Erythropoietin-independent Erythrocyte Production: Signals through gp130 and c-*kit* Dramatically Promote Erythropoiesis from Human CD34$^+$ Cells

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Summary

Erythropoietin (EPO) is the primary humoral regulator of erythropoiesis and no other factor has previously been reported to support proliferation and terminal maturation of erythroid cells from hemopoietic stem cells. Here we show that stimulation of glycoprotein (gp130) by a combination of recombinant human soluble interleukin 6 receptor (sIL-6R) and IL-6 but not sIL-6R or IL-6 alone can support proliferation, differentiation, and terminal maturation of erythroid cells in the absence of EPO from purified human CD34$^+$ cells in suspension culture containing stem cell factor (SCF). A number of erythroid bursts and mixed erythroid colonies also developed in methylcellulose culture under the same combination. The addition of anti-gp130 monoclonal antibodies but not anti-EPO antibody to the same culture completely abrogated the generation of erythroid cells. These results clearly demonstrate that mature erythroid cells can be emerged from hemopoietic progenitors without EPO in vitro. Together with the previous reports that human sera contain detectable levels of sIL-6R, IL-6, and SCF, current data suggest that gp130 signaling in association with c-*kit* activation may play a role in human erythropoiesis in vivo.

Glycoprotein (gp130)[1] is the signal transducing receptor component commonly employed by receptor complexes for the cytokines of the IL-6 family, i.e., IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and IL-11 (1–5). Homodimerization of gp130 induced by IL-6 upon binding IL-6R and heterodimerization of LIFR-gp130 induced by CNTF, LIF, or OSM are believed to trigger signaling leading to cellular response through the activation of, for instance, Janus kinases-STAT3 (for acute phase response factor or signal transducer and activator of transcription; 3) and Ras/mitogen-activated protein kinase pathways (6, 7). Recent studies (1–8) have shown that gp130 is expressed in a wide variety of cells, whereas expression of the ligand-binding receptor chains for the IL-6 family of cytokines shows somewhat restricted distribution and does not necessarily parallel that of gp130. The ubiquitously expressed gp130 in a broad range of tissues suggests that it may play a vital role in vivo. To date, the physiological role of gp130 remains largely unknown, since gp130 has been studied primarily in cultured cell lines.

A soluble form of IL-6 receptor (sIL-6R), lacking the transmembrane and the intracytoplasmic domains, has been shown to be present in human serum and to bind IL-6 with a binding affinity similar to that of the entire IL-6R molecule. gp130 associates with both membrane-anchored IL-6R and sIL-6R when the receptor is occupied by IL-6 and transduces signals (2, 6). We have recently shown that gp130 signaling, initiated by a complex of sIL-6R/IL-6, in the presence of stem cell factor (SCF) potently stimulates the ex vivo expansion of human primitive progenitor cells (9). To address the physiological role of gp130 on human hemopoiesis in more detail, we have examined the effects of gp130 stimulation on the proliferation and differentiation of purified human CD34$^+$ cells by focusing on erythropoiesis.

[1]*Abbreviations used in this paper:* BM, bone marrow; EPO, erythropoietin; FBS, fetal bovine serum; gp, glycoprotein; MNC, mononuclear cells; SCF, stem cell factor; sIL-6R, soluble IL-6R.

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Materials and Methods

Cell Preparation. Human umbilical cord blood, collected according to institutional guidelines, was obtained during normal full-term deliveries. Human bone marrow (BM) was obtained from healthy volunteers with informed consent. Mononuclear cells (MNC) were separated by Ficoll/Paque density gradient centrifugation after depletion of phagocytes with silica (Immuno Biological Laboratories, Fujioka, Japan) (10). $CD34^+$ cells were purified from MNC by using Dynabeads M-450 CD34 and DETACHaBEAD CD34 (Dynal, Oslo, Norway). 85–95% of the cells separated were $CD34^+$ by fluorescence-activated cell sorting (Ortho Diagnostic Systems, Inc., Westwood, MA) analysis.

Receptor and Cytokines. Recombinant human IL-6 and sIL-6R were prepared as described (11). Recombinant human SCF was supplied by Amgen Inc. (Thousand Oaks, CA). Recombinant human IL-3 and erythropoietin (EPO) were generously provided by Kirin Brewery Co. (Tokyo, Japan). All the cytokines were pure recombinant molecules and were used at concentrations that induced optimal response in methylcellulose culture of human hemopoietic cells. These concentrations are 100 ng/ml of SCF, 200 U/ml of IL-3, and 2 U/ml of EPO.

Preparation of Abs. Preparation of anti–human gp130 mAbs (GPX7, GPX22, and GPZ35) has been described (12, 13). The three mAbs recognize different epitopes on gp130 and were shown to inhibit IL-6–mediated biological response through inhibition of the IL-6–induced association of gp130 and IL-6 receptors. Anti–human IL-6R (PM1) mAb was prepared as described (14). PM1 was shown to inhibit IL-6–mediated biological response through inhibition of the binding of IL-6R to IL-6. Rabbit anti–human EPO Ab (IgG K-5) was provided by Kirin Brewery Co. (15).

Suspension Culture. 2,000 $CD34^+$ cells were cultured in 1 ml of culture mixture containing α-medium (Flow Laboratories, Rockville, MD), 20% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT), 1% crystallized and deionized fraction V BSA (Sigma Chemical Co., St. Louis, MO), and different combinations of cytokines in 24-well tissue plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere flushed with 5% CO_2/5% O_2/90% N_2. For serum-free suspension culture, FBS and BSA were replaced by 2% pure BSA, 10 μg/ml of insulin, 200 μg/ml of transferrin (all from Sigma Chemical Co.), 0.01 mM 2-ME (Eastman Organic Chemicals, Rochester, NY), and 40 μg/ml of low density lipoprotein (Sigma Chemical Co.) using a modification of a previously reported method (16). Cultures were demi-depopulated weekly by the removal of half the culture volume, which was then replaced by newly prepared culture mixture with additional feeding of the same combinations of cytokines. Cells in the collected medium were washed, counted in a hemocytometer, cytocentrifuged, and stained. Anti-gp130 mAbs, anti-IL-6R mAb, or anti-EPO Ab were added at the beginning of the culture for blocking studies.

Clonal Culture. The purified $CD34^+$ cells and BM MNC were incubated in triplicate at concentrations of 500 cells/ml for $CD34^+$ cells and 2.5×10^4 cells/ml for BM MNC in methylcellulose culture as previously reported (17, 18). 1 ml of culture mixture containing cells, α-medium, 0.9% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% FBS, 1% BSA, 0.05 mM 2-ME, and various combinations of cytokines with or without sIL-6R was plated in each 35-mm standard nontissue culture dish (Nunc, Naperville, IL) and incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Serum-free methylcellulose culture contained components identical to those in serum-containing culture except 1% pure BSA, 300 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 BSA and FBS (19). All cultures were scored at day 7 for CFU-E–derived colony and day 14 for BFU-E and CFU-Mix–derived colonies according to the criteria as reported previously (10, 17, 18).

Cytochemical and Immunological Staining. Cytocentrifuge preparations from suspension culture and methylcellulose culture were stained for the observation of cellular morphology. Staining with May-Grünwald-Giemsa and benzidine were performed by conventional methods. Immunostaining with the alkaline phosphatase antialkaline phosphatase method using mAbs of antiglycophorin A and antihemoglobin α chain (Cosmo Bio., Tokyo, Japan) were carried out as described previously (20). Briefly, cytocentrifuged samples were fixed with buffered formalin-acetone at 4°C, washed with Tris-buffered saline (Wako, Osaka, Japan), and preincubated with normal rabbit serum to saturate the Fc receptors on the cell surface. After washing, the samples were successively incubated with mouse mAbs and rabbit anti–mouse IgG Ab (Medical and Biological Laboratories, Nagoya, Japan); they were then reacted with calf intestinal alkaline phosphatase-mouse monoclonal antialkaline phosphatase complex (Dako, Osaka, Japan). Alkaline phosphatase activity was detected with naphthol AS-TR phosphate sodium salt ($C_{18}H_{13}ClNO_5PN_{a2}$; Sigma Chemical Co.) and Fast red TR salt (both from Sigma Chemical Co.) in pH 7.6, 40 mmol/liter barbital buffer (Wako) containing levamisole (Sigma Chemical Co.) to inhibit nonspecific alkaline phophatase activity. Positive cells were stained with reddish granules.

Statistical Analysis. For statistical comparison in scoring the number of erythroid cells and colonies, Student's *t* test was applied. The significant level was set at 0.05.

Results

Dose-dependent Effect of sIL-6R and IL-6 on Erythroid Cell Generation from $CD34^+$ Cells. When $CD34^+$ cells isolated from human cord blood MNC were cultured with sIL-6R in the presence of IL-6 and SCF for 14 d, total cell number dramatically increased in accordance with the concentration of sIL-6R (Fig.1 *A*). This increase in a dose-dependent manner began at 80 ng/ml of sIL-6R and reached a plateau at 1,280 ng/ml. It is interesting to note that a number of erythroid cells were observed on the cytospin preparations from the cultures containing sIL-6R. The nature of erythroid cells was confirmed by benzidine staining and immunostainings with mAbs of antiglycophorin A and antihemoglobin α. Erythroid cells, including erythroblasts and erythrocytes, detectable at 80 ng/ml of sIL-6R, increased dramatically in a similar fashion to the total cell increase. Morphological analysis showed that ~70% of total cells were erythroid cells at a concentration of sIL-6R >1,280 ng/ml. In the absence of IL-6, however, sIL-6R failed to increase the total cell number and no erythroid cells were found (Fig. 1 *B*). The increase in the total cell and erythroid cell numbers was also dependent on the concentration of IL-6, and maximal increase was obtained at concentrations exceeding 50 ng/ml when 1,280 ng/ml of sIL-6R was present (Fig. 1 *C*). In contrast, in the absence of sIL-6R, increase in the total cell number by IL-6 was much lower and no erythroid cells were detected even at concentrations >50 ng/ml (Fig. 1

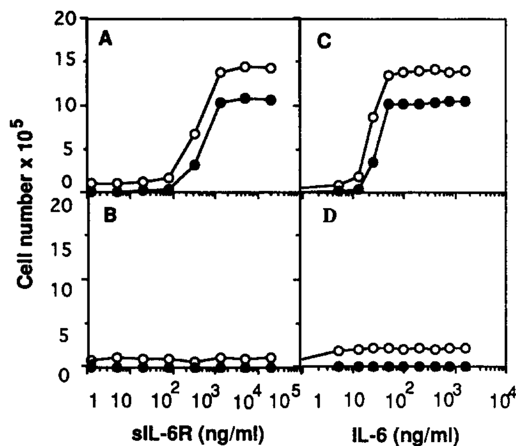

Figure 1. Proliferative activity of sIL-6R and IL-6 on the generation of total cells (*open circles*) and erythroid cells (*filled circles*) from human CD34$^+$ cells in the presence of SCF in serum-containing suspension culture. 2,000 cord blood CD34$^+$ cells were initiated in all cultures and results were examined at day 14. Growth of CD34$^+$ cells under various concentrations of sIL-6R in the presence (*A*) or absence (*B*) of 50 ng/ml of IL-6. Growth of CD34$^+$ cells under various concentrations of IL-6 with SCF in the presence (*C*) or absence (*D*) of 1,280 ng/ml of sIL-6R.

D). The same results were also obtained when CD34$^+$ cells purified from human BM MNC were used. These results clearly indicate that sIL-6R is functional and capable of stimulating the expansion of erythroid as well as total cells from CD34$^+$ cells in serum-containing culture in the presence of IL-6 with SCF. They also show that sIL-6R at 1,280 ng/ml and IL-6 at 50 ng/ml appear to be the optimal concentrations for the generation of erythroid cells.

Erythroid Cell Production from CD34$^+$ Cells by sIL-6R, IL-6, and SCF in Serum-free Suspension Culture. To examine the sIL-6R/IL-6 complex–induced erythropoiesis in more detail, and to exclude the possible influences of an unknown factors(s) existing in FBS on the results obtained in serum-containing culture, serum-free suspension culture of the CD34$^+$ cells over a period of 3 wk was carried out using the optimal concentrations of sIL-6R and IL-6 determined above. The characteristics of expanded erythroid cells were examined weekly. A more significant synergy between sIL-6R and IL-6 in the increase of total and erythroid cells in the presence of SCF was observed in the serum-free cultures. A combination of sIL-6R, IL-6, and SCF in serum-free culture promoted 38-, 530- and 2,170-fold expansion of total cell number at days 7, 14, and 21 of culture, respectively, whereas SCF alone or in combination with IL-6 showed only a 2.2- or 7.5-fold expansion even at day 21 of culture. Differential cell counts of expanded cells on the cytocentrifuge preparations indicated the presence of mainly blast cells at day 7 of culture (Fig. 2 *a* for representative staining). A high proportion of erythroid cells was observed in serum-free suspension cultures with the combination of sIL-6R, IL-6, and SCF at days 14 and 21. The erythroid cells were positively immunostained with mAbs of antiglycophorin A (Fig. 2 *b*) and antihemoglobin α (Fig. 2 *c*). Some of the erythroid cells differentiated to normoblast and enucleated erythrocyte stages (Fig. 2 *d*). At day 21 of culture, most of the erythroid cells differentiated to normoblast stage, and many enucleated erythrocytes were observed. Weekly analyses of the absolute number of erythroid cells generated in serum-free suspension cultures with various combinations of cytokines and sIL-6R are summarized in Table 1. The combination of sIL-6R, IL-6, and SCF enhanced the number of cells in the suspenion culture, in which erythroid cells were predominant. No other combination except that of SCF and EPO could induce such a dramatic increase. Approximately 79 and 69% of the cells generated by this combination were erythroid cells on days 14 and 21 of culture, respectively. The total number of erythroid cells produced by the combination of sIL-6R, IL-6, and SCF was significantly higher than that of other groups (p <0.01–0.0001), and was about 4- and 3.5-fold larger than that produced by EPO alone or by a combination of sIL-6R, IL-6, and EPO, respectively on day 14. The respective increase observed on day 21 was about 150- and 50-fold. Predominant effects of the combination of sIL-6R, IL-6, and SCF on the generation of erythroid cells were also observed with BM CD34$^+$ cells. As is well documented, a combination of EPO and SCF also significantly stimulates the generation of erythroid cells, but appears in a growth pattern different from that of sIL-6R, IL-6, and SCF. More than 90% of the generated cells by EPO and SCF are erythroid cells at day 7, whereas significant production of erythroid cells induced by a combination of sIL-6R, IL-6, and SCF was observed at day 14. At day 21, the total number of erythroid cells produced in the two groups was comparable, and although statistical differences were not reached, a combination of sIL-6R, IL-6, and SCF appeared to induce a higher number of erythroblasts, whereas EPO with SCF generated more enucleated erythrocytes.

These results indicate that the dramatic generation of erythroid cells by sIL-6R/IL-6 needs the combination with SCF. To examine the possible synergy of sIL-6R/IL-6 with other cytokines, suspension culture of CD34$^+$ cells supplemented with different combinations of sIL-6R and IL-6 with various cytokines was carried out. sIL-6R/IL-6 in combination with IL-3 also generated erythroid cells, but to a lesser extent compared with the combination with SCF (Table 1). No erythroid cells were detected in the cultures containing sIL-6R/IL-6 together with either G-CSF, GM-CSF, IL-1β, TGF-β, insulinlike growth factor 1 (IGF-1), macrophage inflammatory protein, platelet derived growth factor, fibroblast growth factor (FGF), or TNF (data not shown).

Erythroid Colony Formation by sIL-6R, IL-6, and SCF. It is likely that the development of a large number of erythroid cells in suspension culture with sIL-6R, IL-6, and SCF has been provided by the proliferation, differentiation, and maturation of immature erythroid progenitors in the CD34$^+$ cell population. To confirm this, we carried out methylcellulose clonal culture of purified CD34$^+$ cells (Table 2). The combination of sIL-6R, IL-6, and SCF in the absence of EPO stimulated not only erythroid bursts but many large erythroid mixed colonies as well, all of which

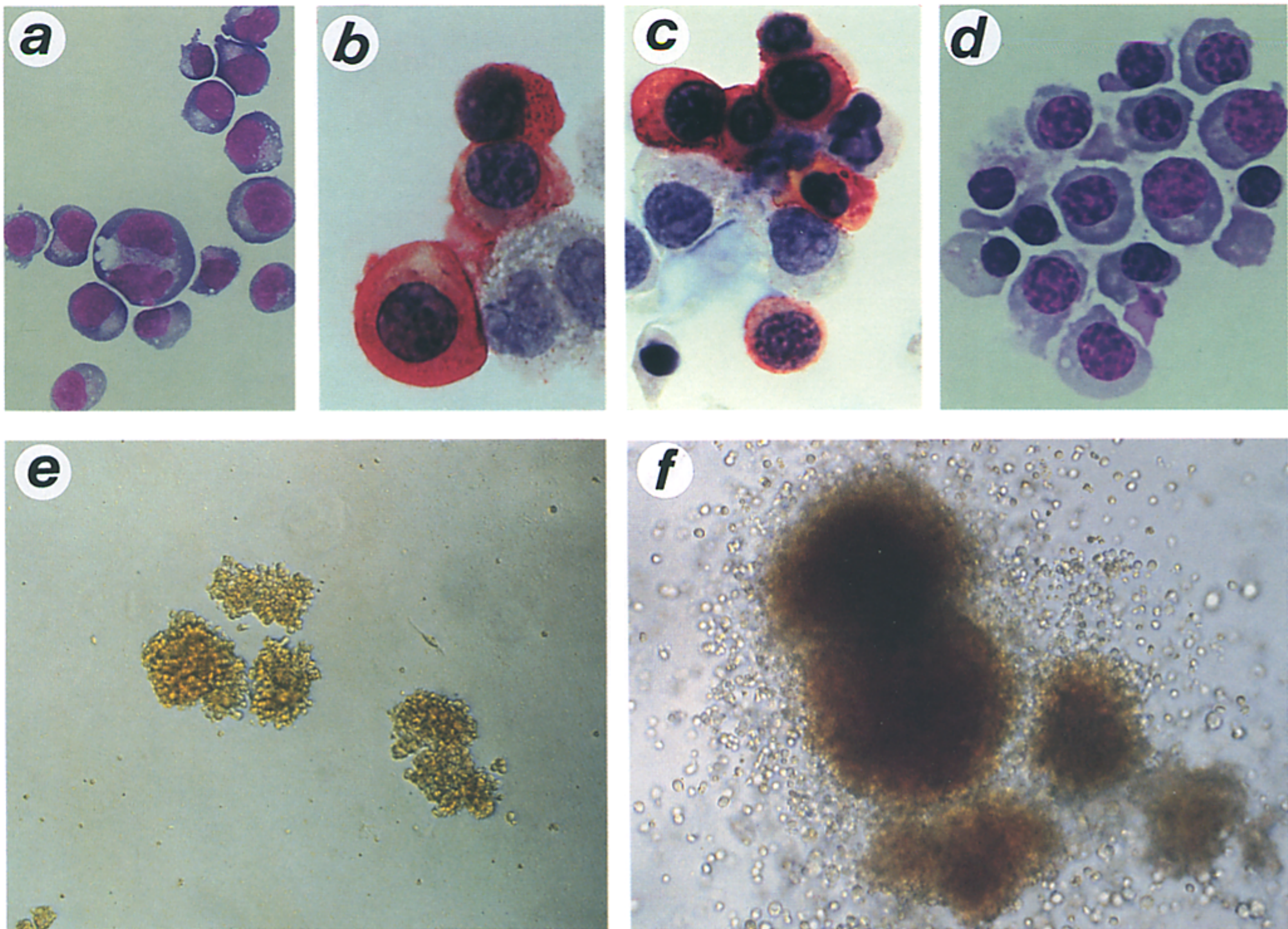

Figure 2. Development of erythroid cells from $CD34^+$ cells in culture with sIL-6R, IL-6, and SCF. Cytocentrifuge preparations of suspension culture were stained with May-Grünwald-Giemsa at day 7 (*a*) and day 14 (*d*), or immunostained with antiglycophorin A mAb (*b*) or antihemoglobin α mAb (*c*) at day 14. In the 14-d methylcellulose culture with sIL-6R, IL-6, and SCF, erythroid burst (*e*) and mixed erythroid colony (*f*) were generated from $CD34^+$ cells. (*a*) ×400; (*b–d*) ×1000; and (*e* and *f*) ×100.

also contained many mature erythroid cells including erythrocytes, as shown in Fig. 2, *e* and *f*. In contrast, neither erythroid bursts nor erythroid mixed colonies were observed in cultures with SCF alone or in combination with IL-6. Generation of erythroid bursts and erythroid mixed colonies by the combination of sIL-6R, IL-6, and SCF was also confirmed in serum-free cultures. The number of erythroid bursts with a combination of sIL-6R, IL-6, and SCF is comparable with that induced by EPO alone or in combination with SCF or IL-3. A combination of EPO with SCF or IL-3 also supported mixed erythroid colony formation, but the colony number was significantly lower than that supplemented with a combination of sIL-6R, IL-6, and SCF ($p < 0.005$). These results suggest that sIL-6R, IL-6, and SCF may act on the same progenitors responsive to EPO, as well as earlier progenitors insensitive to EPO.

When we cultured BM MNC which contain $CD34^-$ mature erythroid progenitors (i.e., CFU-E), efficient formation of erythroid colonies in addition to a number of erythroid bursts and erythroid mixed colonies was also observed in the presence of sIL-6R, IL-6, and SCF in both serum-containing and serum-free conditions (Table 2). The number of erythroid colonies induced by this combination was comparable to that induced by a combination of EPO and SCF, suggesting equal responsiveness of CFU-E to EPO or to the sIL-6R/IL-6 complex in the presence of SCF. It is interesting to note that sIL-6R and IL-6 without SCF also induced a small number of erythroid colonies from BM MNC in both serum-containing and serum-free cultures, implying that a part of CFU-E may respond to a complex of sIL-6R/IL-6 in the absence of SCF.

These results in clonal studies together with those obtained in suspension culture clearly indicated that the combination of sIL-6R, IL-6, and SCF in the absence of EPO can support proliferation, differentiation, and terminal maturation (end-terminal nuclear condensation/enucleation) of not only immature erythroid progenitors in the $CD34^+$ cell population but also of mature erythroid progenitors in BM MNC.

Effect of Anti-gp130 mAb, Anti-IL-6R mAb, and Anti-EPO Ab on the Generation of Erythroid Cell. To test whether

Table 1. *Generation of Erythroid Cells from $CD34^+$ Cells in Serum-free Suspension Culture*

Days in culture	Day 7			Day 14			Day 21		
Cell number (× 10^4)	Total	E-blast	Erythrocyte	Total	E-blast	Erythrocyte	Total	E-blast	Erythrocyte
IL-6	0.09 ± 0.04	0	0	0	0	0	0	0	0
sIL-6R	0.06 ± 0.05	0	0	0	0	0	0	0	0
IL-6 + sIL-6R	0.19 ± 0.08	0	0	0	0	0	0	0	0
SCF	0.6 ± 0.5	0	0	0.2 ± 0.1§	0	0	0.4 ± 0.4*	0	0
SCF + IL-6	1.2 ± 0.3	0	0	4.4 ± 0.5§	0	0	1.5 ± 0.5*	0	0
SCF + IL-6 + sIL-6R	7.6 ± 2.7	2.9 ± 1.0	0	106.2 ± 10.1	64.0 ± 6.5	20.2 ± 1.9	434.6 ± 122.6	286.0 ± 80.0	12.6 ± 3.5
EPO	1.7 ± 0.8	1.7 ± 1.2	0	21.5 ± 7.8‡	20.0 ± 5.9‡	1.5 ± 1.0§	1.9 ± 0.9*	1.2 ± 4.8*	0.7 ± 0.5*
EPO + IL-6	1.7 ± 1.1	1.7 ± 0.7	0	23.0 ± 8.4‡	21.2 ± 9.1‡	1.8 ± 0.6§	2.0 ± 0.3*	0.9 ± 0.2*	1.1 ± 0.7*
EPO + IL-6 + sIL-6R	2.0 ± 0.3	1.9 ± 0.3	0	23.0 ± 5.1‡	22.0 ± 4.2‡	1.0 ± 0.2§	5.6 ± 1.1*	5.4 ± 0.8*	0.2 ± 0.1*
IL-3	0.5 ± 0.2	0	0	1.8 ± 0.3	0	0	0.4 ± 0.1*	0	0
IL-3 + IL-6	0.8 ± 0.5	0	0	1.7 ± 1.0	0	0	2.5 ± 0.6*	0	0
IL-3 + IL-6 + sIL-6R	1.0 ± 0.3	0	0	5.0 ± 0.9	2.3 ± 0.8§	0	5.0 ± 2.1*	2.5 ± 1.2*	0
EPO + SCF	9.4 ± 3.1	8.4 ± 1.8*	0.5 ± 0.4	99.8 ± 21.4	68.2 ± 17.8	26.6 ± 3.9	300.0 ± 98.8	240.3 ± 41.6	35.6 ± 14.9

2,000 $CD34^+$ cells purified from human cord blood were initiated in all cultures. Differential cell count was determined in May-Grünwald-Giemsa and antiglycophorin A mAb staining. Erythroid blasts (E-blast, including proerythroblast, erythroblast, and normoblast) and erythrocytes were calculated based on their proportion on cytocentrifuge preparations and the total cell number generated by each combination. Data are represented by mean ± SD from three separate experiments.
Significantly different from SCF+IL-6+sIL-6R (* $P < 0.01$, ‡ $P < 0.001$, and § $P < 0.0001$).

Table 2. *Generation of Erythroid Colonies from Cord Blood CD34+ Cells and Adult BM MNC in Methylcellulose Culture*

Target cells	Stimuli	Serum-containing			Serum-free		
		E	B	E-Mix	E	B	E-Mix
$CD34^+$ cells	SCF	0	0	0	0	0	0
	IL-6 + SCF	0	0	0	0	0	0
	sIL-6R + IL-6	0	0	0	0	0	0
	sIL-6R + IL-6 + SCF	0	28 ± 65	102 ± 21	0	8 ± 4	45 ± 4
	EPO	0	35 ± 15	0	0	6 ± 2	0
	EPO + SCF	0	44 ± 12	14 ± 4*	0	14 ± 3	4 ± 1‡
	EPO + IL-3	0	41 ± 9	8 ± 7*	0	15 ± 7	5 ± 4*
BM MNC	SCF	0	0	0	0	0	0
	IL-6 + SCF	0	0	0	0	0	0
	sIL-6R + IL-6	31 ± 5	0	0	5 ± 2	0	0
	sIL-6R + IL-6 + SCF	121 ± 3	76 ± 12	162 ± 31	71 ± 9	36 ± 10	45 ± 5
	EPO	102 ± 15	73 ± 21	0	70 ± 14	40 ± 8	0
	EPO + SCF	118 ± 11	93 ± 19	16 ± 4*	81 ± 10	49 ± 5	4 ± 2*
	EPO + IL3	98 ± 21	99 ± 15	8 ± 5*	76 ± 9	48 ± 11	2 ± 3*

500 $CD34^+$ cells/ml or 2.5 × 10^4/ml of BM MNC were initiated in triplicate culture and scored at day 7 for CFU-E–derived colonies, and day 14 for BFU-E and CFU-Mix–derived colonies. Results represent mean ± SD from triplicate cultures.
Significantly different from sIL-6R + IL-6 + SCF (*P <0.005, ‡P <0.0001).
E, CFU-E-derived erythroid colonies; *B*, BFU-E-derived erythroid bursts; *E-Mix*, CFU-Mix–derived erythroid mixed colonies.

membrane-anchored gp130 was involved in sIL-6R/IL-6–mediated erythropoiesis, we examined the effects of anti-gp130 mAbs (GPX7, GPX22, and GPZ35), anti-IL-6R mAb, and anti-EPO neutralizing Ab on the development of erythroid cells from $CD34^+$ cells. The addition of anti-gp130 mAbs to the suspension culture with sIL-6R, IL-6, and SCF completely abrogated the production of erythroid cells, whereas these mAbs had no effect on EPO-dependent production of erythroid cells (Fig. 3 *A*). The addition of anti-IL-6R mAb at a concentration of 10 μg/ml to the culture resulted in almost complete inhibition (Fig. 3 *B*). The relatively lower efficiency of anti-IL-6R mAb compared to that of anti-gp130 mAb may be explained by the presence of many more molecules of sIL-6R added to the culture than those of cell surface gp130 expressed on 2,000 $CD34^+$ cells. By contrast, although an anti-EPO Ab almost completely blocked EPO-dependent production of erythroid cells, it failed to inhibit the generation of erythroid cells induced by the combination of sIL-6R, IL-6, and SCF (Fig. 3 *C*). Methylcellulose clonal culture of $CD34^+$ cells also indicated that anti-gp130 mAbs but not anti-EPO Ab completely blocked the development of both erythroid burst and erythroid mixed colony formation stimulated by the combination of sIL-6R, IL-6, and SCF. These results clearly demonstrate that the observed effects of sIL-6R and IL-6 were provided through the interaction of the sIL-6R/IL-6 complex to membrane-anchored gp130 on the target cells. Our results also indicated that the generation of erythroid cells from $CD34^+$ cells by gp130 signaling in association with c-*kit* activation is independent of EPO.

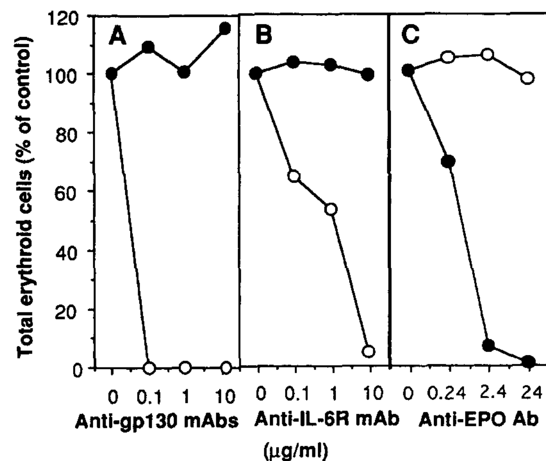

Figure 3. Effects of various concentrations of anti-human gp130 mAbs (*A*), anti-human IL-6R mAb (*B*), and anti-human EPO Ab (*C*) on the generation of erythroid cells from $CD34^+$ cells in suspension culture with a combination of sIL-6R, IL-6, and SCF (*open circles*) or a combination of EPO and SCF (*filled circles*). The mAbs were added at the beginning of the culture and results were determined at day 14. The wells without mAbs were estimated as control experiments. Total erythroid cells including erythroblast, normoblast, and erythrocyte were calculated based on the total cell number and the proportion of the erythroid cells determined on the cytospin slides. Data represent the ratio of the total erythroid cells in each well treated with mAbs to that obtained with control and are expressed as percent (%) control.

Discussion

EPO has been shown as the principal factor that controls proliferation, differentiation, and apoptosis of human erythroid progenitor cells through its special cell surface receptor (EPOR) signaling. Several cytokines such as SCF, IL-3, GM-CSF, IGF-1, and hepatocyte growth factor (HGF) were reported to enhance the proliferation and/or maturation of erythroid progenitors in vitro, but failed to do so without EPO, suggesting the central role of EPO in erythropoiesis (21–28). No factor so far has been reported to possess activity similar to that of EPO, especially on relatively late stage erythroid progenitors. In the present paper, we demonstrate that combined signals through gp130 and c-*kit* could stimulate proliferation and maturation of immature as well as mature erythroid progenitors independent of EPO, giving rise to a number of erythroblasts and erythrocytes. This indicates that EPOR signaling may not be obligatory for proliferation, differentiation, and terminal maturation of normal human erythroid progenitor cells in vitro.

Significant production of erythroid cells in culture with sIL-6R in the presence of.IL-6 and SCF and lack of this production in culture without sIL-6R are reminiscent of the previously reported fact that sIL-6R confers IL-6 responsiveness to cells that show no expression of IL-6R but do express gp130. This view was supported by our immunostaining and flow cytometric analysis experiments in which all $CD34^+$ cells from cord blood and proliferating cells in the sIL-6R/IL-6/SCF culture expressed gp130, but in which most of the $CD34^+$ cells appeared to be negative for IL-6R staining (our unpublished data). Thus, gp130 may be expressed ubiquitously in erythroid progenitors. Activation of gp130, which can be initiated by a complex of sIL-6R/IL-6 but not IL-6 alone as shown in the present study, may transduce signals independent of EPOR to stimulate the development of erythroid cells. Recent studies (6, 7, 29) have indicated that activation of JAK2 kinase is associated with gp130 and EPOR signal transduction, implying that gp130 and EPOR may share a common pathway for intracellular signaling in erythroid grogenitors. It is interesting to note that the observed synergy between gp130 and c-*kit* signalings on erythropoiesis is reminiscent of recent findings by Ip et al. (30) that CNTF signals through gp130 synergize with FGF, which uses Kit-like receptor tyrosine kinase, for the terminal differentiation of neuronal progenitor cells. This suggests that synergistic signals through the gp130 receptor family and the receptors with tyrosine kinase may have an important role in the development of not only blood cells but neurons as well.

The dramatic erythropoiesis supported by the coactivation of both gp130 and c-*kit* signal pathways in the absence of EPO might suggest that gp130 and c-*kit* signalings play a crucial role in the proliferation and maturation of erythroid cells in vivo. In fact, previous studies have demonstrated that mice with *W* or *Steel (Sl)* mutations developed severe anemia with a repressed number of CFU-E in fetal liver (28, 31–33); a recent gene-targeting experiment has shown that impaired erythropoiesis was observed in gp130-deficient mice (34). Detailed hemopoietic analysis of IL-6–deficient mice by Bernad et al. (35) also revealed that while having essential hematological parameters and high BFU-E in BM and spleen in steady-state condition, the IL-6–deficient mice experienced severe anemia, a slow recovery rate, and higher mortality after induction of anemia, suggesting that erythroid differentiation is impaired by the absence of IL-6. Our present data also suggest that receptors presented in soluble form as well as expressed on cell surface play an important role in the development of hemopoiesis. It has been reported that the physiological significance of sIL-6R is indicated by its detection in human sera (36, 37). sIL-6R present in sera is biologically active in terms of its ability to bind IL-6 and eventually to stimulate gp130 (2, 8). A detectable effect of sIL-6R on the generation of erythroid cells from $CD34^+$ cells in vitro was observed at concentrations of 80 ng/ml, which seems likely to be within the physiological range. IL-6 and SCF are also detectable in human sera. In addition, not only IL-6 but also both soluble and membrane-bound forms of SCF are produced by BM stroma cells that may anchor hemopoietic stem cells and immature progenitors and support their proliferation in BM (37–40). Thus, the stimulators for initiating the activation of both gp130 and c-*kit* signal pathways seem sufficient in human physiological state. Taken together, current results suggest that gp130 signaling in combination with SCF play important roles on normal erythropoiesis in vivo.

Our present data provide new insights into mechanisms that control proliferation, differentiation, and terminal maturation of human erythroid cells. More recently, Wu et al. (41) have demonstrated that whereas EPO or EPOR-deficient mice died of failure of erythrocyte generation in murine definitive fetal liver erythropoiesis, low levels of EPO- and EPOR-independent erythropoiesis do occur in primitive erythropoiesis at the yolk sac stage, suggesting that another mechanism may play a critical role. Further studies are needed to elucidate (*a*) whether the novel mechanism with gp130, as demonstrated in this study, contributes to human erythropoiesis in vivo and (*b*) its possible different role and cooperation with EPO.

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