

3. Molecules

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)–dependent *in vitro* and *in vivo* proliferation and differentiation of all hematopoietic progenitor cells in hGM-CSF receptor transgenic mice

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To examine the relation between receptor expression and differentiation of hematopoietic cells, we produced transgenic mice that constitutively expressed the human granulocyte-macrophage colony stimulating factor (hGM-CSF) receptor at almost all stages of hematopoietic cell development. The high-affinity GM-CSF receptor is species specific, allowing analysis of the specific effects of hGM-CSF in our mouse model. Proliferation and differentiation of hematopoietic progenitor cells from transgenic mice were analyzed by means of methylcellulose colony-forming assay and *in vivo* treatment with hGM-CSF, respectively. We found that hGM-CSF supported various types of colonies, including granulocyte-macrophage, mast cell, megakaryocyte, blast cell, and mixed hematopoietic colonies, whereas mouse GM-CSF supported only granulocyte-macrophage colonies. In addition, hGM-CSF generated erythrocyte colonies in the absence of erythropoietin. Furthermore, *in vivo* administration of hGM-CSF to transgenic mice resulted in a dose-dependent increase in reticulocytes and white blood cells in the peripheral blood. The spleens of the mice showed gross enlargement, mainly caused by an increase of erythroid cells and their progenitors. Taken together, these results indicate that hGM-CSF receptor-mediated signals can support the growth of cells of all hematopoietic cell lineages if this receptor is present on the cell surface. This implies that the differentiation of hematopoietic progenitor cells is not determined by exogenous cytokine stimulation (instruction model) but by an intrinsic cell program in which cytokines simply select cells that express the appropriate receptor (stochastic model). (*J Allergy Clin Immunol* 1997;100:S79-86.)

Key words: GM-CSF receptor, hematopoietic progenitor cells, transgenic mice

Hematopoiesis is a multistage developmental process in which pluripotent hematopoietic stem cells give rise to cells of various lineages, including granulocytes, macrophage, erythrocytes, megakaryocytes, and lymphocytes.

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Abbreviations used

BFU-E:	Erythroid burst-forming unit
CFU-E:	Erythrocyte colony-forming unit
FCS:	Fetal calf serum
GM:	Granulocyte-macrophage
hGM-CSF:	Human granulocyte-macrophage colony-stimulating factor
LGLs:	Large granular lymphocytes
MHC:	Major histocompatibility complex
NK:	Natural killer
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcriptase polymerase chain reaction

It is widely accepted that the growth and differentiation of progenitor cells are stimulated by cytokines. However, it remains unknown whether the differentiation of progenitor cells is induced by exogenous cytokines (instruction model) or whether cytokines simply promote the survival of already committed cells (stochastic model).^{1,2} If signal transduction through a lineage-restricted cytokine receptor is a key inductive event in cell commitment, artificial expression of the receptor at an early stage of development would promote differentiation to a particular lineage. To examine the relation between receptor expression and differentiation of hematopoietic cells, we generated transgenic mice that expressed the receptor for human granulocyte-macrophage colony stimulating factor (hGM-CSF) in almost all cell lineages during hematopoiesis.³

The hGM-CSF receptor was selected for two reasons. First, mouse GM-CSF has been shown to bind to its specific receptor, expression of which is restricted to the granulocyte-macrophage (GM) lineages, and which only supports the proliferation and maturation of GM progenitor cells into granulocytes and monocytes or macrophages.^{4,5} Second, the hGM-CSF receptor expressed on mouse cells interacts only with hGM-CSF alone in a species-specific manner,⁶ allowing specific assessment of

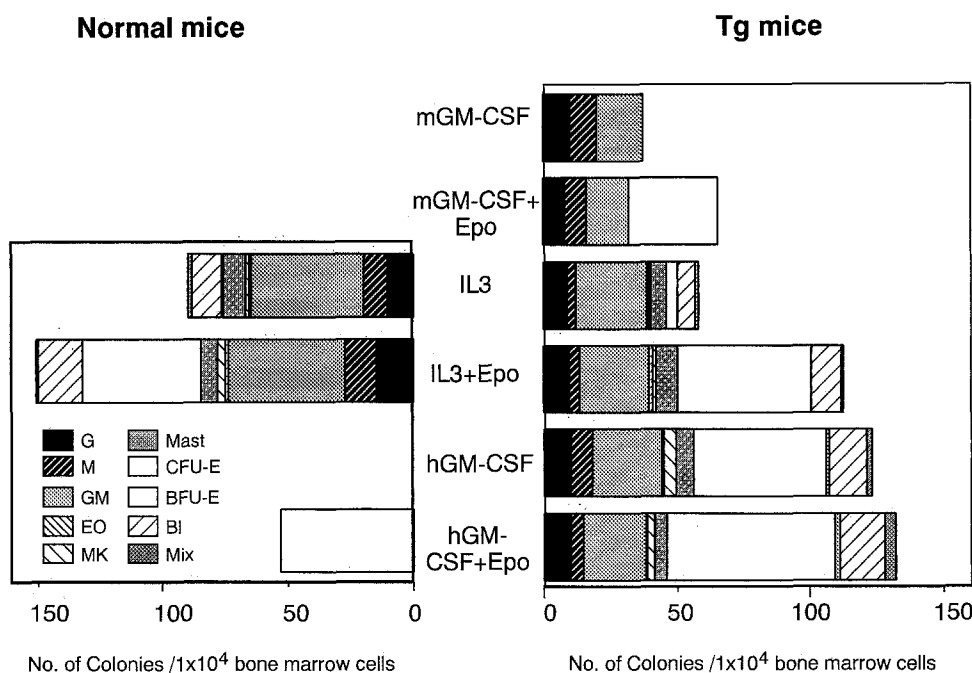


FIG. 1. Colony-forming assay of bone marrow cells from transgenic mice and normal mice. The experiments not described in this figure showed that the effects of mouse GM-CSF and mouse GM-CSF plus erythropoietin on colony numbers and types among normal mice were the same as those among transgenic mice. *G*, granulocyte colonies; *M*, macrophage colonies; *GM*, granulocyte-macrophage colonies; *EO*, eosinophil colonies; *MK*, megakaryocyte colonies; *Mast*, mast cell colonies; *CFU-E*, erythrocyte colonies; *BFU-E*, erythroid burst-forming units; *BI*, blast cell colonies; *Mix*, mixed hematopoietic colonies; *Epo*, erythropoietin.

the effects of hGM-CSF on cells of various lineages that express this receptor in transgenic mice.

Using transgenic mice, we characterized the proliferation and differentiation of hematopoietic progenitor cells with an *in vitro* methylcellulose colony-forming assay and *in vivo* hGM-CSF treatment, respectively.⁷

METHODS

Generation of transgenic mice

Transgenic mice were generated as described elsewhere.³ In brief, fragments of hGM-CSF receptor α and β complementary DNA were inserted into the pLG1 expression vector,⁸ which contained a 1.2 kilobase mouse major histocompatibility complex (MHC) L-locus gene (H2-Ld) promoter and an 0.8 kilobase rabbit β -globin intron upstream of the cloning sites. Transgenic mice were produced by means of the standard oocyte injection method on animals of the C3H/HeN strain.⁹ After production of the transgenic mice, we confirmed transcription and expression of the transgene by means of reverse transcriptase polymerase chain reaction (RT-PCR) and flow cytometry.³

Colony assay of hematopoietic progenitor cells

Methylcellulose clonal culture was performed with a modification of a technique described previously.¹⁰ One milliliter of culture medium that contained 1×10^4 bone marrow cells or 1×10^5 spleen cells, α -minimal essential medium, 1.2% (1500 centipoises) methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% fetal calf serum (FCS; HyClone Laboratories, Logan, Utah), 1% deionized fraction V bovine serum albumin

(Sigma, St. Louis, Mo.), 1×10^{-4} mol/L mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.), and hematopoietic growth factors was plated in 35 mm suspension culture dishes (#171099, Nunc, Naperville, Ill.). These dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air. Unless otherwise specified, the concentrations of growth factors used in this study were 100 ng/ml for rat stem cell factor, 10 ng/ml for mouse IL-3, and 2 U/ml for human erythropoietin, values in the range of optimal concentrations for colony formation.^{11,12} Colony types were determined according to reported criteria¹³ on days 5 to 17 of culture by *in situ* observation with an inverted microscope.

Analysis of erythroid colonies

To rule out the possibility that hGM-CSF would induce the production of endogenous erythropoietin, we added 24 μ g/ml of rabbit anti-erythropoietin antibody to cultures (gift from Kirin Brewery, Tokyo Japan). The transcription of erythroid-specific transcription factors in erythroid burst-forming units (BFU-E) was analyzed by means of RT-PCR as described previously.⁷ Single BFU-E colonies were picked up with a micropipette from cultures with hGM-CSF alone or with both IL-3 and erythropoietin. The PCR primers used were the gift of Dr. M. Yamamoto (University of Tsukuba, Japan).

In vivo administration of hGM-CSF

Administration of hGM-CSF was conducted according to a modification of methods described previously.^{14,15} In brief, transgenic mice bearing the hGM-CSF receptor were given subcutaneous injections of 0.2 ml of phosphate-buffered saline

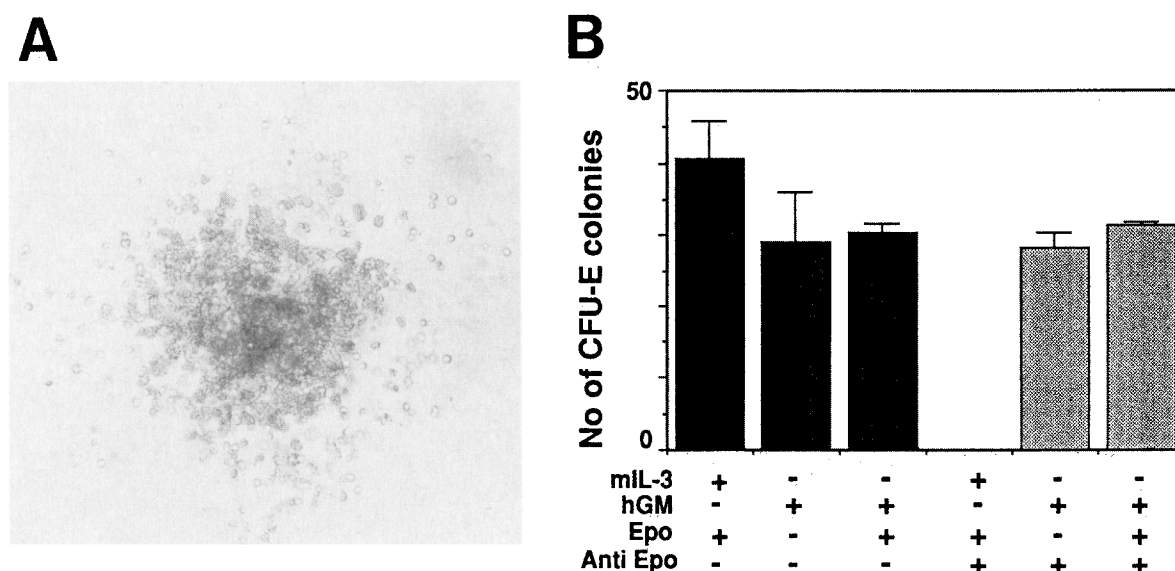


FIG. 2. Erythroid colony formation by bone marrow cells from transgenic mice in the absence of erythropoietin (Epo). **A**, Photomicrograph of BFU-E colonies derived from bone marrow cells of transgenic mice in the presence of hGM-CSF. **B**, Addition of anti-erythropoietin antibody to cultures with hGM-CSF did not affect the number of CFU-E colonies, whereas the same antibody completely suppressed formation of erythroid colonies induced by IL-3 and erythropoietin.

solution containing 1% C3H/HeN serum, or they were given 20, 100, 500, or 2500 ng of hGM-CSF twice a day at 12-hour intervals for 7 days.

Examination of tissues

After hGM-CSF treatment, we determined the white blood cell count, red blood cell count, reticulocyte count, platelet count, hemoglobin concentration, and hematocrit of peripheral blood, as described previously.⁷ Spleen cells and thymic cells were prepared in α -minimal essential medium by means of repeated pipetting and passage through a 50 μ m nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, N.J.). Cytochrome preparations were made from the spleen cell suspensions with a cytochrome (Cytospin II; Shandon Southern Instruments, Sewickley, Pa.) and were stained with May-Grünwald-Giemsa stain. Hepatic mononuclear cells were isolated by means of a previously described method.¹⁶

Flow cytometry

After red blood cells were depleted, peripheral blood, bone marrow, spleen, thymus, and hepatic mononuclear cells were washed twice with the staining solution (phosphate-buffered saline solution containing 5% FCS and 0.02% sodium azide). Then 1×10^6 cells were incubated at 4°C for 30 minutes in 100 μ l of staining solution with 500 ng of fluorescein isothiocyanate-labeled rat anti-mouse CD8 α antibody (Ly-2, clone 53-6.7; Pharmingen, San Diego, Calif.) combined with 250 ng of phycoerythrin-labeled rat anti-mouse CD4 antibody (L3T4, clone RM4-5; Pharmingen) or with 500 ng of phycoerythrin-labeled rat anti-mouse NK1.1 antibody (clone PK136; Pharmingen) and 1 ng of fluorescein isothiocyanate-labeled hamster anti-mouse CD3 ϵ antibody (clone 145-2C11; Pharmingen). After staining, the cells were washed twice in staining solution and then incubated at 4°C for 10 minutes in 300 μ l of staining solution containing 2 mg/ml of 7-aminoactinomycin D (Sigma). Debris, erythrocytes, and dead cells were excluded from anal-

ysis by means of gating for forward and side scatter and 7-aminoactinomycin D. Flow cytometry was performed with a Becton-Dickinson FACScan fluorescence-activated cell sorter (Becton-Dickinson, San Jose, Calif.) and LYSIS II software.

Cytotoxicity assay

The target cells for the cytotoxicity assays were natural killer (NK)-resistant P815 cells and NK-sensitive YAC-1 cells. Target cells (3×10^6) were labeled with 100 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ for 60 minutes at 37°C in RPMI 1640 medium containing 10% FCS, washed three times with medium, and subjected to cytotoxicity assays. Labeled target cells (10^4 /well) were incubated in a total volume of 200 μ l with effector cells in RPMI 1640 medium in 96-well, round-bottom microtiter plates. The plates were centrifuged after for 4 hours, and the supernatant was harvested and counted with a gamma counter. Cytotoxicity was calculated as the percentage of released counts that remained after subtraction of spontaneous release.

RESULTS

Establishment of transgenic mice

We used the H2-Ld promoter, which was expressed by almost all tissues, including both immature and mature hematopoietic cells. Thirteen lines of transgenic mice were established, and two lines expressed both α and β proteins.³ The ribonucleic acid (RNA) transcripts of the transgenes were examined by RT-PCR using hGM-CSF receptor α and β primers. There were high levels of α and β RT-PCR products in the bone marrow, spleen, kidney, heart, liver, and lung. Low levels were found in the brain and skeletal muscle.³ This pattern of transgene expression was almost the same as that of intrinsic MHC class I expression.

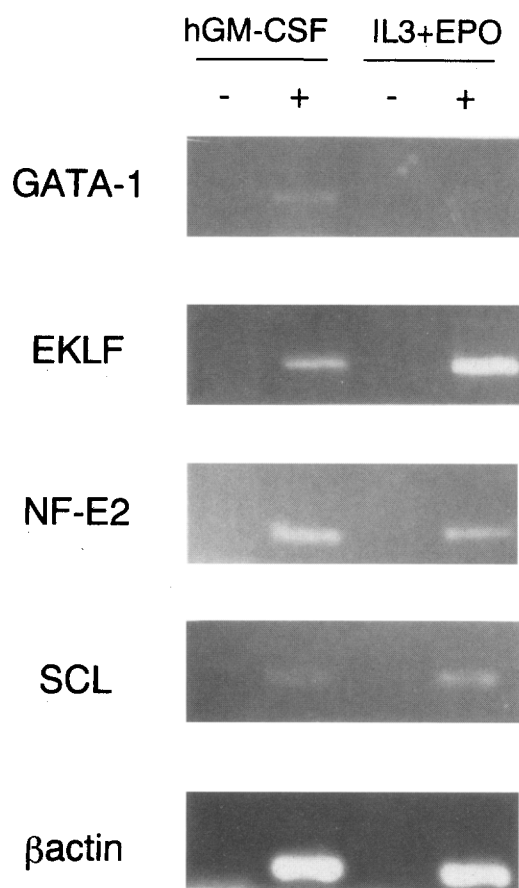


FIG. 3. RT-PCR analysis of the expression of erythroid-specific transcription factors in BFU-E colonies. Each BFU-E colony was picked up with a micropipette from cultures with hGM-CSF or IL-3 plus erythropoietin. The lane marked with a minus sign is the PCR product obtained with mock complementary DNA (no reverse transcriptase in the complementary DNA synthesis reaction).

hGM-CSF stimulates colony formation by bone marrow cells from transgenic mice

To characterize the functional properties of cells that expressed the hGM-CSF receptor, the effects of hGM-CSF on *in vitro* colony formation were examined by use of bone marrow cells derived from transgenic mice and a methylcellulose colony-forming assay. We first examined the concentration dependence of the response of total colony formation to hGM-CSF. We found that hGM-CSF stimulated colony formation in a concentration-dependent manner and that the number of colonies was maximal at 10 ng/ml.³ Therefore, hGM-CSF was used at 10 ng/ml in subsequent studies. In contrast, hGM-CSF did not stimulate colony formation by bone marrow cells from normal littermates of the mice.

hGM-CSF supports development of erythroid cells from transgenic mice

We examined the effects of hGM-CSF on colony numbers and the types of colonies derived from bone

TABLE I. Peripheral blood cells in transgenic mice given injections of hGM-CSF for 1 or 2 weeks

Dose of hGM-CSF	0 ng	1 week	2 weeks
		500 ng	500 ng
WBC ($\times 10^3/\mu\text{l}$)	3.2 ± 0.8	24.5 ± 8.6	46.4 ± 4.6
Differential count (%)			
Neutrophils (Stab)	1.8 ± 0.9	1.0 ± 1.1	2.8 ± 0.8
(Seg)	30.5 ± 4.0	13.7 ± 8.2	22.3 ± 2.4
Lymphocytes	66.5 ± 3.7	86.1 ± 4.7	68.0 ± 3.3
Eosinophils	0.5 ± 0.4	0.8 ± 0.6	5.7 ± 2.8
Monocytes	0.8 ± 0.7	1.6 ± 0.8	1.2 ± 0.3
RBC ($\times 10^5/\mu\text{l}$)	85.8 ± 3.1	88.0 ± 5.6	$95.8 \pm 4.0^*$
Hematocrit (%)	43.9 ± 2.7	49.0 ± 3.1	$51.4 \pm 4.0^*$
Hemoglobin concentration (g/dl)	14.6 ± 0.6	14.6 ± 0.8	$17.4 \pm 1.2^*$
Reticulocytes (% of total RBC)	2.4 ± 0.9	8.6 ± 2.7	6.3 ± 1.6
Platelets ($\times 10^3/\mu\text{l}$)	992.7 ± 86.0	433.6 ± 128.8	710.5 ± 84.1

*The RBC counts, hematocrit, and hemoglobin concentration showed significant changes after 2 weeks of hGM-CSF treatment ($p < 0.01$).

marrow cells. Human GM-CSF supported the formation of various types of colonies, including GM, eosinophil, mast cell, megakaryocyte, BFU-E, erythrocyte colony-forming unit (CFU-E), blast cell, and mixed hematopoietic colonies, whereas mouse GM-CSF yielded only GM colonies (Fig. 1). The effect of hGM-CSF more closely resembled that of mouse IL-3 than that of mouse GM-CSF. It should be noted, however, that as many as 60% of mixed colonies formed by hGM-CSF contained erythrocytes in the absence of erythropoietin. It was interesting that hGM-CSF alone also supported the proliferation of both BFU-E and CFU-E colonies, which are respectively derived from early and late progenitors restricted to the erythroid lineage (Fig. 2A). In contrast, mouse IL-3 alone did not support erythroid colonies (Fig. 1). To rule out the possibility that hGM-CSF induced endogenous production of erythropoietin, we added anti-erythropoietin antibody to cultures. Addition of this antibody did not reduce the number of CFU-E colonies produced by hGM-CSF, whereas the antibody completely suppressed formation of CFU-E colonies in response to mouse IL-3 and erythropoietin (Fig. 2B). The erythroid colonies were confirmed to express messenger RNA for erythroid-specific transcription factors (Fig. 3).

We also performed serum-free culture of bone marrow cells to exclude the possibility that the effects of hGM-CSF were related to a combined action with unknown factors contained in FCS. The results obtained were similar to those in the presence of 30% FCS.³

In vivo hGM-CSF treatment increases circulating white blood cells and reticulocytes

We characterized the *in vivo* effects of hGM-CSF on hematopoiesis among transgenic mice.⁷ Transgenic mice

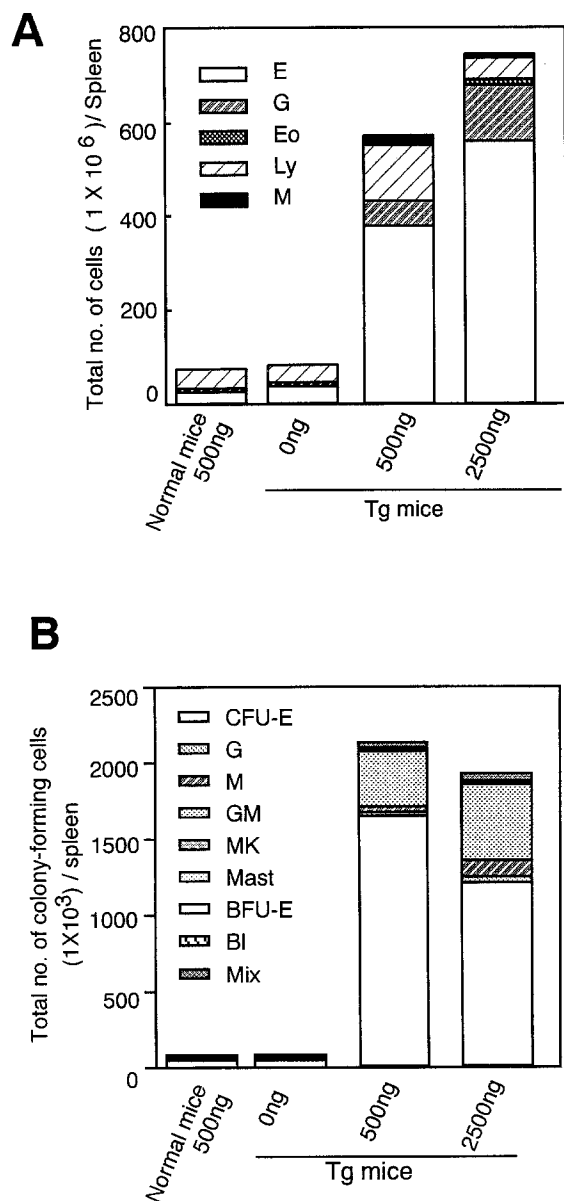


FIG. 4. Dose-response effects of hGM-CSF on the number of constituent cells (**A**) and hematopoietic progenitors (**B**) in spleens of transgenic mice as assayed by means of methylcellulose clonal culture. Treatment with hGM-CSF increased the number of both immature and mature erythroid cells. (From Nishijima I et al. Blood 1997;90:1031-8.) See Fig. 1 for abbreviations.

and their littermates were given injections of 20 to 2500 ng of hGM-CSF or phosphate-buffered saline solution containing serum twice a day for 7 days. After this treatment, both the transgenic mice and the littermates remained healthy. Examination of peripheral blood on day 8 showed that the white blood cell count increased in transgenic mice, but not in the littermates, in accordance with the dose of hGM-CSF injected. Transgenic mice given injections of 500 ng of hGM-CSF showed a sevenfold increase in white blood cells (Table I).

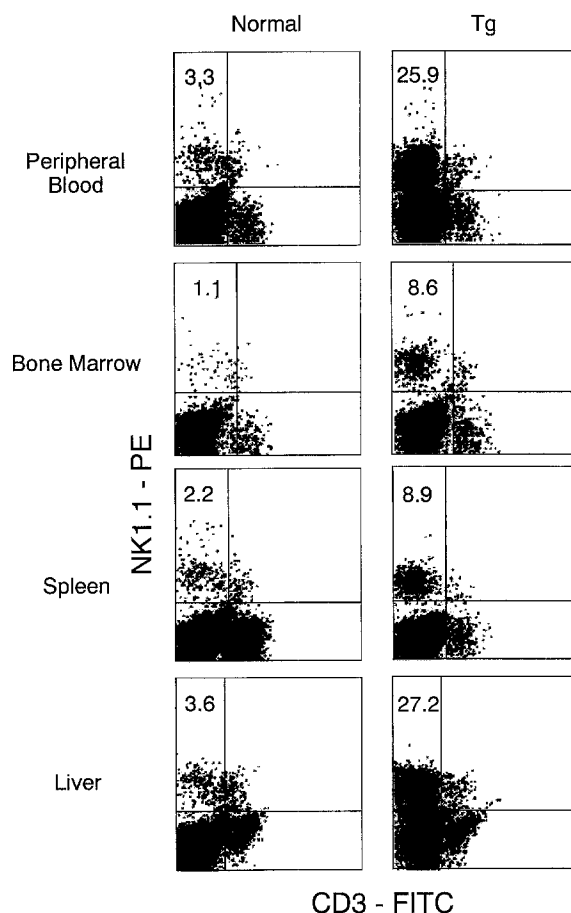


FIG. 5. Findings from flow cytometric analysis of cells from peripheral blood, bone marrow, spleen, and liver of normal littermates (*left*) and transgenic mice (*right*) treated with 500 ng hGM-CSF. Cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD3 and phycoerythrin (PE)-labeled anti-NK1.1. Number of CD3⁺ NK1.1⁺ cells increased in peripheral blood, bone marrow, spleen, and liver of transgenic mice treated with hGM-CSF. (From Nishijima I et al. Blood 1997;90:1031-8.)

Morphologic estimation of absolute numbers showed that numbers of granulocytes, eosinophils, monocytes, and lymphocytes were increased, lymphocytes showing the most statistically significant increase. The ratio and absolute number of reticulocytes increased approximately threefold in transgenic mice with administration of 500 ng or more of hGM-CSF. Red blood cell count, hematocrit, and hemoglobin concentration showed no statistically significant changes after 1 week of hGM-CSF injections, but the numbers increased by 10% to 20% after 2 weeks of hGM-CSF treatment ($p < 0.01$) (Table I). Thus hGM-CSF also increased the red blood cell count, but the change was slower than the change in white blood cell count. The platelet count decreased by as much as 50% in transgenic mice after injection of 500 ng of hGM-CSF. Because the platelet count did not decrease when we injected hGM-CSF into splenectomized transgenic mice (un-

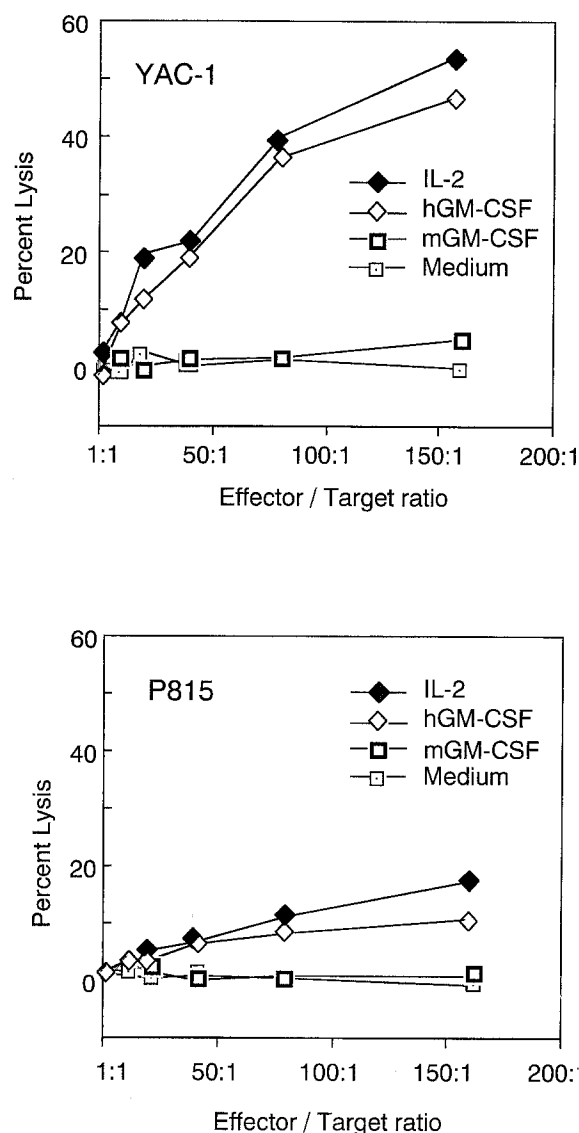


FIG. 6. Cytotoxicity of spleen mononuclear cells from transgenic mice induced by hGM-CSF. Target cells used in the cytotoxicity assay were NK-resistant P815 cells and NK-sensitive YAC-1 cells. The cytotoxicity of peripheral NK cells was induced by hGM-CSF.

published observation), this decrease in circulating platelets may be explained with promotion of their destruction in the spleen.

In vivo hGM-CSF treatment increases erythroid cells in the spleen

After 1 week of hGM-CSF treatment, splenomegaly was prominent among the transgenic mice.⁷ The spleen weight of transgenic mice given injections of 500 ng or 2500 ng of hGM-CSF increased six- to ninefold over that of transgenic mice given phosphate-buffered saline solution containing serum or normal littermates treated with 500 ng of hGM-CSF.⁷ The number of spleen cells increased in

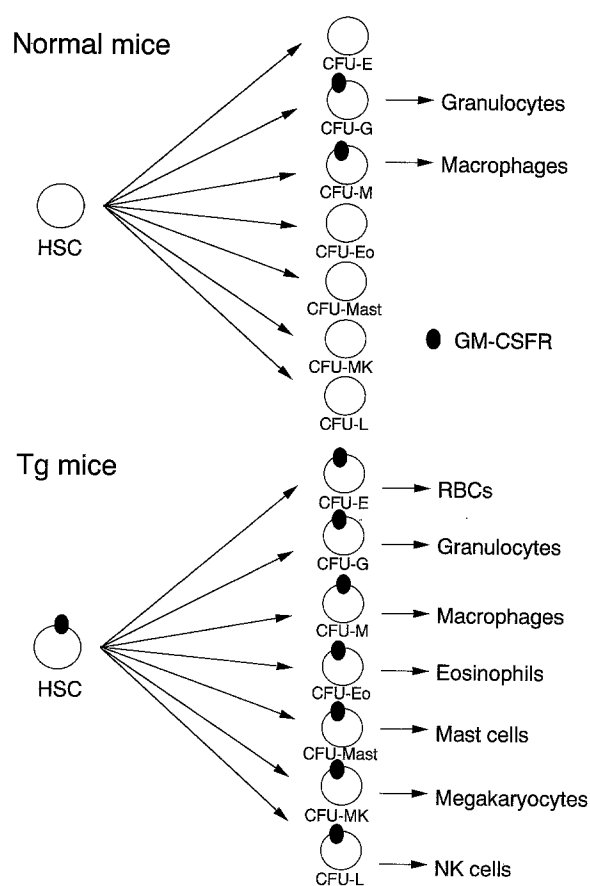


FIG. 7. Two models that illustrate the possible relation between receptor expression and differentiation of hematopoietic cells. The results of in vitro and in vivo studies indicate that the differentiation of hematopoietic progenitor cells is not induced by exogenous cytokine stimulation (instruction model) but rather is determined by the intrinsic cell program with cytokines simply selecting cells that express the appropriate receptor (selection model). *HSC*, hematopoietic stem cell; *Tg*, transgenic.

parallel with the weight (Fig. 4A). Morphologic examination showed that numbers of all types of blood cells increased to various extents, but the most statistically significant increase was in the number of erythroid cells: approximately 150- to 200-fold after administration of 500 ng or more of hGM-CSF. All types of hematopoietic progenitors increased after injection of 500 ng or more of hGM-CSF (Fig. 4B). In particular, CFU-E (late erythroid progenitors) expanded approximately 150- to 200-fold.

In vivo hGM-CSF treatment decreases the number of CD4⁺ CD8⁺ double-positive cells in the thymus

Although the number of CD4⁺ and CD8⁺ single-positive cells in the peripheral blood of transgenic mice increased after administration of hGM-CSF, the number of thymic T cells decreased in a statistically significant

way. Histologic examination of thymic tissues showed that the cortex and the medulla had undergone marked involution in transgenic mice given injections of 500 ng of hGM-CSF compared with those given phosphate-buffered saline solution containing serum.⁷ Flow cytometric analysis indicated that the number of CD4⁺ CD8⁺ double-positive cells decreased in the thymus after injection of 500 ng of hGM-CSF (see Yasuda et al. in this supplement^{16a}). We found that CD4⁻ CD8⁻ double-negative cells and CD4⁺ or CD8⁺ single-positive cells, but not CD4⁺ CD8⁺ double-positive cells, from the thymus of transgenic mice could proliferate in culture medium containing hGM-CSF. However, addition of hGM-CSF to a transgenic mouse fetal thymic organ culture system resulted in inhibition of the differentiation of CD4⁻ CD8⁻ double-negative cells into double-positive cells.¹⁷ Taken together with these in vitro observations, the results suggest that hGM-CSF inhibits in vivo thymic differentiation of CD4⁻ CD8⁻ double-negative cells into CD4⁺ CD8⁺ double-positive cells but stimulates the growth of mature CD4⁺ or CD8⁺ single-positive cells, resulting in an increase of T cells in the peripheral blood.

In vivo hGM-CSF treatment increases NK1.1-positive cells in peripheral hematopoietic organs

An interesting finding was that the peripheral blood of transgenic mice treated with hGM-CSF contained a statistically significant number of large granular lymphocytes (LGLs), whereas only a few LGLs were detected in the blood of littermates given injections of 500 ng of hGM-CSF or transgenic mice given injections of phosphate-buffered saline solution containing serum. The percentage and absolute number of LGLs in the peripheral blood of transgenic mice increased in a dose-dependent manner with injection of hGM-CSF. It is generally accepted that LGLs are typical morphologic features of NK cells or $\gamma\delta$ T cells. With an anti-mouse NK1.1 antibody in combination with an anti-CD3 antibody, we investigated whether these LGLs possess the phenotypic characteristics of NK cells. Because C3H/HeN transgenic mice do not possess NK1.1 antigen,¹⁸ flow cytometric analysis for the detection of NK1.1⁺ cells was performed with newly generated transgenic mice derived from the (C57B1/6J $\times$ C3H/HeN) F₃ strain. These transgenic mice showed similar peripheral blood changes to those of C3H/HeN transgenic mice given hGM-CSF. The white blood cell count increased and there was a high proportion of LGLs. Flow cytometric analysis showed that the number of CD3⁻ NK1.1⁺ cells increased in a statistically significant way in the peripheral blood, bone marrow, spleen, and liver (Fig. 5) but not in the thymus of transgenic mice treated with 500 ng of hGM-CSF compared with their littermates.⁷ The percentage of CD3⁻ NK1.1⁺ cells was similar in all samples examined, suggesting that this represented the same NK cell population. Furthermore, most of the FACS-sorted CD3⁻ NK1.1⁺ cells were morpho-

logically identical to LGLs in peripheral blood smear preparations, suggesting that hGM-CSF induced in vivo expansion of NK cells.

DISCUSSION

We generated transgenic mice that constitutive expressed the hGM-CSF receptor and analyzed hGM-CSF-dependent in vitro colony formation by hematopoietic cells and the in vivo response to hGM-CSF treatment. Our in vitro results indicated that unlike mouse GM-CSF, which only acts on cells of the GM lineages, hGM-CSF stimulates early progenitors as well as lineage-committed progenitors that express the hGM-CSF receptor. An interesting finding was that erythroid colony formation, which normally requires the erythropoietin receptor signal, was supported by hGM-CSF in the absence of erythropoietin. Thus it appears that the hGM-CSF receptor signal has the capability to support proliferation of cells of the erythroid lineage. We also found that hGM-CSF induced in vivo expansion of various types of blood cells and their progenitors that express the hGM-CSF receptor, thereby supporting our in vitro observations. Furthermore, the development of lymphoid cells, including T and NK cells in transgenic mice, was affected by hGM-CSF treatment. It has been proposed that NK cells can differentiate from bone marrow cells in culture medium containing IL-2.¹⁹ Peripheral NK cells proliferate and gain cytotoxic activity in the presence of IL-2,²⁰ and hGM-CSF can induce cytotoxicity of peripheral NK cells (Fig. 6). It is tempting to speculate that hGM-CSF has the potential to substitute for IL-2 and stimulate NK progenitor cells in our transgenic mouse system.

These in vitro and in vivo results indicate that hGM-CSF does not substantially alter the developmental program of hematopoietic progenitors but instead promotes the proliferation of cells that express its receptor. This implies that the differentiation of hematopoietic progenitor cells is not induced by cytokines (instruction model) but rather is determined by the intrinsic programming of the cell. It also implies that cytokines support simply selected cells that express the appropriate receptor (stochastic model) (Fig. 7). This view also is supported by findings concerning the expression of receptors for more lineage-restricted cytokines, such as the erythropoietin receptor,^{21,22} macrophage colony-stimulating factor receptor,²³ and IL-5 receptor.²⁴

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