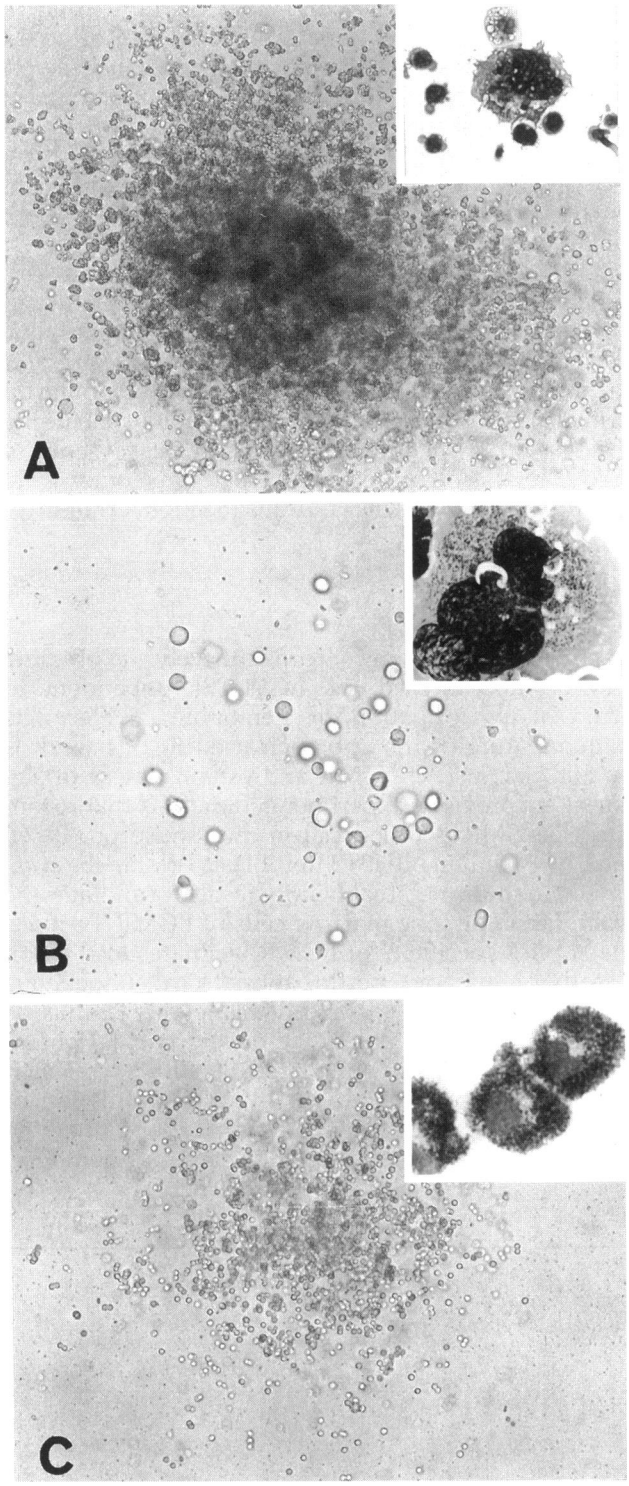

Figure 5. Photomicrographs of various typical colonies derived from bone marrow cells of Tg-mice in the presence of hGM-CSF. (A) Day 10 macroscopic GEMM colony (×40) and May-Grünwald Giemsa-staining in inset (×400); (B) Day 5 megakaryocyte colony (×100) and May-Grünwald Giemsa-staining in inset (×1000); (C) Day 14 mast cell colony (×100) and May-Grünwald Giemsa-staining in inset (×1000).

etic progenitors could be monitored. Based on the strict species-specificity of GM-CSF between mice and humans, we assumed that the constitutive expression of hGMR in various tissues does not affect the process of mouse development.

Using this system, we found that bone marrow cells from Tg-mice expressing both hGMRα and β subunits, but not normal or hGMRα or β Tg-mice, have the potential to form colonies in response to hGM-CSF in methylcellulose semisolid medium. Therefore, hGMR is functional and capable of transducing proliferative signals in mouse cells if α and β subunits are present. When culturing bone marrow cells derived from hGMR Tg-mice, we found that hGM-CSF stimulates colony formation of early progenitors as well as lineage committed progenitors, whereas mGM-CSF supports mainly GM colonies. Thus, like mIL-3, hGM-CSF functions as a multi-CSF in hGMR Tg-mice. The number of GM colonies stimulated by hGM-CSF was much the same as that stimulated by mIL-3 or mGM-CSF. It appears that progenitors of GM lineage expressing mIL-3R and mGMR in the Tg-mice are equally responsive to hGM-CSF, through ectopically expressed hGMR.

hGM-CSF, however, stimulated two to three times larger numbers of MK, Mix, or Blast colonies than did mIL-3. The difference between hGM-CSF and mIL-3 in their potential to form MK, Mix, or Blast colonies was even more pronounced in 5-FU–treated hGMR Tg-mice. In addition, the size of the colonies supported by hGM-CSF was much larger than those formed by either mIL-3 or mGM-CSF. These results suggest that hGM-CSF acts on the same progenitors stimulated by mIL-3 as well as earlier progenitors insensitive to mIL-3. The early progenitors enriched by 5-FU treatment probably contain populations of cells lacking functional IL-3R (Ogata *et al.*, 1992). Most likely, in the Tg-mice, earlier progenitor cells that lack the functional mIL-3R acquired the ability to form colonies in response to hGM-CSF, through ectopically expressed hGMR. As SCF and mIL-3 act synergistically in the development of early hemopoietic progenitors (Shiohara *et al.*, 1993), we examined the effects of SCF on hGM-CSF– or mIL-3–dependent colony formation. Unlike mIL-3, SCF had no synergistic effect on hGM-CSF and the number of Mix colonies by hGM-CSF alone was comparable to the combination of mIL-3 and SCF. Taken together, these results indicate that hGMR is expressed in early progenitors, which also express c-*kit* but not functional IL-3R.

We also found that hGM-CSF by itself stimulates colony formation of mast cells and of erythrocytes, a function of which is believed to require an additional signaling pathway distinct from that triggered by common β of IL-3R/GMR/IL-5R (Liboi *et al.*, 1993). GM-CSF of both mice and humans does not support proliferation and differentiation of mast cells, whereas

Table 2. Colony formation by bone marrow cells in serum free culture

Factors	No. of colonies[a]/10^4 bone marrow cells								
	G	M	GM	MK	Mast	B	Mix	Blast	Total
Transgenic mice									
mIL-3	6.3 ± 3.1	2.3 ± 1.5	24.3 ± 1.2	0.3 ± 0.6	2.6 ± 4.6	0	5.7 ± 2.9	1.3 ± 1.2	43.7 ± 4.7
mGM-CSF	9.3 ± 0.6	0.7 ± 0.6	14.7 ± 3.8	0	0	0	0	0	25.0 ± 4.6
hGM-CSF	6.3 ± 0.6	1.7 ± 1.2	21.7 ± 5.5	3.6 ± 0.6	2.0 ± 2.9	9.7 ± 1.5	15.7 ± 3.2	4.0 ± 2.6	64.0 ± 7.0
Normal mice									
hGM-CSF	0	0	0	0	0	0	0	0	0

Values are mean ± SD of triplicate plates containing 10^4 bone marrow cells. hGM-CSF 10 ng/ml; mGM-CSF 10 U/ml; mIL-3 10 ng/ml.
[a]Abbreviations: see Table 1.

IL-3 supports proliferation and differentiation of mast cell in mice, but not in humans (Saito *et al.*, 1988). Because mice but not humans carry the IL-3R–specific β-chain, βIL-3, we reasoned that mouse mast cells may proliferate through a growth signal specific to the βIL-3; however, our results do not support this hypothesis, because hGM-CSF also supports mast cells to form colonies from bone marrow cells of hGMR Tg-mice in semisolid medium and to proliferate in liquid medium. This indicates that mast cell growth does not require the βIL-3–specific signaling pathway. The lack of response of mast cells to mGM-CSF is likely due to the lack of mGMRα expression in normal mice.

Interestingly, erythroid colony formation that normally requires the EPOR signal was also supported by hGM-CSF in the absence of added EPO with hGMR-expressing cells. It appears that hGMR has the capacity to support proliferation of cells of erythroid lineage. Using bone marrow cells of mIL-5Rα Tg-mice, mIL-5 was found to support all lineages of hemopoietic cells, except erythroid lineage (Takagi and Miyajima, unpublished data). The lack of erythrocyte formation in mIL-5Rα Tg-mice may be due to lack of expression of the β subunit at a later stage of erythrocyte differentiation. It should also be noted that BA/F3 cells transfected with EPOR proliferate in response to either mIL-3 or EPO, whereas only EPO induces globin synthesis (Liboi *et al.*, 1993), indicating a difference in signaling pathways of IL-3 and EPO. The discrepancy between our Tg-mice and BA/F3 cells remains to be resolved.

Our observations are important because unlike mGM-CSF, the activity of which is restricted mainly to cells of GM lineage, hGM-CSF stimulates early progenitors as well as lineage-committed progenitors, if hGMR is expressed on the target cells. However, compositions of colonies formed by hGM-CSF are similar to those formed by mIL-3. These results indicate that hGM-CSF does not significantly alter the developmental program of hemopoietic progenitors, rather it amplifies cells expressing hGMR. Several possibilities can be considered for the role of cytokine in hemopoietic

Table 3. Colony formation by bone marrow cells of 5-FU-treated hGMR Tg-mice

	No. of colonies[a]/5 × 10^4 bone marrow cells						
	G	M	GM	MK	Mix	Blast	Total
Transgenic mice							
SCF	0	0	0	0	0	0	0
mIL-3	0.7 ± 0.6	0.3 ± 0.6	1.7 ± 1.2	0.7 ± 0.6	0.3 ± 0.6	0	3.7 ± 1.5
mIL-3 + SCF	1.3 ± 0.6	0.7 ± 0.6	4.7 ± 0.6	0.3 ± 0.6	4.3 ± 1.7	1.0 ± 1.7	12.3 ± 2.3
mGM	0.7 ± 1.2	0.3 ± 0.6	0.7 ± 0.6	0	0	0	1.7 ± 2.1
mGM + SCF	0.3 ± 0.6	0.3 ± 0.6	1.7 ± 1.2	0	0	0.3 ± 0.6	2.3 ± 1.5
hGM	0.6 ± 1.2	0.3 ± 0.6	1.7 ± 0.6	2.7 ± 0.6	3.7 ± 1.2	3.7 ± 0.6	12.7 ± 1.7
hGM + SCF	1.7 ± 2.1	0.3 ± 0.6	4.0 ± 1.0	2.0 ± 0.6	3.3 ± 1.2	2.7 ± 2.3	14.0 ± 1.0
Normal mice							
hGM-CSF	0	0	0	0	0	0	0

Values are mean ± SD of triplicate plates containing 5 × 10^4 bone marrow cells of 5-FU-treated mice from two independent experiments. hGM-CSF 10 ng/ml; mGM-CSF 10 U/ml; mIL-3 10 ng/ml; mSCF 100 ng/ml.
[a]Abbreviations: see Table 1.

Table 4. Replating experiment of blast colonies derived from Tg-mice bone marrow cells

Colony no.	Total cell count	Cytokine in secondary culture	Number of secondary colonies per 1/2 primary colonies								Total replating efficiency (%)
			G	M	GM	MK	Mast	Mix	Bl	Total	
1	128	IL-3	4	0	15	0	3	2	1	25	39
		hGM-CSF	8	0	4	0	6	9	2	29	45
2	98	IL-3	5	0	12	1	2	1	2	23	47
		hGM-CSF	6	0	4	1	3	6	3	23	47
3	185	IL-3	9	1	35	0	8	7	6	66	71
		hGM-CSF	5	0	27	2	9	11	8	62	67
4	320	IL-3	12	8	16	1	0	2	2	41	26
		hGM-CSF	12	29	16	1	0	6	0	64	40
5	198	IL-3	10	3	13	1	2	6	3	38	38
		hGM-CSF	3	4	10	5	3	6	5	36	36
6	146	IL-3	3	1	7	4	1	5	0	21	29
		hGM-CSF	4	0	10	3	2	5	4	28	38
7	132	IL-3	3	0	11	0	0	2	0	16	24
		hGM-CSF	2	0	17	0	2	7	2	30	45
8	148	IL-3	2	0	23	1	3	6	0	35	47
		hGM-CSF	3	1	19	6	2	4	0	35	47
9	74	IL-3	6	0	3	2	3	5	2	21	56
		hGM-CSF	7	0	7	7	1	10	1	33	89
10	159	IL-3	16	1	49	1	1	4	1	73	92
		hGM-CSF	24	1	49	3	2	16	0	95	119
11	97	IL-3	8	0	3	0	3	2	3	19	39
		hGM-CSF	5	1	2	0	3	2	3	16	33
12	88	IL-3	2	0	3	0	2	2	0	9	20
		hGM-CSF	4	0	2	0	2	3	1	12	27
13	72	IL-3	1	1	18	5	3	8	0	36	100
		hGM-CSF	0	0	8	5	1	19	2	35	97
14	52	IL-3	2	0	9	2	3	1	0	17	65
		hGM-CSF	0	1	9	1	0	7	0	18	69
15	74	IL-3	0	0	5	1	0	1	0	7	19
		hGM-CSF	0	0	7	0	3	5	0	15	41
16	116	IL-3	3	0	8	0	4	3	8	26	45
		hGM-CSF	1	1	8	1	11	9	3	34	59

Blast cell colonies derived from hGMRTg-mice were individually picked up from cultures on day 8, and replated in secondary culture medium containing hGM-CSF or mIL-3. Data represent the number of colonies on day 16 of secondary culture. Colony no. 1–10 are derived from primary culture with hGM-CSF, and no. 11–16 are derived from primary culture with mIL-3.

cell development. The "selection" model assumes that lineage of progenitor cells is determined by the intrinsic program of the cell and the cytokine simply selects cells expressing the matched receptor. The "instruction" model predicts that the cytokine plays an important role in the determination of lineage of progenitor cells. Our finding that hGM-CSF neither instructs nor restricts the process of cell differentiation favors the selection model. The replating of Blast colonies derived from the culture with hGM-CSF formed various types of colonies, which also confirmed that the commitment of earlier progenitors is decided by itself and not by hGM-CSF. This view is also supported by recent reports that expression of receptors for more lineage-restricted cytokines such as EPOR (Dubart *et al.*, 1994), CSF-1R (M-CSFR) (Pharr *et al.*, 1994), or IL-5R (Takagi and Miyajima, unpublished data) in early hemopoietic progenitors enhances colony formation composed of cells of various lineages.

Taken together, our results demonstrate that hemopoietic progenitor cells have a set of intracellular signaling molecules that can be shared by various cytokine receptors. Inasmuch as GMR expression is limited to particular stages of hemopoietic development, the potential of the cell to proliferate in response to GM-CSF appears to be controlled by the expression of GMR. Our observation that progenitor cells can be stimulated via artificially expressed hGMR is in keeping with this view. However, hGMR signals do not significantly affect developmental potential of progenitor cells, thereby indicating that commitment of the cell to various lineages is not simply determined by

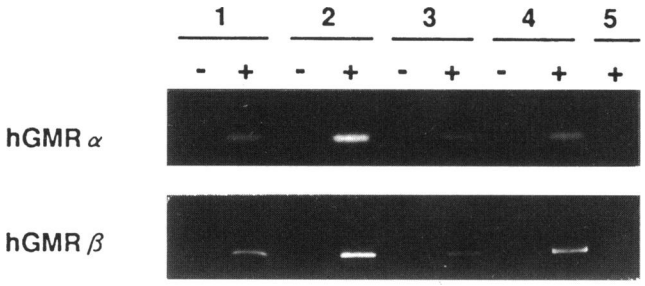

Figure 6. Analysis of hGMR$\alpha\beta$ mRNA in Mix or mast cell colonies by RT-PCR. Each colony was picked up with a micropipette from cultures with hGM-CSF. mRNA was prepared and analyzed for hGMR α and β mRNA expression by RT-PCR, as described in MATERIALS AND METHODS. Mix colony (lanes 1 and 2) and mast cell colony (lanes 3 and 4) derived from Tg-mice. Mix colony derived from normal mice (lane 5). Lane marked (−) is the PCR product of a mock cDNA (no reverse transcriptase included in cDNA synthesis reaction).

receptor expression. More detailed cellular and molecular analyses will need to be done to better understand mechanisms underlying the commitment of hemopoietic progenitor cells.

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