A Novel Mechanism of Cooperation between c-Kit and Erythropoietin Receptor

Reuben Kapur‡ and Lei Zhang

From the Herman B Wells Center for Pediatric Research, Department of Pediatrics, James Whitcomb Riley Hospital for Children, Indiana University School of Medicine, Indianapolis, Indiana 46202

Optimal production of red cells in vivo requires collaboration between c-Kit, erythropoietin receptor (Epo-R), and GATA-1. However, the mechanism(s) of collaboration remain unclear. Utilizing an embryonic stem cell-derived erythroid progenitor cell line from mice deficient in GATA-1, we have examined the role of c-Kit and Epo-R in erythroid cell proliferation, survival, and differentiation. In the absence of GATA-1, we demonstrate an essential role for c-Kit in survival and proliferation of erythroid progenitors via the regulation of Bcl-2 expression. In addition, we demonstrate that Epo-R and Stat5 are regulated by a second, novel mechanism. We demonstrate that c-Kit stimulation by stem cell factor is essential for the maintenance of Epo-R and Stat5 protein expression, which results in significantly enhanced Bcl-xL induction and survival of erythroid progenitors in response to Epo stimulation. Restoration of GATA-1 function results in terminal erythroid maturation and up-regulation of Epo-R and Bcl-xL expression, leading also to significantly enhanced survival of terminally differentiating erythroid progenitors in the presence of only Epo. These results demonstrate that c-Kit and Epo-R have unique role(s) during distinct phases of erythroid maturation, and both stem cell factor and Epo contribute to the regulation of the Epo-R-Stat5-Bcl-xL pathway to ensure optimal survival, proliferation, and differentiation of erythroid progenitors.

The survival, proliferation, and differentiation of erythroid progenitor cells depends to a large extent on the signals emanating from c-Kit, a receptor tyrosine kinase, and the erythropoietin (Epo) receptor (Epo-R), a member of the hematopoietin receptor family (1, 2). Mutant mice that lack the expression of c-Kit (dominant white spotting, or W, mutants) or Epo-R demonstrate severe deficiencies in erythroid cell development (1, 2). c-Kit-deficient mice exhibit a severe reduction of colony-forming unit-erythroid (CFU-E) progenitors in the fetal liver and die of anemia around day 16 of gestation (1, 3). Epo-R-deficient mice also demonstrate a significant decrease in CFU-E progenitors and die of anemia between days 13 and 15 of gestation (2). The data suggest that committed erythroid progenitors cannot survive, proliferate, or differentiate unless both the c-Kit and the Epo-R signal transduction pathways are functional. Recent studies have suggested that Epo-R may contribute to this process by preventing committed erythroid progenitors from undergoing apoptosis (4, 5). The role of c-Kit in the process of erythroid cell development alone or in combination with Epo-R remains unclear. Recent studies have demonstrated that erythroid precursors from mice deficient in the expression of Epo-R can be rescued by in vitro infection with an Epo-R-containing retrovirus but only in the presence of stem cell factor (SCF) (6). From these data, it has been inferred that an interaction between Epo-R and c-Kit, which was previously demonstrated in a cultured cell line (7), is required for erythroid colony formation, presumably at a stage prior to that at which Epo-R-deficient precursors are arrested.

Bcl-2 family proteins are likely to be key effectors of growth factor receptor-mediated survival signals (8). The balance of antiapoptotic Bcl-2, Bcl-xL, A1, and MCL1 and proapoptotic (Bax, Bad, Bak, and Bcl-xS) Bcl-2 family proteins in the cell is critical in determining its ability to survive and subsequently proliferate and differentiate (9). The balance of these observations, both Bcl-xL and Bcl-2 are involved in regulating erythroid progenitor cell survival (10–15). It is uncertain during which phase of erythroid cell maturation Bcl-xL and Bcl-2 function. Many cytokines induce the expression of antiapoptotic Bcl-2 family proteins, although the mechanisms involved are not clear (16–18). When and how c-Kit and Epo-R induce the expression of Bcl-2 and/or Bcl-xL in erythroid cells remains poorly defined. In particular, the role of c-Kit in regulating the expression of Bcl-2 and Bcl-xL during erythroid cell development remains unknown.

Recent studies have shown that Stat5 may in part be responsible for regulating the expression of Bcl-xL in erythroid progenitor cells (19). Mutants of mice that lack both Stat5a and Stat5b demonstrate embryonic anemia (19). In vivo deficiency of Stat5 does not result in embryonic lethality (19, 20), suggesting that other factors may regulate the expression of Bcl-xL, either independently or cooperatively with Stat5 during embryogenesis. Stat5 is activated by Epo-R (21) and other cytokine receptors including those for thrombopoietin; granulocyte-macrophage colony-stimulating factor; granulocyte colony-stimulating factor; interleukin-2, -3, and -5; prolactin; and growth hormone.

In addition to a role for growth factors in inducing the expression of antiapoptotic genes, hematopoietic cells also use internal programs to ensure their survival. The transcription...
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factor GATA-1, which is abundant in erythroid progenitor cells, is essential for maturation of erythroblasts (22–24). Although GATA-1 is expressed in multipotential progenitor cells prior to the commitment to a single lineage, disruption of the GATA-1 gene produces maturation arrest relatively late in erythroid development (22–24). GATA-1-deficient embryos are embryonic lethal at the yolk sac stage (24). They appear colorless; proerythroblast-like cells are present in the yolk sac and circulation. Upon in vitro differentiation of GATA-1-deficient embryonic stem (ES) cells, definitive erythroid precursors are arrested at the proerythroblast stage and undergo apoptosis (25, 26).

The generation of an erythroid cell line (G1E-ER2) from GATA-1-deficient ES cells provides an excellent model to study the function of c-Kit and Epo-R in erythroblasts in the context of GATA-1 function (27). G1E-ER2 cells express erythroid but not myeloid genes (27). These cells proliferate continuously in culture as developmentally arrested erythroid precursors. G1E-ER2 cells stably express a conditional (estrogen-responsive) form of GATA-1, and upon exposure to β-estradiol they undergo synchronous erythroid maturation (15). Utilizing G1E-ER2 cells, recent studies have shown that GATA-1 can induce the expression of Bcl-xL and cooperate with Epo to promote the survival of these cells, although the mechanism of Bcl-xL expression remains to be determined (15). In the present study, we demonstrate an essential role for c-Kit in proliferation and survival of erythroid progenitors via the induction of Bcl-2 expression. We also demonstrate that c-Kit synergizes with Epo-R in enhancing proliferation and survival of erythroid progenitors in the absence of GATA-1 function by inducing the expression of Epo-R, Stat5, and Bcl-xL. Restoration of GATA-1 function results in terminal erythroid differentiation, down-regulation of c-Kit and Bcl-2, and up-regulation of Epo-R and Bcl-xL, leading to significantly enhanced survival of differentiating erythroid progenitors. These results suggest that c-Kit and Epo-R have unique roles during distinct phases of erythroid cell maturation and describe an essential role for c-Kit in proliferation and in inducing the expression of critical genes involved in regulating survival of erythroid progenitors by Epo, including Epo-R, Stat5, and Bcl-xL.

EXPERIMENTAL PROCEDURES

Cell Line, Antibodies, and Flow Cytometric Analysis—G1E-ER2 cells have been described previously (15, 28). Unless otherwise specified, G1E-ER2 cells were grown in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) with 15% heat-inactivated embryonic stem cell serum (HyClone, Logan, UT), recombinant erythropoietin (Epo) (2 units/ml) (Amgen, Thousand Oaks, CA), and recombinant rat SCF (50 ng/ml) (Amgen, Thousand Oaks, CA).

Phycoerythrin-conjugated monoclonal antibodies were directed against Ter119. All of the phycoerythrin-conjugated monoclonal antibodies, including the isotype control antibodies, were purchased from Pharmingen (San Diego, CA). G1E-ER2 cells (1 × 10⁶) were incubated at 4 °C for 30 min with 1 μg of the primary monoclonal antibody. Cells were washed three times with phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma) and analyzed by a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA).

Effects of SCF and Epo on Proliferation and Survival of G1E-ER2 Cells—The effect of SCF and Epo on proliferation of G1E-ER2 cells was assayed using thymidine incorporation. 96-Well tissue culture plates were plated with 5 × 10⁴ cells/well for 48 h, either in the presence or absence of recombinant rat SCF and/or Epo. Subsequently, 1.0 μCi of [3H]thymidine (Amersham Pharmacia Biotech) was added to each well for 6–8 h at 37 °C. Cells were then harvested using an automated cell harvester (96-well harvester; Brandel, Gaithersburg, MD), and thymidine incorporation was determined in a scintillation counter. The effect of SCF and/or Epo on cell death (apoptosis and necrosis) of G1E-ER2 cells was assayed by staining the cells with annexin-fluorescein isothiocyanate-conjugated and propidium iodide according to the manufacturer's instructions (Pharmingen, San Diego, CA). 24-Well tissue culture plates were utilized for these studies. G1E-ER2 cells were plated at 5 × 10⁶ cells/well for 48 h, either in the presence or absence of SCF and/or Epo. Subsequently, cells were harvested and stained with annexin-fluorescein isothiocyanate and propidium iodide and analyzed by flow cytometry.

Western Blotting—Western blotting was performed according to standard protocols. 1–2 × 10⁶ G1E-ER2 cells were plated in duplicate six-well tissue culture plates for 48 h at 37 °C in the presence or absence of SCF and/or Epo. Thereafter, cells were harvested and lysed in lysis buffer (10 mmol/liter K₃HPO₄, 1 mmol/liter EDTA, 5 mmol/liter EGTA, 10 mmol/liter MgCl₂, 1 mmol/liter Na₂VO₄, 50 mmol/liter β-glycerol phosphate, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin) at 4 °C for 30 min. Cell lysates were centrifuged for 30 min at 10,000 × g at 4 °C. An equal amount of protein was fractioned on 10% polyacrylamide/SDS gel and electrophoretically transferred to nitrocellulose membrane. Expression of c-Kit was determined by using a 1:1000 dilution of an anti-c-Kit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Expression of Epo-R was determined by using a 1:1000 dilution of an anti-Epo-R antibody (Santa Cruz Biotechnology). Expression of Bcl-xL and Bcl-2 was determined by using a 1:1000 dilution of anti-Bcl-2 (Santa Cruz Biotechnology) and anti-Bcl-xL (Pharmingen, San Diego, CA) antibody. The expression of Erk and p38 was determined by using anti-Erk and p38 antibodies (all purchased from New England Biolabs, Beverly, MA).

Western blotting was performed according to the manufacturer's instructions (New England Biolabs, Beverly, MA).

RESULTS

Stimulation of c-Kit by SCF Results in Proliferation of G1E-ER2 Cells but Impairment of GATA-1-induced Differentiation—Previous work, including the analysis of mice deficient in the expression of c-Kit and Epo-R, has suggested an essential function for c-Kit and Epo-R in erythroid cell development (1, 2). To determine the role(s) of c-Kit and Epo-R in erythroid cell development, we first examined proliferation in G1E-ER2 cells stimulated with either SCF or Epo or with the combination of these two cytokines in the absence or presence of GATA-1 function. As expected, in cultures in which no cytokines were added, G1E-ER2 cells demonstrated no proliferation after 48 h. Stimulation of G1E-ER2 cells with SCF alone, in the absence of GATA-1, resulted in a significant increase in DNA synthesis in comparison with unstimulated cells (68,144 ± 13,765 versus 455 ± 194 cpm (mean ± S.D.), respectively, p < 0.0001) (Fig. 1A). Interestingly, stimulation of these cells with Epo alone resulted in only a modest increase in DNA synthesis in comparison with unstimulated cells (5144 ± 2711 versus 455 ± 194 cpm, mean ± S.D., respectively, p < 0.0001) (Fig. 1A). DNA synthesis in response to Epo could not be augmented by higher Epo concentration (data not shown). Stimulation of G1E-ER2 cells with combined Epo and SCF resulted in significantly greater proliferation in comparison with cells stimulated with SCF alone (92,013 ± 17,923 versus 68,144 ± 13,765 cpm (mean ± S.D.), respectively, p < 0.0001) (Fig. 1A). These results demonstrate an essential and nonredundant role for c-Kit in promoting G1E-ER2 cell proliferation in the absence of GATA-1 function.

We next examined the effect of c-Kit and Epo-R activation on proliferation of G1E-ER2 cells in the presence of GATA-1 expression. β-Estradiol-induced GATA-1-expressing G1E-ER2 cells were left unstimulated or were stimulated with SCF or Epo or with the combination of these two cytokines as described above. Western blot analysis was performed according to the manufacturer's instructions (New England Biolabs, Beverly, MA).
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DNA synthesis over cytokine-unstimulated cells was also apparent following stimulation with Epo alone (780 ± 133 versus 356 ± 170 cpm (mean ± S.D.), respectively, p < 0.01) (Fig. 1B). Combined stimulation of GATA-1-expressing cells with SCF and Epo also demonstrated significantly greater proliferation in comparison with cells stimulated with SCF alone (6006 ± 1356 versus 1181 ± 478 cpm, mean ± S.D., respectively, p < 0.01) (Fig. 1B).

Previous studies have shown that the expression of GATA-1 by adding β-estradiol in the presence of Epo triggers synchronous erythroid maturation in G1E-ER2 cells, G1 phase cell cycle arrest, followed by the appearance of benzidine-staining cells (15). Consistent with these observations, in our hands, the expression of GATA-1 in the presence of Epo also resulted in the emergence of benzidine-positive cells (data not shown) and induction of cell surface protein, Ter119 (Fig. 2). However, GATA-1-induced differentiation, as measured by the induction of Ter119, was significantly reduced in the presence of SCF compared with Epo stimulation (20.3 ± 3 versus 72.6 ± 7.5% Ter119 expression (mean ± S.D.), respectively, p < 0.005) (Fig. 2). These results demonstrate that in contrast to the effect of c-Kit activation on G1E-ER2 cell proliferation in the absence of GATA-1, in the presence of GATA-1 expression, SCF significantly impairs erythroid differentiation.

Stimulation of c-Kit by SCF Maintains the Survival of G1E-ER2 Cells in the Absence of GATA-1 but Not in the Presence of GATA-1 Expression—A reasonable hypothesis to explain the differential effects of Epo and SCF on proliferation and differentiation of G1E-ER2 cells would be that these two cytokines maintain the survival of G1E-ER2 cells during distinct stages of maturation, which may be regulated by GATA-1 (Fig. 7). To test this, we examined apoptosis in G1E-ER2 cells following stimulation with either SCF or Epo or with the combination of these two cytokines in the absence or presence of GATA-1 function. Following no cytokine treatment, factor-starved G1E-ER2 cells lacking the expression of GATA-1 demonstrated significantly greater cell death in comparison with cells cultured in the presence of either SCF or Epo or with the combination of these two after 48 h (Fig. 3A). Cell death of G1E-ER2 cells in the presence of SCF alone was significantly less relative to cultures stimulated with Epo alone (28 ± 3.4 versus 49 ± 3.7% (mean ± S.D.), respectively, p < 0.001) (Fig. 3A). Further, combined stimulation of these cells with both SCF and Epo resulted in significantly less cell death relative to cultures stimulated with SCF alone (13 ± 3.5 versus 28 ± 3.4% (mean ± S.D.), respectively, p < 0.001) (Fig. 3A).

In contrast to significantly less cell death of SCF-treated cells in the absence of GATA-1, only a modest decrease in cell death was observed in SCF treated cultures in the presence of GATA-1 (92 ± 2.2 versus 96 ± 2.4% (mean ± S.D.), respectively, p < 0.007) (Fig. 3B). In contrast, the addition of Epo to GATA-1-expressing cultures was associated with significantly less cell death of G1E-ER2 cells relative to SCF-stimulated cultures (61 ± 9.1 versus 92 ± 2.2% (mean ± S.D.), respectively, p < 0.01) (Fig. 3B). Further, analogous to the results observed in the absence of GATA-1 (see Fig. 3A), stimulation of GATA-1-expressing cells with SCF and Epo resulted in least cell death (Fig. 3B). These results demonstrate that in the absence of GATA-1, c-Kit-mediated antiapoptotic signals appear to be essential in maintaining the survival of G1E-ER2 cells. In contrast, Epo-R-mediated antiapoptotic signals play a dominant role in maintaining the survival of GATA-1-induced terminally differentiating G1E-ER2 cells. Combined stimulation of c-Kit and Epo-R is essential for maximum survival of G1E-ER2 cells, both in the absence and presence of GATA-1 function (Fig. 7).

Stimulation of c-Kit by SCF Induces Bcl-2 but Not Bcl-xL Expression in G1E-ER2 Cells—To determine the mechanism of differential survival of G1E-ER2 cells in response to SCF and Epo stimulation during distinct stages of erythroid cell maturation, we examined the expression of antiapoptotic gene family members Bcl-2 and Bcl-xL in the absence and presence of GATA-1 function. G1E-ER2 cells grown in log phase in the presence of SCF and Epo were factor-starved for 5 h and stimulated with cytokines (Fig. 4). 48 h later, cells were harvested and lysed, and an equal amount of protein was subjected to immunoblot analysis using an anti-Bcl-2 or anti-Bcl-xL antibody. In the absence of GATA-1, stimulation of G1E-ER2 cells
with SCF resulted in significant expression of Bcl-2 (Fig. 4, lane 2). In contrast, stimulation of these cells with Epo was associated with complete loss of Bcl-2 protein (Fig. 4, lane 3). Combined stimulation with SCF and Epo was associated with significant levels of Bcl-2 protein after 48 h of culture (Fig. 4, lane 4). However, stimulation of G1E-ER2 cells with Epo resulted in significant Bcl-xL expression (Fig. 4, lane 3). Surprisingly, stimulation of c-Kit with SCF resulted in a significant decrease in the expression of Bcl-xL after 48 h (Fig. 4, lane 2). Consistent with the survival data noted above, combined stimulation of c-Kit and Epo-R significantly increased the expression of Bcl-xL relative to Epo stimulation alone (Fig. 4, lane 4). These data demonstrate that in the absence of GATA-1 expression, survival of G1E-ER2 cells by SCF is associated with the expression of Bcl-2. In contrast, Epo-mediated survival of these cells is associated with the induction of Bcl-xL. Further, maximum survival in cultures stimulated concurrently with SCF and Epo is associated with the combined antiapoptotic effects of both Bcl-2 and Bcl-xL (Fig. 7).

We next examined the antiapoptotic effects of Bcl-2 and Bcl-xL in GATA-1-induced terminally differentiating G1E-ER2 cells in the presence of SCF or Epo or the combination of these two cytokines. Stimulation of these cells with SCF resulted in neither Bcl-2 nor Bcl-xL expression (Fig. 4, lane 5). Further, the
lack of Bcl-2 induction noted earlier in response to Epo in the absence of GATA-1 was also apparent in the presence of GATA-1 expression. (Fig. 4, lane 6). These data suggest that GATA-1, either directly or indirectly, down-regulates the expression of Bcl-2, which is associated with complete loss of survival in G1E-ER2 cells stimulated with SCF (Fig. 7).

In contrast to the apparent lack of Bcl-2 effects on the survival of terminally differentiating G1E-ER2 cells in the presence of SCF or Epo, expression of GATA-1 significantly enhanced the expression of Bcl-xL in the absence of Epo but not SCF (Fig. 4, lane 6). Epo-mediated increase in Bcl-xL expression in G1E-ER2 cells was significantly greater than that observed in cells stimulated with Epo in the absence of GATA-1 expression (Fig. 4, lane 3). The increase in Bcl-xL expression under these conditions was correlated with enhanced survival of G1E-ER2 cells in the presence of Epo but not SCF noted earlier (see Fig. 3B). Analogous to our previous observation in the absence of GATA-1, combined stimulation of GATA-1-expressing G1E-ER2 cells with SCF and Epo also resulted in enhanced survival via the induction of both Bcl-xL and Bcl-2 (Fig. 4, lane 7), although the level of Bcl-2 was less than that observed in the absence of GATA-1. These results demonstrate that the expression of Bcl-xL is associated with the survival of terminally differentiating G1E-ER2 cells. Combined expression of Bcl-2 and Bcl-xL is associated with further augmenting the survival of terminally differentiating G1E-ER2 cells. Further, unlike the lack of cooperation noted between GATA-1 and SCF in inducing the expression of Bcl-2, Epo in collaboration with GATA-1 significantly enhances the expression of Bcl-xL and survival of terminally differentiating G1E-ER2 cells (Fig. 7).

**Stimulation of c-Kit by SCF Does Not Activate Stat5 but Maintains Its Protein Expression—**Stat5 binds to the Bcl-xL promoter and induces the expression of Bcl-xL in response to Epo stimulation in HCD57 cells (19). To explain the observation that c-Kit stimulation by SCF in GATA-1-nonexpressing cells does not maintain the expression of Bcl-xL but stimulation with Epo-R significantly enhances the expression of Bcl-xL, we hypothesized that activation of Stat5 in G1E-ER2 cells is mediated differentially via Epo-R but not c-Kit stimulation. To test this hypothesis, c-Kit-expressing G1E-ER2 cells were factor-starved for 5–6 h and stimulated with SCF or Epo or with the combination of these two cytokines (Fig. 5A). Thereafter, cells were lysed, and immunoblot analysis was performed with an antiphospho-Stat5 antibody. Consistent with previously published observations in HCD57 cells (19), stimulation of G1E-ER2 cells with Epo resulted in Stat5 activation (Fig. 5A, lanes 5 and 6). Consistent with our hypothesis and with the lack of Bcl-xL induction noted in response to c-Kit activation, stimulation of G1E-ER2 cells with SCF did not activate Stat5 (Fig. 5A, lanes 2 and 3). These data suggest that GATA-1, either directly or indirectly, down-regulates the expression of Bcl-2, which is associated with complete loss of survival in G1E-ER2 cells stimulated with Epo (Fig. 7).

We next hypothesized that the expression of Bcl-xL after stimulation of both c-Kit and Epo-R and the survival of G1E-ER2 cells in the absence of GATA-1 and these cytokines might in part be due to the regulation of the Stat5 protein by SCF. In this model (Fig. 7), we propose that regulation of Stat5 occurs in two steps. First, stimulation of c-Kit by SCF induces the expression of Stat5 protein in G1E-ER2 cells, followed by phosphorylation and subsequent activation of Stat5 protein via the activation of Epo-R by Epo. To test if c-Kit stimulation by SCF was associated with the induction of expression of Stat5, G1E-ER2 cells grown in the presence of SCF and Epo were washed three times in medium containing no growth factors and stimulated with either SCF or Epo or with the combination of these two cytokines (Fig. 5B). 48 h later, cells were harvested and lysed, and an equal amount of protein was subjected to immunoblot analysis. Consistent with our hypothesis, activation of c-Kit by SCF is essential for maintaining the expression of Stat5 in G1E-ER2 cells (Fig. 5B, lane 2). Stimulation of these cells with Epo alone lead to a significant decrease in the expression of Stat5 protein after 48 h of culture (Fig. 5B, lane 3). As a loading control, the expression of mitogen-activated protein kinase family members Erk and p38 was examined. The expression of these proteins remained similar in cultures stimulated with either SCF or Epo (Fig. 5B, middle and lower panels). These results demonstrate a novel mechanism of cooperation between c-Kit and Epo-R in erythroid cells, whereby stimulation of c-Kit by SCF is an essential prerequisite for
activation of Stat5 via Epo stimulation and resulting enhanced Bcl-x\textsubscript{L} expression and survival (Fig. 7).

GATA-1 Modulates the Expression of c-Kit and Epo-R during Terminal Stages of Erythroid Maturation—To further explore the differential responsiveness of G1E-ER2 cells to SCF and Epo in the absence and presence of GATA-1 function, we examined the consequence(s) of GATA-1 in modulating the expression of c-Kit and Epo-R. We hypothesized that GATA-1 either directly or indirectly modulates the expression of c-Kit and Epo-R during terminal stages of erythroid differentiation, rendering these cells nonresponsive to SCF but not Epo. To test this, we examined the expression of c-Kit and Epo-R in G1E-ER2 cells by immunoblot analysis in the absence and presence of GATA-1 expression. G1E-ER2 cells grown in the presence of SCF and Epo were starved for 5 h and stimulated with either SCF or Epo or with the combination of these two cytokines in the absence or presence of \(\beta\)-estradiol. 48 h later, cells were harvested and lysed, and an equal amount of protein was subjected to immunoblot analysis. Stimulation of G1E-ER2 cells by SCF in the absence of GATA-1 resulted in significant c-Kit expression after 48 h of culture (Fig. 6, lane 2). However, stimulation of these cells in the presence of Epo down-regulated the expression of c-Kit after 48 h (Fig. 6, lane 3). In contrast, induction of GATA-1 significantly down-regulated the expression of c-Kit in the presence of Epo (Fig. 6, lane 5). These data demonstrate that restoring the function of GATA-1, either directly or indirectly down-regulates the expression of c-Kit in G1E-ER2 cells. Therefore, loss of Bcl-2 expression and survival noted earlier in G1E-ER2 cells in the presence of SCF may in part be due to the loss of c-Kit protein in terminally differentiating G1E-ER2 cells (Fig. 7).

We next examined the effect of GATA-1 induction on Epo-R expression. As seen in Fig. 6, stimulation of G1E-ER2 cells with SCF in the absence of GATA-1 resulted in significant expression of Epo-R after 48 h of culture (Fig. 6, lane 2). In contrast, a significant loss of Epo-R was observed in cells stimulated with Epo alone (Fig. 6, lane 3). However, restoration of GATA-1 in the presence of Epo significantly enhanced the level of Epo-R in these cells (Fig. 6, lane 6). Thus, analogous to described previously role for GATA-1 and Epo in enhancing the expression of Bcl-x\textsubscript{L} in terminally differentiating G1E-ER2 cells (see Fig. 4B, lane 6), these data also demonstrate cooperativity between GATA-1 and Epo in inducing the expression of Epo-R (Fig. 7).

DISCUSSION

SCF is crucial for the development of CFU-E, since mice lacking SCF or its receptor c-Kit exhibit significant reduction of fetal liver CFU-E progenitors and suffer severe anemia (1, 3). The survival of CFU-E progenitors also depends on Epo, suggesting that committed erythroid progenitors cannot proliferate or mature further unless both c-Kit and the Epo-R signal transduction pathways are functional. Our results demonstrate an essential and nonredundant function for SCF/c-Kit in proliferation and survival of proerythroblasts. In contrast, we demonstrate that stimulation of Epo-R signaling does not induce proliferation, although it maintains the survival of proerythroblasts. These observations are consistent with a previously reported role for Epo in the survival of CFU-Es without affecting their cell cycle status (29–31). Interestingly, the combined stimulation of G1E-ER2 cells with SCF and Epo significantly enhanced the proliferation of these cells. In this regard, we and others have previously shown that SCF can replace Epo in supporting the growth of HCD57 cells, an Epo-dependent erythroid cell line (7, 32). In these cells, SCF rapidly induced tyrosine phosphorylation of both c-Kit and Epo-R. These data suggest that c-Kit may activate the Epo/Epo-R signal transduction pathway via tyrosine phosphorylation of the Epo-R and that, in turn, the activated Epo-R may induce further proliferation and maturation of committed erythroid progenitors. We believe that this may in part be the mechanism of increased proliferation noted in G1E-ER2 cells in response to simultaneous activation of both c-Kit and Epo-R.

The mechanism of Epo and SCF regulation of red cell production is not clear. The maintenance of cell survival may constitute one such mechanism, since we demonstrate that the maximum number of erythroid progenitors rescued from apoptosis depends on simultaneous activation of both c-Kit and Epo-R. The graded response of G1E-ER2 cells to Epo and SCF could in part be due to variation in the antiapoptotic signaling that progenitors require; a number of distinct antiapoptotic pathways may interact additively or synergistically to rescue maximum numbers of progenitors stimulated with Epo and SCF. Support for this model comes from our studies demonstrating induction of two separate antiapoptotic proteins, Bcl-2 and Bcl-x\textsubscript{L}, by c-Kit and Epo-R, respectively (Fig. 7). We show that the combined induction of both Bcl-2 and Bcl-x\textsubscript{L} by two completely separate cytokines is associated with maximum survival of G1E-ER2 cells both at the proerythroblast stage (in the absence of GATA-1) as well as in terminally differentiated erythroid cells (in the presence of GATA-1). Our results also demonstrate that SCF-induced Bcl-2 expression is predomi-
nantly associated with the survival of proerythrobasts. In contrast, Epo-induced Bcl-xL is associated with the survival of terminally differentiated erythroblast cells.

The production of definitive erythroid lineage cells to a large extent is controlled by Epo (33). Epo prevents the apoptotic cell death of definitive erythroid progenitors. The antiapoptotic effect of Epo on definitive erythroid progenitors has been observed from late erythroid progenitors (CFU-E) up to the onset of hemoglobinization (4, 34–36). Epo-deprived apoptotic cell death is observed less at the end of maturation when maximum hemoglobin synthesis occurs. On the other hand, studies performed in mice lacking Bcl-xL have clearly demonstrated that apoptotic cell death in Bcl-xL−/− mice occurs only at the end of terminal differentiation in both primitive and definitive erythropoiesis (37). Therefore, it is reasonable to propose that accumulation of Bcl-xL resulting from Epo stimulation probably prevents the apoptotic cell death of terminally differentiated erythroblast cells. Consistent with this hypothesis, our results in G1E-ER2 cells demonstrate maximum induction of Bcl-xL in terminally differentiated erythroid cells. Further, our data show that the lack of Bcl-xL induction, noted in response to SCF stimulation of GATA-1-expressing G1E-ER2 cells resulted in significantly reduced differentiation. The data demonstrate an essential role for Bcl-xL in maintaining the survival of differentiated erythroid cells. However, the accumulation of Bcl-xL in terminally differentiated cells cannot be the only mechanism for the antiapoptotic effect of Epo, since some Bcl-xL−/−colonies contain a significant proportion of viable cells that partially hemoglobinize (15, 37). Therefore, it is possible that other death-antagonizing genes also play a role in the survival of terminally differentiated erythroid cells. Our data would suggest that c-Kit-induced Bcl-2 expression may partly be responsible for partial survival of hemoglobinized colonies seen in Bcl-xL−/−mice (Fig. 7).

A significant increase in the expression of Bcl-xL during terminal differentiation is intriguing. Clearly, this increase is mediated either directly or indirectly via the induction of GATA-1. How GATA-1 up-regulates the expression of Bcl-xL remains to be determined. One possible explanation might involve the modulation of c-Kit and Epo-R. GATA-1 has previously been shown to directly bind the Epo-R promoter and induce its expression (38–40). It is conceivable that the enhanced expression of Bcl-xL during terminal stages of erythroid maturation may be an indirect effect of increased Epo-R expression by GATA-1. Further, during terminal stages of erythroid maturation GATA-1 alone is not sufficient to induce the expression of Bcl-xL; rather, GATA-1 cooperates with Epo in this process. Consistent with these observations, our results demonstrate a significant increase in the expression of Epo-R in GATA-1-expressing cells compared with GATA-1-nonexpressing cells. This increase in the expression of Epo-R may partly account for the increase in the expression of Bcl-xL in these cells.

In addition to the previously described mechanism of cross-talk between c-Kit and Epo-R in inducing proliferation of erythroid progenitors (7, 32), we describe a novel mechanism via which c-Kit and Epo-R may augment the proliferation and survival of proerythroblasts. We demonstrate that the expression of Epo-R and Stat5 is dependent upon the activation of c-Kit by SCF. Although some survival of G1E-ER2 cells is observed in the presence of Epo stimulation alone, stimulation by both SCF and Epo significantly enhances the survival of these cells. We demonstrate that the increase in the survival of proerythroblasts in the presence of both SCF and Epo is partly due to the increase in the expression of Epo-R and Stat5 by SCF. We propose that c-Kit stimulation by SCF is essential for the expression of Epo-R and Stat5, resulting in optimal activation of Epo-R and Stat5 by Epo, followed by Bcl-xL induction and enhanced survival of these cells (Fig. 7). We hypothesize that the lack of this process may in part be responsible for reduced numbers of CFU-Es noted in mutants of SCF and c-Kit. Further support for this mechanism of cooperation between c-Kit and Epo-R comes from studies performed in a human leukemic cell line, HML/SE (41). These studies demonstrated an increase in the Epo-R mRNA in response to stimulation by SCF (41). These studies also showed that SCF can activate the human Epo-R promoter containing the GATA and Sp1 binding sites, and mutations in the Sp1 binding site resulted in abrogation of Epo-R mRNA in response to SCF stimulation. Together, these results may also help explain an absolute requirement for both Epo and SCF in the formation of CFU-E colonies from fetal livers of Epo-R-deficient mice infected in vitro with a retrovirus expressing the wild-type Epo-R (6). Taken together, we demonstrate a novel mechanism for controlling the Epo-R-Stat5-Bcl-xL signaling pathway in erythroid cells by regulating the expression of Epo-R and Stat5 via the activation of c-Kit.

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REFERENCES
1. Russel, E. S. (1979) Adv. Genet. 20, 357–459
2. Wu, H., Liu, X., Jaenisch, R., and Lodish, H. F. (1995) Cell 83, 59–67
3. Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., and Besmer, P. (1989) Genes Dev. 3, 816–826
4. Koury, M. J., and Bondurant, M. C. (1990) Science 249, 378–381
5. Kelley, L. L., Koury, M. J., Bondurant, M. C., Koury, S. T., Sawyer, S. T., and Wickrema, A. (1996) Blood 82, 2340–2352
6. Wu, H., Klingmuller, U., Acuario, A., Hsiao, J. G., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1806–1810
7. Wu, H., Klingmuller, U., Besmer, P., and Lodish, H. F. (1995) Nature 377, 242–246
8. Chao, D. T., and Korsmeyer, S. J. (1998) Annu. Rev. Immunol. 16, 395–419
9. Olkvi, Z. N., and Korsmeyer, S. J. (1994) Cell 79, 189–192
10. Matsumiya, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., and Loh, D. Y. (1995) Science 267, 1506–1510
11. Benito, A., Silva, M., Grillot, D., Nunez, G., and Fernandez-Luna, J. L. (1994) Blood 83, 837–838
12. Silva, M., Grillot, D., Benito, A., Richard, C., Nunez, G., and Fernandez-Luna, J. L. (1994) Blood 83, 1576–1582
13. Lacroux, V., Varlet, P., Mayeux, P., Parieu, A., Gisselbrecht, S., Kahn, A., and Lacroix, C. (1997) Blood 90, 3050–3055
14. Gregoli, P. A., and Bondurant, M. C. (1997) Blood 90, 630–640
15. Gregory, T., Yu, C., Ma, A., Orkin, S. H., Blobel, G. A., and Weiss, M. J. (1999) Blood 94, 87–96
16. Kozopas, K. M., Yang, T., Buchanan, H. L., Zhou, P., and Craig, R. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3516–3520
17. Lin, E. Y., Oroshsky, A., Berger, M. S., and Prystowsky, M. B. (1993) J. Immunol. 141, 1979–1988
18. Miyazaki, T., Liu, J. Z., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barossumian, E. L., Permutt, R. M., and Taniguchi, T. (1995) Cell 81, 223–231
19. Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C., and Lodish, H. F. (1999) Cell 98, 181–191
20. Teglund, S., McKay, C., Schuetz, e., van Deursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Groseveld, G., and Bile, J. N. (1998) Cell 93, 841–850
21. Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999) Trends Endocrinol. Metab. 10, 18–23
22. Penney, L., Lin, C. S., Robertson, E., Klein, W. H., Tsai, S. F., D’Agati, V., Orkin, S. H., and Costantini, F. (1995) Development 121, 163–172
23. Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C., and Orkin, S. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12355–12358
24. Weiss, M. J., Keller, G., and Orkin, S. H. (1994) Genes Dev. 8, 1184–1197
25. Weiss, M. J., and Orkin, S. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9623–9627
26. Weiss, M. J., Yu, C., and Orkin, S. H. (1997) Mol. Cell. Biol. 17, 1642–1651
27. Kapur, R., Cooper, R., Xiao, X., Weiss, M. J., Donovan, P., and Williams, D. A. (1999) Blood 94, 1915–1925
28. Dranoff, G., Crawford, A. D., Sadelaian, M., Ream, B., Rashid, A., Bronson, R. T., Dickerson, G. R., Bachurski, C. J., Mark, E. L., Whitsett, J. A., and Mulligan, R. C. (1994) Science 264, 713–716
29. Landschulz, K. T., Boyer, S. H., Noyes, A. N., Rogers, O. C., and Frelin, L. P. (1992) Blood 79, 2749–2758
31. Kelley, L. L., Green, W. F., Hicks, G. G., Bondurant, M. C., Koury, M. J., and Ruley, H. E. (1994) Mol. Cell. Biol. 14, 4183–4192
32. Kapur, R., Majumdar, M., Xiao, X., McAndrews-Hill, M., Schindler, K., and Williams, D. A. (1998) Blood 91, 879–889
33. Ihle, J. N., Quelle, F. W., and Miura, O. (1993) Semin. Immunol. 5, 375–389
34. Spivak, J. L., Pham, T., Isaacs, M., and Hankins, W. D. (1991) Blood 77, 1228–1233
35. Yu, H., Bauer, B., Lipke, G. K., Phillips, R. L., and Van Zant, G. (1993) Blood 81, 373–384
36. Koury, M. J., Kelley, L. L., and Bondurant, M. C. (1994) Ann. N. Y. Acad. Sci. 718, 259–267
37. Motoyama, N., Kimura, T., Takahashi, T., Watanabe, T., and Nakano, T. (1999) J. Exp. Med. 189, 1691–1698
38. Chin, K., Oda, N., Shen, R., and Noguchi, C. T. (1995) Nucleic Acids Res. 23, 3041–3049
39. Zon, L. I., Youssoufian, H., Mather, C., Lodish, H. F., and Orkin, S. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10638–10641
40. Chiba, T., Ikawa, Y., and Todokoro, K. (1991) Nucleic Acids Res. 19, 3843–3848
41. Sato, T., Watanabe, S., Ishii, E., Tsuji, K., and Nakahata, T. (1998) J. Biol. Chem. 273, 16921–16926
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Reuben Kapur and Lei Zhang

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