Analysis of Interleukin 6 Receptor and gp130 Expressions and Proliferative Capability of Human CD34$^+$ Cells

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Summary

We recently demonstrated that stimulation of gp130 by a combination of soluble interleukin 6 receptor (sIL-6R) and IL-6 but not IL-6 alone significantly stimulates the ex vivo expansion of primitive hematopoietic progenitors and the generation of erythroid cells from human CD34$^+$ cells in the presence of stem cell factor (SCF). Here, we show that gp130 is found low positively on most CD34$^+$ cells, whereas IL-6R is expressed on only 30–50% of these cells. Although most of the colonies generated from FACS®-sorted CD34$^+$IL-6R$^+$ cells were granulocyte/macrophage (GM) colonies, CD34$^+$IL-6R$^-$ cells gave rise to various types of colonies, including erythroid bursts, GM, megakaryocytes, and mixed colonies in methylcellulose culture with a combination of IL-6, sIL-6R, and SCF. Similar results were obtained in culture supplemented with a combination of IL-3, IL-6, SCF, granulocyte colony–stimulating factor, erythropoietin, and thrombopoietin. A limiting dilution analysis of long-term culture–initiating cells (LTC-IC) showed that the CD34$^+$IL-6R$^-$ cells contained a larger number of LTC-IC than did the CD34$^+$IL-6R$^+$ cells. In a serum-free suspension of CD34$^+$IL-6R$^-$ cells, the addition of sIL-6R to the combination of IL-6 and SCF dramatically increased the total and multipotential progenitors, whereas CD34$^+$IL-6R$^+$ cells failed to do so under the same conditions. These results indicate that most of the erythroid, megakaryocytic, and primitive human hematopoietic progenitors are included in the IL-6R$^-$ populations, and the activation of gp130 on these progenitors can be achieved by a complex of IL-6–sIL-6R, but not by IL-6 alone. The present culture system using IL-6, sIL-6R, and SCF may provide a novel approach for ex vivo expansion of human primitive hematopoietic progenitors.

Intercellular communication in the hemopoietic system is mediated by soluble factors called IL or cytokines. These molecules exert their biological functions through specific receptors expressed on the surface of target cells. The cloning of genes encoding the receptors for cytokines regulating the hematopoietic system has revealed that the majority of cytokine receptors falls into a hematopoietic cytokine receptor superfamily. Most cytokine receptor systems in this family, except for the receptors of erythropoietin (EPO)[1], granulocyte colony-stimulating factor (G-CSF), and thrombopoietin (TPO), consist of a multichain complex, a ligand-specific receptor chain (α chain), and a signal transducing chain (β chain), the latter of which is often common to several receptor complexes (1–3). The cytokines that share a β chain often mediate similar functions on various cells. In addition to functional redundancy, each cytokine has its own unique functions in certain cell types, suggesting that cellular responsiveness is largely determined by the regulated expression of the ligand-specific receptors.

[1]*Abbreviations used in this paper:* BL, blast cell; BFU, burst-forming cells; CFU, colony-forming cells; CNTF, ciliary neurotrophic factor; E, erythroid; Eo, eosinophil; EPO, erythropoietin; FBS, fetal bovine serum; G, granulocyte; G-CSF, granulocyte colony–stimulating factor; sIL-6R, soluble IL-6R; LIF, leukemia inhibitory factor; LTC-IC, long-term culture–initiating cells; M, macrophage; Meg, megakaryocyte; Mix, mixed; NMC, nonphagocytic mononuclear cells; OSM, oncostatin M; SA–PE, PE-conjugated streptavidin; SCF, stem cell factor; TPO, thrombopoietin.

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The IL-6 receptor system is composed of two functionally different chains: a ligand-binding chain (IL-6R) and signal-transducing gp130, the latter of which is shared by receptors of IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin 1 (3–5). In the IL-6R interaction, IL-6 first binds to the IL-6R, and this complex (IL-6–sIL-6R) then associates with gp130, leading to its homodimerization, and transduces the signal. A soluble form of IL-6R (sIL-6R), lacking a transmembrane and cytoplasmic region, has been shown to bind IL-6 with a binding affinity similar to that of the entire IL-6R molecule and to induce homodimerization of gp130 upon IL-6 binding (1, 3, 6, 7).

We recently demonstrated that a complex of IL-6–sIL-6R, but not IL-6 alone, can activate gp130 and transduce the functional signals to stimulate the ex vivo expansion of hemopoietic progenitors from human $CD34^+$ cells in the presence of stem cell factor (SCF) (8). We have also observed the significant production of erythroid cells from $CD34^+$ cells in cuture with sIL-6R in the presence of IL-6 and SCF and lack of this production in culture without sIL-6R (9). These results lead us to test the hypothesis that the IL-6–sIL-6R complex confers IL-6 responsiveness to primitive hemopoietic progenitors and erythroid progenitors that show no expression of IL-6R but do express gp130. In this study, we examine the expression of gp130 and IL-6R on $CD34^+$ cells by flow cytometry, and demonstrate that gp130 is ubiquitously expressed on $CD34^+$ cells, whereas IL-6R displays a limited expression. Our comparison of the proliferation and differentiation capabilities of FACS®-sorted $CD34^+IL\text{-}6R^+$ cells with those of $CD34^+IL\text{-}6R^-$ cells using methylcellulose clonal culture, suspension culture, and a limiting dilution analysis of long-term culture–initiating cells (LTC-IC) reveals that most of the erythroid, megakaryocytic, and primitive hematopoietic progenitors are included in the $IL\text{-}6R^-$ populations. The findings obtained in this study provide new information concerning the mechanism that controls the development of human hematopoietic stem/progenitor cells and have potential clinical application.

Materials and Methods

Cell Preparation. Human umbilical cord blood samples, collected according to our institutional guidelines, were obtained during normal full-term deliveries. Human bone marrow cells were aspirated from the posterior iliac crest of healthy adult volunteers after informed consent was obtained. Nonphagocytic mononuclear cells (NMC) were separated by Ficoll–Paque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation after depletion of phagocytes with silica (Immuno Biological Laboratories, Fujioka, Japan) (10).

Receptor, Cytokines, and Antibodies. rhIL-6 and sIL-6R were prepared as described previously (11). rhSCF was kindly provided by Amgen Biologicals (Thousand Oaks, CA). rhTPO, IL-3, and EPO were generously provided by Kirin Brewery (Tokyo, Japan). G-CSF was kindly provided by Chugai Pharmaceutical Co. (Tokyo, Japan). All the cytokines were pure recombinant molecules and were used at concentrations that induced optimal response in methylcellulose culture of human hematopoietic cells. These concentrations are 100 ng/ml of SCF, 100 ng/ml of IL-6, 200 U/ml of IL-3, 100 ng/ml of G-CSF, 2 U/ml of EPO and 4 ng/ml of TPO.

The preparation of anti–human gp130 mAb (AM64) and anti–human IL-6R mAb (MT18) has been described previously (12, 13). The anti-gp130 and anti–IL-6R mAbs were biotinylated with *N*-hydroxysuccinimido-biotin (Pierce Chemical Co., Rockford, IL). FITC-conjugated mouse IgG1 mAb specific for CD34 (8G12) (CD34-FITC), FITC- and biotin-conjugated irrelevant mouse IgG1 mAbs, and PE-conjugated streptavidin (SA–PE) were provided by Becton Dickinson & Co. (San Jose, CA). PE-conjugated goat anti–mouse IgG Ab and PE-cyanine 5-succinimidylester–conjugated SA (SA–PE/Cy5) were purchased from DAKO A/S (Glostrup, Denmark).

Serum-free Suspension Culture. The sorted cells were incubated in serum-free suspension culture as we recently described (8) with a minor modification of a previously reported method (14). 1 ml of culture mixture containing 200 sorted cells, α-medium (Flow Laboratories, Rockville, MD), 2% deionized pure BSA (Sigma Chemical Co., St. Louis, MO), 10 μg/ml of insulin (Sigma Chemical Co.), 200 μg/ml of transferrin (Sigma Chemical Co.), 0.01 mM 2-ME (Eastman Organic Chemicals, Rochester, NY), and 40 μg/ml of low-density lipoprotein (Sigma Chemical Co.), and different combinations of cytokines were incubated in 24-well tissue plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere flushed with 5% CO_2, 5% O_2, and 90% N_2. At weekly intervals, cultures were demidepopulated by the removal of half the culture volume, which was then replaced by newly prepared medium with the same combinations of cytokines. Cells in the collected media were washed, counted, cytocentrifuged, and stained. Total progenitor cells generated at each time point in the suspension culture were evaluated by culturing a fraction of the expanded cells in the clonal assay as described below.

Clonal Culture. The sorted cells and their progenies in suspension culture were incubated in triplicate at concentrations of 200 cells/ml for the sorted $CD34^+IL\text{-}6R^-$ and $CD34^+IL\text{-}6R^+$ cells and $0.4\text{–}10 \times 10^3$ cells/ml for cultured cells in methylcellulose culture as previously reported (15, 16). 1 ml of culture mixture containing cells, α-medium, 0.9% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT), 1% deionized fraction V BSA (Sigma Chemical Co.), 0.05 mM 2-ME, and various combinations of cytokines was plated in 35-mm Lux standard nontissue culture dishes (Nunc) and incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. For megakaryocyte colony formation, the culture contained components identical to those in serum-containing culture with the exception of FBS replaced by human platelet–poor plasma. Serum-free methylcellulose culture contained components identical to those in serum-containing culture except that 1% pure BSA, 200 μg/ml of human transferrin, 160 μg/ml of soybean lecithin (Sigma Chemical Co.), and 96 μg/ml of cholesterol (Nacalai Tesque, Kyoto, Japan) replaced the BSA and FBS (17). A combination of early and late acting cytokines including SCF, IL-6, IL-3, G-CSF, and EPO, was used to determine the expanded progenitors generated in suspension culture at each time point. All cultures were scored at day 14 according to criteria reported previously (10, 15, 16). A combination of TPO, SCF, and IL-3 was used for the determination of megakaryocytic progenitors in the plasma-containing culture, and the megakaryocyte colonies were scored at day 11. To assess the accuracy of the in situ identification of colonies, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visual-

ization, spread on glass slides using a cytocentrifuge (Cytospin II; Shandon Southern Instruments Inc., Sewickley, PA), and stained for morphological examination. Staining with May–Grünwald–Giemsa was performed. Immunostaining with the alkaline phosphatase antialkaline phosphatase method using mAbs against glycoprotein IIb/IIIa complex (Nichirei Co., Tokyo, Japan) was carried out as described previously (18). The abbreviations used for the colony types are as follows: G, granulocyte colonies; M, macrophage colonies; GM, granulocyte-macrophage colonies; E, erythroid bursts; Eo, eosinophil colonies; Meg, megakaryocyte colonies; Mix, mixed colonies; and BL, blast cell colonies.

Flow Cytometry and Cell Sorting. Cell staining with CD34, gp130, and IL-6R markers was performed with NMC obtained from human cord blood. 2×10^6 NMC were suspended in 200 μl PBS with 2.5% FBS. Incubation with antibodies was carried out on ice for 30 min. For two-dimensional flow-cytometric analysis with CD34 and IL-6R, NMC were first incubated with biotinylated anti–IL-6R mAb and then incubated simultaneously with CD34-FITC and SA–PE. For three-dimensional analysis with CD34, IL-6R, and gp130, NMC were first incubated with anti–IL-6R mAb without labeling and then reacted with PE goat anti–mouse IgG Ab. To block surplus second antibody, normal mouse serum was added and further incubated with NMC. Finally, the NMC were stained with biotinylated anti-gp130 mAb followed by staining with SA–PE/Cy5 and CD34-FITC. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson & Co.). A morphologic gate including ~25% of the events and all the $CD34^+$ cells was determined on two-parameter histograms (side scatter and forward scatter). Compensation for two-color– or three-color–labeled samples was set up with single-stained samples. Positivity or negativity for IL-6R and gp130 antigens among $CD34^+$ cells was determined using control cells that were stained with the FITC-, PE-, and PE/Cy5–labeled irrelevant mouse IgG1 mAb as their isotype-matched controls. Cell sorting was performed on a flow cytometer (EPICS Elite; Coulter Electronics Inc., Hialeah, FL). The purity of sorted $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ populations as verified by reanalysis was >95%. For clone sorting, the sorted cells were resorted singly into 96-well flat-bottomed plates (Nunc) with a FACStar Plus equipped with an automated cell deposition unit (ACDU; Becton Dickinson & Co.). The clone-sorted cells were cultured in each well containing 200 μl serum-containing methylcellulose culture medium supplemented with SCF, IL-6, IL-3, G-CSF, and EPO.

A Limiting Dilution Analysis of LTC-IC. To prepare feeders, mononuclear cells obtained from the adult bone marrow donor were used as described (19–21). The mononuclear cells were first cultured at a concentration of 2×10^7 cells per T25 flask in α-medium containing 20% FBS and 0.05 mM 2-ME. After culturing for 2 wk, >80% of the confluent stromal layers were irradiated (15 Gy of 250-kV peak x rays) and trypsinized. The cells were resuspended in LTC medium (MyeloCult H5100; Stem Cell Technologies Inc., Vancouver, Canada) supplemented with 10^{-6} M hydrocortisone and seeded in 96-well flat-bottomed microwell plates at 5×10^4 cells per well for reestablishing the stromal feeder layer. The following day, the sorted cells were plated into each of the 96 wells at six different dilutions (range 12–400 cells/well) with a total volume of 200 μl/well. At weekly intervals, half of the nonadherent cells were removed, and at the same time half of the medium was replaced. After 5 wk, the nonadherent cells and the adherent cells suspended by treatment with trypsin were washed and plated in clonal methylcellulose culture supplemented with SCF, IL-6, IL-3, G-CSF, and EPO to determine the total clonogenic cell content of each LTC.

Results

Expression of IL-6R and gp130 on Human Cord Blood $CD34^+$ Cells. To examine the expression of IL-6R and gp130 on human $CD34^+$ cells, cord blood NMC were prepared for flow-cytometric analysis. The expression of CD34 was first analyzed on NMC within the low forward- and side-scatter properties. On a population of $CD34^+$ cells determined based on the isotype-matched control, then, the expression of IL-6R and gp130 was analyzed. IL-6R was identified on 30–50% of total $CD34^+$ cells, whereas the remainder (50–70%) of the $CD34^+$ cells were $IL\text{-}6R^-$ (Fig. 1 *A*). In contrast, most of the $CD34^+$ cells were shown to express gp130 low positively (Fig. 1 *B*). When the expression of IL-6R and gp130 on $CD34^+$ cells was characterized on three-dimensional flow cytometric analysis, most of the $CD34^+$ cells were $gp130^{low}$, whereas more than half of the $CD34^+$ $gp130^{low}$ cells did not express IL-6R (Fig. 1 *C*). These results clearly indicate that two populations of $gp130^{low}$ $IL6R^+$ and $gp130^{low}IL\text{-}6R^-$ cells were present in human cord blood $CD34^+$ cells.

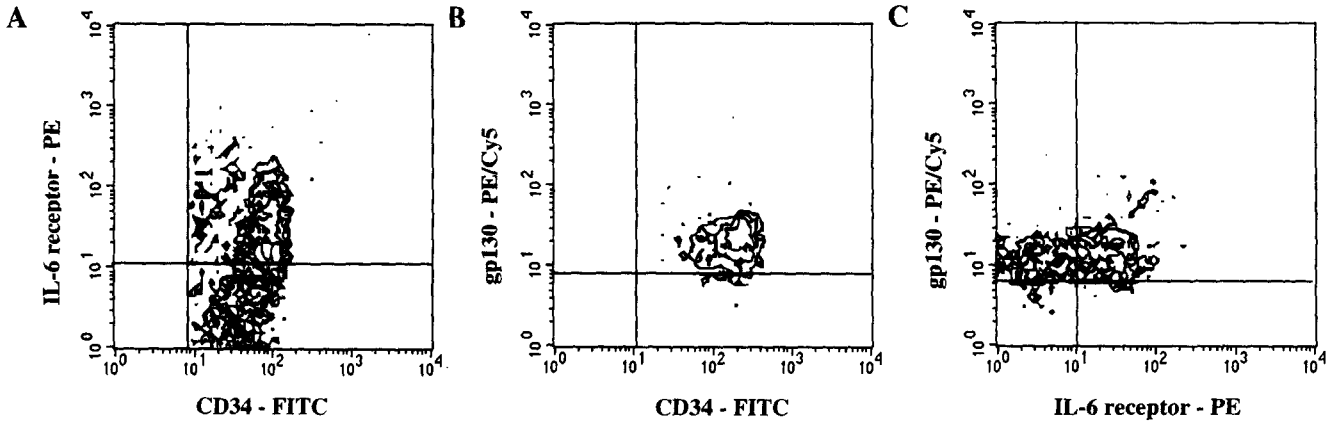

Figure 1. Flow cytometric analysis of human cord blood $CD34^+$ cells. (*A*) CD34–FITC and IL-6R–biotin–SA–PE (*IL-6 receptor-PE*) profile; (*B*) CD34–FITC and gp130–biotin–SA–Cy5 (*gp130-PECy5*) profile; (*C*) IL-6R–PE and gp130–Cy5 profile on $CD34^+$ cells. The $CD34^+$ cells were determined on CD34–FITC histogram. Positivity or negativity for antigens was determined using their isotype-matched controls.

Table 1. *Colony Formation from CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ Cells in Serum-free Methylcellulose Culture Supplemented with IL-6, sIL-6R and SCF*

Cell type	Factors	Number of colonies per 200 cells							
		G	M	GM	E	Mix	Meg	BL	Total
CD34$^+$IL-6R$^+$	SCF	0	0	0	0	0	0	5 ± 3	5 ± 3
	SCF–IL-6	0	2 ± 2	0	0	0	0	6 ± 1	9 ± 3
	SCF–IL-6–sIL-6R	7 ± 2	10 ± 4	3 ± 1	1 ± 1	0	0	1 ± 1	22 ± 5
CD34$^+$IL-6R$^-$	SCF	0	0	0	0	0	0	2 ± 2	2 ± 2
	SCF–IL-6	0	0 ± 1	0	0	0	0	3 ± 3	4 ± 3
	SCF–IL-6–sIL-6R	5 ± 3	4 ± 2	1 ± 1	42 ± 15	32 ± 6	3 ± 2	3 ± 2	90 ± 19

Cells were cultured in the presence of designated factor combinations, and colonies were scored at day 14. The number of colonies indicates mean ± SD of triplicate cultures.

Effects of sIL-6R, IL-6, and SCF on Colony Formation of CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ Cells in Serum-free Methylcellulose Culture. The data shown in Fig. 1 indicating that a significant proportion of CD34$^+$ cells express gp130 but not IL-6R suggested that those cells may be responsive to IL-6–sIL-6R but not IL-6 alone. To examine whether CD34$^+$IL-6R$^-$ cells were functionally distinct from CD34$^+$ IL-6R$^+$ cells in response to IL-6 or sIL-6R–IL-6, a cell sorting of both populations was performed, and the 200 sorted cells were cultured in serum-free methylcellulose culture containing 100 ng/ml of IL-6, 1 μg/ml of sIL-6R, and 100 ng/ml of SCF (Table 1). SCF alone or in combination with IL-6 induced only a small number of colonies from both CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ cells, in accordance with our previous studies (8). An increase in the total number of colonies was observed when sIL-6R was added to the culture of both types of cells. The addition of sIL-6R to the culture of CD34$^+$IL-6R$^+$ cells induced the increase of the number of colonies, most of which were GM colonies. A dramatic effect of sIL-6R was observed in the culture of CD34$^+$IL-6R$^-$ cells. The addition of sIL-6R to the culture of CD34$^+$IL-6R$^-$ cells in the presence of SCF and IL-6 increased the number of colonies by 22.5-fold with a plating efficiency of ~50%. Interestingly, various types of colonies including E bursts, Meg, and Mix colonies in addition to GM colonies, were derived from the CD34$^+$IL-6R$^-$ cells.

Hemopoietic Colony-forming Capacity of CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ Cells. These results suggest that the CD34$^+$ cells may be classified into two distinct subgroups of CD34$^+$ IL-6R$^-$ and CD34$^+$IL-6R$^+$ cells with different functional capabilities. To test this hypothesis, 200 sorted CD34$^+$ IL-6R$^+$ and CD34$^+$IL-6R$^-$ cells were cultured in serum-containing methylcellulose cultures with SCF, IL-3, IL-6, G-CSF, and EPO. Table 2 shows the results of two representative experiments. In both experiments, the cloning efficiency of CD34$^+$IL-6R$^-$ cells was higher than that of CD34$^+$IL-6R$^+$ cells, and >90% of the colonies formed from CD34$^+$IL-6R$^+$ cells were GM colonies, including Eo colonies, whereas CD34$^+$IL-6$^-$ cells gave rise to various types of colonies including not only G/M and Eo colonies, but also E bursts and Mix colonies. We simultaneously carried out plasma-containing methylcellulose culture supplemented with SCF, IL-3, and TPO for the precise detection of Meg

Table 2. *Colony Formation from CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ Cells in Clonal Culture*

Experiment	Cell type	Cloning efficiency	Type of colonies						
			G	M	GM	Eo	E	Mix	BL
		%							
1	CD34$^+$IL-6R$^+$	39.5	29.1	57.8	10.5	1.7	0.0	0.8	0.0
	CD34$^+$IL-6R$^-$	60.2	28.3	13.6	20.5	2.5	19.9	13.6	1.7
2	CD34$^+$IL-6R$^+$	56.5	28.6	48.7	17.1	3.5	0.9	0.0	1.2
	CD34$^+$IL-6R$^-$	72.7	10.1	4.8	7.0	1.0	46.1	28.1	3.1

200 cells were cultured in serum-containing methylcellulose culture with SCF, IL-6, IL-3, G-CSF, and EPO, and colonies were scored on day 14. The cloning efficiency and the percentage of each type of colonies indicate the mean of triplicate cultures.

Table 3. *Colony Formation from Clone-sorted CD34$^+$IL-6$^+$ and CD34$^+$IL-6R$^-$ Cells*

Cell type	Number of clone-sorted cells	Number of colonies					
		G	M	GM	E	Mix	Total
CD34$^+$IL-6R$^+$	95	1	11	14	0	0	26
CD34$^+$IL-6R$^-$	96	2	2	4	12	26	46

Clone-sorted CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ cells by FACStar® PLUS equipped with ACDU were cultured in serum-containing methylcellulose culture with SCF, IL-6, IL-3, G-CSF, and EPO, and colonies were scored at day 14.

colonies. Whereas CD34$^+$IL-6R$^+$ cells produced no Meg colonies, 13 ± 1 Meg colonies were generated from 200 CD34$^+$IL-6R$^-$ cells. These results indicate that IL-6R expression on CD34$^+$ cells might be limited to mainly GM colony-forming cells (CFU-GM), whereas CD34$^+$IL-6R$^-$ cells contain various types of progenitors, including E burst-forming cells (BFU-E), Meg colony-forming cells (CFU-Meg), and Mix colony-forming cells (CFU-Mix), in addition to CFU-GM.

We further carried out the methylcellulose culture of single cells clone sorted by use of FACStar® PLUS ACDU in the presence of SCF, IL-3, IL-6, G-CSF, and EPO to determine the colony-forming capacity of CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ cells more precisely. As shown in Table 3, most colonies derived from single CD34$^+$IL-6R$^+$ cells were also GM colonies,whereas the CD34$^+$IL-6R$^-$ cells generated various types of colonies such as E bursts, Meg, and Mix colonies in addition to GM colonies. When clone-sorted cells were cultured in the presence of TPO, SCF, and IL-3, 96 CD34$^+$IL-6R$^+$ cells produced only one Meg colony, whereas 10 Meg colonies were generated from 96 CD34$^+$IL-6R$^-$ cells. These results in the culture of clone-sorted cells confirmed the observations shown in Table 2.

The Frequency of LTC-IC in CD34$^+$IL-6R$^{+/-}$ Populations. The result that CD34$^+$IL-6R$^-$ cells produced a significantly larger number of Mix colonies suggests that more primitive progenitors may be contained in the CD34$^+$IL-6R$^-$ population than in the CD34$^+$IL-6R$^+$ population. We then assayed the frequency of LTC-IC, which are believed to reflect primitive hematopoietic progenitor cells, in each sorted population. CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ cells were distributed into microwells containing preestablished irradiated adherent layer cells. For each evaluation, six cell concentrations were used with 12–24 replicates per concentration. The frequency of negative wells (no clonogenic progenitors detectable 5 wk later) was then determined. The frequency of LTC-IC in the starting population was calculated by Poisson statistics and the weighted mean method with iterative procedures to determine the best linear fit (22) (Fig. 2). The frequency of LTC-IC in a population of CD34$^+$IL-6R$^+$ cells was 1:1,264, whereas that in a population of CD34$^+$IL-6R$^-$ cells was 1:199, indicating that the CD34$^+$IL-6R$^-$ population included more primitive progenitor cells than the CD34$^+$IL-6R$^+$ population.

Expansion of Clonogenic Progenitor Cells in Serum-free Suspension Cultures of CD34$^+$IL-6R$^+$ and CD34$^+$IL-6R$^-$ Cells. Our previous study demonstrated that the expansion of primitive progenitor cells in a CD34$^+$ population can be achieved with the combined signals through gp130 and c-Kit initiated by sIL-6R, IL-6, and SCF (8). Therefore, our current observation that most of the primitive progenitors belong to the population of CD34$^+$IL-6R$^-$ cells suggests that hematopoietic progenitor cells in the CD34$^+$IL-6R$^-$ cells may be expanded more effectively by the stimulation

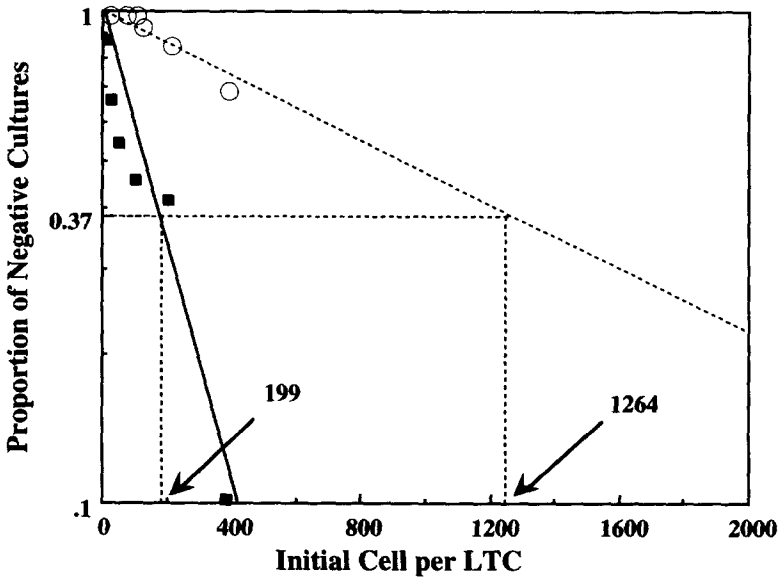

Figure 2. Quantitation of LTC-IC by limiting dilution analysis. Six dilutions of CD34$^+$IL-6R$^+$ cells (*circles*) and CD34$^+$IL-6R$^-$ cells (*squares*) were cultured over irradiated stromal layers, and the number of clonogenic cells detectable after 5 wk was determined. In this experiment, the frequency of LTC-IC was 1:1,264 cells in CD34$^+$ IL-6R$^+$ cells (*dotted line*) and 1:199 cells in CD34$^+$IL-6R$^-$ cells (*solid line*).

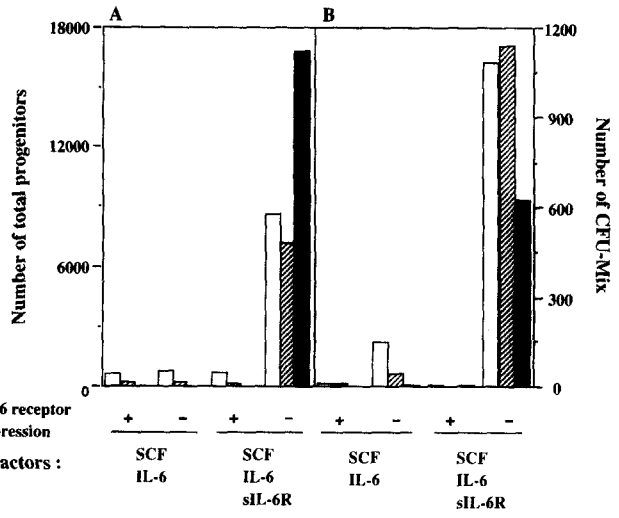

Figure 3. Generation of total progenitors (*A*) and CFU-Mix (*B*) from 3×10^2 $CD34^+IL\text{-}6R^+$ cells containing 167 progenitors and no CFU-Mix, and 3×10^2 $CD34^+IL\text{-}6R^-$ cells containing 218 progenitors and 56 CFU-Mix in suspension cultures supplemented with SCF and IL-6 in the presence or absence of sIL-6R at day 7 (*open bars*), day 14 (*hatched bars*), and day 21 (*solid bars*). The data presented are from a single experiment. Similar data were obtained in two additional experiments.

of IL-6, sIL-6R, and SCF. To examine this possibility, sorted $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ cells were cultured in serum-free suspension culture supplemented with a combination of sIL-6R, IL-6, and SCF. The results showed that significant expansion of hematopoietic progenitors from $CD34^+IL\text{-}6R^-$ cells but not $CD34^+IL\text{-}6R^+$ cells occurred (Fig. 3). The addition of sIL-6R to the combination of SCF and IL-6 increased the generation of progenitor cells from $CD34^+IL\text{-}6R^-$ cells. The increase of total and multipotential progenitors was 11.1- and 7.4-fold at day 7, 25.3- and 27.1-fold at day 14, and 540.6- and 103.5-fold at day 21 of suspension culture, respectively. In contrast, in the suspension culture of $CD34^+IL\text{-}6R^+$ cells, the addition of sIL-6R failed to stimulate the progenitor expansion. This result suggests that our previous observation of the expansion of primitive progenitors from $CD34^+$ cells by sIL-6R, IL-6, and SCF was mainly provided by the highly proliferative capability of the $CD34^+IL\text{-}6R^-$ cell subpopulation, and that the $CD34^+IL\text{-}6R^-$ cell population can be a good target cell population for ex vivo expansion using IL-6, sIL-6R, and SCF.

Discussion

The unparalleled expression pattern of gp130 and IL-6R on human cord blood $CD34^+$ cells was demonstrated by our flow-cytometric studies. Most of the $CD34^+$ cells were found to express gp130 in accordance with previous reports that gp130 is ubiquitously expressed in all tissues examined (7, 23, 24). By contrast, half to two thirds of the $CD34^+$ cells lacked the expression of IL-6R. The $CD34^+IL\text{-}6R^-$ cells may have other ligand-specific receptor(s) requiring gp130 as a signal transducer. In our recent studies, IL-11 and LIF were shown to have comparable effects with IL-6 on colony formation from cord blood $CD34^+$ cells in the presence of SCF, and no stimulatory effects were observed with OSM and CNTF (25). These suggest that the expression of receptors for IL-11 and LIF reveal a similar distribution to that of IL-6R on $CD34^+$ cells, and that $CD34^+$ cells express little or no receptors for OSM and CNTF. However, there remains a possibility that unknown ligand-specific receptor(s) using gp130 may present on the $CD34^+$ $IL\text{-}6R^-$ cells.

The characterization of the two distinct subpopulations of $CD34^+$ cells was carried out using sorted $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ cells. In serum-containing clonal culture supplemented with SCF, IL-3, IL-6, G-CSF, and EPO, or plasma-containing clonal culture supplemented with TPO, SCF, and IL-3, most of the colonies generated from the $CD34^+IL\text{-}6R^+$ cells were GM colonies, whereas $CD34^+$ $IL\text{-}6R^-$ cells gave rise to various types of colonies, including E bursts, Meg, and Mix colonies, in addition to GM colonies. Furthermore, LTC-IC assay showed that the $CD34^+IL\text{-}6R^-$ population contained a larger number of LTC-IC than did the $CD34^+IL\text{-}6^+$ population. Similar data were obtained from experiments using bone marrow $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ cells (data not shown). These results indicate that $CD34^+IL\text{-}6R^+$ progenitors are already committed for myeloid lineage, whereas $CD34^+$ $IL\text{-}6R^-$ cells contain myeloid, erythroid, megakaryocytic, multipotential progenitors and more primitive hematopoietic progenitor cells. This model can explain our previous observation that cord blood $CD34^+$ cells formed various types of colonies, including GM, E bursts, Meg, and Mix colonies in serum-free clonal culture supplemented with IL-6, sIL-6R, and SCF, whereas a combination of SCF and IL-6 induced only a small number of GM colonies (8). It has been shown that IL-6 in combination with IL-3 or SCF induces the proliferation of murine 5-fluorouracil–resistant multipotential hematopoietic progenitor cells, suggesting the expression of IL-6R on murine primitive hematopoietic progenitor cells (26). Therein may lie the difference between the IL-6R expression pattern on murine and human hematopoietic cells.

The essential role of gp130 in hematopoiesis in vivo was confirmed in gp130-deficient mice (27). The mutant embryos had greatly reduced numbers of pluripotential and committed hematopoietic progenitors such as spleen colony-forming cells (CFU-S), CFU-GM, BFU-E, and CFU-Meg in the liver, and some showed severe anemia due to impaired maturation of erythroid cells. The in vivo role of c-Kit in hematopoiesis has been well documented in W-mutant mice (28). The mice revealed decreased numbers of CFU-S and erythroid progenitors. In addition, human serum contains detectable levels of SCF, IL-6, and sIL-6R, as well as a functional complex of IL-6–sIL-6R (29–31). Taken together with the in vitro data, the synergistic effects of gp130 and c-Kit signaling play a vital role in the proliferation and differentiation of hematopoietic progenitor cells in vivo.

There has been great interest in ex vivo expansion of human primitive hematopoietic progenitor cells for clinical

application, including gene therapy. So far, however, the ex vivo expansion methods reported previously may be insufficient because primitive hematopoietic progenitors differentiated to mature oligopotent progenitors during expansion. In this study, significant expansion of not only total progenitors but also multipotential hematopoietic progenitors was obtained from sorted $CD34^+IL\text{-}6R^-$ cells in serum-free suspension culture supplemented with IL-6, sIL-6R, and SCF. Because it is conceivable that human primitive hematopoietic progenitor cells express both gp130 and c-Kit, coactivation of the gp130 and c-Kit signal pathway by the three factors may provide a novel approach for expansion of human primitive hematopoietic progenitor cells.

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