receptor (c-Mpl) gene, respectively, as a growth-signal generator.

In vitro effects of the EPO-driven SAG on Ba/F3

Bicistronic retroviral vectors were generated which express the EPO-driven SAG or wild-type EPOR (EPORwt) gene as the first cistron and the EYFP gene as the second cistron. The vectors were infected into Ba/F3 cells. Ba/F3 is a mouse pro-B cell line and the cells require IL-3 for growth. YFP-positive cells were isolated (>98% purity) and stimulated by rhEPO at various concentrations (Figure 2A). All the cells acquired the ability of EPO-dependent growth and were able to proliferate even in the absence of IL-3. Ba/F3 cells expressing either EPORwt, EPORGCR, or EPORMpl reached the maximum growth levels by adding 1–100 ng/ml EPO (Figure 2A). Endogenous EPO will not induce a significant proliferative response of the cells, since the physiological range of serum EPO concentrations is below 0.1 ng/ml.

We compared the EPO- and steroid-driven SAGs in terms of their ability to expand Ba/F3 cells. The Ba/F3 cells expressing either of the two EPO-driven SAGs proliferated in the presence of EPO to the same extent as the parental Ba/F3 cells in the presence of IL-3. Of note, the EPO-driven SAG (EPORGCR) expanded Ba/F3 cells by around 10^4-fold more than the steroid-driven counterpart (ΔGCRTmR) after 2 weeks of culture (Figure 2B), indicating that the molecular switch using the EPOR is more efficient than that using the tamoxifen receptor despite the inclusion of the same signal generator (GCR) in the SAGs. Thus, we used EPO-driven SAGs for subsequent experiments.

In vitro effects of the EPO-driven SAGs on human CD34$^+$ cells

To examine whether GCR or c-Mpl is the more suitable signal generator of the EPO-driven SAG, human cord blood CD34$^+$ cells were used as targets. CD34$^+$ cells were transduced with bicistronic retroviral vectors which express the EPO-driven SAG as the first cistron and the EYFP gene as the second cistron. After transduction, 27.3 ± 4.7% of the cells fluoresced (YFP-positive). The transduced CD34$^+$ cells were then cultured in liquid medium in the presence of EPO. The fraction of YFP-positive cells increased over time, and virtually all (>95%) of the cells became YFP-positive during a 2-week culture with EPO. This suggests that the EPO-driven SAGs are able to confer a growth advantage on human CD34$^+$ cells. As shown in Figure 3, although the cells transduced with EPORwt proliferated most quickly, the cell number already began to decrease within 2 weeks after the culture initiation. The cells transduced with EPORGCR grew slowly compared with the others, but began to decrease in number by week 3. On the other hand, the cells transduced with EPORMpl proliferated the longest (1 month) in the presence of EPO and the cell number increased by 10^4-fold over this period.

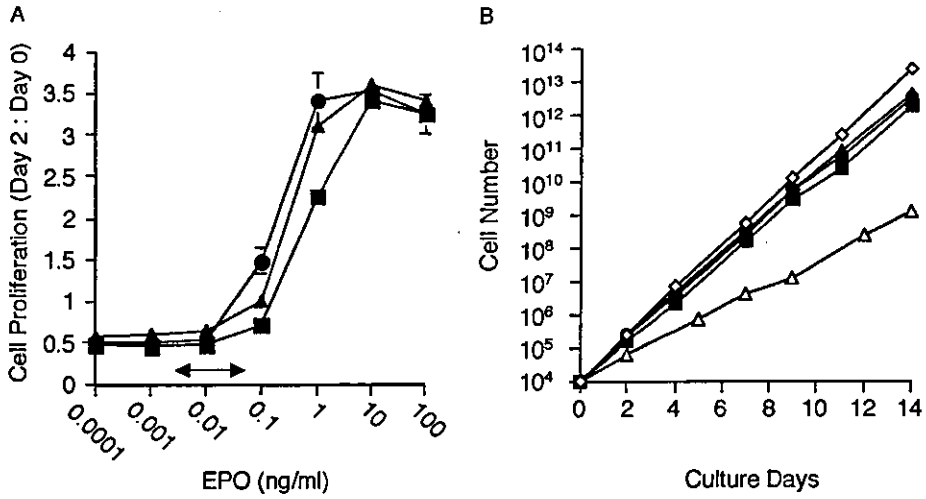

Figure 2. The EPO-driven SAG efficiently stimulates Ba/F3 cell growth. (A) EPO-dependent growth of Ba/F3 cells by introduction of the EPO-driven SAG. BaF3 cells were transduced with the EPORwt (solid triangles), EPORGCR (solid squares) or EPORMpl gene (solid circles) each along with the EYFP gene by bicistronic retroviral vectors. YFP-positive cells were sorted (>98%) and treated with EPO at various concentrations. The proliferation assay (see Materials and Methods) was performed on days 0 and 2, and the ratio of day 2 A_{490}-A_{650} to day 0 A_{490}-A_{650} (means ± SD of triplicate) is shown. The arrow indicates the physiological range of EPO concentrations in human plasma. (B) The EPO-driven SAG triggers higher levels of cell proliferation than the steroid-driven SAG. The parental Ba/F3 cells (open diamonds) were cultured in the presence of IL-3 (10 ng/ml). Ba/F3 cells transduced with the EPORwt (solid triangles), EPORGCR (solid squares), or EPORMpl gene (solid circles) were cultured in the presence of rhEPO (10 ng/ml). Ba/F3 cells transduced with the ΔGCRTmR gene (open triangles) were cultured in the presence of tamoxifen (10^{-7} M). Accumulative data were calculated by means of a triplicate experiment. Experiments were repeated three times and a representative one is shown

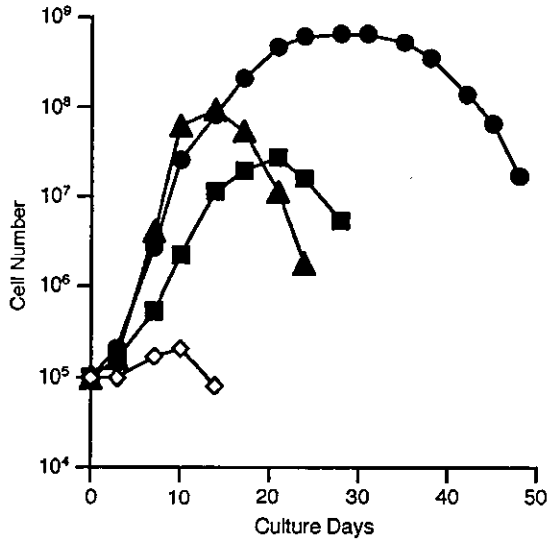

Figure 3. The EPORMpl is the most potent amplifier for human cord blood CD34+ cells. Human cord blood CD34+ cells were transduced with the EPOwt (solid triangles), EPORGCR (solid squares), or EPORMpl gene (solid circles) each along with the EYFP gene by bicistronic retroviral vectors. Untransduced cells are also shown (open diamonds). The cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO. Virtually all the cells (>95%) became YFP-positive by week 2. Accumulative data were calculated by means of a triplicate experiment. Experiments were repeated three times and a representative one is shown

Characterization of the c-Mpl signal of SAG

The transduced CD34+ cells were then examined for the expression of c-Kit, a primitive hematopoietic cell marker, by flow cytometry (Figure 4). The c-Kit+ fraction decreased over time, implying that the cells were differentiated during culture. The c-Kit+ fraction in the cells transduced with EPORMpl, however, was relatively high (33%) at week 3 in liquid culture, whereas the c-Kit+ fraction decreased to 10% or lower in the cells transduced with EPORwt or EPORGCR at the same time point. These results demonstrate that the c-Mpl signal preserved more c-Kit+ immature hematopoietic cells than the other signals.

To examine the EPO-driven SAGs for their ability to expand hematopoietic progenitor cells, CD34+ cells transduced with the EPO-driven SAGs were cultured in semisolid (methylcellulose) media in the presence of multiple cytokines (IL-3, IL-6 and SCF) or EPO alone. Table 1 summarizes the results. The cells transduced with the EPO-driven SAGs formed many colonies in the presence of EPO and almost all of them (94–100%) contained the provirus as assessed by individual colony PCR. In contrast, 25–38% of the colonies formed by cells in the presence of multiple cytokines contained the provirus. This result shows that the EPO-driven SAGs are able to confer an EPO-dependent growth advantage at the level of clonogenic progenitor cells. The cells transduced with the EPO-driven SAGs before (day 0) and after (day 7) liquid culture with EPO were placed in semisolid media in the presence of EPO without other cytokines, and the resultant myeloid and erythroid colonies were counted. As shown in Figure 5, during the liquid culture with EPO, the transduction by EPORMpl resulted in the highest levels of clonogenic progenitor cell expansion by more than 10-fold.

We then examined whether cells transduced with the EPO-driven SAGs would show any specific lineage

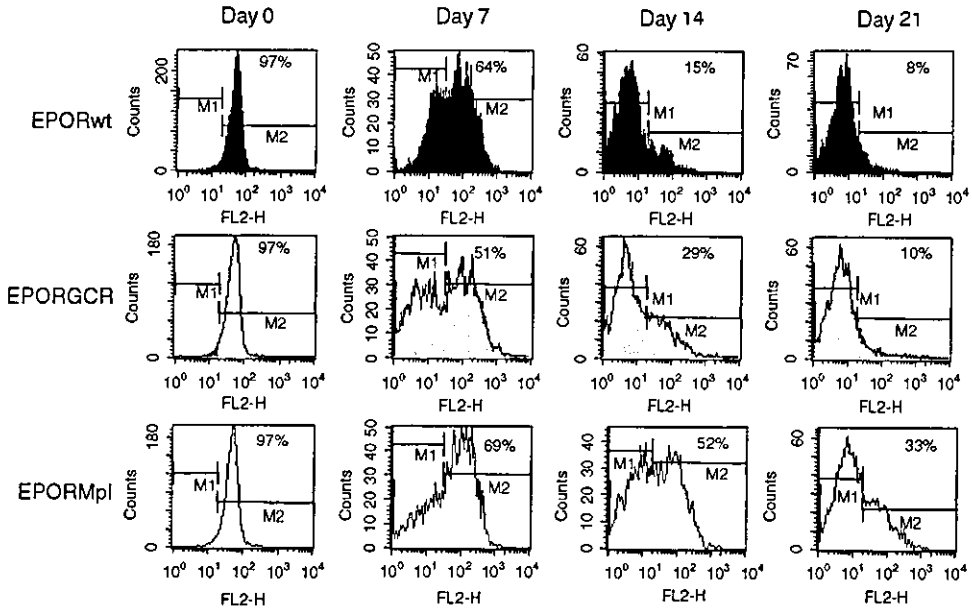

Figure 4. The EPOR-Mpl preserves c-Kit+ cells most efficiently. Human cord blood CD34+ cells were transduced with the EPOwt (black), EPORGCR (gray), or EPORMpl gene (white) by the same retroviral vectors as in Figure 3. The cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO. On the indicated days, aliquots of the cells were examined for c-Kit expression by flow cytometry. The percentage of c-Kit+ cells is shown. Experiments were repeated four times and a representative profile is shown

Table 1. Colony formation by human cord blood CD34$^+$ cells transduced with the EPO-driven SAGs

	IL-3 (100 ng/ml) IL-6 (100 ng/ml) SCF (100 ng/ml)		EPO (20 ng/ml)	
Transgene	Number of colonies*	Provirus-positive colonies†	Number of colonies*	Provirus-positive colonies†
EPORwt-YFP	62 ± 11	5/16 (31%)	15 ± 3	15/16 (94%)
EPORGCR-YFP	54 ± 8	6/16 (38%)	24 ± 1	16/16 (100%)
EPORMpl-YFP	54 ± 9	4/16 (25%)	31 ± 6	15/16 (94%)
YFP	49 ± 4	8/16 (50%)	12 ± 1	9/16 (56%)
Untransduced	53 ± 4	ND	17 ± 1	ND

*Colony number out of 200 cells is shown. Each value represents mean ± SD of triplicate culture.
†Individual colony DNA was subjected to PCR for the proviral YFP and genomic β-actin sequences and the ratio of the provirus-positive colony number to the β-actin-positive colony number is shown.

preference after liquid culture with EPO. The transduced CD34$^+$ cells were cultured in liquid medium containing EPO. During the culture, the expression of various differentiation markers was examined by flow cytometry (Figure 6). As expected, the erythroid marker (glycophorin A) was expressed in almost all (93%) cells transduced with EPORwt at day 14. The myeloid marker (CD15) was expressed in 24% of cells transduced with EPORGCR at day 7 (data not shown), but fell to 1% by day 14. Thus, EPORGCR induced very few cells to differentiate toward the myeloid lineage despite the inclusion of the GCR moiety as a signal generator. One reason may be that a point mutation (Y703F) was introduced into the GCR cDNA to attenuate the granulocytic differentiation signal (Figure 1) [3]. On the other hand, cells transduced with EPORMpl expressed all of these markers at relatively high levels at day 14; the megakaryocytic marker (CD41) (46%), glycophorin A (58%) and CD15 (11%). Thus, the cells expanded by the c-Mpl signal showed the most balanced expression of myeloid, erythroid, and megakaryocyte markers. We therefore decided to utilize EPORMpl as an SAG for subsequent *in vivo* experiments in mice.

In vivo expansion of gene-modified cells

Finally, we examined the efficacy of the EPOMpl-type SAG *in vivo* in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the MSCV-based vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced cells were transplanted into irradiated mice and, after hematopoietic reconstitution, YFP expression was examined in the peripheral blood by flow cytometry to see whether the EPOMpl-transduced cells would increase in response to EPO administration. In mice, however, even drawing a small volume of blood will result in the elevation of endogenous EPO concentrations [23,24]. We also confirmed that sequential blood drawing caused an elevation of endogenous serum EPO concentrations in mice (data not shown). Therefore, drawing blood from the transplanted mice may result in the expansion of transduced hematopoietic cells. To avoid development of anemia due to blood drawing, we

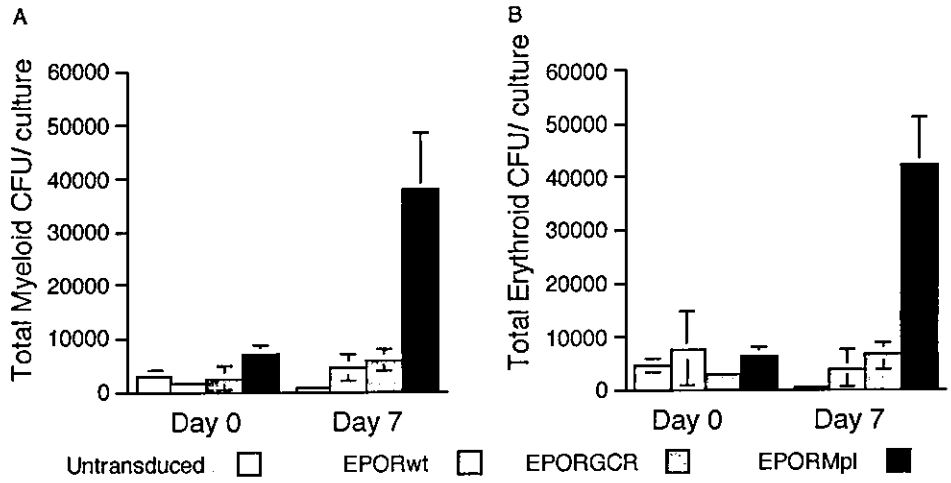

Figure 5. The EPOR-Mpl expands clonogenic progenitor cells most efficiently. Human cord blood CD34$^+$ cells were transduced with the EPOwt, EPORGCR or EPORMpl gene by the same retroviral vectors as in Figure 3. The untransduced and transduced cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO for 7 days. The cells before (day 0) and after (day 7) the liquid culture were plated in methylcellulose medium in the presence of EPO alone and the resultant colonies were counted. (A) Total myeloid clonogenic progenitor cell (colony-forming units, CFU) numbers per culture. (B) Total erythroid CFU numbers per culture. Means ± SD of a triplicate experiment are shown. Experiments were repeated three times and a representative one is shown

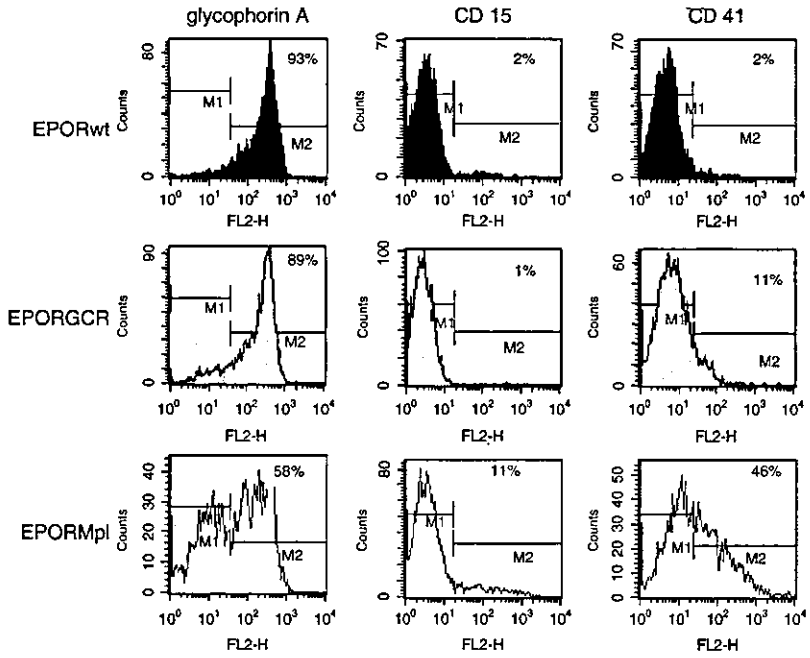

Figure 6. The CD34$^+$ cells expanded by the EPOR-Mpl show the most balanced expression of multilineage surface markers. Human cord blood CD34$^+$ cells were transduced with the EPOwt, EPORGCR, or EPORMpl gene by the same retroviral vectors as in Figure 3. After 14-day liquid culture with 10% FBS and 10 ng/ml EPO, the transduced cells were examined for the expression of glycophorin A (erythroid marker), CD15 (myeloid marker), and CD41 (megakaryocyte marker) by flow cytometry. The percentages of marker-positive cells are shown. Experiments were repeated four times and a representative profile is shown

transfused mice at the time of blood drawing. As a result, the mice did not develop anemia, and thus the elevation of endogenous EPO concentration was prevented.

In the group receiving EPORMpl, YFP-positive cells increased in response to the EPO administration (n = 6), although YFP-positive cells remained unchanged without EPO administration (n = 4) (Figure 7A). A significant increase (paired *t*-test, $p < 0.05$) in YFP-positive cells was observed 4 weeks after the initiation of EPO administration. The increase was attributable to that in granulocytes and monocytes (data not shown). We could not detect any significant change in other lineages. The increase seemed transient, as a significant increase was no longer observed at further time points. On the other hand, in the control group receiving YFP alone without EPORMpl, YFP-positive cells remained unchanged at around 10% in the peripheral blood regardless of EPO administration (n = 6 for a subgroup with EPO, n = 6 for a subgroup without EPO; Figure 7B).

Discussion

Although a few HSC gene therapy trials have proven successful [25,26], most attempts have been hampered by the low efficiency of gene transfer into HSCs. To overcome the problem, we have previously developed a method of selective *in vivo* amplification of transduced hematopoietic cells using a 'selective amplifier gene' (SAG) which encodes a fusion protein consisting of a growth-signal generator and its molecular switch. The prototype SAG encodes a fusion protein between the GCR and the estrogen or tamoxifen receptor, and confers a growth advantage on gene-modified hematopoietic cells in an estrogen- or tamoxifen-inducible fashion *in vivo* [6,7]. In the present study, we developed a new generation SAG which utilizes the EPOR as a molecular switch instead of the steroid receptor. The EPO-driven SAG encodes a fusion protein between the extracellular plus transmembrane domain of the EPOR and the cytoplasmic domain of the GCR or c-Mpl. The results reported here indicated that the SAG utilizing the EPOR as a molecular switch is more efficient for hematopoietic cell proliferation than that utilizing the steroid (or tamoxifen) receptor despite the inclusion of the same signal generator in the SAGs.

Cytokine receptors generate the growth signal through ligand-induced dimerization. Dimerization is necessary but not sufficient for optimal signal generation [27,28]. The EPO-driven SAG might have allowed more effective ligand-induced conformation change than the steroid-driven SAG. Similar to our chimeric receptors, Blau *et al.* developed a cell growth switch that is a cytokine receptor-FK506 binding protein (FKBP) fusion gene to confer inducible proliferation to transduced cells [29,30]. In their system, cytokine receptor signal is turned on by treatment with a synthetic dimerizer FK1012 or its derivatives. However, it remains unclear whether their chimeric protein would allow effective ligand-induced conformation change to the same extent as the EPO-driven SAG.

We also showed that the c-Mpl signal expanded clonogenic progenitor cells (CFU) far more efficiently than the EPOR or GCR signal. In addition, the cells expanded

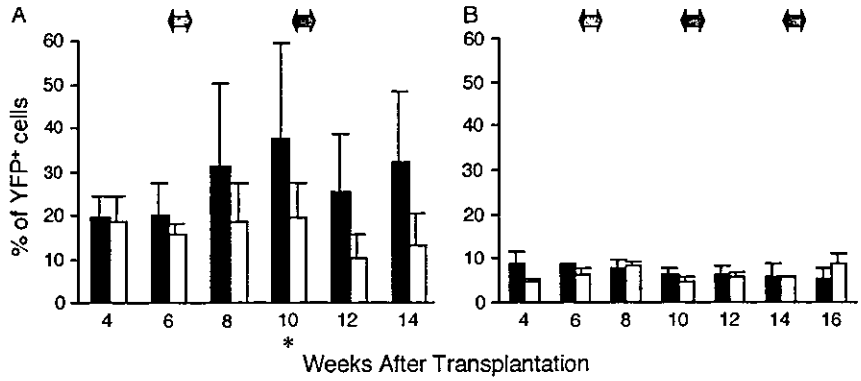

Figure 7. The gene-modified hematopoietic cells can be expanded by treatment with EPO *in vivo* in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the retroviral vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced cells were transplanted into irradiated mice. The percentages of YFP-positive cells in the peripheral blood are shown for the EPOR-Mpl group (A) or the YFP control group (B). In each group, mice were divided into two subgroups: EPO-treated subgroup (solid bars, 200 IU/kg, three times a week, n = 6 each for A and B) and EPO-untreated subgroup (open bars, n = 4 for A and n = 6 for B). The gray arrows in A and B indicate the week of EPO administration. Means ± SD of each subgroup are shown. The increase in YFP-positive cells in the EPO-treated mice was statistically significant at week 10 (4 weeks after the initiation of EPO administration) (*$p < 0.05$)

by the c-Mpl signal showed the most balanced expression of myeloid, erythroid, and megakaryocyte markers. Other investigators have also shown that the c-Mpl signal is able to efficiently support the growth of transduced murine bone marrow cells [31]. Taken together, the intracellular signal from c-Mpl may be suitable for reliable expansion of immature hematopoietic cells.

We have demonstrated that EPORMpl can confer an EPO-dependent growth advantage on the transduced hematopoietic cells *in vivo* in a mouse transplantation model. It should be noted that EPORMpl contains the human c-Mpl and may not have worked well in mouse cells. It would be more predictive to examine the efficacy of the EPORMpl in nonhuman primates. We are evaluating the efficacy of EPOMpl-type SAGs in the setting of a nonhuman primate transplantation protocol. In mice, the increase of transduced cells with EPORMpl seemed transient, as was the case with chimeric genes reported by other investigators [32,33]. The method may not result in the selection of transduced cells at the HSC level. The long terminal repeat (LTR) promoter may not express the transgene in HSCs. Alternatively, the c-Mpl signal may not induce proliferation of HSCs. Thus, the selection of transduced cells may occur only within the differentiated progeny of transduced HSCs, not at the level of transduced HSCs themselves. In order to obtain clinically relevant effects, repeated EPO administration would be required. Polycythemia may take place, but it can be treated by occasional phlebotomy safely. Given that our earlier version of SAG utilized estrogen receptor as a molecular switch, we believe that EPO is much safer than estrogen to turn on a molecular switch, since side effects induced by estrogen may not be well treated or controlled.

With the EPO-driven SAG, therapeutic effects may result from continuously elevated levels of endogenous EPO in patients with chronic anemia such as thalassemia. When anemia is ameliorated and endogenous EPO levels return to physiological levels, the positive selection system is then 'automatically' turned off. This 'leave it to patients' system would be convenient. However, a safety concern may be raised regarding leukemogenesis, as the SAG proliferation signal is persistently turned on *in vivo* by endogenous EPO, although physiological levels of EPO will not induce a significant proliferative response. Since a set of EPO-mimetic peptides or a modified EPO such as the erythropoiesis stimulating protein (NESP) has been developed [34,35], it may be possible to develop an EPO-driven SAG containing a mutant EPOR which does not bind to endogenous EPO but binds to the EPO-mimetic peptides or modified EPO.

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References

1. Dunbar CE, Cottler-Fox M, O'Shaughnessy JA, *et al.* Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood* 1995; **85**: 3048–3057.
2. Ito K, Ueda Y, Kokubun M, *et al.* Development of a novel selective amplifier gene for controllable expansion of transduced hematopoietic cells. *Blood* 1997; **90**: 3884–3892.
3. Matsuda KM, Kume A, Ueda Y, *et al.* Development of a modified selective amplifier gene for hematopoietic stem cell gene therapy. *Gene Ther* 1999; **6**: 1038–1044.
4. Xu R, Kume A, Matsuda KM, *et al.* A selective amplifier gene for tamoxifen-inducible expansion of hematopoietic cells. *J Gene Med* 1999; **1**: 236–244.
5. Nagashima T, Ueda Y, Hanazono Y, *et al.* New selective amplifier genes containing c-Mpl for hematopoietic cell expansion. *Biochem Biophys Res Commun* 2003; **303**: 170–176.

6. Kume A, Koremoto M, Xu R, *et al. In vivo* expansion of transduced murine hematopoietic cells with a selective amplifier gene. *J Gene Med* 2003; **5**: 175–181.
7. Hanazono Y, Nagashima T, Takatoku M, *et al. In vivo* selective expansion of gene-modified hematopoietic cells in a nonhuman primate model. *Gene Ther* 2002; **9**: 1055–1064.
8. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci U S A* 1990; **87**: 6934–6938.
9. Suzanne LK, Donald NC, William W, *et al.* Proliferation of multipotent hematopoietic cells controlled by a truncated erythropoietin receptor transgene. *Proc Natl Acad Sci U S A* 1996; **93**: 9402–9407.
10. Brandt JR, Avner ED, Hickman RO, *et al.* Safety and efficacy of erythropoietin in children with chronic renal failure. *Pediatr Nephrol* 1999; **13**: 143–147.
11. Itri LM. The use of epoetin alfa in chemotherapy patients: a consistent profile of efficacy and safety. *Semin Oncol* 2002; **29**: 81–87.
12. Borge OJ, Ramsfjell V, Cui L, *et al.* Ability of early acting cytokines to directly promote survival and suppress apoptosis of human primitive $CD34^+$ $CD38^-$ bone marrow cells with multilineage potential at the single-cell level: key role of thrombopoietin. *Blood* 1997; **90**: 2282–2292.
13. Solar GP, Kerr WG, Zeigler FC. *et al.* Role of c-mpl in early hematopoiesis. *Blood* 1998; **92**: 4–10.
14. Kimura S, Roberts AW, Metcalf D, *et al.* Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci U S A* 1998; **95**: 1195–1200.
15. Kaushansky K. Mpl and the hematopoietic stem cell. *Leukemia* 2002; **16**: 738–739.
16. Gurney AL, Wong SC, Henzel WJ, *et al.* Distinct regions of c-Mpl cytoplasmic domain are coupled to the JAK-STAT signal transduction pathway and Shc phosphorylation. *Proc Natl. Acad Sci U S A* 1995; **92**: 5292–5296.
17. Pear WS, Nolan GP, Scott ML, *et al.* Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci. U S A* 1993; **90**: 8392–8396.
18. Kralovics R, Sokol L, Prchal JT. Absence of polycythemia in a child with a unique erythropoietin receptor mutation in a family with autosomal dominant primary polycythemia. *J Clin Invest* 1998; **102**: 124–129.
19. Hawley RG, Lieu FH, Fong AZ, *et al.* Versatile retroviral vectors for potential use in gene therapy. *Gene Ther* 1994; **1**: 136–138.
20. Takatoku M, Kametaka M, Shimizu R, *et al.* Identification of functional domains of the human thrombopoietin receptor required for growth and differentiation of megakaryocytic cells. *J Biol Chem* 1997; **272**: 7259–7263.
21. Huang Z, Tamura M, Sakurai T, *et al. In vivo* transfection of testicular germ cells and transgenesis by using the mitochondrially localized jellyfish fluorescent protein gene. *FEBS Lett* 2000; **487**: 248–251.
22. Hanenberg H, Xiao XL, Dilloo D, *et al.* Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat Med* 1996; **2**: 876–882.
23. Oishi A, Sakamoto H, Shimizu R, *et al.* Evaluation of phlebotomy-induced erythropoietin production in the dog. *J Vet Med Sci* 1993; **55**: 51–58.
24. Chapel SH, Veng-Pedersen P, Schmidt RL, *et al.* Receptor-based model accounts for phlebotomy-induced changes in erythropoietin pharmacokinetics. *Exp Hematol* 2001; **29**: 425–431.
25. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al.* Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 2000; **288**: 669–672.
26. Aiuti A, Slavin S, Aker M, *et al.* Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 2002; **296**: 2410–2413.
27. Livnah O, Stura EA, Middleton SA, *et al.* Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science* 1999; **283**: 987–990.
28. Remy I, Wilson IA, Michnick SW. Erythropoietin receptor activation by a ligand-induced conformation change. *Science* 1999; **283**: 990–993.
29. Blau CA, Peterson KR, Drachman JG, *et al.* A proliferation switch for genetically modified cells. *Proc Natl Acad Sci U S A* 1997; **94**: 3076–3081.
30. Richard RE, Wood B, Zeng H, *et al.* Expansion of genetically modified primary human hemopoietic cells using chemical inducers of dimerization. *Blood* 2000; **95**: 430–436.
31. Zeng H, Masuko M, Jin L, *et al.* Receptor specificity in the self-renewal and differentiation of primary multipotential hemopoietic cells. *Blood* 2001; **98**: 328–334.
32. Jin L, Zeng H, Chien S, *et al. In vivo* selection using a cell-growth switch. *Nat Genet* 2000; **26**: 64–66.
33. Neff T, Horn PA, Valli VE, *et al.* Pharmacologically regulated *in vivo* selection in a large animal. *Blood* 2002; **100**: 2026–2031.
34. Wrighton NC, Farrell FX, Chang R, *et al.* Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* 1996; **273**: 458–464.
35. Macdougall IC. Novel erythropoiesis stimulating protein. *Semin Nephrol* 2000; **20**: 375–381.

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ORIGINAL ARTICLE

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Phase II study of the combination of vinorelbine and cisplatin in advanced non-small-cell lung cancer

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Abstract *Purpose*: To evaluate the efficacy and safety of combination chemotherapy with cisplatin and vinorelbine for the treatment of previously untreated patients with advanced non-small-cell lung cancer (NSCLC). *Patients and methods*: Eligible patients were those with measurable NSCLC. They were treated with two or more cycles of a regimen consisting of vinorelbine 25 mg/m^2 on days 1 and 8 and cisplatin 80 mg/m^2 on day 1 every 3 weeks. *Results*: A total of 45 patients were enrolled. The response rate was 51.1% (23/45; 95% CI 35.8% to 66.3%). The median survival was 286 days with a 1-year survival rate of 40%. The median number of treatment cycles was 2. The major toxic effect was neutropenia of grade 3 or higher (84%). Nonhematological toxicities, including vomiting (62%), were mild (grade 2 or less). There were no treatment-related deaths. *Conclusion*: The high response rate and good tolerability proved this combination therapy to be a safe and effective treatment for advanced NSCLC.

Keywords Non-small-cell lung cancer · Vinorelbine · Cisplatin · Phase II study

Introduction

Vinorelbine ditartrate [1], a vinca alkaloid derivative, shows antitumor activity mainly by inhibiting microtubule polymerization in tumor cells just as other vinca alkaloid drugs do [2, 9]. Clinical studies of vinorelbine (VNR) have shown a good therapeutic outcome in non-small-cell lung cancer (NSCLC) and breast cancer, and a reduction in peripheral neuropathy that occurs frequently with vinca alkaloids [5, 7, 10, 12]. The combination of VNR and cisplatin (CDDP) (VP therapy) has shown a synergistic effect in vitro, while the main side effects are different between the drugs [4]. A phase I-II study has demonstrated efficacy of this combination in NSCLC [3]. VP therapy is considered a promising combination regimen for NSCLC on account of its higher response rate and longer survival compared with VNR or CDDP alone, or CDDP combined with vindesine [8, 17].

In clinical studies performed in Europe and the US, patient compliance rate was as low as 50% or less with regard to VNR when VP therapy, as VNR 25 mg/m^2 weekly and CDDP 80 mg/m^2 on day 1, was repeated every 4 weeks. This indicates the need to reconsider the dosing schedule of VNR [17]. Another dosing schedule for VP therapy (VNR 20 to 30 mg/m^2 on days 1 and 8 and CDDP 80 mg/m^2 on day 1 every 3 weeks) showed almost complete compliance and was found to be beneficial since the response rate was 28.3% to 56.7% and the survival 9.2 to 10.6 months [6, 13, 15, 17].

VP therapy is an effective regimen against advanced NSCLC. A multicenter joint phase III study is being planned in Japan to compare four regimens for advanced NSCLC: CDDP plus irinotecan used as a reference arm, CDDP plus VNR every 3 weeks, CDDP plus gemcitabine and carboplatin plus paclitaxel. A phase II study of VP therapy has not been conducted in Japan. We therefore carried out a phase II study of VNR 25 mg/m^2 on days 1 and 8 plus CDDP 80 mg/m^2 on day 1 given every 3 weeks in advanced NSCLC to evaluate the efficacy and safety of VP therapy.

Patients and methods

Patient selection

Patients eligible for the study were those admitted to our hospital between August 1999 and October 2001 who were histologically or

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cytologically diagnosed as having NSCLC and who were in clinical stage III or IV with unresectable disease, or in whom radiotherapy with curative intent was not possible, including those who had pleural effusion and dissemination, those with intrapulmonary metastasis within the ipsilateral lobe, those in whom the irradiation field exceeded one-half of one lung, those with metastasis to the contralateral hilar lymph nodes, and those with reduced lung function. None of the patients had received prior therapy. Other eligibility criteria included expected survival of 12 weeks, age ≤ 75 years, Eastern Cooperative Oncology Group performance score (PS) of 0–2, measurable lesions, adequate hematological function (WBC ≥4000/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥10 g/dl), renal function (serum creatinine ≤ 1.5 mg/dl, creatinine clearance ≥60 ml/min), and hepatic function (total serum bilirubin ≤ 1.5 mg/dl, serum GOT and serum GPT less than twice the upper limit of normal). Written informed consent was obtained from every patient with the statement that the patient was aware of the investigational nature of this treatment regimen. Pretreatment evaluation included medical history, physical examination, complete blood count, serum biochemical analyses, chest roentgenogram, electrocardiogram and urinalysis. All patients underwent radionuclide bone scan and computerized tomography of the brain, thorax, and abdomen.

Treatment

The anticancer drugs were administered via the intravenous route, VNR 25 mg/m^2 (Navelbine, Kyowa Hakko Kogyo) on days 1 and 8 and CDDP 80 mg/m^2 (Randa, Nippon Kayaku) on day 1. This combination therapy repeated every 3 weeks constituted a cycle of treatment. The minimal number of cycles to be evaluated was two. On day 8, the physician examined the patient and evaluated the development of adverse events, and if leukocytes had decreased to below 2000/mm^3, platelets had decreased to below 75,000/mm^3 or fever with infection had occurred, administration of VNR on that day was withheld at the discretion of the physician. To proceed with the second and subsequent cycles, patients were required to have a neutrophil count ≥1500/mm^3 and a platelet count ≥100,000/mm^3. Those patients receiving granulocyte colony-stimulating factor (G-CSF) were observed for 3 days after the final dose of G-CSF to ensure that their neutrophil count was 1500/mm^3 or more. Serum creatinine levels were required to be below the upper limit of normal and serum GOT/GPT levels below twice the upper limit of normal. In the presence of liver dysfunction due to apparent liver metastasis, however, serum GOT and GPT levels were required to be below three times the upper limit of normal. If fever occurred or if the PS advanced to grade 3 or worse, the subsequent cycle was postponed until the temperature fell below 38°C or until the PS returned to 2 or less. In the presence of grade 2 peripheral neuropathy dosing was temporarily postponed; with improvement to grade 1 or less treatment was cautiously resumed, but medication was discontinued if 6 weeks passed without any improvement. Peripheral neuropathy (including transient) grade 3 or higher required discontinuation of treatment. For the third and subsequent cycles, VNR or CDDP was decreased by 25% in accordance with the treatment-related adverse events observed during the preceding cycle. Steroid and HT_3-antagonist were administered to prevent nausea and vomiting.

Target population size and interim analysis

Simon's two-stage minimax design [16] was used to estimate the number of patients required for interim and final analyses at a threshold response rate (P_0) of 0.20, an expected response rate (P_1) of 0.40, $\alpha = 0.05$ and $\beta = 0.10$. If the interim analysis revealed 6 responding patients out of 24, recruitment would be continued until the target population size was achieved. The combination therapy was considered effective if 14 or more of 45 patients showed response in the final analysis.

Since an interim response rate of 48.1% (13/27) [11] was obtained, it was necessary to enroll up to 45 patients for the final analysis.

Evaluation of response and toxicity

Response and toxicity were evaluated on the basis of tumor images obtained by CT and other techniques, laboratory data and subjective/objective symptoms before, during and after administration of the study drugs and during the period from completion of treatment to the final analysis. Measurable disease parameters were determined every 4 weeks by various means such as computerized tomography. Evaluation was made in compliance with Response Evaluation Criteria in Solid Tumors (RECIST) guidelines [14] for antitumor activity and with NCI Common Toxicity Criteria version 2 for safety. The Institutional Ethical Review Committee gave approval to the study.

Results

Patient characteristics

Table 1 gives characteristics of the patients included. Their median age was 59.5 years (range 35 to 75 years). Male, PS 1 and adenocarcinoma predominated. There were 26 patients (58%) with stage IV disease and 19 (42%) with stage IIIB disease.

Treatments administered

The total number of cycles administered was 126 with a median of two per patient (ranging from one to four cycles; Table 2) and 43 patients received two cycles or more. In the two patients who received fewer than two cycles, treatment was discontinued because of CDDP-induced renal dysfunction in one and patient refusal in the other. Patients who completed two cycles or more accounted for 96% of patients (43/45). Except the two patients who received only one cycle, the every-3-week

Table 1 Patient characteristics

Eligible patients (*n*)	45
Age (years)	
Median	59.5
Range	35–75
Sex (*n*)	
Male	34
Female	11
Performance status (*n*)	
0	11
1	32
2	2
Histology (*n*)	
Adenocarcinoma	30
Squamous cell carcinoma	9
Other	6
Stage (*n*)	
IIIB	19
IV	26

Table 2 Efficacy of treatment ($n=45$)

No. of cycles	
Median	2.0
Range	1–4
Response	
Partial response	23
No change	21
Not evaluable	1
Response Rate (%)	51.1
95% CI (%)	35.8–66.3
1-year survival rate (%)	40

dosing schedule was adhered to by 88% of patients (38/43) in the second cycle, 68% (17/25) in the third and 92% (12/13) in the fourth, with a total of 83% (67/81). Only in two cycles was VNR withheld on day 8. The dose of VNR was reduced in 9% of dose administrations (22/250) and the does of CDDP was reduced in 8% (10/126). The planned dose intensities were 16.7 mg/m^2 per week for VNR and 26.7 mg/m^2 per week for CDDP while the actual dose intensities were 16.4 and 24.7 mg/m^2 per week, respectively. The median delivered dose intensity for CDDP (day 1) and VNR (days 1 and 8) of each course together was 90% or more (Table 3).

Efficacy of treatment

Of the 45 patients, 23 showed a partial response, 21 showed no change and 1 was not evaluable (Table 2). The response rate was 51.1% (23/45; 95% CI 35.8% to 66.3%; Table 2). The nonevaluable patient died of sudden hemoptysis on the 22nd day after the start of the second cycle (43rd day after the start of treatment) and could not be evaluated. Ten patients were alive at the time of this report. The time to progressive disease was 172 days and the median survival was 286 days (95% CI 248 to 404 days; Table 2). The 1-year survival rate was 40%.

Table 3 Median delivered dose intensity

	Median dose intensity (%)			
	Course 1	Course 2	Course 3	Course 4
CDDP	100	98.8	96	92.3
VNR				
Day 1	100	98.6	95.5	93.8
Day 8	97.8	98.6	95.5	93.8

Toxicities

Table 4 lists toxicities observed during the study. Hematological and blood biochemical reactions included a high incidence of leukopenia and neutropenia, i.e. leukopenia and neutropenia of grade 3 or higher occurred in 73% of patients (33/45) and 84% (38/45), respectively. Neutropenia-associated fever was limited to two patients. All neutropenic patients recovered upon treatment with G-CSF. Platelets decreased in 4% of patients (2/45). Creatinine was temporarily elevated in 15.6% (7/45).

Subjective and objective symptoms observed were of grade 2 or less and included vomiting in 77.8% of patients (35/45), hiccough in 33.3% (15/45), constipation in 40% (18/45), diarrhea in 22% (10/45), rash in 31.1% (14/45) and injection site reaction in 26.7% (12/45). All of these toxicities disappeared or improved with symptomatic treatment. There were no toxic deaths.

Discussion

As for the VP regimen for advanced NSCLC, the every-3-week dosing schedule has been tried in several medical facilities [6, 13, 15, 17]. Table 5 summarizes the clinical outcomes of every-3-week VP therapy reported in the literature and in this study. Response rates range from 28% to 57% and median survival is approximately 10 months. The results are similar among the studies.

In 96% of patients (43/45), two or more cycles of VP therapy were administered. The every-3-week dosing

Table 4 Toxicities ($n=45$)

Toxicity	Grade (Common Toxicity Criteria)				Grade 3/4 (%)
	1	2	3	4	
Leukopenia	4	3	25	8	33 (73%)
Neutropenia	2	2	13	25	38 (84%)
Anemia	12	3	1	4	5 (11%)
Thrombocytopenia	5	1	2	0	2 (4%)
Creatinine	5	2	0	0	–
Vomiting	29	6	0	0	–
Hiccough	15	0	0	0	–
Constipation	13	5	0	0	–
Diarrhea	9	1	0	0	–
Rash	10	4	0	0	–
Neuropathy	4	0	0	0	–
Injection site reaction	4	8	0	0	–
Alopecia	3	0	0	0	–

Table 5 Outcomes of studies of VP therapy (VNR days 1 and 8, CDDP day 1, every 3 weeks)

Reference	VNR (mg/m^2)	CDDP (mg/m^2)	Response	Median survival time (months)
4	25	80	28.3% (28/99)	9.2
10	25	80	56.7% (42/74)	10
11	20–25	80	46.7% (14/30)	10.6
1	30	80	36.2% (47/130)	–
Present study	25	80	51.1% (23/45)	9.6

schedule was adhered to in 85% of all cycles administered. In cycles in which noncompliance was seen, medication was postponed to the 4th to 5th week because, in most cases, the neutrophil count in the 3rd week failed to meet the criterion for going on to subsequent cycles. The planned dose intensity was almost attained since the actual dose intensity was 16.4 mg/m^2 per week for VNR and 24.7 mg/m^2 per week for CDDP, accounting for 98% and 93% of the planned values, respectively [13].

Most adverse reactions were hematological. In particular, leukopenia and neutropenia of grade 3 or worse occurred in 73% and 84% of 45 patients, respectively. Others have reported the incidence of leukopenia of grade 3 or worse to be 8% to 33% [6, 13, 17]. Although the difference in patient characteristics hinders simple comparison and analysis of these data, it can be said that leukopenia was more frequent in our study. The leukocyte count improved rapidly upon treatment with G-CSF. Nonhematological toxicities were mild and adverse reactions of grade 3 or higher were not noted.

The combination of VNR 25 mg/m^2 on days 1 and 8 and CDDP 80 mg/m^2 on day 1 was administered every 3 weeks to 45 patients with advanced NSCLC in this phase II study. The response rate was 51.1%; the main adverse effect was neutropenia. The high response rate and good tolerability indicate that this combination therapy is a safe and effective treatment for advanced NSCLC. Its usefulness will be further verified in phase III studies.

References

1. Adam Z, Coupkova H, Kolek V, et al (1995) Vinorelbine and cisplatin in the treatment of advanced non-small-cell lung cancer: results of a multicenter Czech study. Acta Med Austriaca 22:120–124
2. Berthaud P, Le Chevalier LT, Ruffie P, et al (1992) Phase I-II study of vinorelbine (Navelbine) plus cisplatin in advanced non-small cell lung cancer. Eur J Cancer 28A:1863–1865
3. Binet S, Fellous A, Lataste H, Krikorian A, Couzinier JP, Meininger V (1989) In situ analysis of the action of navelbine on various types of microtubules using immunofluorescence. Semin Oncol 16 [Suppl 4]:5–8
4. Bretti S, Berruti A, Gorzegno G, et al (1996) Multicenter phase II trial of intermediate dose cisplatin and vinorelbine in inoperable non-small cell lung cancer patients. Lung Cancer 14:353–360
5. Canobbio L, Boccardo F, Pastorino G, et al (1989) Phase II study of Navelbine in advanced breast cancer. Semin Oncol 16 [Suppl 4]:33–36
6. Chevalier TL, Brisgand D, Douillard JY, et al (1994) Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small cell lung cancer: results of a European multicenter trial including 612 patients. J Clin Oncol 12:360–367
7. Depierre A, Lemarie E, Dabouis G, Garnier G, Jacoulet P, Dalphin JC (1989) Efficacy of Navelbine (NVB) in non-small cell lung cancer (NSCLC). Semin Oncol 16 [Suppl 4]:26–29
8. Depierre A, Lemarie E, Dabouis G, Garnier G, Jacoulet P, Dalphin JC (1991) A phase II study of Navelbine (vinorelbine) in the treatment of non-small cell lung cancer (NSCLC). Am J Clin Oncol 14:115–119
9. Fellous A, Ohayon R, Vacassin T, et al (1989) Biochemical effects of Navelbine on tubulin and associated proteins. Semin Oncol 16 [Suppl 4]:9–14
10. Frontini L, Candido P, Cattaneo MT, et al (1996) Cisplatin-vinorelbine combination chemotherapy in locally advanced non-small cell lung cancer. Tumori 82:57–60
11. Gebbia V, Caruso M, Valenza R, et al (1994) Vinorelbine plus cisplatinum for the treatment of stage IIIB and IV non-small cell lung carcinoma. Anticancer Res 14:1247–1250
12. Gomi K, Ohno H, Nomura K, Okabe M, Kobayashi K, Niitani H (1986) Kinetic analysis of combination effect of Navelbine (KW-2307) with cisplatin against human lung adenocarcinoma PC-12 cell in culture. Jpn J Cancer Res 83:532–539
13. Kusunoki Y, Furuse K, Yamori S, et al (1995) Randomized phase II study of vinorelbine (VRB) vs vindesine (VDS) in previously untreated non-small cell lung cancer (NSCLC) (abstract 1071). Proc Am Soc Clin Oncol 14:353
14. Mori K, Kamiyama Y, Kondoh T, Tominaga K (2002) Phase II study of combined vinorelbine plus cisplatin for advanced non-small cell lung cancer (an interim report). Jpn Cancer Chemother 29:405–410
15. Potier P (1989) The synthesis of navelbine prototype of a new series of vinblastine derivatives. Semin Oncol 16 [Suppl 4]:2–4
16. Simon R (1989) Optimal two-stage designs for phase II clinical trial. Control Clin Trial 10:1–10
17. Wozniak AJ, Crowley JJ, Balcerzak SP, et al (1998) Randomized trial comparing cisplatin with cisplatin plus vinorelbine in the treatment of advanced non-small-cell lung cancer: a Southwest Oncology Group study. J Clin Oncol 16:2459–2465

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Ectopic cyclin D1 expression blocks STI571-induced erythroid differentiation of K562 cells

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Abstract

Bcr-Abl tyrosine kinase inhibitor induces apoptosis and erythroid differentiation of K562 cells. During this erythroid differentiation, c-Myc and cyclin D1 transcripts are transiently downregulated. Accordingly, we studied the effect of cyclin D1 overexpression on erythroid differentiation. After treatment with 250 nM STI571, 90% of K562 and 25% of K562/D1 cells underwent erythroid differentiation. The basal expression of glycophorin A in K562/D1 cells was markedly diminished compared with that by parental cells. STI571 treatment failed to induce glycophorin A expression in K562/D1 cells. During STI571 treatment, ERK activity was downregulated in parental cells, while it was constantly activated in K562/D1 cells. These results suggest that ectopic expression of cyclin D1 causes the resistance of K562 cells to erythroid differentiation by modulating ERK regulation.

Keywords: Cyclin D1; STI571; CML; K562; Erythroid differentiation; MAPK

1. Introduction

Cellular differentiation and cell cycle progression are closely interrelated. Under normal circumstances, terminally differentiated cells lose the capacity to proliferate, while stem cells or progenitor cells retain this capability. The cell cycle regulatory mechanism was intensively studied during 1990s [1], and it was found that most of the cell cycle-related genes are tightly regulated during the process of hematopoietic cell differentiation [2,3]. Cyclin D1 is one of the regulators of G_0 to G_1 and G_1 to S progression [4], and cyclin D1 overexpression has been reported in several cancers and hematopoietic malignancies [5–7]. Cyclin D1 binds with Cdk4 and Cdk6 to promote their kinase activity, which leads to the phosphorylation of various target proteins, including retinoblastoma protein [8]. Phosphorylated retinoblastoma protein loses its repressor activity against the E2F1 transcription family [9]. Since E2Fs regulate genes that are necessary for progression into S phase, overexpression of cyclin D1 may cause acceleration of the cell cycle. Interestingly, cyclin D1 has been demonstrated to bind with several transcription factors and modulate their transcriptional activity. Such direct binding by cyclin D1 may also contribute to the control of cell proliferation and differentiation [10–14]. c-Myc is also known to be a cell cycle accelerator, and it has been demonstrated to interfere with cellular differentiation by ectopic overexpression [15]. Recently, c-Myc has been demonstrated to modulate the transcription of cell cycle inhibitor, $p21^{Cip1/WAF1}$, not by binding to the E-box, but by directly binding to another transcription factor [16–19]. This suggests that cell cycle-regulating genes are also involved in the regulation of cell differentiation.

We previously demonstrated that a benzaquinoid ansamycin antibiotic, herbimycin A, transiently downregulated the transcription of c-Myc and cyclin D1 during the erythroid differentiation of K562 cells [20]. Herbimycin A is a potent inhibitor of Bcr-Abl kinase. Recently, a more

Abbreviations: Cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated protein kinase; K562/D1, cyclin D1-transfected K562; K562/Myc, c-Myc-transfected K562; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI3-K, phosphatidylinositol 3-kinase

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specific Bcr-Abl tyrosine kinase inhibitor, STI571 (imatinib mesylate), has been developed [21] and its clinical usefulness has been demonstrated [22,23]. Like herbimycin A, STI571 induces the erythroid differentiation of K562 cells, and this is modulated by the MEK–ERK activity [24]. Accordingly, we studied the effect of ectopic cyclin D1 expression on STI571-induced erythroid differentiation, and found that cyclin D1 could impair differentiation of K562 cells to the same extent as observed in cells with c-Myc overexpression. Consistent activation of ERK by cyclin D1-overexpressing cells during STI571 treatment may lead to the inhibition of erythroid differentiation.

2. Materials and methods

2.1. Cells and culture conditions

K562 cells, derived from the leukemic cells of a chronic myeloid leukemia (CML) patient in blastic crisis, were obtained from the Riken cell bank (Tsukuba, Japan). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA) in a 5% CO_2 incubator with 100% humidity. For the present experiments, exponentially growing cells (2×10^5 ml^{-1}) were suspended in 20 ml of culture medium containing either 800 nM herbimycin A (Sigma, St. Louis, MO, USA) or 250 nM of STI571 (a gift from Novartis, Basel, Switzerland). Creation of c-Myc-transfected K562 cells (K562/Myc) and cyclin D1-transfected K562 cells (K562/D1) was described elsewhere [25]. Briefly, pcDNA3 (Invitrogen, Carlbad, CA, USA) containing human cyclin D1 cDNA was transfected by electropolation. Two cloned cell lines (#18 and #24) were used for the experiments. The expression levels of cyclins D2 and D3 in parental and transfected cells were equal by RT-PCR (data not shown). Viable cells were counted by the erythrosine B dye exclusion assay (Nacalai Tesque Inc., Kyoto, Japan).

2.2. Detection of erythroid differentiation

2.2.1. Cytochemical assays

Erythroid differentiation of K562 cells was assessed by detecting heme or hemoglobin via a specific reaction using benzidine/hydrogen peroxide solution. Benzidine dihydrochloride (2 mg/ml in 0.5% acetic acid) was mixed with 30% hydrogen peroxide (5 μl/ml) and added directly to an equal volume (10 μl) of cell suspension in a centrifuge tube. After incubation at room temperature for 5 min, the percentage of benzidine-positive cells was calculated after counting at least 600 cells under a phase-contrast microscope.

2.2.2. Glycophorin A expression

Glycophorin A expression on the cell surface was detected by the direct immunofluorescence method. An FITC-conjugated monoclonal antibody for glycophorin A and control IgG-κ were purchased from DAKO (Carpinteria, CA, USA). Cells (10^6) were harvested, washed once with PBS containing 2% fetal calf serum, and incubated in the dark with the antibodies for 45 min at 4 °C. After washing three times with PBS containing 2% fetal calf serum, the cells were analyzed by flow cytometry (FACs Caliber; Becton Dickinson, San Jose, CA, USA).

2.3. Immunoblot analysis

Cells were washed with ice-cold TBS buffer (25 mM Tris–HCl (pH 8.0), 150 mM NaCl), and lysed in the lysis buffer (Cell Signaling Technology Inc., MA, USA). Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and size-separated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a submerged electroblotting chamber. Membranes were blocked in TBS buffer containing 0.1% Tween 20 and 5% skin milk for 1 h at room temperature. ERK and phosphorylated ERK, and MEK and phosphorylated MEK were studied using the each detection kit (Cell Signaling Technology Inc.). Anti-cyclin D1 and anti-c-Myc (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used for each blotting. Briefly, incubation with the primary antibodies was performed overnight at 4 °C using the dilutions recommended by the manufacturer. Then incubation was done for 1 h at room temperature with anti-rabbit Ig conjugated with horseradish peroxidase, after which proteins were detected by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA).

3. Results

3.1. Growth of K562/cyclin D1 cells

Parental K562, K562/D1(clones 18 and 24), K562/Myc, and K562/mock cells were incubated with 800 nM herbimycin A or 250 nM STI571 for 48 h, and viable cells were counted by the erythrosine B dye exclusion assay. The growth of K562/D1(#18) cells with no treatment was equal to that of K562 cells (Fig. 1). As shown in Fig. 1A, the growth of herbimycin A-treated cells was severely inhibited, especially during the latter half of treatment from 24 to 48 h. The growth of each cell line was equally inhibited. But, the growth of all cell lines was scarcely affected by STI571 treatment (Fig. 1B).

3.2. Changes of cyclin D1 expression during treatment with inhibitors

We previously demonstrated that cyclin D1 transcription was transiently suppressed during treatment with herbimycin A [20]. So we assessed the cyclin D1 protein by immunoblotting in this study. Basal cyclin D1 expression

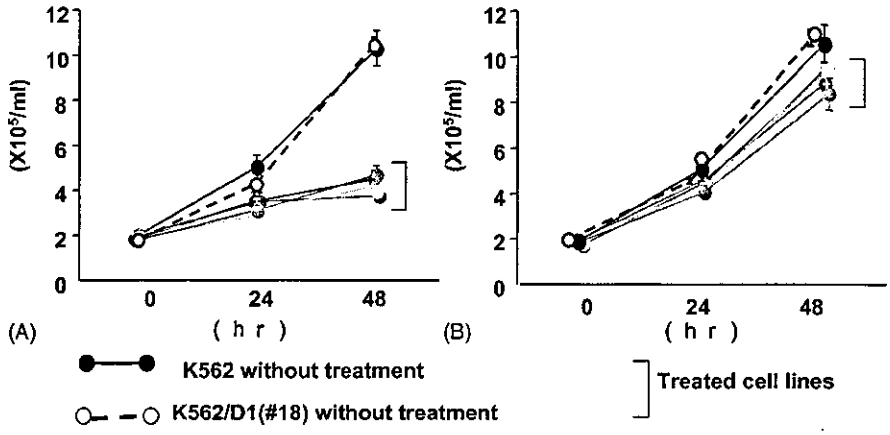

Fig. 1. Growth inhibition of each cell line by herbimycin A and STI571. (A) Herbimycin A (800 nM) inhibited the proliferation of cells. (B) STI571 (250 nM) slightly reduced cell proliferation, but the inhibition was weak compared with that due to 800 nM herbimycin A. K562, K562/D1(#18), K562/D1(#24), K562/Myc and K562/mock cells were studied. Proliferations of these cells under the each treatment were shown by lines in the blanketed area.

in K562/D1(#18) cells was markedly higher than K562 cells (Fig. 2A), and cyclin D1 expression in herbimycin A-treated K562 cells was decreased at 3 h and no product was observed at 6 h (Fig. 2A). Unlike cyclin D1 mRNA, reappearance of cyclin D1 was not observed in the parental cells. In K562/D1 cells, however, high expression of cyclin D1 lasted throughout the treatment period. The changes of cyclin D1 expression during STI571 treatment were similar to the changes caused by herbimycin A treatment, although the downregulation of cyclin D1 occurred earlier and was more marked than with herbimycin A.

Next, we studied the c-Myc protein (Fig. 2B). Unlike our previous finding about c-Myc mRNA during herbimycin A treatment, transient downregulation of c-Myc protein was not observed in herbimycin A-treated parental K562 cells. There was also no transient change of c-Myc expression in herbimycin A-treated K562/D1 and K562/mock cells. In contrast, treatment with STI571 induced the transient downregulation of c-Myc from 3 to 12 h, and c-Myc expression subsequently returned to the initial level by 48 h. There were no differences of the c-Myc expression pattern between each cell line during STI571 treatment.

3.3. K562/cyclin D1 cells showed resistance to erythroid differentiation

The effect of Bcr-Abl inhibitors on erythroid differentiation was studied by detecting benzidine-positive cells. As demonstrated in Fig. 3, herbimycin A and STI571 induced erythroid differentiation in 60 and 90% of K562

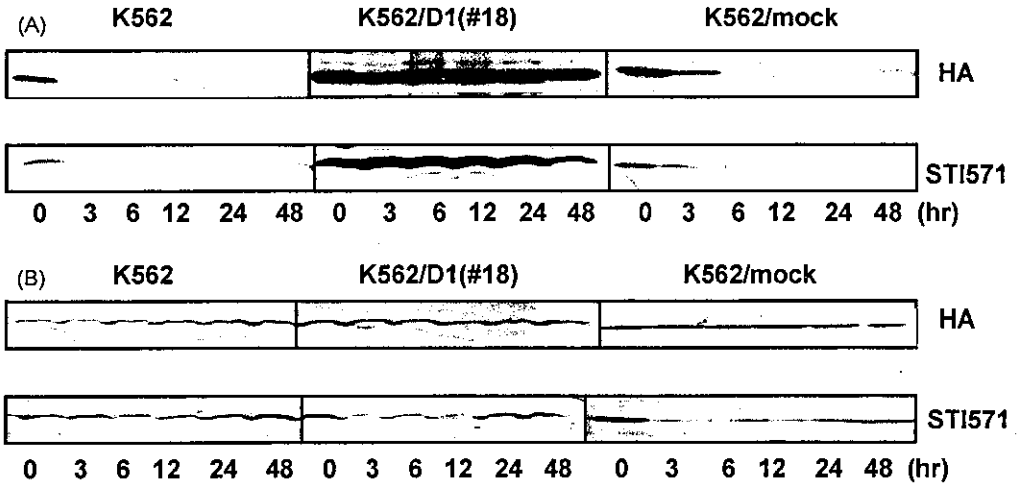

Fig. 2. Changes of cyclin D1 and c-Myc on immunoblotting during the induction of differentiation. (A) Immunoblot of cyclin D1. Cyclin D1 was downregulated in K562 cells and K562/mock cells at 3–6 h of herbimycin A and STI571 treatment, but herbimycin A and STI571 failed to downregulate cyclin D1 in K562/D1 cells. (B) Immunoblot of c-Myc. There were no marked changes in the expression of c-Myc by each cell line during herbimycin A treatment, but STI571 transiently downregulated c-Myc from 6 to 24 h. Recovery of c-Myc was observed at 48 h in each cell line. K562 and K562/D1(#18) were studied on the same blot. Detection of cyclin D1 and c-Myc were studied using same protein samples. HA: herbimycin A.

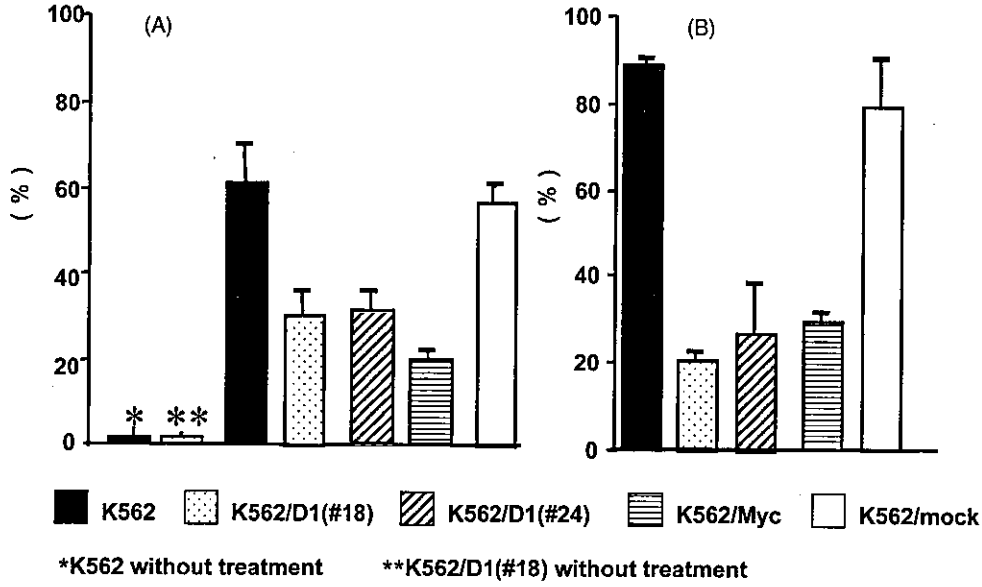

Fig. 3. Inhibition of erythroid differentiation by overexpression of c-Myc and cyclin D1. Cells were treated with 800 nM herbimycin A or 250 nM STI71 for 48 h. Erythroid differentiation was studied by benzidine staining. (A) Inhibition of erythroid differentiation induced by herbimycin A. Erythroid differentiation was reduced in K562/D1 and K562/Myc cells compared with parental K562 cells and K562/mock cells. (B) Inhibition of erythroid differentiation induced by STI571. Up to 90% of K562 cells underwent erythroid differentiation, while less than 30% of K562/D1 and K562/Myc cells showed differentiation.

cells, respectively, but K562/D1 cells only showed 30% erythroid differentiation after culture with herbimycin A. STI571 also failed to cause the differentiation of K562/D1 cells, since only 20% of cells were benzidine-positive. The inhibition of differentiation was comparable between K562/D1 and K562/Myc cells. These results clearly demonstrated that K562/D1 cells were resistant to the induction of erythroid differentiation by Bcr-Abl tyrosine kinase inhibitors.

To confirm the resistance of K562/D1 cells to erythroid differentiation, surface glycophorin A expression was studied (Fig. 4). K562 cells and K562/mock cells without any treatment showed weak expression of glycophorin A (Fig. 4A), but glycophorin A expression on K562/D1 cells was completely suppressed (Fig. 4A). This suggested that ectopic expression of cyclin D1 in K562 cells was able to reverse erythroid differentiation partly. Both herbimycin A (Fig. 4B) and STI571 (Fig. 4C) induced the erythroid

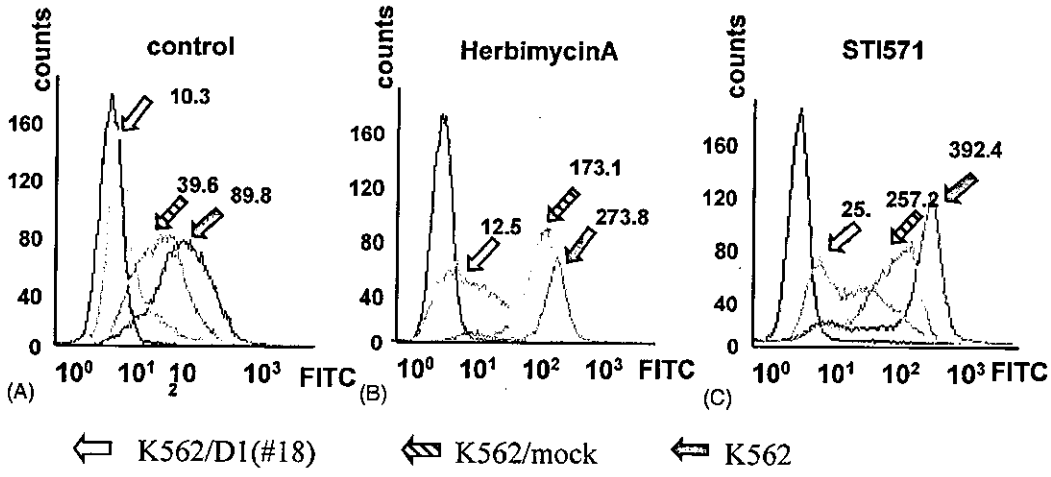

Fig. 4. Expression of glycophorin A. Cells were treated with 800 nM herbimycin A or 250 nM STI71 for 48 h. (A) Expression of glycophorin A in K562/D1 cells was markedly reduced compared with that in K562 and K562/mock cells. (B) Expression of glycophorin A after herbimycin A treatment. K562 and K562/mock cells strongly expressed glycophorin A, but its expression was inhibited in K562/D1 cells. (C) Expression of glycophorin A after STI571 treatment. STI571 induced stronger glycophorin A expression than herbimycin A. Although the expression of glycophorin A was strongly induced in K562 and K562/mock cells, its expression was blocked in K562/D1 cells.

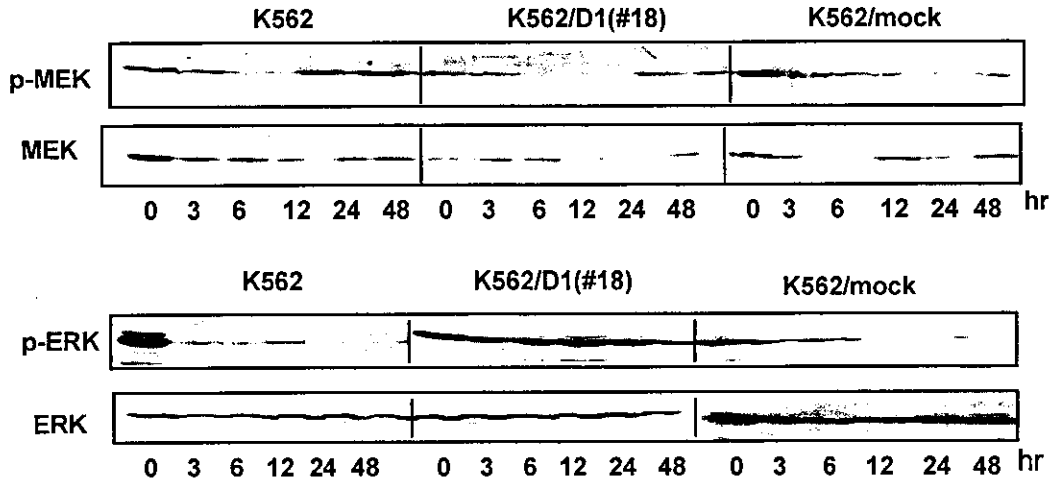

Fig. 5. Changes of MEK1 and ERK activity during erythroid differentiation. Each cell line was treated with 250 nM STI 571, and MEK and ERK activity were studied by detecting phosphorylated MEK and ERK. In all three cell lines, MEK activity was transiently downregulated in a similar manner. ERK activity was immediately downregulated in K562 and K562/mock cells, but the ERK activity of K562/D1 cells was not affected but slight reduction at 48 h.

differentiation of K562 cells, and the mean intensity of glycophorin A expression after exposure to these agents was 273.8 and 392.4, respectively. These results coincided with the detection of erythroid differentiation by benzidine-positive cells. Glycophorin A expression was also suppressed in K562/D1 cells. The mean peak expression of glycophorin A in K562/D1 cells exposed to herbimycin A and STI571 was 12.5 and 25.0, respectively.

3.4. K562/D1 cells show resistance to inactivation of ERK

The MAPK pathway is one of the important pathways controlled by the Bcr-Abl fusion gene, so we studied whether the MEK-to-ERK pathway was altered during STI571 treatment. Exposure to STI571 downregulated MEK activity in K562, K562/D1 and K562/mock cells from 3 to 12 h, and MEK activity recovered from 24 to 48 h (Fig. 5). Next, we studied the changes of ERK activity. Phosphorylated ERKs were largely downregulated in K562 and K562/mock cells, and no subsequent change was observed. In contrast, no downregulation of phosphorylated ERKs was observed in K562/D1 cells (Fig. 5). These data indicate that the signal transduction from Bcr-Abl to MEK–ERK was blocked by STI571 in K562 cells, while ERK was activated independently from MEK in cyclin D1-overexpressing cells.

4. Discussion

In this study, we demonstrated that the overexpression of cyclin D1 by K562 cells confers resistance to STI571-induced erythroid differentiation, and this inhibition of differentiation was comparable to that seen in cells overexpressing c-Myc. In general, the differentiation of hematopoietic cells coincides with the loss of proliferative activity, but the growth rate of each cell line during STI571 treatment was mostly similar to that of parental K562 cells (Fig. 1B). Unlike herbimycin A treatment, 250 nM STI571 caused almost no inhibition of proliferation, but STI571 induced erythroid differentiation more strongly than herbimycin A. Therefore, erythroid differentiation was actively induced by STI571 treatment, rather than occurring as a consequence of cytotoxicity. Moreover, differentiation was actually suppressed by cyclin D1 overexpression, while the growth of STI571-treated K562/D1 cells was similar to that of parental cells.

Expression of cyclin D1 following stimulation by growth factors is mainly regulated by the MAPK and PI3-K pathways [26–28]. Since the MAPK and PI3-K pathways are the two main routes stimulated by Bcr-Abl chimeric genes [29], the downregulation of cyclin D1 by STI571 treatment of parental K562 cells seems to be rational. In fact, we demonstrated that MEK1 activity was downregulated by STI571 in both K562 cells and K562/D1 cells. The changes in the activity of ERK, the downstream target of MEK1, were interesting. In K562 cells, ERK activity was downregulated from the early stage of STI571 treatment, but its activity in K562/D1 cells was constantly kept in high level and reduced slightly at 48 h. This persistence of ERK activity in K562/D1 cells may have contributed to interfering with STI571-induced differentiation. Our previous data also support this possibility because constant MEK1 activation renders K562 cells resistant to STI571-induced erythroid differentiation, while the induction of ERK-specific phosphatase potentiates the differentiation-inducing activity of STI571 [24]. Thus, ectopic expression of cyclin D1 may alter this MEK–ERK and cyclin D1 circuit, leading to the repression of differentiation.

Recently, it has demonstrated that the overexpression of cyclin D1 alters differentiation [14], and this action may be closely related not only to its effect on Cdk4 but also to the regulation of transcription factors. In skeletal muscle

cells, differentiation-associated genes (regulated by MEF2 transcription factor) are repressed by Cdk4–cyclin D1 kinase [14]. The proliferating cells in small intestinal crypts express cyclin D1, while the BETA2/NeuroD-dependent differentiation-related genes are repressed through the binding of cyclin D1 to a transcriptional co-factor (p300) [12]. Cyclin D1 has been demonstrated to bind to thyroxine and estrogen receptors [10,11,13], and it modulates the transcriptional activity of these receptors in both a ligand-dependent and -independent manner. These findings show that cyclin D1 has a potential to modulate the transcription of gene expression, so the overexpression of cyclin D1 in K562 cells may interfere with the transcription of genes that are required for the induction of erythroid differentiation.

In conclusion, we demonstrated that cells overexpressing cyclin D1 show resistance to STI571-induced erythroid differentiation. STI571 is expected to bring several benefits by its clinical application. Since our study suggested one of mechanisms of resistance to STI571, we are now studying the effects of concomitant treatment with other drugs to overcome the resistance [30].

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Contributions. T. Kawano interpreted and analyzed the data, provided drafting of the article, gave final approval, collected and assembled the data. J. Horiguchi-Yamada provided critical revisions and important intellectual content, gave final approval, and provided administrative support. S. Saito gave final approval, and provided administrative support. S. Iwase gave final approval, and obtained a funding source. Y. Furukawa gave final approval, and provided study materials/patients. Y. Kano provided critical revisions and important intellectual content, gave final approval, and provided study materials/patients. H. Yamada contributed to the concept and design, interpreted and analyzed the data, gave final approval, obtained a funding source, collected and assembled the data.

References

[1] Sherr CR. Cell cycle control and cancer. Harvey Lect 2000–2001;96:73–92.
[2] Horiguchi-Yamada J, Yamada H, Nakada S, Ochi K, Nemoto T. Changes of G1 cyclins, cdk2, and cyclin A during the differentiation of HL60 cells induced by TPA. Mol Cell Biochem 1994;132:31–7.
[3] Furukawa Y, Kikuchi J, Nakamura M, Iwase S, Yamada H, Matsuda M. Lineage-specific regulation of cell cycle control gene expression during haematopoietic cell differentiation. Br J Haematol 2000;110:663–73.
[4] Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochem Biophy Acta 2002;1062:73–87.
[5] Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, et al. Expression and amplification of cyclin genes in human breast cancer. Oncogene 1993;8:2127–33.
[6] Tsuruta H, Sakamoto H, Onda M, Terada M. Amplification and overexpression of *EXP1* and *EXP2*/cyclin D1 genes in human esophageal carcinomas. Biochem Biophys Res Commun 1993;196:1529–36.
[7] Oka K, Ohno T, Kita K, Yamaguchi M, Takakura N, Nishii K, et al. *PRAD1* gene over-expression in mantle-cell lymphoma but not in other low-grade B-cell lymphomas, including extranodal lymphoma. Br J Haematol 1994;86:786–91.
[8] Sherr CJ. Cancer and cycles. Science 1996;274:1672–7.
[9] Nevins JR. The Rb/E2F pathway and cancer. Hum Mol Genet 2001;10:699–703.
[10] Zwijsen RM, Buckle RS, Hijmans EM, Loomans CJ, Bernards R. Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. Genes Dev 1998;12:3488–98.
[11] McMahon C, Suthiphongchai T, DiRenzo J, Ewen ME. P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. Proc Natl Acad Sci USA 1999;96:5382–7.
[12] Ratineau C, Petry MW, Mutoh H, Leiter AB. Cyclin D1 represses the basic helix-loop-helix transcription factor, BETA2/NeuroD. J Biol Chem 2002;277:8847–53.
[13] Lin HM, Zhao L, Cheng SY. Cyclin D1 is a ligand-independent co-repressor for thyroid hormone receptors. J Biol Chem 2002;277:28733–41.
[14] Lazaro JB, Bailey PJ, Lassar AB. Cyclin D–cdk4 activity modulates the subnuclear localization and interaction of MEF2 with SRC-family coactivators during skeletal muscle differentiation. Genes Dev 2002;16:1792–805.
[15] Hoffman B, Amanullah A, Shafarenko M, Liebermann DL. The proto-oncogene c-*myc* in hematopoietic development and leukemogenesis. Oncogene 2002;21:3414–21.
[16] Gartel AL, Ye X, Goufman E, Shianov P, Hay N, Najmabadi F, et al. Myc represses the p21(WAF1/CIP1) promoter and interacts with SP1/Sp3. Proc Natl Acad Sci USA 2000;98:4510–5.
[17] Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, et al. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc Natl Acad Sci USA 2000;97:3260–5.
[18] Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature 2002;419:729–34.
[19] Horiguchi-Yamada J, Fukumi S, Saito S, Nakayama R, Iwase S, Yamada H. DNA topoisomerase II inhibitor, etoposide, induces p21WAF1/CIP1 through down-regulation of c-Myc in K562 cells. Anticancer Res 2002;22:3827–32.
[20] Yamada H, Iwase S, Nagai M, Nemoto T, Sekikawa T, Takahara S, et al. Herbimycin A down-regulates messages of cyclin D1 and c-myc during erythroid differentiation of K562 cells. Int J Hematol 1996;65:31–40.
[21] Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996;2:561–6.
[22] Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Eng J Med 2001;344:1031–7.
[23] Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med 2001;344:1038–42.
[24] Kawano T, Horiguchi-Yamada J, Iwase S, Furukawa Y, Kano Y, Yamada, H. Inactivation of ERK accelerates erythroid differentiation of K562 cells induced by herbimycin A and STI571 while activation of MEK1 interferes with it. Mol Cell Biochem, in press.

[25] Akiyama M, Yamada O, Akita S, Urashima M, Horiguchi-Yamada J, Ohno T, et al. Ectopic expression of c-myc fails to overcome downregulation of telomerase activity induced by herbimycin A, but ectopic hTERT expression overcomes it. Leukemia 2000;14:1260–5.
[26] Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 1996;271:20608–16.
[27] Aktas H, Cai H, Cooper GM. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. Mol Cell Biol 1997;17:3850–7.
[28] Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem 1998;273:29864–72.
[29] Maru Y. Molecular biology of chronic myeloid leukemia. Int J Hematol 2001;73:308–22.
[30] Kano Y, Akutsu M, Tsunoda S, Mano H, Sato Y, Honma Y, et al. In vitro cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. Blood 2001;97:1999–2007.

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Inactivation of ERK accelerates erythroid differentiation of K562 cells induced by herbimycin A and STI571 while activation of MEK1 interferes with it

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Abstract

K562 cells contain a Bcr-Abl chimeric gene and differentiate into various lineages in response to different inducers. We studied the role of the mitogen-activated protein kinase (MAPK) kinase 1 (MEK1)/extracellular signal-regulated kinase (ERK) pathway during the erythroid differentiation of K562 cells induced by tyrosine kinase inhibitors (herbimycin A or STI571), using genetically modified cells (constitutively MEK1-activated K562: K562/MEK1, and inducible ERK-inactivated K562: K562/CL100). Basal expression of glycophorin A was markedly reduced in K562/MEK1 cells compared with that in parental cells, while it was augmented in K562/CL100 cells. Herbimycin A and STI571 differentiated K562 cells accompanying with the transient down-regulated ERK. Moreover, the erythroid differentiation was markedly suppressed in K562/MEK1 cells, and early down-regulation of ERK activity was not observed in these cells. In contrast, the induction of ERK-specific phosphatase in K562/CL100 cells potentiated erythroid differentiation. Once the phosphatase was induced, the initial ERK activity became repressed and its early down-regulation by the inhibition of Bcr-Abl was marked and prolonged. These results demonstrate that the erythroid differentiation of K562 cells induced by herbimycin A or STI571 requires the down-regulation of MEK1/ERK pathway. (Mol Cell Biochem **258**: 25–33, 2004)

Key words: STI571, herbimycin A, MEK, ERK, erythroid differentiation, K562

Introduction

Differentiation therapy is one of the promising strategies for the treatment of leukemia, especially in elderly patients who cannot tolerate intensive chemotherapy or bone marrow transplantation. Great success has been achieved with all-trans retinoic acid therapy for promyelocytic leukemia [1], but the available drugs are not effective enough for clinical application against other types of leukemia. This is partly because of our poor understanding regarding the differentiation of leukemic cells.

K562 cells are a human erythroleukemia cell line that was derived from a patient with chronic myelogenous leukemia in blastic crisis. This pluripotent cell line is useful for studying the molecular mechanisms that regulate the lineage commitment of leukemic cells. Various compounds are known to induce the differentiation of K562 cells into different lineages [2]. For example, K562 cells are differentiated into the

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megakaryocytic lineage by phorbol 12-myristate 13-acetate (PMA) [3], while herbimycin A [4], hemin, Ara C [5], and butyrate [6] all induce erythroid differentiation. K562 cells have a Bcr-Abl chimeric gene. This chimeric protein promotes tyrosine kinase activity, and mediates various growth signals that lie down-stream [7]. Through the association of Grb2 and SOS with the Bcr-Abl fusion protein, Ras proto-oncogene is activated and this leads to the activation of several signal transduction systems [7]. The Raf/MAPK pathway is one of the major signaling pathways from Bcr-Abl and is critical for the growth of CML cells, because an inhibitor of the association between Bcr-Abl with Grb2 strongly inhibits the proliferation of CML cells [8]. Modulation of the Bcr-Abl/Raf/MEK1/ERK system seems to be an important regulator for the differentiation of CML cells as well as for control of proliferation. PMA, which activates the MEK1/ERK pathway and induces the expression of cyclin D1 and $p21^{cip1}$, leads K562 cells into differentiation towards megakaryocytic lineage [9], while the MEK1 inhibitor PD098059 has been demonstrated to enhance erythroid differentiation (10). However, these relationships may differ depending on either the inducer or the signal modulator. In activation of Grb2 by a specific peptide could inhibit proliferation of K562 cells, but failed to induce erythroid differentiation [8]. K562 cells transfected with an active mutant of MEK1 or MEK2 demonstrated a differentiated phenotype compatible with megakaryocytes after PMA treatment by adhering to the culture flask with a spreading morphology [11]. Moreover, their proliferation was slightly down-regulated and α-globin gene expression was markedly suppressed [11]. On the other hand, activated Ras-transfected K562 cells were unexpectedly showed a benzidine positive phenotype, although the MEK/ERK pathway was activated [12]. Moreover, Woessmann *et al.* showed the requirement of ERK activation in hemin-induced erythroid differentiation [13].

Herbimycin A, a benzoquinoid ansamycin antibiotic, was originally isolated as a tyrosine kinase inhibitor. Honma *et al.* demonstrated that it caused K562 cells to differentiate into erythroid cells [4]. We previously demonstrated that herbimycin A transiently down-regulated c-myc and cyclin D1 during erythroid differentiation of K562 cells [14] and that this was also associated with the partial down-regulation of telomerase activity [15]. Recently, a more specific Bcr-Abl kinase inhibitor (STI571) has been developed [16], and it has been demonstrated to suppress the growth of CML cells both *in vivo* and *in vitro* [17]. Here we studied the relationships between inactivation of Bcr-Abl and the MEK1/ERK pathway during erythroid differentiation using genetically modulated cell lines and these Bcr-Abl specific inhibitors. We found that inactivation of ERK was critical for determining the erythroid differentiation of K562 cells induced by inhibition of Bcr-Abl activity.

Materials and methods

Cells and culture conditions

K562 cells were obtained from the Riken cell bank (Tsukuba, Japan). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA) in a 5% CO_2 incubator with 100% humidity. For the experiments, exponentially growing cells (2×10^5/ml) were suspended in 20 ml of culture medium containing 800 nM herbimycin A (Sigma, St. Louis, MO, USA) and 250 nM of STI571 (a gift from Novartis; Basel, Switzerland).

Construction of expression vectors and establishment of cell lines

A constitutive active MEK1 expression vector was purchased from Upstate biotechnology (Lake Placid, NY, USA). It contains the activated rat MEK1cDNA (S218D/S222D) under the control of CMV promoter [18], and it is demonstrated to function properly in human cells elsewhere [19]. The plasmid was transfected into K562 cells by electroporation, as previously reported [15]. After transfection, cells were selected using neomycin and single-cell colonies were isolated in semi-solid methyl cellulose medium.

For experiments on ERK-specific phosphatase [20], the LacSwitch™ II Inducible Mammalian Expression System (purchased from STRATAGENE, La Jolla, CA, USA) was used. ERK specific-phosphatase cDNA was constructed using the RT-PCR method. Briefly, total RNA was isolated from parental K562 cells, and an oligo-dT primed first-strand cDNA was made using MMTV-reverse transcriptase (Gibco BRL). The primers for PCR amplification were as follows; Forword primer: 5-TGAGGCCGGCCATGGTCA-TGGAAG-3, Reverse primer: 5-CCTTTCAGCAGCTGG-GAGAGG-3. PCR products were ligated into the TA cloning vector (Invitrogen, Carlsbad, CA, USA). After isolation of a clone, the insert was confirmed by sequencing using an ABI sequencing kit (Applied Biosystems, Foster City, CA, USA). The isolated CL100-cDNA was transferred into the pOPRSV/MCS operator vector at the Spe1 and EcoRV sites and then transfected into K562 cells, which had been pretransfected with the pCMVLacI repressor vector. Double vector-transfected K562 cells were selected in 800 μg/ml G418 (Gibco) and 1200 μg/ml hygromycin (Gibco) selection medium. For induction of CL100-cDNA, the cells were treated with 2 mM IPTG at 3 h before the experiments.

Detection of erythroid differentiation

Cytochemical assays

K562 cells with erythroid differentiation that contained heme or hemoglobin were detected by a specific reaction using a benzidine/hydrogen peroxide solution. Benzidine dihydrochloride (2 mg/ml in 0.5% acetic acid) was mixed with 30% hydrogen peroxide (5 μl/ml) and added directly to an equal volume (10 μl) of cell suspension in a centrifuge tube. The mixture was incubated at room temperature for 5 min and the percentage of benzidine-positive cells was calculated under microscopy after counting at least 600 cells.

Glycophorin A analysis

Glycophorin A on the cell surface was examined by a direct immunofluorescence method. An FITC-conjugated monoclonal antibody for glycophorin A and control IgG-κ were purchased from DAKO (Carpinteria, CA, USA). Then 10^6 cells were harvested, washed once with 2% fetal calf serum containing PBS, and incubated in the dark with the antibodies for 45 min at 4°C. After washing three times with 2% fetal calf serum containing PBS, the cells were analyzed by flow cytometry (FACS caliber; Becton Dickinson, Franklin Lakes, NJ, USA).

Immunoblot analysis

Cells were washed with ice-cold TBS buffer (25 mM Tris-HCl (pH 8.0), 150 mM NaCl), and lysed in the lysis buffer supplied with p38MAK kinase assay kit (Cell Signaling Technology, Inc. MA, USA). Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and size-separated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a submerge electroblotting chamber. Membranes were blocked in TBS buffer containing 0.1% Tween-20 and 5% skim milk for 1 h at room temperature. Detection of ERK and phosphorylated-ERK was done using the phosphorylated ERK detection kit (purchased from Cell Signaling Technology, Inc.). Briefly, incubation with the primary antibodies was done overnight at 4°C using dilutions recommended by the manufacturer. And after incubation for one hour at room temperature with anti-rabbit Ig conjugated with horseradish peroxidase, proteins were detected by an enhanced chemiluminescence system.

Results

Herbimycin A and STI571 induce erythroid differentiation of K562 cells

We previously demonstrated that 800 nM herbimycin A induced the erythroid differentiation of K562 cells [14]. Therefore, we investigated whether STI571 could also induce erythroid differentiation. K562 cells were treated with either 800 nM herbimycin A or 250 nM STI571 for 48 h, after which the percentage of benzidine-positive cells and the intensity of glycophorin A expression on the cell surface were studied. As demonstrated in Fig. 1, 56.7 and 93.5% of K562 cells were stained with benzidine (Figs 1A and 1B) after herbimycin A or STI571 treatment, respectively. FACS analysis also showed marked induction of glycophorin A on the cell surface by herbimycin A and STI571 (Fig. 1C).

Herbimycin A and STI571 transiently down-regulate the ERK signaling pathway in the early phase of treatment

One of the signals from Bcr-Abl is transduced through the Raf/MAPK pathways, and such signaling is considered to play a critical role in leukemogenesis. When we studied the changes of ERK activation during differentiation, herbimycin A suppressed ERK activation from 3–6 h. ERK activity began to return from 12 h and became completely normal at 24–48 h (Fig. 2). STI571 (250 nM) also changed ERK activity in the same manner as herbimycin A, but inactivation occurred earlier than with herbimycin A treatment and weak reactivation only occurred at 12 h (Fig. 2). No phosphorylated ERK was observed at 24–48 h.

Characterization of K562/MEK1 and K562/CL100 cells

Because the ERK signaling pathway was altered during erythroid differentiation induced by herbimycin A and STI571, we established cell lines in which ERK was either genetically activated (K562/MEK1) or suppressed (K562/CL100) without the need to using chemical inhibitors. The cellular growth rate and the expression of glycophorin A were studied. Proliferation of K562/MEK1 cells was similar to that of parental K562 cells (Fig. 3A), while the basal expression of glycophorin A was markedly suppressed (Fig. 3C). The mean level of glycophorin A expression by parental K562 cells was 37.9, but that of K562/MEK1 cells was 8.6. In contrast, the growth rate of K562/CL100 cells was slightly, but significantly, inhibited at 72 h after the induction of phosphatase by IPTG (Fig. 3B). Moreover, basal glycophorin A expression on K562/CL100 cells was stronger than that by parental K562 cells (Fig. 3C). The mean level of glycophorin A expression on K562/CL100 cells without exposure to IPTG was 98.2, while that after induction with IPTG was 118.6.

K562/MEK1 cells are resistant to induction of erythroid differentiation by herbimycin A or STI571

Because the basal expression of glycophorin A was reduced in K562/MEK1 cells, we studied the erythroid differentiation