

Differential expression of granulocyte-macrophage colony-stimulating factor and IL-3 receptor subunits on human CD34⁺ cells and leukemic cell lines

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Cytokines transduce their signals through specific receptors. Receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and IL-5 share the common signal transducing subunit (β c), whereas the α subunits function as specific ligand binding components. In this study we prepared specific mouse monoclonal antibodies against human GM-CSF receptor- α subunit (hGMR α) by immunizing mice with Ba/F3 cells transfected with hGMR α complementary DNA. Using these anti-hGMR α antibodies in combination with antibodies against IL-3 receptor- α (IL-3R α), β c subunits, and c-kit, we examined expression patterns and modulation of these receptor subunits on several human hematopoietic cells, including CD34⁺ cells and leukemic cell lines. GMR α and IL-3R α were expressed on GM-CSF- and IL-3-responsive cell lines, such as TF-1 and UT-7, whereas the expression levels were much lower on UT-7E, a GM-CSF- and IL-3-unresponsive subline of UT-7. The GMR α subunit was expressed only on mature granulocytes and monocytes, and IL-3R α was expressed on monocytes but not on mature granulocytes, and none of these subunits were expressed on lymphocytes. For CD34⁺ cells, GMR α was expressed more abundantly on CD34⁺CD33^{high} cells than on CD34⁺CD33^{low} cells, whereas IL-3R α was expressed more abundantly on CD34⁺CD33^{low} cells than on CD34⁺CD33^{high} and CD34⁺CD33^{neg} cells. Slight but significant expression of the β c subunit was detected on CD34⁺ cells. Expression of not only GMR α and IL-3R α subunits but also c-kit was specifically downregulated by 48-hour incubation with their respective ligands. Receptor transmodulation between GM-CSF, IL-3, and stem cell factor (or kit ligand) was not detected on CD34⁺ cells in 48-hour cultures. We also detected upregulation of these α subunits by IL-1 α and interferon- γ on leukemic cell lines. Our study showed expression levels for each receptor subunit—including GMR, IL-3R, and c-kit on human bone marrow and peripheral blood cells and leukemic cell lines—and revealed differential regulation of the expression of the receptor subunits. (J ALLERGY CLIN IMMUNOL 1995;96:1083-99.)

Key words: GM-CSF receptor, IL-3 receptor, c-kit, CD34, anti-receptor antibody, hematopoietic stem cell

The hematopoietic system is a complex system involving proliferation and differentiation of cells and is regulated by a network of cytokines.¹⁻⁵

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Among these cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 play important roles in regulating both proliferation and differentiation of hematopoietic precursor cells. IL-3 acts on the early progenitors, such as multipotent (CFU-GEMM), early erythroid (BFU-E), granulocyte-macrophage (CFU-GM), and megakaryocyte (CFU-Meg) progenitors, whereas GM-CSF exerts similar effects but acts on more differentiated progenitors than IL-3.^{6, 7} On the other hand, stem cell factor (SCF) is also indispensable for stromal cell-dependent proliferation of hematopoietic stem cells, as well as erythroid and mast cell precursors.⁶ It has been asserted that SCF plays a permissive role for

Abbreviations used

β c:	Common β chain
DMEM:	Dulbecco's modified eagle medium
DMSO:	Dimethyl sulfoxide
Epo:	Erythropoietin
FCS:	Fetal calf serum
FITC:	Fluorescein isothiocyanate
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
GMR α :	GM-CSF receptor- α
IFN- γ :	Interferon- γ
IL-3R α :	IL-3 receptor- α
IMDM:	Iscove's modified DMEM
mAb:	Monoclonal antibody
PB:	Peripheral blood
SBA:	Soy bean agglutinin
SCF:	Stem cell factor

growth in the early stage of multipotential progenitors and potentiates the stimulatory activities of other cytokines. Other hematopoietic growth factors, including G-CSF, IL-6, and IL-11, aside from their cell lineage-specific effects, enhance the effects of GM-CSF, IL-3, and SCF by shortening the G_0 phase of the cell cycle.^{6,8-10}

Molecular cloning of cytokine receptors¹¹⁻¹⁴ has revealed that in human and mouse systems, GM-CSF receptor (GMR), IL-3R, and IL-5R are composed of $\alpha\beta$ heterodimers, sharing a common β (β c) subunit.^{4,5,15,16}

Until recently, the examination of expressions and functions of hematopoietic growth factor receptors on hematopoietic progenitor cells has been hindered because of the lack of purification techniques for these cells and specific antibodies. Recently, several techniques have been established for the purification of hematopoietic progenitors, such as fluorescence-activated cell sorting,¹⁷⁻¹⁹ immunomagnetic beads, and panning with antibody-coated plate methods.^{20,21} Thus the direct assessment of the expression of cytokine receptors on these hematopoietic progenitor cells has become possible. In murine systems Thy-1^{low}, Lin⁻, Sca-1⁺ cells were identified as candidates for hematopoietic stem cells,¹⁷ whereas in human systems CD34⁺CD33⁻ cells contain hematopoietic stem cell populations.^{18,19} Although the action of cytokines on early hematopoietic progenitors has been extensively investigated, little is known about the expression of cytokine receptors on these early progenitor cells and their regulatory mechanism.

Therefore it seems important to examine the expression patterns and transmodulation of these cytokine receptors on such early hematopoietic progenitor cells.

In this study we prepared specific monoclonal antibodies (mAbs) against hGMR α and examined the expression of GMR α , IL-3R α , and β c subunits and c-kit on human leukemic cell lines, normal peripheral blood (PB) and bone marrow cells; we also examined the relationship between their expression and responsiveness to the ligands and the up- and downmodulation by their own ligands and several other factors. We showed that purified CD34⁺ cells elicited differentially regulated expression patterns of GMR α and IL-3R β subunits in relation to expression of differentiation antigen and under the influence of several cytokines.

METHODS

Cell lines, media, and cytokines

The murine IL-3-dependent cell line, BA/F3²² and its derivatives were cultured in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum, (FCS), 50 mmol/L 2-mercaptoethanol, and 100 U/ml murine IL-3 (mIL-3).²³ BA/F3- α is a BA/F3 transformant expressing the α subunit of human GM-CSF receptor (hGMR α).¹⁵ Murine NIH3T3 cells and their derivatives were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. NIH3T3- α is an NIH3T3 transformant expressing hGMR α .¹⁵ A multifactor-dependent human hematopoietic cell line, TF-1²⁴ (provided by Dr. Kitamura; DNAX Research Institute, Calif.), was maintained in RPMI-1640 medium supplemented with 10% FCS and 1 ng/ml hGM-CSF. A human megakaryoblastic leukemia cell line UT-7 and its subline UT-7E cells were provided by Dr. Komatsu (Jichi Medical School, Tochigi, Japan).^{25,26} UT-7 cells were cultured in Iscove's modified DMEM (IMDM) supplemented with 10% FCS with 1 ng/ml hGM-CSF. UT-7E cells were established through passage by culturing UT-7 cells with human erythropoietin (Epo), and they lost responsiveness to hGM-CSF and hIL-3 but responded well to human Epo.²⁶ They were cultured in IMDM supplemented with 10% FCS and 1 U/ml human Epo. EOL3, U937, HL60, and K562 cells were cultured in RPMI-1640 medium supplemented with 10% FCS. Recombinant mIL-3 (rmIL-3) was produced in the silkworm,²³ and rhGM-CSF was kindly provided by Dr. Kastelein. Recombinant human SCF was kindly provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Recombinant human Epo was a gift from Amgen (Thousand Oaks, Calif.), rhIL-3 from Sandoz Pharmaceutical Co. Ltd., rhIL-1 α from Ohtsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). Interferon- γ (IFN- γ) (Immunomax) was purchased from Shionogi Pharmaceutical Co. Ltd (Tokyo, Japan).

Generation of mAbs

BALB/c mice were immunized with BA/F3- α cells by injection into their foot pads.²⁷ The regional (popliteal) lymph node cells or splenic cells were fused with the murine myeloma cell line PAI at a 10:1 ratio with electrofusion (high-voltage cell fusion apparatus; BM-850; Kurabo) or the polyethylene glycol method. The hybridoma cells were selected with hypoxanthine, aminopterin, and thymidine-containing medium.²⁸⁻³⁰ Supernatants of hybridomas were screened by flow cytometry as described below. Hybridomas, which produced antibodies that bound to BA/F3- α but not to BA/F3 cells, were selected and cloned. They were adapted to RPMI-1640 medium supplemented with 10% FCS and 10% bone marrow-conditioned H1 (Boehringer-Mannheim, Mannheim, Germany) and re-cloned by using the limiting dilution method. They were rescreened for their reactivity to NIH3T3- α but not to NIH3T3 cells. Three clones (GMA1, GMA2, and GMA3) were obtained from popliteal lymph node cell hybridomas, and one clone (GMA4) was obtained from splenic cell hybridomas. Antibodies were purified by using 50% ammonium sulfate fractionation, followed by protein A-affinity column purification (AffiGel Protein A MAPS II kit; Bio-Rad Laboratories, Life Science Group, Hercules, Calif.). Subclasses of the mAbs were determined by using the monoclonal typing kit (Mouse Ab-STAT; Funakoshi, Japan). All of the antibodies (GMA1 to GMA4) were classified as IgG₁.

Screening of hybridoma supernatants with flow cytometry

Cells (1 to 5×10^5) were washed with phosphate-buffered saline containing 5% FCS and 0.1% sodium azide (staining buffer) and incubated with 50 μ l of hybridoma supernatant for 60 minutes at 4°C. Then, after the cells were pelleted and washed with staining buffer, they were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-murine IgG and FITC-conjugated anti-murine IgM antibodies (Sigma Chemical, St. Louis, Mo.) for 45 minutes at 4°C. Cells were then washed three times with staining buffer. Cells incubated without hybridoma supernatant but with fresh hybridoma culture media were used as negative controls. Fluorescence intensity was measured with an EPICS cell sorter (Coulter Corp., Miami, Fla.) immediately after staining.

Other mAbs

Anti-human IL-3R α chain antibody (N3A) and anti-human β c antibodies (CRS1, 2, and 3) were prepared as previously reported.^{29,30} Anti-c-kit antibody (ACK-2) was provided by Dr. S. Nishikawa (Kyoto University), which was prepared against murine c-kit but was shown to cross-react with human c-kit.³¹ HPCA-1 (CD34; IgG₁ κ) and phycoerythrin-conjugated Leu-M9 (CD33; IgG1 κ) were purchased from Becton-Dickinson Immunocytometry Systems (San Jose, Calif.). Phycoerythrin-

conjugated HLA-DR class II (IOT 2a; IgG2b- κ) was purchased from Immunotech S.A. (Marseille, France). The second antibodies, FITC-conjugated anti-mouse IgG and FITC-conjugated anti-rat IgG, were purchased from Zymed (South San Francisco, Calif.).

Preparation of PB and bone marrow cells

Heparinized PB and bone marrow samples were obtained from healthy human volunteers, after their informed consent had been obtained. PB and bone marrow leukocytes were prepared by using 1.2% dextran-saline sedimentation, followed by washing with phosphate-buffered saline containing 10% FCS and 0.1% sodium azide. Cells were pelleted for staining with the monoclonal anti-receptor antibodies.

Flow cytometric analysis

Separated blood cells were suspended in phosphate-buffered saline containing 10 mg/ml human γ -globulin (Beriglobin P; Hoechst, Japan), 5% FCS, and 0.1% sodium azide, incubated on ice for 15 minutes to block binding to Fc receptors and nonspecific sites. They were then pelleted and incubated with each anti-receptor antibody (3 μ g/ml) for 60 minutes on ice, washed twice, and subsequently treated with FITC-conjugated second antibodies (5 μ g/ml) for 45 minutes on ice. After washing twice, cells were analyzed with a FACScan (Becton-Dickinson Immunocytometry Systems). For negative controls, cells were stained with isotype-matched mouse IgG or rat IgG (Sigma) in place of anti-receptor antibodies. For double staining, cells were stained with the first antibodies, washed twice, blocked with about 300-fold molar excess (1 mg/ml) of mouse IgG, and then stained with the phycoerythrin-conjugated second antibodies.

Separation of CD34⁺ cells

Heparinized bone marrow cells were diluted 1:1 with IMDM and were suspended with a Pasteur pipette. The light density cell fraction was separated by using Histopaque (Sigma) density gradient centrifugation (400 g for 20 minutes). From the fraction, adherent cells and T lymphocytes were simultaneously depleted by using a modification of the soy bean agglutinin (SBA) method^{20,21} with SBA MicroCELLector flasks (Applied Immune Science, Inc., Menlo Park, Calif.). SBA^{neg} cells were allowed to adhere to the flasks coated with anti-CD34 antibody (Applied Immune Science, Inc.) for 60 minutes at room temperature and washed; and the CD34⁺ cells were recovered, according to the manufacturer's instructions. The separated cells were tested with another anti-CD34 antibody (My-10, Becton Dickinson) with flow cytometry, and it was ascertained that about 96% of them were CD34⁺.

Liquid culture

The separated bone marrow CD34⁺ cells were incubated in liquid culture for 48 hours at a starting concen-

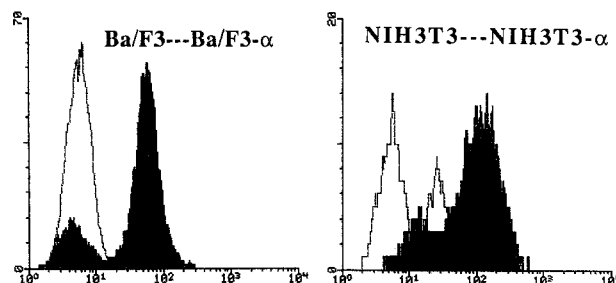


FIG. 1. Expression of hGMR α on BA/F3- α and NIH3T3- α cells. Cells were stained with anti-hGMR α mAb (GMA-1). Cells were incubated with 3 mg/ml anti-hGMR α antibody (GMA-1), washed, and incubated with 5 mg/ml of FITC-conjugated anti-mouse IgG or anti-rat IgG antibody (shaded area). For negative control (blank area), parental cells were stained with the same antibody.

tration of 10^6 cells/ml. The culture medium was composed of IMDM supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin, Sigma). The cultures were stimulated with or without growth factors, including rhGM-CSF (10 ng/ml), rhIL-3 (10 ng/ml), or rhSCF (100 ng/ml). The growth factors were added once at the start of the culture.

RESULTS

Establishment of hybridomas producing mAbs against hGMR α

Mice were immunized with BA/F3- α , BA/F3 cells stably transformed with the hGMR α cDNA, and then hybridomas were generated as described in the Methods section.

Thirty-four of 151 hybridomas obtained from the regional lymph node cells produced antibodies reacting with BA/F3- α cells. Seven of the 34 clones produced antibodies that did not react with original BA/F3 cells. The seven clones were re-cloned with the limiting dilution method, and the supernatants were re-screened with BA/F3, BA/F3- α , NIH3T3, and NIH3T3- α cells. Three appropriate clones (GMA-1, GMA-2, and GMA-3) were obtained from hybridomas of lymph node cells; and one clone (GMA-4) was obtained from hybridomas of spleen cells. Stable transformants with the hGMR α cDNA, BA/F3- α , and NIH3T3- α , revealed various degrees of fluorescence intensity with the antibodies, probably because of the different expression levels, whereas original cells showed background intensity (Fig. 1). Then we used these antibodies as specific anti-hGMR α antibodies. All of the four mAbs bound almost equally to BA/F3- α cells and NIH3T3- α cells. The fluorescence intensities detected on BA/F3- α cells are higher than those detected on NIH3T3- α cells, probably because of the larger numbers of recep-

tors expressed on the former cells. These antibodies did not neutralize GM-CSF-dependent growth of TF-1 (data not shown).

Expression of GMR α and other receptor subunits on leukemic cell lines

Anti-GMR α antibody (GMA-1), in combination with anti-IL-3R α (N3A), anti- β c (CRS-1), and anti-c-kit (ACK-2) antibodies, was used to examine the expression of cytokine receptors on several human leukemic cell lines including TF-1, UT-7, UT-7E, EOL3, HL-60, U937, and K562 cells (Fig. 2). The levels of GMR α expression varied among these leukemic cell lines. Among growth factor-dependent human leukemic cell lines, both TF-1^{24,32} and UT-7 cells expressed all of the four receptor subunits including GMR α , IL-3R α , β c, and c-kit. The expression levels of GMR α and c-kit on UT-7 cells were higher than those on TF-1 cells.²⁵ In contrast, UT-7E cells, a GM-CSF- and IL-3-unresponsive subline derived from UT-7 cells, expressed much lower levels of the four receptor subunits. Among growth factor-independent cell lines, EOL-3 cells expressed high levels of GMR α and IL-3R α , whereas HL60 and U937³² cells expressed only detectable levels of GMR α . On the other hand, K562 cells did not express detectable levels of any of these four receptor subunits, in accordance with the finding that they did not respond to these cytokines.

Cytokines induce concentration-dependent downregulation of their corresponding receptor subunits

TF-1 and UT-7 cells were stimulated for 24 hours with a panel of different cytokines, including GM-CSF (1 ng/ml or 10 ng/ml), IL-3 (10 ng/ml), SCF (100 ng/ml), and Epo (20 U/ml). As shown

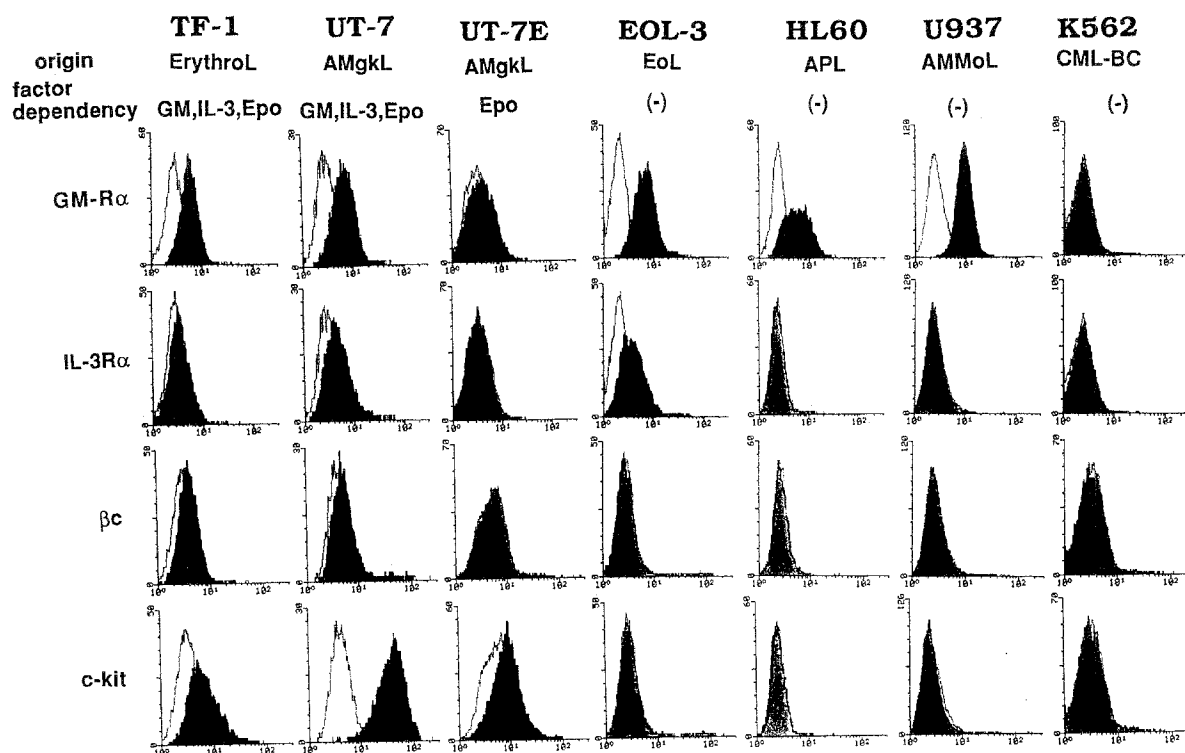


FIG. 2. Expression of GMR α , IL-3R α , β c, and c-kit on human leukemic cell lines. Cells were incubated with 10 mg/ml human γ -globulin for blocking of Fc receptors, then with 3 μ g/ml anti-receptor antibodies, washed, and incubated with 5 μ g/ml FITC-conjugated anti-mouse IgG or anti-rat IgG antibody (*shaded area*). For negative control (*blank area*), cells were stained with the same isotype murine IgG or rat IgG in place of anti-receptor antibodies. Stained cells were analyzed on FACScan.

in a representative experiment with UT-7 cells (Fig. 3), a high concentration of GM-CSF induced downregulation of GMR α but did not induce significant changes in IL-3R α or c-kit expression. On the other hand, a high concentration of IL-3 (10 ng/ml) induced reduction in expression of IL-3R α but did not induce significant changes in expression of the other three subunits. SCF (100 ng/ml) markedly downregulated the expression of c-kit and slightly downregulated IL-3R α expression, although it did not affect the expression of GMR α and β c. Epo slightly downregulated IL-3R α expression, but did not affect the expression of the other three subunits.

Regulation of GMR α expression by other cytokines

According to the previous reports, IL-1 α augments the proliferative response of TF-1 cells to GM-CSF through upregulation of high-affinity receptors.^{29, 33} We examined the effects of IL-1 α and IFN- γ on the expression of these four subunits. Both of them markedly increased expression of

GMR α and c-kit and slightly increased expression of β c and IL-3R α subunits (Fig. 4).

Expression of GMR, IL-3R subunits, and c-kit receptor on PB and bone marrow cells

The dextran-sedimented leukocytes from normal PB and bone marrow cells were examined with the anti-receptor antibodies as described above. Fig. 5 shows the expression patterns of the receptor subunits on each population. Total bone marrow populations were subdivided into three populations, based on forward scatter and side scatter intensity on flow cytometric analysis. The G population was mainly composed of mature granulocytes, and the M population was composed of monocytes (and large blasts), whereas the L population was composed of lymphocytes (and small blasts). Cells of the G population expressed only the GMR α subunit and no detectable levels of IL-3R α and β c. The M population contained cells expressing GMR α and IL-3R α . Low levels of β c subunits were expressed on both G and M popu-

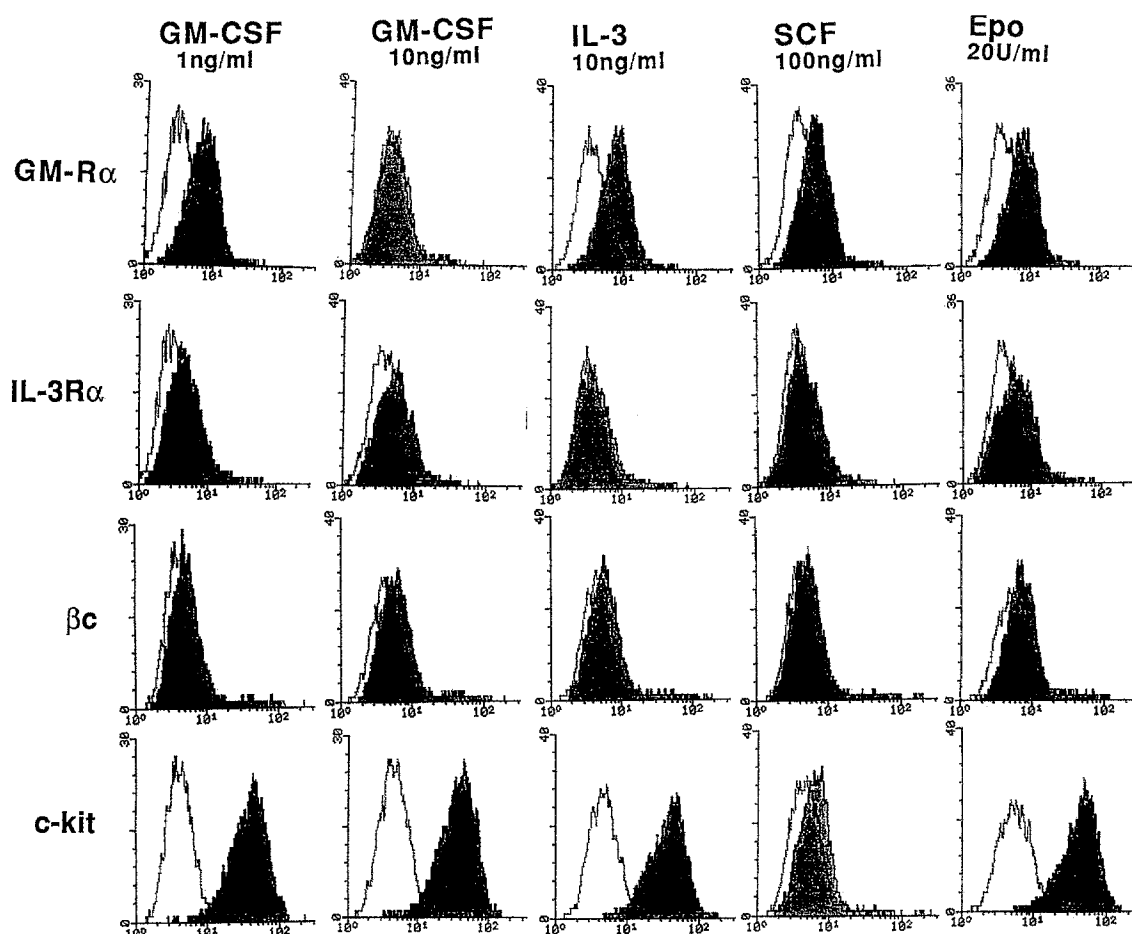


FIG. 3. Downregulation of GMR α , IL-3R α , and β c subunits and c-kit on UT-7 cells by cytokines. UT-7 cells were cultured with 1 ng/ml hGM-CSF. Cells were washed and incubated with the indicated cytokines for 24 hours. After culture, cells were stained as Fig. 2. Basic experiments were repeated four times, and similar results were obtained. The same experiments were done with TF-1 cells, and similar downmodulation was observed.

lation cells. Cells of the L population expressed none of these three receptor subunits.

Expression of receptor subunits on CD34⁺ cells

We examined the expression of these receptor subunits on early progenitor cells. CD34⁺ cells were purified from bone marrow mononuclear cells with anti-CD34 antibody-coated panning plates^{20, 21} and stained with anti-receptor antibodies. Samples from three normal volunteers exhibited similar patterns, and Fig. 6 shows a representative analysis. The separated SBA⁺CD34⁺ cells expressed a low to moderate level of CD34 and low to high levels of CD33 and c-kit. The purified CD34⁺ cell populations contain cells expressing GMR α , IL-3R α , and β c subunits. Fig. 7 shows the double-staining patterns of CD34⁺ cells with anti-

receptor antibodies and anti-CD33 antibody. Three populations of CD34⁺ cells could be distinguished on the basis of different expression levels of the GMR α subunit and CD33 antigens. The largest population of the CD33^{high} cells and the second largest population of CD33^{low to negative} cells expressed undetectable levels of GMR α . The third cell population was characterized by high CD33 expression and an intermediate to high density of GMR α ; only a few cells of the fourth population expressed a low level of CD33 and a detectable level of GMR α . As for the IL-3R α subunit expression, the major populations, which were CD33-positive or CD33-negative, expressed no detectable levels of IL-3R α . However, we detected one population of cells that expressed a high level of IL-3R α and a low to medium level of CD33. We also examined the expression of β c and detected

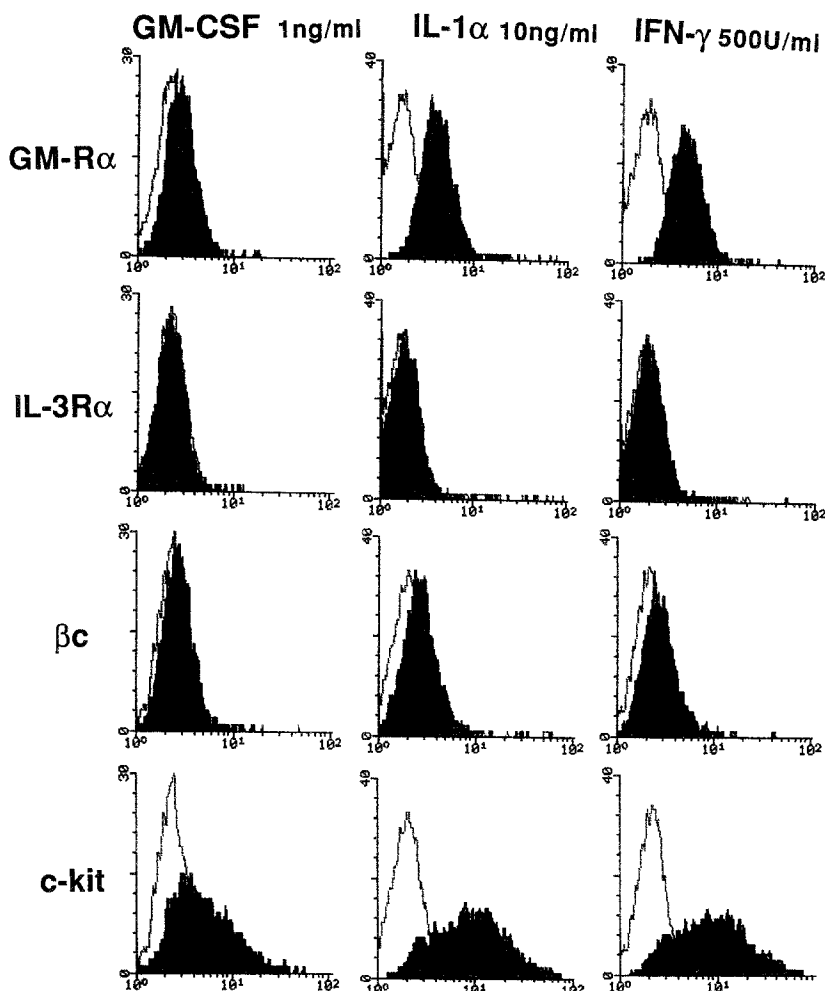


FIG. 4. Upregulation of receptor subunit on TF-1 cells by IL-1 α and IFN- γ . TF-1 cells were cultured with 1 ng/ml hGM-CSF. Cells were washed and incubated with IL-1 α (10 ng/ml) or IFN- γ (500 U/ml) for 24 hours. After culture, cells were stained as Fig. 2.

only a slight shift of fluorescent intensity compared with the negative control.

Furthermore, we divided the CD34⁺ cells into two populations on the basis of forward scatter and side scatter on the analysis program and analyzed the expression patterns of receptor subunits on each subpopulation. Fig. 8 shows that R1 (smaller sized CD34⁺) cells represent most of the CD33^{low to med} cells and contain most of the CD33^{med} GMR α ^{high} and CD33^{med} IL-3R α ^{high} populations. On the other hand, R2 (larger sized CD34⁺) cells represent most of the CD33^{high} population and contain most of the CD33^{high} GMR α ^{med} and CD33^{high} IL-3R α ^{med} populations. The differences in β c subunit expression between these populations were not as clear as those in expression of GMR α or IL-3R α subunits.

Effects of several cytokines on expression of GMR, IL-3R subunits, and c-kit receptor

Because CD34⁺ cells proliferate in response to IL-3, GM-CSF, and SCF, we examined the modulation of cytokine receptors by several cytokines (Fig. 9). The CD34⁺ cells were incubated with or without cytokines for 48 hours and then examined by using double staining with anti-receptor antibodies and anti-CD33 antibody. When incubated with 10 ng/ml GM-CSF, the relative proportion of CD33^{high} GMR α ^{med} population decreased and that of the GMR α ^{neg to low} population increased, whereas that of the CD33^{med} IL-3R α ^{high} population was only slightly decreased. On the other hand, when incubated with 10 ng/ml IL-3, the proportion of CD33^{high} GMR α ^{med} population increased only slightly, whereas almost all of the CD33^{med} IL-3R α ^{high} cells disappeared. When in-

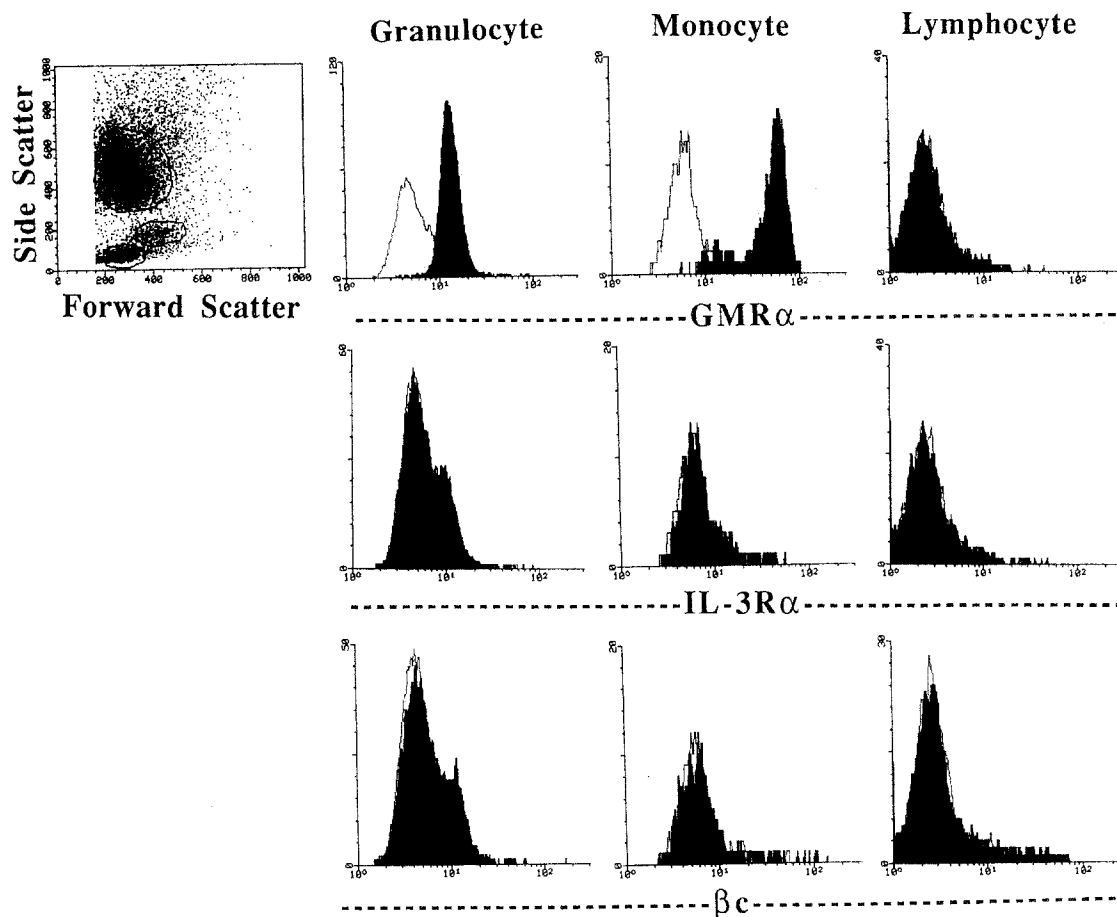


FIG. 5. Expression of GMR α , IL-3R α , and βc subunits on human bone marrow cells. Human bone marrow cells were sedimented with 1.5% dextran-saline, washed, and stained as in Fig. 2. Cell populations gated on the basis of forward scatter and side light scatter represent mature granulocytes, monocytes (and large blasts), and lymphocytes (and small blasts).

cubated with 10 ng/ml SCF, the proportions of both CD33^{high}GMR α ^{med} and CD33^{med}IL-3R α ^{high} populations were not affected, whereas the population of c-kit^{high} population decreased markedly (Fig. 9). The expression levels of βc decreased slightly when incubated with GM-CSF or IL-3 but were not affected when incubated with SCF. Furthermore, we could not detect any upregulation of receptor subunits in these conditions.

DISCUSSION

Detection of receptor subunits with specific mAbs

In this study we established specific mAbs against hGMR α , and in combination with anti-IL-3R α and βc antibodies,²⁸⁻³⁰ analyzed the expression patterns of these receptor subunits on several normal and leukemic hematopoietic cells with flow

cytometry. Despite the argument of some reports,^{34,35} the fluorescence intensities stained with these anti-receptor antibodies correlated well with numbers of receptor subunits and biologic responsiveness.^{32,33,36} TF-1 and UT-7 cells, on which we detected significant levels of GMR α , IL-3R α , and βc expression (Fig. 2), responded well to both GM-CSF and IL-3.^{25,32,33,36} The numbers of high-affinity and low-affinity GMRs expressed on TF-1 cells were 1700 and 9460 per cell as determined by Kitamura et al.³³ and 2000 and 8600 per cell as determined by Khwaja et al.³⁷ by binding assay, respectively. Our flow cytometric study showed that TF-1 cells expressed high fluorescent intensity of GMR α and low intensity of GMR β , compatible with results of these studies. Interestingly, the UT-7E cell line, a subline of UT-7, which had lost responsiveness to GM-CSF and IL-3,²⁶ expressed

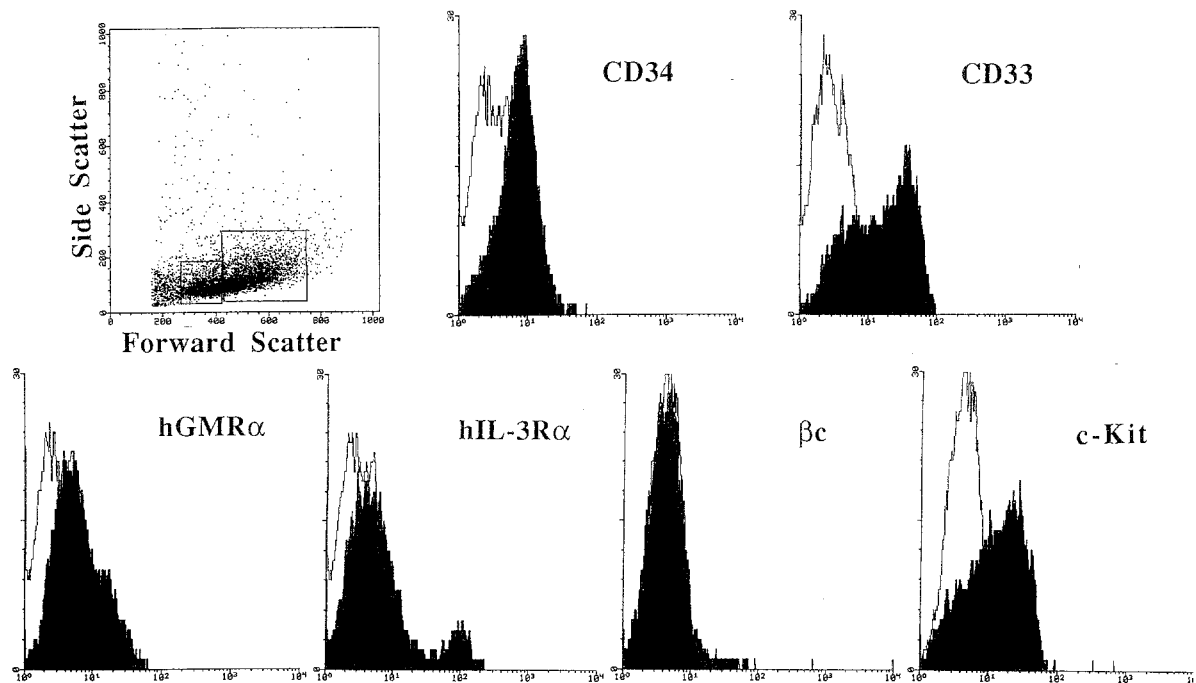


FIG. 6. Expression of GMR α , IL-3R α , β c, c-kit, and surface antigens on separated bone marrow CD34⁺ cells. Bone marrow cells were separated by the panning method. Light density cells on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, were selected for nonadherence on SBA-coated plates and adherence on an anti-CD34 antibody-coated plate. The purity of CD34⁺ cells was more than about 96%. CD34⁺ cells were distributed in the range of lymphocyte and monocyte fractions on the basis of forward scatter and side scatter. They were stained with anti-receptor antibodies as in Fig. 2.

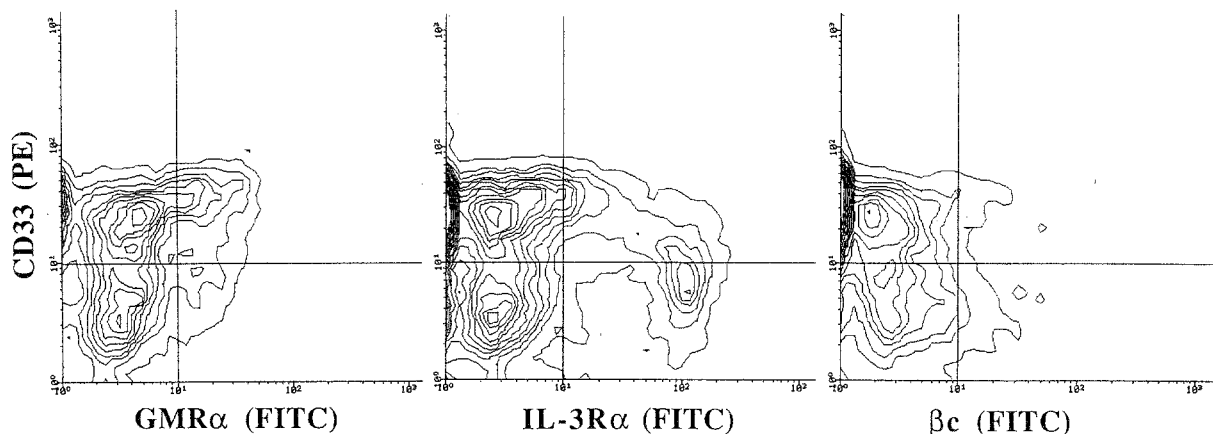


FIG. 7. Two-dimensional analysis of CD34⁺ cells for expression of GMR α , IL-3R α , and β c subunits and CD33 antigen. CD34⁺ cells were stained with anti-receptor antibodies as in Fig. 1., blocked with 300-fold excess (1 mg/ml) of mouse or rat IgG, and incubated with Phycoerythrin-conjugated anti-CD33 antibody. Cells were represented by 3% to 5% probability contour plotting. PE, Phycoerythrin.

much lower levels of GMR α , IL-3R α , and β c subunits than the parental UT-7 cells²⁵ (Fig. 2). We did not detect the expression of IL-3R α on HL-60 cells, but another study, in which the same

antibodies were used, revealed the expression of both GMR α and IL-3R α on HL60 cells (Yoshinari Watanabe. Personal communication, DNAX Research Institute, Calif.). These differences might

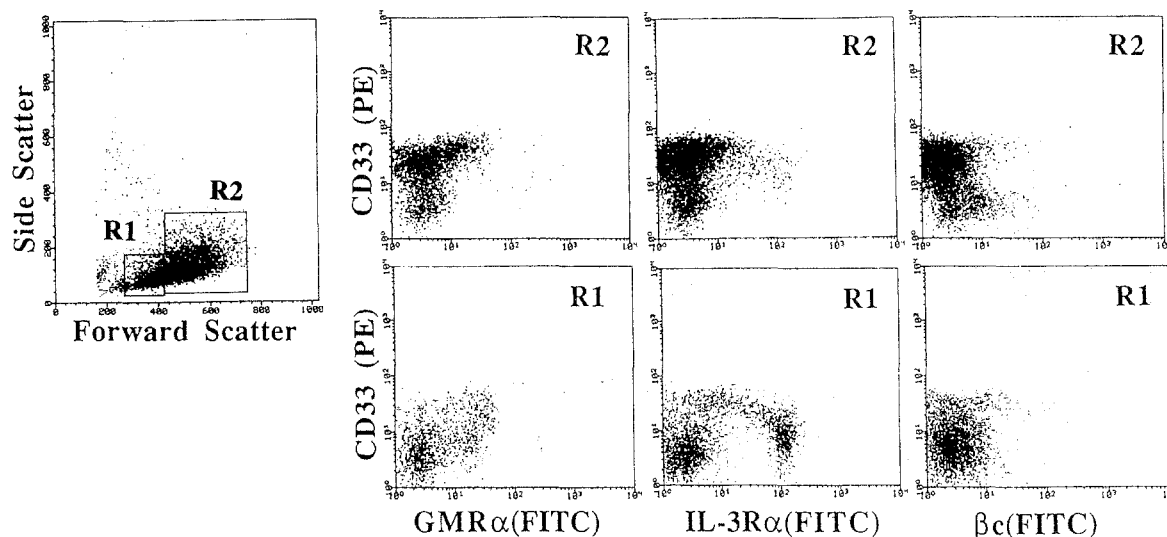


FIG. 8. Differential analysis of CD34⁺ cells on the basis of cell size. CD34⁺ cells, stained as in Fig. 7, were separated on the basis of forward scatter and side scatter. R1 and R2 represent smaller and larger size populations of CD34⁺ cells, respectively.

be explained by the heterogeneity of the HL60 sublines examined, which is often the problem on the repetitive passages and the frozen storage with dimethyl sulfoxide (DMSO).³⁸ Recently, the comparison between parental and DMSO-induced HL60 cells revealed that DMSO-induced, differentiated HL60 cells had larger numbers of GMRs but did not proliferate in response to GM-CSF.³⁹ There seem to be several stages of differentiation for HL60 cells with respect to receptor expression levels and responsiveness.

Receptor expression and signal transduction

Among cytokine receptor subfamilies, IL-3R, GMR, and IL-5R share a common β subunit. Unique α subunits constitute low-affinity receptors and are responsible for specific binding to the ligands,¹¹ whereas the β subunit alone does not bind the ligands but is essential for signal transduction.^{12, 40} Because the β subunit transduces signals in response to ligands, the functions of GMR and IL-3R are overlapping,⁴¹ and the differences in responsiveness to each ligand may be explained in part by the different distribution of each α subunit. It is also suggested that binding of these cytokines to their α subunits leads to tight association with the β subunits and then activation of signaling pathways through conformational changes of the β subunits⁴² (Muto et al. Unpublished data). Experiments with cytoplasmic deletion mutants of GMR revealed that distinct cytoplasmic regions of β are responsible for pro-

liferative response and activation of the *c-myc* gene, association and activation of JAK kinase families,⁴³ or activation of the Ras pathway, leading to induction of *c-fos/c-jun* genes.^{40, 44}

Differentiation lineages and receptor expression

As mentioned above, the target cells of GM-CSF and IL-3 are somewhat different in their lineages and differentiation stages. GM-CSF activates relatively more differentiated cells than IL-3,⁴⁵ whereas IL-3 is capable of stimulating megakaryocyte precursors but is incapable of enhancing neutrophil activation,⁴⁵ which is compatible with the different expression patterns of each α subunit. Our previous studies showed that β c and IL-3R α are expressed on various lineages of cells expressing myeloid differentiation markers, such as CD13, CD14, CD15, CD33, and CD34.³⁰ In this study GMR α expression was detected on mature granulocytes and monocytes but not on lymphocytes in PB and bone marrow cells, whereas IL-3R α was expressed only on monocytes in PB and bone marrow (Fig. 5). These receptor distributions on specific lineages coincide with their responsiveness to respective cytokines and binding studies.⁴⁵⁻⁴⁷ DiPersio et al.⁴⁷ showed that neutrophils express a single class of high-affinity GMR, although the reason that low-affinity GMRs were not detected on neutrophils remains unknown. IL-3R α was not detected on neutrophils, which

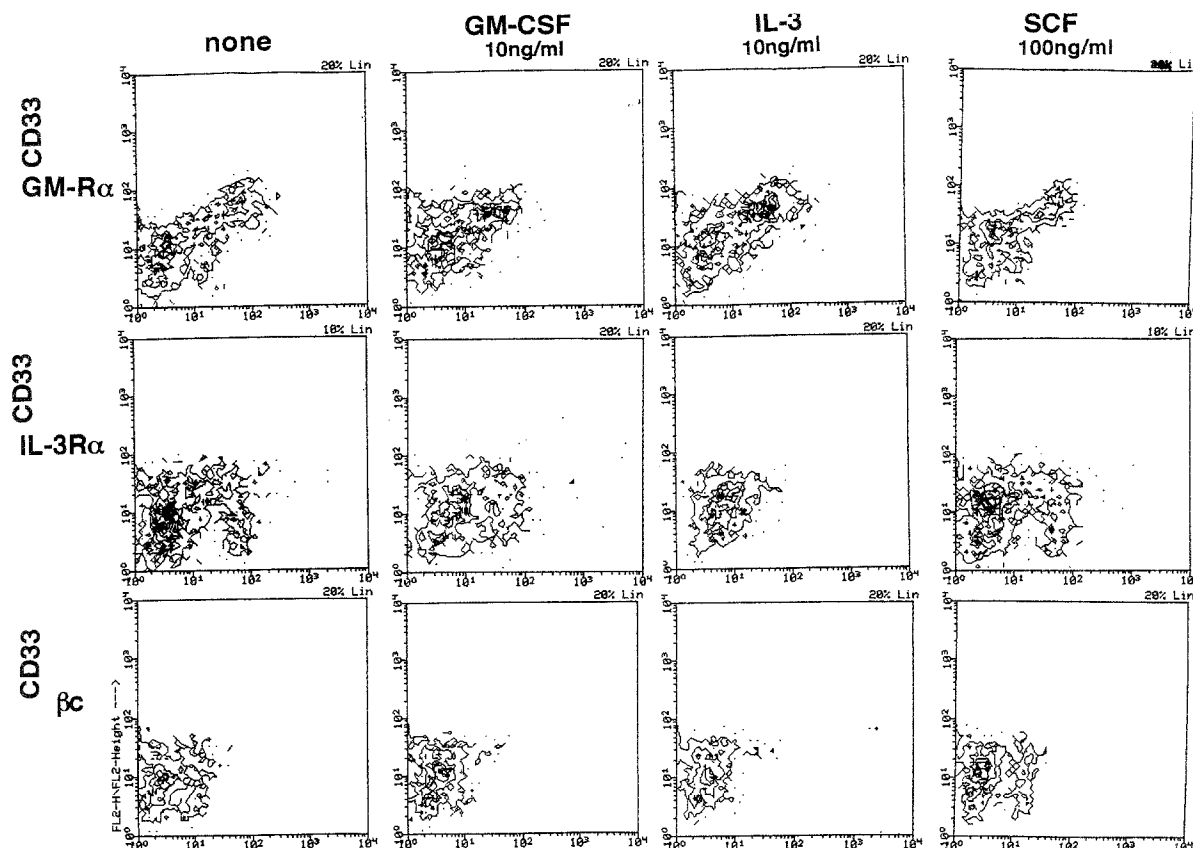


FIG. 9. Effects of cytokines on expression of GMR α , IL-3R α , and β c subunits. Purified CD34⁺ cells were incubated with or without cytokines (GM-CSF [10 ng/ml], IL-3 [10 ng/ml], or SCF [100 ng/ml]) in IMDM plus 10% FCS for 48 hours. Then cells were stained as in Fig. 4. Cells were represented by 20% probability contour plotting.

is compatible with the fact that IL-3 has no effect on their activation and receptor transmodulation.⁴⁸

Hematopoietic stem cells and cytokine receptor expression

CD34 antigen is expressed on approximately 1% of human bone marrow cells, and CD34⁺ cells contain multipotent progenitors. The purified CD34⁺ cells are very heterogeneous with respect to their differentiation stages and proliferative activities, because the cells express several levels of early and more mature markers such as CD33, CD13, HLA-DR, CD38, CD45, and CD71⁴⁹⁻⁵¹ and differentiate into various lineages of cells.^{49, 52} Previous research¹⁹ indicated that 37% of bone marrow CD34⁺ cells formed in vitro colonies in the presence of phytohemagglutinin-stimulated leukocyte-conditioned medium and Epo and that CD34⁺CD33⁺ bone marrow cells formed larger numbers of granulocyte-macrophage and macrophage colonies than CD34⁺CD33⁻ cells. They also reported that CD34⁺CD33^{+/+} cells differentiated

to CD34⁻CD33⁺ cells, which formed granulocyte/macrophage or eosinophil colonies in the presence of IL-3, G-CSF, or IL-5.⁵³ However, colony-forming cells in long-term bone marrow cultures are derived from CD34⁺CD33⁻ cells rather than CD34⁺CD33⁺ cells.¹⁸ CD34⁺ cord blood cells also respond to combinations of GM-CSF + IL-3,⁵⁴ SCF + IL-3, or GM-CSF + Epo^{55, 56} and generate large numbers of differentiated cells. In the murine system, most blast cells express receptors for all four CSFs, including GM-CSF, G-CSF, M-CSF, and IL-3.^{48, 57, 58} In contrast to human systems, in which GM-CSF enhanced the responsiveness of the monocytic progenitor cells to M-CSF,⁵⁹ the effects of CSF combinations on murine committed progenitor cells might be sometimes inhibitory. That is, GM-CSF, in combination with M-CSF, induced fewer macrophage colonies than M-CSF alone.⁶⁰

Our data show that bone marrow CD34⁺CD33⁻ cells express fewer GMR α and IL-3R α subunits than CD34⁺CD33⁺ cells (Fig. 7), although CD34⁺

CD33^{low} cells express a much higher level of IL-3R α . These expression patterns of receptor subunits coincide with the data from colony-forming activities. IL-3 and GM-CSF can induce strong multiplication of CD34⁺ cells,^{49, 54, 61-64} and CD34⁺ cells contain pluripotent stem cells, which respond well to IL-3 and, to some extent, to GM-CSF.⁴⁹ Furthermore, Testa et al.⁶⁵ reported that high-affinity IL-3Rs (2700/cell) and GMRs (300/cell) were detected on purified CD34⁺CD33⁻ cells by using a binding assay (grossly) in accordance with our findings of high IL-3R and low GMR expression on CD34⁺CD33^{neg to low} cells. However, there are some arguments about receptor expression and responsiveness in hematopoietic progenitor cells. Wognum et al.⁶⁶ reported that among rhesus monkey bone marrow and PB cells, RhLA-DR^{high} CD34⁺ cells expressed high GMRs, whereas RhLA-DR^{low} CD34⁺ cells expressed negative or low GMRs. Sorted CD34⁺ cells with high GMR expression did not form colonies in response to GM-CSF but differentiated into monocyte precursors. In contrast, CD34⁺ cells with low to intermediate levels of GMR expression proliferated in response to GM-CSF. Most colony-forming cells, in response to GM-CSF, were contained in the subset of CD34⁺ cells with undetectable levels of GMRs.

Modulation of receptor expression by other cytokines

Previous reports, concerning the relationship between cytokines and receptor expression, showed several types of interactions.* These include (1) receptor downmodulation by their own ligands,⁷² (2) cross-competition between cytokines with receptors that share common subunits,^{35, 41, 67-69} (3) transmodulation by other cytokines,^{48, 65, 70-73} and (4) differentiation-inducing effects of cytokines, leading to changes in expression of cytokine receptors.

First, we showed in this report that receptor downmodulation by their own ligands also occurred on CD34⁺ cells (Fig. 9). Internalization and recycling of GMRs were observed^{70, 74} but not absolutely required for GM-CSF-induced signal transduction.⁷⁵

Second, in human systems cross-competition between receptor binding of GM-CSF and IL-3 has been reported on normal,^{35, 41, 67, 76, 77} and leukemic^{35, 68, 69, 78} cells. The degree of cross-competition between GM-CSF and IL-3 depends on the

relative abundance of each α subunit.⁷⁹ According to the binding assays reported previously and our flow cytometry analyses, the numbers of β c subunits expressed on human hematopoietic cells are much smaller than those of the α subunits. Then, competition for binding with the β c chain to form heterodimers occurs. In contrast, the number of β chains (AIC2B) is much larger than those of the α subunits in murine systems, and the IL-3R α subunit interacts with not only AIC2B but also AIC2A; then, no cross-competition has been observed.

Third, for transmodulation, several phenomena on different cells and in different conditions were reported.^{48, 65, 80, 81} Total murine bone marrow cells show hierarchical patterns of receptor transmodulation.⁴⁸ Furthermore, in murine erythroid and myeloid cell lines, β c (AIC2A and AIC2B) transcription was transiently induced by Epo, M-CSF, IL-3, GM-CSF, and SCF.⁸¹ In addition, the cascade transactivation model was proposed on purified human hematopoietic progenitors⁶⁵ and lineage marker-negative (Lin⁻) murine bone marrow progenitor cells.⁸⁰ These models suggest that the receptor expressions on immature progenitor cells may be regulated differently than those on mature cells.^{65, 80}

Some of the synergistic effects of cytokines are ascribed in part to upregulation of the receptors,* but the exact mechanism of receptor transmodulation⁴⁶ awaits analysis of expression of each subunit by specific mAbs.

Furthermore, the role of receptor transmodulation remains undetermined. Receptor downmodulation on bone marrow precursor cells results in activation of the receptors,⁴⁸ and the inability of GM-CSF, M-CSF, and G-CSF to mimic the effect of IL-3 is due to their inability to downmodulate IL-3R. However, the phenomena may also be explained by the difference in cellular distribution between IL-3R and GMR, because their biologic effects are almost the same on the cells expressing both receptors.

Furthermore, IL-1 α ^{29, 33, 82} and tumor necrosis factor- α ⁷¹ exert their synergistic effects on some human cell lines by upregulating GMR and IL-3R through increased expression of the β c subunit.⁸⁴ Our previous study showed that tumor necrosis factor- α upregulated β c expression and G-CSF upregulated IL-3R α on CD34⁺ cells.³⁰ Transforming growth factor- β downmodulates GMR, IL-3R, and G-CSF-R on murine cell lines and inhibits

*References 35, 41, 46, 48, 65, and 67-73.

*References 29, 33, 65, 80, 82, and 83.

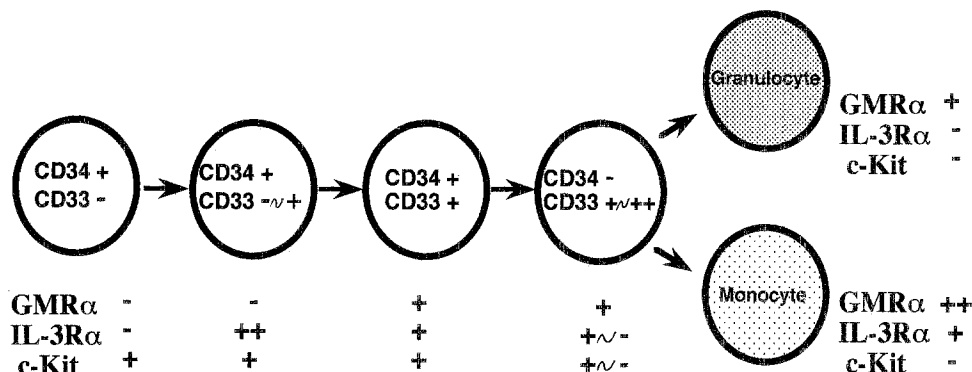


FIG. 10. Expression patterns of GMR, IL-3R, and c-kit along with differentiation into granulocyte and monocyte lineages.

growth of immature hematopoietic progenitor cells.⁸³ IFN- γ upregulates the βc subunit in monocytes⁷³ and synergizes with SCF or other hematopoietic growth factors in supporting multipotential progenitor cells.^{85, 86} This study showed marked downmodulation by their own ligands⁸⁷ in 24-hour cultures of UT-7 cells and in 48-hour cultures of CD34⁺ cells, but no remarkable upregulation was detected (Fig. 9).

Differentiation induction and cytokine receptor expression

Finally, differentiation induced by cytokines affects the expression of receptors.^{80, 88-90} In response to GM-CSF and G-CSF and G-CSF, FDCP-MixA4 cells differentiated into mature neutrophils, which had an increased level of GMR but lost proliferative response to GM-CSF.⁹⁰ Incubation of Lin⁻ murine bone marrow cells with IL-3 and GM-CSF for 7 days induced differentiation and upregulation of GMR.⁸⁰ We have not examined the effects of differentiation on receptor expression in these 48-hour culture conditions. However, considering the relationship between receptor subunits and CD33 antigen expression on CD34⁺ cells and mature cells, we may propose the patterns of receptor expression in relation to differentiation stages (Fig. 10). The most immature progenitors, CD34⁺CD33⁻ cells, express neither GMR α nor IL-3R α , and slightly differentiated CD34⁺CD33^{low} cells express the highest level of IL-3R α but no detectable GMR α . More differentiated progenitor cells with the CD34⁺CD33^{high} phenotype express a medium level of IL-3R α and GMR α . Granulocytic precursors lose IL-3R α and express an increased level of GMR α during differentiation, whereas monocytic precursors retain IL-

3R α and GMR α until they become mature monocytes. We could not determine the changing pattern of βc subunit expression from our data. βc and c-kit expression were also reported to be controlled during development.⁹¹⁻⁹⁵

Specificity and redundancy of cytokine receptor signals

Expression of exogenous receptors into hematopoietic cells with retrovirus vectors revealed the specificity and redundancy of cytokine receptor signals. Several reports support the concept of common signal transduction mechanisms among different receptors. Retrovirally expressed *c-fms* (CSF-1-R) induces the proliferative response to M-CSF of the IL-3-dependent mast cells⁹⁶ or 32DC13 cells.⁹⁷ GMR, G-CSF-R, or Epo receptor can deliver a proliferative signal to BA/F3 cells,⁴ and epidermal growth factor receptor on 32D cells⁹⁸ or GH-R on FDC-P1 cells⁹⁹ can deliver proliferative signals. These results seem to support the redundancy of cytokine receptor signals in several cell lines. However, other reports suggest the presence of lineage-restriction and receptor-specific signals. Fetal liver cells that express *c-fms* can form smaller erythroid colonies with less hemoglobinization than in the presence of Epo,¹⁰⁰ and *c-fms* expression cannot induce growth of granulocyte precursor cells or activation of granulocytes.¹⁰⁰ The 32DC13 cells that express *c-fms* can grow without differentiation in the presence of M-CSF.⁹⁷ The interchangeability of receptors for growth and differentiation signals depends on the type of cells. BA/F3 cells can grow in response to several cytokine signals, whereas CTLL2 cells cannot.^{4, 100} Like DMSO-induced HL60 cells for GM-CSF-induced growth, responsiveness to cytokine

signals is determined by the presence or absence and the activated status of the cellular signaling pathways. Furthermore, the interchangeability of receptor signals is also determined by the types of receptors themselves. Receptors in the same subfamily have common signaling subunits and show common features of activities on hematopoietic cells,⁶ except for some minor differences. In contrast, receptors of different subgroups cannot replace the signals, just as IL-6R cannot replace the IL-3 signal, or Epo receptor cannot replace the GMR/IL-3R signal.

Conclusion

Regulation of expression of cytokine receptors in a stage- and cell lineage-specific manner has long been recognized. In addition, their expression is transiently affected by the action of their own or other cytokines in the microenvironment. Our model (Fig. 10) will be further corrected by more detailed studies in which progenitor cell separating techniques, flow cytometry with these antibodies, binding assay, and analysis of receptor gene expression are used. For these studies of receptor expression and modulation, specific anti-receptor mAbs, such as those prepared by us²⁸⁻³⁰ or by others,¹⁰¹ will be particularly useful for examining the differential expression of receptor subunits.

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