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Inhibition of *abl* Oncogene Tyrosine Kinase Induces Erythroid Differentiation of Human Myelogenous Leukemia K562 Cells

Yoshio Honma,[1] Junko Okabe-Kado,[1] Takashi Kasukabe,[1] Motoo Hozumi[1] and Kazuo Umezawa[2]

[1]*Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Saitama 362 and*
[2]*Faculty of Science and Technology, Keio University, Hiyoshi, Yokohama 223*

The human chronic myelogenous leukemia cell line K562 expresses a structurally altered c-*abl* protein with tyrosine kinase activity. Erythroid differentiation of K562 cells was induced by tyrosine kinase inhibitors, but not by other kinase inhibitors. Treatment of K562 cells with 5′d(TACTGGCCGCTG-AAGGGC)3′, complementary to the second exon (codons 2 to 7) of c-*abl* mRNA, inhibited cell growth and induced benzidine-positive cells in a dose-dependent manner. However, exposure to the sense oligomer did not induce erythroid differentiation of the cells. These results suggest that inhibition of *abl* tyrosine kinase activity is closely related to induction of erythroid differentiation of K562 cells. A multidrug-resistant subline (K562R) was induced to undergo erythroid differentiation by tyrosine kinase inhibitors such as genistein or herbimycin A as effectively as the parent K562 cells were. Therefore, tyrosine kinase inhibitors might be useful as cancer chemotherapeutic agents against some multidrug-resistant leukemias having abnormally high activity of oncogene tyrosine kinase(s).

Key words: K562 — Differentiation — *abl* oncogene — Antisense oligomer — Tyrosine kinase inhibitor

There is general agreement on the importance of oncogenes in causing malignancy.[1] The use of compounds which inhibit the activity of an oncogene product may provide a new strategy to control some tumors. The transforming activity of *src* family oncogenes is closely related to their tyrosine kinase activity. Thus, specific inhibitors for tyrosine kinases could be useful as antitumor agents against some types of tumors for which tyrosine kinase activities have been implicated as determinants of the oncogenic state.

The Philadelphia translocation t(9;22)(q34,q11) is found in over 90% of cases of chronic myelogenous leukemia and in a much smaller proportion of patients with acute lymphoid leukemia.[2,3] A structurally altered c-*abl* protein (p210) has been detected in leukemia cells with the translocation. The tyrosine kinase activity of p210 may promote proliferation and might be responsible for maintaining the leukemic cells at the blast stage.

The human chronic myelogenous leukemia cell line K562 expresses a structurally altered c-*abl* protein (p210) with high activity of tyrosine kinase. Erythroid differentiation of the K562 cells was induced by herbimycin A, which was found to reduce intracellular phosphorylation by tyrosine kinase.[4] However, no linkage between erythroid differentiation of the cells and the function of p210 has been established. In the present investigation, we examined the effect of various protein kinase inhibitors and *abl* antisense oligodeoxynucleotide on the growth and differentiation of K562 cells.

Abbreviation: p210, structurally altered *c-abl* protein with a molecular weight of 210,000.

MATERIALS AND METHODS

Cells and culture conditions The K562 cell line was originally established from a pleural effusion of a patient with chronic myelogenous leukemia in terminal crisis.[5] Erythroid differentiation of this cell line has been shown with various inducers.[6–8] The multidrug-resistant K562 subline (K562R) was established by culturing in stepwise increasing concentrations of adriamycin. The stock culture of K562R cells was maintained in the presence of 3.4×10^{-8} *M* adriamycin. Cells (5×10^4/ml) were suspended in 2 ml of RPMI-1640 medium supplemented with 10% fetal bovine serum. The cell number was counted with a Model ZM Coulter Counter (Hialeah, USA) 4 days after treatment.

Drugs Herbimycin A was a generous gift from Dr. Y. Uehara, National Institute of Health in Japan, Tokyo. Erbstatin was prepared as described previously.[9] Genistein, ML9, A3, H7, staurosporin, sphingosine were purchased from Funakoshi Chemical Co., Tokyo. Vincristine sulfate was from Shionogi Co., Osaka. Adriamycin and actinomycin D were from Sigma Chemical Co.

Oligodeoxynucleotides The 18-base pair oligodeoxynucleotides used in the present investigation were synthesized at Takara Biomedicals, Tokyo and had the following sequences: human *abl* antisense, 5′-TACTGG-CCGCTGAAGGGC-3′; human *abl* sense, 5′-GCCCTT-

CAGCGGCCAGTA-3′.[10] Oligodeoxynucleotides were added after seeding and every 48 h thereafter.

Benzidine staining Erythroid differentiation was scored by benzidine staining by the procedure reported previously.[7]

Assay of c-*abl* mRNA RNA was extracted using a concentrated NaI solution and mRNA was selectively bound to nitrocellulose sheets.[11] The sheets were washed with RNase-free water and 70% ethanol, and the immobilized mRNA was hybridized to ^{32}P-labeled *abl* DNA probe. The *abl* mRNA levels were quantitated by densitometric scanning of the autoradiographs.

RESULTS

Effects of various protein kinase inhibitors on differentiation of K562 cells Several inhibitors of protein kinase were tested for ability to induce erythroid differentiation of K562 cells. The cells were treated with the concentration of inhibitor required for 50% inhibition of cell growth for 4 days. Among the inhibitors tested, only tyrosine kinase inhibitors could induce erythroid differentiation of the cell (Table I). Induction of the benzidine-positive cells was dose-dependent and could be observed at concentrations of herbimycin A or genistein that did not inhibit cell growth. Even high concentrations of the other kinase inhibitors failed to induce benzidine-positive cells (data not shown). The results suggest that differentiation of K562 cells is closely related to inhibition of tyrosine kinase activity.

Differentiation of the antisense oligomer-treated cells To determine directly whether *abl* tyrosine kinase has a role

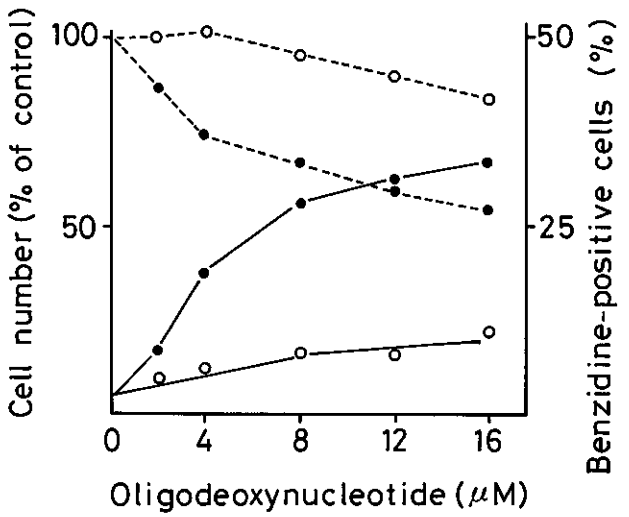

Fig. 1. Effect of an *abl* antisense oligomer on growth and differentiation of K562 cells. The cells were cultured with various concentrations of the *abl* antisense oligomer (●) or the *abl* sense oligomer (○) for 6 days. Solid line, % of benzidine-positive cells; broken line, cell number.

Table I. Effect of Various Protein Kinase Inhibitors on Induction of Differentiation of K562 Cells

Inhibitor	Specificity of inhibitor[a]	Growth inhibition	Induction of differentiation
		IC_{50}[b] (μg/ml)	B^+ cells[c] (%)
Herbimycin A	TK	0.08	57.4±1.3[d]
Genistein	TK	6.75	54.1±2.1
Erbstatin	TK	2.71	11.8±1.2
ML9	MLC	1.35	2.1±0.3
A3	PKA, PKG	8.75	1.9±0.1
H7	PKC, PKA, PKG	56.1	1.4±0.1
Staurosporin	PKC, MLC	0.03	1.5±0.1
Sphingosine	PKC	0.91	1.8±0.1
None			1.7±0.1

a) TK, tyrosine kinase; MLC, myosin light chain kinase; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PKC, protein kinase C.
b) IC_{50}, concentration of drug required for 50% inhibition of cell growth. Cells were treated with the inhibitor for 4 days.
c) Percentage of cells staining with benzidine at IC_{50}.
d) Averages±SD for 4 separate experiments.

in the control of growth and differentiation of K562 cells, the cells in culture were treated with the *abl* antisense oligomer. This approach has been used successfully to study the effect of *abl* gene on proliferation of hematopoietic cell proliferation.[10] A dose-dependent inhibition of proliferation was found with a roughly linear plot. Erythroid differentiation of the *abl* antisense oligomer-treated cells was induced in a dose-dependent manner (Fig. 1). When the cells were treated with the IC_{50} concentration of the antisense oligomer for 6 days, 35% of the cells became benzidine-positive, whereas only 1.8% of the untreated cells was benzidine-positive. Viability of the treated cells was more than 95%, just like that of untreated cells. Erythrodifferentiation was observed at concentrations of the antisense oligomer that did not inhibit growth (Fig. 1). These results suggest that the antisense oligomer induces differentiation of benzidine-positive cells without cell killing. An 18-base *abl* sense sequence corresponding to the antisense sequence served as a control. However, exposure of K562 cells to the *abl* sense oligomer had only a slight effect on their proliferation or differentiation (Fig. 1). The effect of the antisense oligomer on the level of c-*abl* mRNA was examined for comparison with the time course of induction of benzidine-positive cells. The reduction of *abl* RNA level could be seen after 1 day of treatment with the antisense oligomer. The induction of benzidine-positive cells was preceded by the reduction of *abl* RNA level when K562 cells were treated with 16 μM antisense oligomer (Fig. 2).

Table II. IC_{50} Values of K562 and K562R Cells

Drug	IC_{50}[a] (M)		Index of resistance[b]
	K562	K562R	
Adriamycin	1.1×10^{-8}	31.0×10^{-8}	28.2
Actinomycin D	0.6×10^{-9}	26.1×10^{-9}	43.5
Vincristine	0.2×10^{-9}	29.3×10^{-9}	146.5
Herbimycin A	1.2×10^{-7}	2.7×10^{-7}	2.3
Genistein	2.5×10^{-5}	1.9×10^{-5}	0.8

a) Cells were treated with drugs for 4 days.
b) IC_{50} of K562R/IC_{50} of K562.

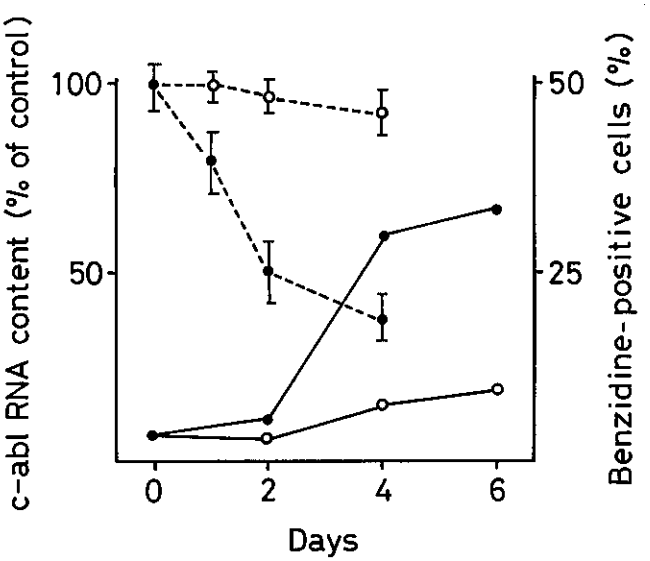

Fig. 2. Expression of *abl* RNA and erythrodifferentiation in K562 cells treated with c-*abl* sense and antisense oligomers. The cells were cultured with 16 μM sense oligomer (○) or antisense oligomer (●). Solid line, % of benzidine-positive cells; broken line, *abl* RNA content. Each value represents the mean ± SD of three determinations.

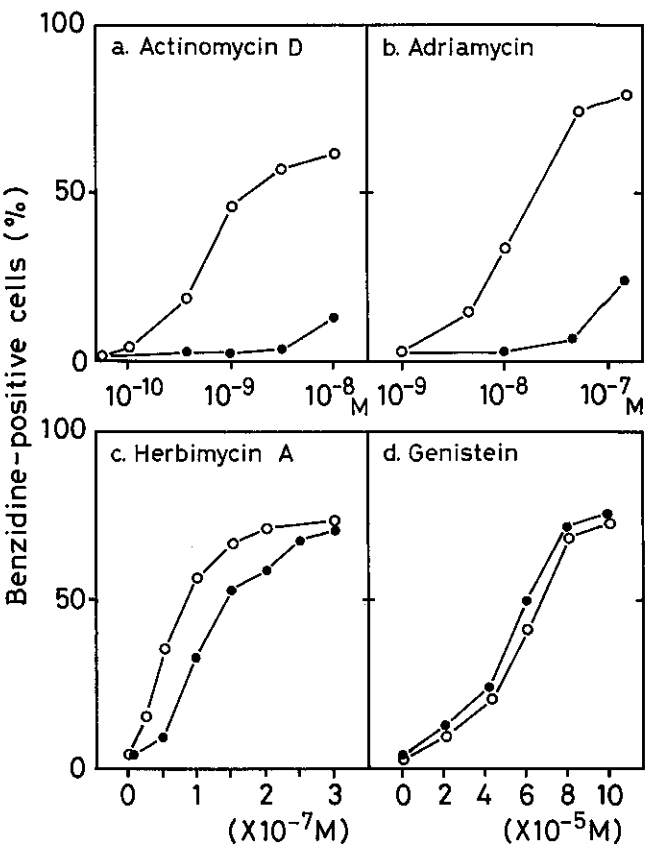

Fig. 3. Erythroid differentiation of K562 and K562R cells induced by adriamycin, actinomycin D, and inhibitors of tyrosine kinase activity. The cells were treated with the drugs for 4 days. ○, parent K562 cells; ●, multidrug-resistant K562R cells.

Effects of tyrosine kinase inhibitors on growth and differentiation of multidrug-resistant K562 cells The parent K562 cells and multidug-resistant variant (K562R) cells were treated with adriamycin, actinomycin D, vincristine, herbimycin A, or genistein for 4 days. With respect to cell growth, the K562R cells were resistant to actinomycin D, adriamycin and vincristine, and the indices of their resistance to actinomycin D, adriamycin and vincristine were 43.5, 28.2 and 146.5, respectively (Table II). However, their sensitivities to tyrosine kinase inhibitors, herbimycin A and genistein, were similar to those of the parent cells (Table II). Next we examined the induction of benzidine-positive cells. Although actinomycin D and adriamycin hardly induced benzidine-positive cells in K562R cell culture, herbimycin A and genistein had similar effects in induction of differentiation in K562R cells and their parent K562 cells (Fig. 3).

DISCUSSION

Genistein, an isoflavone, is a specific inhibitor of tyrosine kinases and the inhibition is competitive with respect to ATP.[12] Herbimycin A, a benzoquinonoid ansamycin, is another type of tyrosine kinase inhibitor.[13] Different mechanisms are involved in their inactivations of tyrosine kinase.[14] The present experiment with various types of protein kinase inhibitors clearly indicates that inhibition of a tyrosine kinase(s) is closely related to induction of erythroid differentiation of K562 cells. Herbimycin A caused reversion of transformed Rous sarcoma virus-infected rat kidney cells to normal morphology.[13] The drug was also effective against various cells transformed by other tyrosine kinase oncogenes, but did not reverse the morphology transformed by oncogenes *raf, ras,* and *myc*.[15] Tyrosine kinase inhibitors might be useful as differentiation-inducing agents for some types of tumors with activated tyrosine kinase oncogenes.

Inhibition of specific gene functions by exposing target cells to synthetic antisense oligomers was used to investigate the role of c-*abl* in normal hematopoiesis.[10] They showed that the p210 level in K562 cells was significantly reduced in the presence of an *abl* antisense oligomer, but not by exposure to the sense oligomer. On the other hand, the mRNA level of beta-microglobulin was unaffected by the exposure to the antisense oligomer. The antisense oligomer is complementary to 18 nucleotides of the second exon of c-*abl* (codons 2 to 7), which is common to both c-*abl* and *bcr/abl* genes for p210. There is no homology between the sequence of the oligomer and those of the other tyrosine kinase genes so far studied, suggesting that the antisense oligomer is unique to human *abl* gene sequences. We have now examined the effect of the same antisense and sense oligomers on induction of erythroid differentiation. K562 cells have two species of c-*abl* mRNAs (6 and 7 kb) and *bcr/abl* mRNA (8 kb), which is experssed predominantly.[2] The amount of *bcr/abl* mRNA was about 70% of total *abl* transcripts in the K562 cells we used in the present experiments (Honma *et al.*, submitted elsewhere). Moreover, the tyrosine kinase activity of p210 is much higher than those of c-*abl* proteins.[2] Therefore, the inactivation of p210 may be an important step to induce erythroid differentiation of K562 cells.

Cross-resistance to anthracyclines and vinca alkaloids has frequently been observed in various tumous with acquired resistance to the drugs. Previously, we demonstrated that butyric acid and hemin induced differentiation of multidrug-resistant K562 (K562/VCR) cells as effectively as that of parent K562 cells.[16] These inducers would seem to have less therapeutic value in the treatment of leukemia because of the unusually high concentration required or their inactivation in the body fluid. A multidrug-resistant K562R subline was also resistant to the abilities of both adriamycin and actinomycin D to inhibit cell proliferation and to induce differentiation. However, the actions of genistein and herbimycin A in inhibiting proliferation and inducing differentiation of K562R cells were independent of the anticancer drug treatment. The sensitivity of K562 cells to growth inhibition by herbimycin A was greater than that of the other leukemia cell lines tested.[4] Therefore, tyrosine kinase inhibitor treatment of Ph^1-positive (*bcr/abl* gene-containing) leukemia cells that are truly resistant to cytotoxic chemotherapeutic drugs may be an alternative therapeutic approach to the control of leukemia.

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