

Role of Ras/ERK-dependent pathway in the erythroid differentiation of K562 cells

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Abbreviations: CML, Chronic myelogenous leukemia; PTK, protein tyrosine kinase; MAPK/ERK-activating kinase, MEK; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activating kinase; SDS, sodium-dodecyl sulfate; PAGE, polyacrylamide gel-electrophoresis

Abstract

The chronic myelogenous leukemic K562 cell line carrying Bcr-Abl tyrosine kinase is considered as pluripotent hematopoietic progenitor cells expressing markers for erythroid, granulocytic, monocytic, and megakaryocytic lineages. Here we investigated the signaling modulations required for induction of erythroid differentiation of K562 cells. When the K562 cells were treated with herbimycin A (an inhibitor of protein tyrosine kinase), *ras* antisense oligonucleotide, and PD98059 (a specific inhibitor of MEK), inhibition of ERK/MAPK activity and cell growth, and induction of erythroid differentiation were observed. The *ras* mutant, pZIPRas^{61leu}-transfected cells, K562-Ras^{61leu}, have shown a markedly decreased cell proliferation rate with approximately 2-fold doubling time, compared with the parental K562 cells, and about 60% of these cells have shown the phenotype of erythroid differentiation. In addition, herbimycin A inhibited the growth rate and increased the erythroid differentiation, but did not affect the elevated activity of ERK/MAPK in the K562-Ras^{61leu} cells. On the other hand, effects of PD98059 on the growth and differentiation of K562-Ras^{61leu} cells were biphasic. At low concentration of PD98059, which inhibited

the elevated activity of ERK/MAPK to the level of parental cells, the growth rate increased and the erythroid differentiation decreased slightly, and at high concentration of PD98059, which inhibited the elevated activity of ERK/MAPK below that of the parental cells, the growth rate turned down and the erythroid differentiation was restored to the untreated control level. Taken together, these results suggest that an appropriate activity of ERK/MAPK is required to maintain the rapid growth and transformed phenotype of K562 cells.

Keywords: K562, erythroid differentiation, Ras/ERK pathway, PD98059

Introduction

The K562 cell line was established from pleural effusion of a patient with chronic myelogenous leukemia (CML) in blast crisis (Lozzio and Lozzio, 1975). CML is characterized by the reciprocal chromosomal translocation t(9;22)(q34;q11), which generates the Philadelphia chromosome (Ph). This event occurs in the pluripotent hematopoietic stem cell and transposes the *c-abl* proto-oncogene on chromosome 9, encoding a protein tyrosine kinase (PTK), to a new position downstream of second exon of the gene *bcr* on chromosome 22. This translocation generates a novel fusion gene, *bcr-abl*, that encodes a chimeric protein, p210 bcr-abl, the PTK activity of which is aberrantly regulated relative to *c-abl* (Konopka *et al.*, 1985). K562 cells are regarded as pluripotent hematopoietic progenitor cells expressing markers for erythroid, granulocytic, monocytic, and megakaryocytic lineages as defined by surface-antigen expression (Leary *et al.*, 1987). The K562 cells have been induced to erythroid differentiation by the treatment with hemin (Rowley *et al.*, 1981), butyric acid (Cioe *et al.*, 1981), antineoplastic drugs such as Ara-C (Luisi DeLuca *et al.*, 1984), daunomycin (Tonini *et al.*, 1987), and tiazofurin (Olah *et al.*, 1988), and tyrosine kinase inhibitors (Honma *et al.*, 1989; Honma *et al.*, 1990; Anafi *et al.*, 1993). However, signaling mechanism of an erythroid differentiation is not clearly understood.

Protein tyrosine phosphorylation was found to be reduced during the hemin-induced erythroid differentiation of K562 cells, and this reduction was suggested to be due to an inhibition of the Bcr-Abl PTK synthesis (Richardson *et al.*, 1987). Inhibition of PTK activity by herbimycin A also resulted in the erythroid differentiation of K562 cells (Honma *et al.*, 1989) and specific inhibition of *abl* PTK activity by human *abl* antisense oligonucleotide and

GP57148, a selective inhibitor of Bcr-Abl PTK (Druker *et al.*, 1996; Carroll *et al.*, 1997), induces K562 cells into erythroid differentiation (Honma *et al.*, 1990; LaMontagne *et al.*, 1998). Therefore, inhibition of Bcr-Abl PTK appears to play an important role in the erythroid differentiation of K562 cells. Altered expression of several genes is associated with the erythroid differentiation of K562 cells. Decrease in *H-ras*, *K-ras* and *N-ras* expression (*H-ras* was affected more dramatically than *K-ras* and *N-ras*) was found in the erythroid differentiated K562 cells after exposure to Ara-C (Delgado *et al.*, 1992). Tiazofurin causes down-regulation of *c-Ki-ras* gene (Olah *et al.*, 1988; Weber *et al.*, 1991). In addition, decrease in *c-myc* gene expression was observed in the K562 cells treated with Ara-C (Bianchi Scarra *et al.*, 1986; Tonini *et al.*, 1987).

Bcr-Abl PTK is expressed in 95% of cases of CML as a consequence of Philadelphia translocation t(9;22)(q34;q11) (Kurzrock *et al.*, 1988). Bcr-Abl PTK can bind and/or phosphorylate a large number of proteins, many of which can be directly linked to signal transduction pathways (Raitano *et al.*, 1997). Similar to other receptor tyrosine kinases, Bcr-Abl activates Ras pathway through Grb-2, Shc and CRKL (Mandanias *et al.*, 1993; Pui *et al.*, 1994; Skorski *et al.*, 1994; Tauchi *et al.*, 1994; Goga *et al.*, 1995; Raitano *et al.*, 1997). The p85 subunit of PI3-kinase is tyrosine phosphorylated in Bcr-Abl expressing cells (Gotoh *et al.*, 1994) and forms complexes with Bcr-Abl through interaction with CBL and CRKL (Sattler *et al.*, 1996), and an increased PI3-kinase activity is seen in Bcr-Abl-transformed fibroblasts (Varticovski *et al.*, 1991) and hematopoietic cells (Skorski *et al.*, 1995; Jain *et al.*, 1996).

Although an inhibition of Bcr-Abl PTK appears to be a primarily causative step to induce K562 cells into erythroid differentiation, modulations of downstream signaling, which are required for induction of erythroid differentiation, is not clearly understood. In the present study, we have shown that the erythroid differentiation of K562 cells could be induced by either inhibition or activation of Ras/ERK pathway, and it seems that an appropriate activity of Ras/ERK pathway is required to maintain the transformed phenotype of chronic myelogenous leukemic cell line, K562.

Materials and Methods

Cell culture

K562 cells were grown in suspension of RPMI medium 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Life Technologies, Inc), 100 units/ml penicillin, and 100 mg/ml streptomycin (Sigma Chemical Co.). The cultured cells were passed twice weekly, seeding at a density of about 2×10^5 cells/ml. In order to treat with various agents, exponential growing K562 cells were collected

by centrifugation at 1,200 g, for 10 min and resuspended in fresh culture medium.

Plasmid and transfection

K562 cells were transfected with pZIPRas^{61leu} (kindly donated by G.M. Cooper, Harvard Medical School). 20 mg of supercoiled plasmid DNA was added to K562 cells (2×10^6) and electroporation was carried out using Electroporator II (Invitrogen) at 1,500 V/cm, 50 μ F condition. Electroporated cells were cultured in G418 (450 mg/ml) containing medium. After 2 weeks, individual G418 resistant colonies were isolated and expanded in the presence of G418 to generate stable transformants.

Measurement of cell growth and erythroid differentiation

Cells (5×10^4 /ml) were treated with various drugs for 3 days. Stock solutions of herbimycin A, PD98059, and wortmannin were made with DMSO at the concentration of 10 mM, 50 mM and 20 mM, respectively. For treatment of *ras* antisense oligonucleotide, cells were incubated in minimal volume of serum-free medium (20 μ l) containing oligonucleotide and Lipofectin (0.2 μ l, Gibco, BRL, Inc.) in a 96-well tissue culture plate for 6 h, followed by an addition of 180 μ l of complete medium. The sequence of *ras* antisense oligonucleotide is 5'-TCC GTC ATC GCT CCT CAG GG-3' (Monia *et al.*, 1992), and the random phosphorothioate oligonucleotide with same length was used as control. For treatment of wortmannin, medium containing wortmannin was refreshed every 6 h. Number of cells was counted with hemocytometer, and cell growth was expressed as % of control.

Hemoglobin-producing cells were scored by benzidine staining (Rowley *et al.*, 1981). The cells were washed once with phosphate-buffered saline, and benzidine solution (benzidine dihydrochloride 2 mg/ml in 0.5% acetic acid) was mixed with 50 μ l/ml of a 3% hydrogen peroxide solution and added to an equal volume of cell suspension. Cells were scored after 5 min as benzidine-positive (blue) or benzidine-negative (yellow) under the microscope.

Assay of ERK/MAPK activity

For an assay of MAP kinase activity, cells (1×10^6) were treated in 12-well tissue culture plate with various drugs for 5 h except *ras* antisense oligonucleotide (for 24 h). ERK/MAPK activity was measured with p44/42 MAP Kinase Assay Kit (New England BioLabs, Inc.) according to manufacturer's protocol. Cells were lysed in 20 mM Tris-HCl buffer, pH7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM γ -glycerophosphate, 1 mM Na_3VO_4 , 1 mg/ml leupeptin, and 1 mM PMSF for 15 min at 4°C. The lysates were centrifuged at 10,000 g for 15 min and the supernatants were used as cell lysates. 200 μ l of cell

lysate (approximately 200 µg protein) were mixed with phospho-MAPK antibody (1:50 dilution) and incubated overnight at 4°C with gentle rocking. Immunoprecipitates were collected by protein A Sepharose beads (10-20 µl) for 2 h at 4°C and beads were washed twice with cold lysis buffer and twice with 500 µl of kinase buffer (20 mM Tris buffer, pH 7.5, containing 5 mM -glycerophosphate 2 mM DTT, 0.1 mM Na₃VO₄, and 10 mM MgCl₂). Kinase assay was performed by incubating the suspended pellet with kinase buffer containing 100 mM ATP and GST-Elk1 fusion protein for 30 min at 30°C. The reactions were terminated with 25 µl 3 × sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue) and the samples were analyzed by 12% SDS-PAGE. Phospho (Ser383)-Elk1 was detected with specific antibody using western blot analysis.

Results

Effect of herbimycin A, *ras* antisense oligonucleotide, PD98059 and wortmannin on growth, differentiation and ERK/MAPK activity of K562 cells

Activation of Ras-dependent pathway and PI3-kinase-dependent pathway is known to play important roles in Bcr-Abl-mediated transformation of hematopoietic cells (Goga *et al.*, 1995; Skorski *et al.*, 1997). We have examined whether the blocking of those pathways leads to growth arrest and erythroid differentiation of K562 cells. Herbimycin A, a PTK inhibitor and an inducer of erythroid differentiation of K562 cells (Honma *et al.*, 1989), inhibited cell growth, induced erythroid differentiation in a dose-dependent manner, and also inhibited ERK/MAPK activity, which is downstream of Ras pathway and regulates

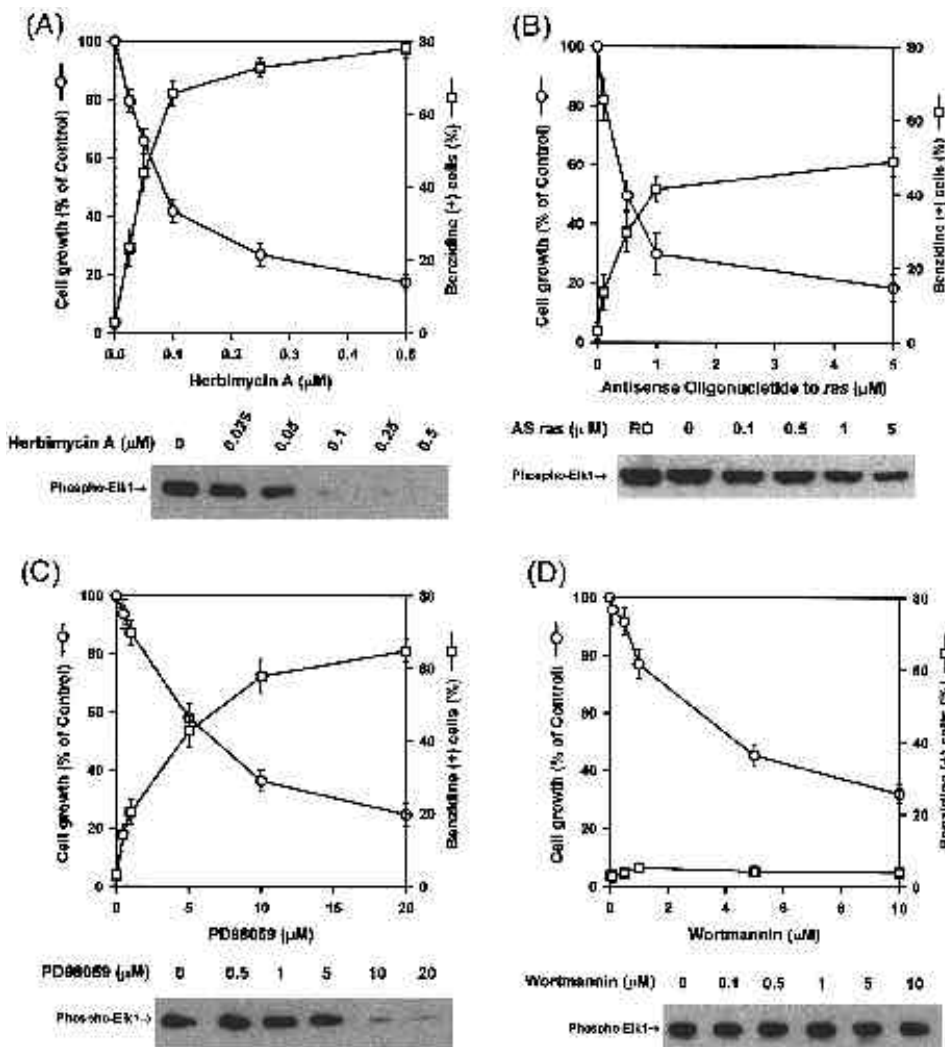


Figure 1. Effects of herbimycin A, *ras* antisense oligonucleotide, PD98059 and wortmannin on the growth, erythroid differentiation and ERK/MAPK activity of K562 cells. K562 cells (5×10^4 /ml) were treated with herbimycin A (A), *ras* antisense oligonucleotide (B), PD98059 (C) and wortmannin (D) for 3 days, and number of cells and benzidine (+) cells was counted with hemocytometer (upper panels). The cell growth was expressed as relative to the untreated control (100%). Shown are the means \pm S.D. of three independent experiments. For assay of ERK/MAPK activity (lower panels), cells (1×10^6) were treated with various drugs for 5 h except *ras* antisense oligonucleotide (AS *ras*, for 24 h; random oligonucleotide, RO, was used as a negative control), and cell lysates (200 µg) were immunoprecipitated with anti-phospho-MAPK antibody. Kinase activity was measured with Elk1-fusion protein. Phosphorylated Elk1-fusion protein was separated by 12% SDS-PAGE and detected with Phospho-specific-Elk1-antibody. DMSO, a vehicle for herbimycin A, PD98059 and wortmannin, did not affect the growth, erythroid differentiation and ERK/MAPK activity of K562 cells at concentrations used here (data not shown).

cell growth (Seger and Krebs, 1995) (Figure 1A). When 0.5 mM herbimycin A was given, ERK/MAPK activity decreased to an almost undetectable level, cell growth decreased to less than 20% of control, and about 80% of cells were differentiated. To see whether the inhibition of cell proliferation and induction of erythroid differentiation of K562 cells are associated with an inhibition of ERK/MAPK activity, Ras/ERK pathway was blocked with *ras* antisense oligonucleotide and PD98059, a specific inhibitor of MEK. Ras antisense oligonucleotide and PD98059 also inhibited both the ERK/MAPK activities and cellular proliferation, and induction of erythroid differentiation (Figure 1B and 1C). In contrast, when K562 cells were treated with wortmannin, an inhibitor of PI3-kinase, erythroid differentiation and inhibition of ERK/MAPK activity were not observed, although cell growth decreased in a dose-dependent manner (Figure 1D). These results suggest that the inhibition of Ras/ERK-dependent pathway, not the PI3-kinase-dependent pathway is associated with the induction of erythroid differentiation of K562 cells.

Effect of active Ras mutant on the growth and erythroid differentiation

To establish the relationship between the Ras/ERK pathway and erythroid differentiation, K562 cells were transfected with pZipRas^{61leu}, an active mutant of *ras* (Figure 2A). The pZipRas^{61leu}-transfected cells, K562-Ras^{61leu}, were found to have a huge cell size, a markedly decreased proliferation with approximately 2-fold doubling time and an increased activity of ERK/MAPK compared with their parental cells (Figure 2B, 2C and 3). Surprisingly, about 60% of these *ras* mutant transfected cells were spontaneously differentiated to erythroid lineage (Figure 3). Since *ras* antisense oligonucleotide and PD98059 inhibit the ERK/MAPK activities and cell growth, and induce erythroid differentiation (Figure 1B

and 1C), spontaneous differentiation of K562-Ras^{61leu} cells to erythroid lineage was unexpected. Therefore, K562-Ras^{61leu} cells provided as a model to examine signal pathways of Bcr-Abl PTK in relationship with differentiation and ERK/MAPK activity. Herbimycin A further inhibited the growth rate and increased the erythroid differentiation, but did not inhibit the activity of ERK/MAPK in K562-Ras^{61leu} cells (Figure 3A), suggesting that there might be an additional mitogenic and transforming signals from Bcr-Abl PTK other than ERK/MAPK pathway. On the other hand, effects of PD98059 on the growth and differentiation of K562-Ras^{61leu} cells were biphasic (Figure 3B). Up to 10 mM of PD98059 concentration, the elevated activity of ERK/MAPK due to *ras*^{61leu} transfection was decreased to the level of parental cells, and a slight increase of the growth rate and a slight decrease of the erythroid differentiation were observed. But at concentration greater than 10 mM of PD98059, the activity of ERK/MAPK was below that of the parental cells and we observed significant decrease of the growth rate and restoration of the erythroid differentiation to the untreated control level, suggesting that an appropriate activity of ERK/MAPK is required to maintain the rapid growth and transformed phenotype of K562 cells.

Discussion

We have studied the role of downstream signaling pathways of Bcr-Abl PTK in erythroid differentiation of K562 cells. Among the cellular events associated with the tumorigenic potential of CML including K562 cells is the constitutive expression of the activated Bcr-Abl PTK (Ben Neriah *et al.*, 1986). The expression of this fusion gene product is associated with an increase in tyrosine phosphorylated proteins that leads to activation of intracellular signaling pathways such as Ras and PI3-kinase

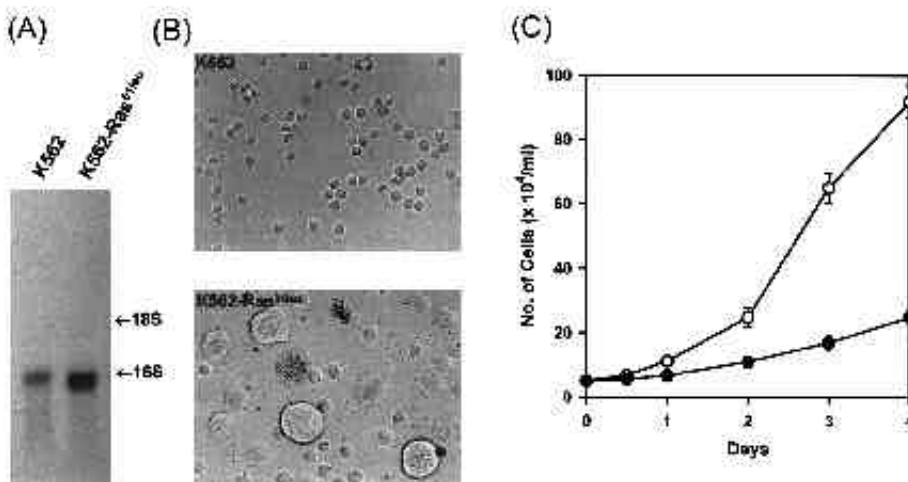


Figure 2. Isolation of pZIPRas^{61Leu}-transfected stable transformants and effect of the overexpressed Ras^{61Leu} on the growth of K562 cells. (A) Northern blot analysis was done to identify the pZIPRas^{61Leu}-transfected K562 cells (K562-Ras^{61leu}). (B) The morphology of K562 (upper panel) and K562-Ras^{61leu} cells (lower panel). (C) K562 (—) and K562-Ras^{61leu} (---) cells (5×10^4 /ml) were seeded and cultured up to 4 days in complete medium. Number of cells was counted with hemocytometer. Shown are the means \pm S.D. of three independent experiments.

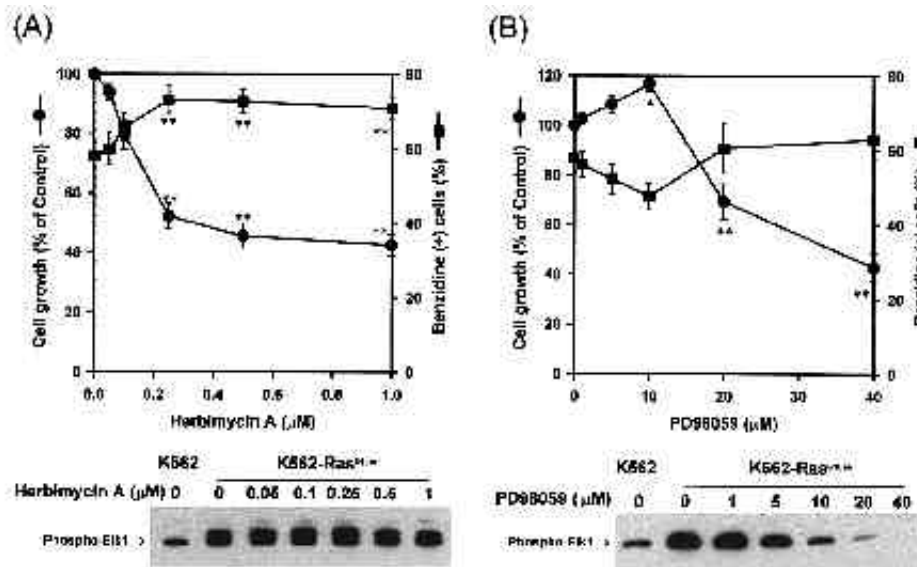


Figure 3. Effects of herbimycin A and PD98059 on the growth, erythroid differentiation and MAP kinase activity of K562-Ras^{61leu} cells. Cells (5×10^4 cells/ml) were treated with herbimycin A (A) and PD98059 (B) for 3 days, and number of cells and benzidine (+) cells was counted with hemocytometer (upper panels). Shown are the means \pm S.D. of three independent experiments. *, $P < 0.05$ and **, $P < 0.01$; significantly different from the each control value. For assay of MAP kinase activity (lower panels), K562-Ras^{61leu} cells (1×10^6) were treated with herbimycin A (A) and PD98059 (B) for 5 h, and assay was performed as described in Figure 1 legend.

(Puil *et al.*, 1994; Skorski *et al.*, 1997). It has been shown that a specific reduction of Bcr-Abl PTK activity with human *abl* antisense oligonucleotide and CGP57148 induces erythroid differentiation of K562 cells (Druker *et al.*, 1996; Carroll *et al.*, 1997), and downregulation of H-ras, K-ras and N-ras expression is associated with the erythroid differentiation induced by Ara-C and tiazofurin (Delgado *et al.*, 1992; Olah *et al.*, 1988; Weber *et al.*, 1991). However, modulation of signaling pathway that is required to induce erythroid differentiation is still not clearly understood. Here, we have demonstrated that both down- and up-regulation of Ras/ERK pathway are associated with the induction of erythroid differentiation of K562 cells.

It has been shown that activation of Ras- and PI3-kinase-dependent pathways play important roles in Bcr-Abl-mediated transformation of hematopoietic cells (Goga *et al.*, 1995; Skorski *et al.*, 1997). Numerous studies have shown that Ras/ERK- and PI3-kinase-dependent pathways are correlated with cell proliferation in various cells (Seger and Krebs, 1995; Toker and Cantley, 1997). Treatment with PTK inhibitors including herbimycin A, human *abl* antisense oligonucleotide and CGP57148, a selective inhibitor of Bcr-Abl PTK (Druker *et al.*, 1996; Carroll *et al.*, 1997; Deininger *et al.*, 1997), leads to growth inhibition and induction of erythroid differentiation of K562 cells (Honma *et al.*, 1989; Honma *et al.*, 1990; LaMontagne *et al.*, 1998), indicating that the inhibition of Bcr-Abl PTK is an important step in induction of erythroid differentiation of K562 cells. In the present study, it is shown that herbimycin A, *ras* antisense oligonucleotide and PD98059 inhibited growth and ERK/MAPK activity of K562 cells and induced erythroid differentiation of K562 cells,

whereas wortmannin inhibited growth of K562 cells, and neither inhibit ERK/AMAPK activity nor induce erythroid differentiation of K562 cells. These results suggest that although both Ras/ERK- and PI3-kinase-dependent pathways are required for cell proliferation of K562 cells, the inhibition of Ras/ERK pathway, not the PI3-kinase dependent pathway, is associated with the induction of erythroid differentiation of K562 cells. Although some reports have shown that Bcr-Abl signal is not propagated to ERK/MAPK pathway but to c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) (Kabarowski *et al.*, 1994; Raitano *et al.*, 1995), other experiments have shown that the Bcr-Abl PTK activates mitogenic signaling pathways including Ras, ERK, and JNK pathways as a primary consequence of expression (Cortez *et al.*, 1997), and we have observed that ERK/MAPK activity of K562 cells was approximately 5-fold higher than that of Jurkat, HL60 and U937 cells, and JNK/SAPK activities were similar among them (unpublished data). Therefore, ERK/MAPK pathway seems to be important in Bcr-Abl-mediated growth stimulation and transformation of hematopoietic cells, and the inhibition of ERK/MAPK pathway appears to be related to the induction of erythroid differentiation.

PI3-kinase is also required for the proliferation of Philadelphia chromosome-positive cells (Skorski *et al.*, 1995) and transformation of hematopoietic cells (Skorski *et al.*, 1997). In our study, wortmannin, an inhibitor of PI3-kinase, inhibits cell growth of K562 cells but not induces erythroid differentiation, suggesting that although PI3-kinase-dependent pathway plays an important role in Bcr-Abl-mediated leukemogenesis, inhibition of PI3-kinase is not associated with the induction of erythroid differentiation of K562 cells.

If inhibition of ERK/MAPK activity leads to induction of erythroid differentiation of K562 cells, erythroid differentiation of K562 cells induced by inhibition of Bcr-Abl PTK would be blocked by transfection with active mutant of H-Ras, which leads to activation of ERK/MAPK pathway (Blenis, 1993). However, our experiment have shown that after transfection of K562 cells with pZIPRas^{61leu}, about 60% of K562-Ras^{61leu} cells were spontaneously differentiated to erythroid lineage, and these cells were found to show a markedly decreased proliferation and an increased activity of ERK/MAPK. Recently, it has been reported that butyrate, an inducer of erythroid differentiation of K562 cells, activates ERK/MAPK (Rivero and Adunyah, 1996), and rapidly increases the transcription and nuclear level of the erythroid transcription factors including NF-E2 and GATA-1 (Chenais *et al.*, 1997; Chenais, 1998) in K562 cells. In addition, activation of Ras/ERK pathway increases the NF-E2 activity in MEL cells (Nagai *et al.*, 1998). These studies may explain the spontaneously erythroid-differentiated phenotype of K562-Ras^{61leu}. Indeed, some reports suggested that *ras* mutations may have little or no role in initiation or progression of common CML (Cogswell *et al.*, 1989; LeMaistre *et al.*, 1989). In the present study, it is shown that herbimycin A further inhibited the growth rate and increased the erythroid differentiation but did not affect the activity of ERK/MAPK in K562-Ras^{61leu}. And PD98059 induced a biphasic response in K562-Ras^{61leu} with the increase of growth rate and inhibition of the erythroid differentiation at low concentration followed by the reversion of growth rate and erythroid differentiation at high concentration. These results suggest that a well-controlled activity of Ras/ERK pathway is important for Bcr-Abl-mediated leukemogenesis, and there might be additional mitogenic and transforming signals from Bcr-Abl PTK other than ERK/MAPK pathway.

In conclusion, the erythroid differentiation of K562 cells could be induced by either inhibition or activation of Ras/ERK pathway, and it seems that an appropriate activity of Ras/ERK pathway is required to maintain the growth and transformed phenotype of chronic myelogenous leukemic cell line, K562.

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