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The differentiation effect of low-dose cytosine arabinoside is disturbed in PU.1-knockdown K562 cells

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Abstract. We recently demonstrated by using PU.1-knockdown K562 (K562 PU.1KD) cells stably expressing PU.1 short inhibitory RNAs and PU.1-overexpressing K562 (K562 PU.1OE) cells, that therapeutic concentrations of 5-aza-2'-deoxycytidine (5-azadC) induce erythroid differentiation of these cells and that the PU.1 expression level is closely associated with the differentiating and apoptotic effects of 5-azadC on K562 cells. In this study, we investigated whether the effects of low-dose cytosine arabinoside (Ara-C), which is another erythroid differentiation inducer in K562 cells, is associated with the expression level of PU.1 in these cells. As a result, we demonstrated that the effect of Ara-C on cell viability and differentiation, as determined by the WST-8 assay and β-globin mRNA expression analysis, respectively, was suppressed in K562 PU.1KD cells compared to their controls. Collectively, these findings suggest that sufficient expression of PU.1 is indispensable for the erythroid differentiation of K562 cells.

Introduction

PU.1 is a member of the Ezb transformation-specific sequence family of transcription factors and is expressed mainly in granulocytic, monocytic and B lymphoid cells (1). The downregulation of PU.1 was reported to play a role in the pathogenesis of various hematological malignancies, including acute myeloid leukemia (2), multiple myeloma (3) and acute lymphoblastic leukemia (4). Furthermore, several studies indicated that downregulation of PU.1 is required for erythroid terminal differentiation (5).

We recently demonstrated that the effects of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-azadC) are correlated with PU.1 expression in PU.1-transgenic chronic myeloid leukemia-derived K562 cells (6). We revealed that therapeutic concentrations of 5-azadC induce erythroid differentiation of these cells. PU.1 expression is closely associated with the effect of this agent and sufficient PU.1 levels were shown to accelerate erythroid differentiation and apoptosis induced by 5-azadC (6).

Several studies indicated that low concentrations of cytosine arabinoside (Ara-C) induce erythroid differentiation of K562 cells (7,8). In this study, we investigated whether the effects of Ara-C are also associated with the expression level of PU.1.

Materials and methods

Cell culture of PU.1-knockdown K562 (K562 PU.1KD) and PU.1-overexpressing K562 (K562 PU.1OE) cells. K562 PU.1KD and K562 PU.1OE cells were previously established in our laboratory (9,10) and were employed in this study. Specifically, we used 2-10 and 3-10 as K562 PU.1KD cells with vec 5 and vec 6 as their control cells and H8 and A2 as K562 PU.1OE cells with vec 1 and vec 2 as their control cells. The K562 PU.1KD cells were maintained in RPMI-1640 medium (Gibco-BRL, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum (HIFBS) and 1 μg/ml puromycin. The K562 PU.1OE cells were grown in RPMI-1640 containing 10% HIFBS and 400 μg/ml neomycin. All the cells were cultured under 5% CO_2 at 37˚C in a humidified atmosphere.

Assessment of cell viability. The proportion of viable cells was determined by a dye reduction assay involving 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8; Dojindo, Kunamoto, Japan), which is a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The effective dose $(ED)_{50}$ values were calculated from the data obtained by the cell growth assays. We selected 7 different Ara-C (Sigma, St. Louis, MO, USA) doses, with the group without Ara-C serving as control, and performed the assays 5 days after addition of Ara-C. The viable cells (%) were calculated as the ratio of the absorbance (490 nm) of Ara-C-treated to that of Ara-C-untreated cells in the cell growth assays. The calculated ratios were analyzed using the website http://www.

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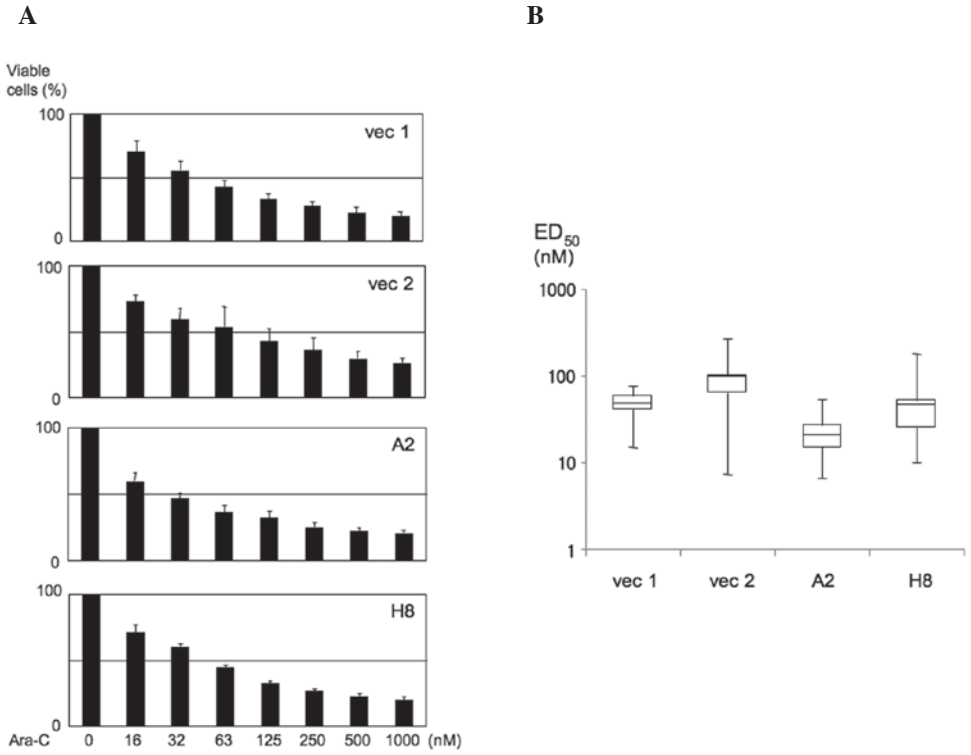

Figure 1. Low-dose cytosine arabinoside (Ara-C) effects on cell viability and effective dose $(ED)_{50}$ values in PU.1-overexpressing K562 (K562 PU.1OE) cells. (A) Effects of Ara-C on cell viability. The indicated amounts of Ara-C were added to K562 PU.1OE cells (A2 and H8) and their controls (vec 1 and vec 2), incubated for 5 days and the WST-8 assay was performed. The percentage of viable cells (%) is shown in the y axis. Data were calculated from 5-6 independent experiments and each experiment was performed in triplicate. (B) ED_{50} values. The ED_{50} values were calculated as described in Materials and methods. The data are presented as boxplots representing the 25th and 75th percentile, median and 5-95 range.

vector.co.jp/soft/win95/edu/se248471.html and the ED_{50} values were obtained.

mRNA expression analysis. cDNAs were prepared from cells using reverse transcription (Transcriptor First Strand cDNA synthesis kit; Roche Diagnostics, Mannheim, Germany). Quantitative polymerase chain reaction (qPCR) was performed using QuantiTect SYBR Green PCR reagent (Qiagen, Miami, FL, USA) according to the manufacturer's protocol and using an Opticon Mini Real-Time PCR instrument (Bio-Rad, Hercules, CA, USA) as previously described (11). The primer sequences were as follows: α-globin: forward, 5'-AAGGTCGGCGCGCACGCT-3' and reverse, 5'-CTCAGGTCGAAGTGCGGG-3'; human β-globin: forward, 5'-CTCATGGCAAGAAAGTGCTCG-3' and reverse, 5'-AATTCTTTGCCAAAGTGATGGG-3'; and GAPDH: forward, 5'-GAAGGTGAAGGTCGGAGT-3' and reverse; 5'-GAAGATGGTGATGGGATTTC-3'. The thermal cycling conditions for α-globin were incubation at 95˚C for 15 min, followed by 35 cycles at 95˚C for 15 sec, at 55˚C for 15 sec and at 72˚C for 15 sec. The thermal cycling conditions for β-globin and GAPDH were incubation at 95˚C for 15 min, followed by 35 cycles at 95˚C for 30 sec, at 55˚C for 30 sec and at 72˚C for 45 sec. The copy number of each sample was calculated as previously described (12).

Statistical analysis. Data are expressed as means ± standard error of the mean and $^{*}P<0.05$ was considered to indicate a statistically significant difference, whereas $^{**}P<0.01$. Comparison of the means was performed with the Student's t-test (http://www.physics.csbsju.edu) between all the controls and each group of K562 PU.1KD or K562 PU.1OE cells.

Results

ED_{50} of Ara-C is increased in K562 PU.1KD cells. We first assessed the viability of K562 PU.1OE cells in the presence of various concentrations of Ara-C, as depicted in Fig. 1A. The range of the Ara-C dose and time of incubation for erythroid differentiation was based on the results published by Sztiller-Sikorska *et al* (8). Based on the data presented in Fig. 1A, we calculated ED_{50} from the Ara-C effect on cell viability. As a result, in K562 PU.1OE A2 cells, the ED_{50} value tended to be decreased (6.55-53.2 nM, median 20.38 nM; P=0.099). However, there was no difference between K562 PU.1OE H8 cells and their controls [H8: 10.1-179.26 nM, median 45.7 nM; vec 1 (control): 14.9-76.24 nM, median 47.80 nM; vec 2 (control): 7.28-267.17 nM, median 95.3 nM] (Fig. 1B).

We then assessed cell viability (Fig. 2A) and ED_{50} value (Fig. 2B) in K562 PU.1KD cells. In contrast to the results of the K562 PU.1OE cells, the ED_{50} value was distinctly increased in 2-10 cells (62.75-250.59 nM, median 137.16 nM; P=0.0042), which exhibited the lowest PU.1 expression among all cell lines (9,10), compared to their controls (vec 5: 18.53-72.06 nM, median 30.47 nM; vec 6: 7.88-28.46 nM,

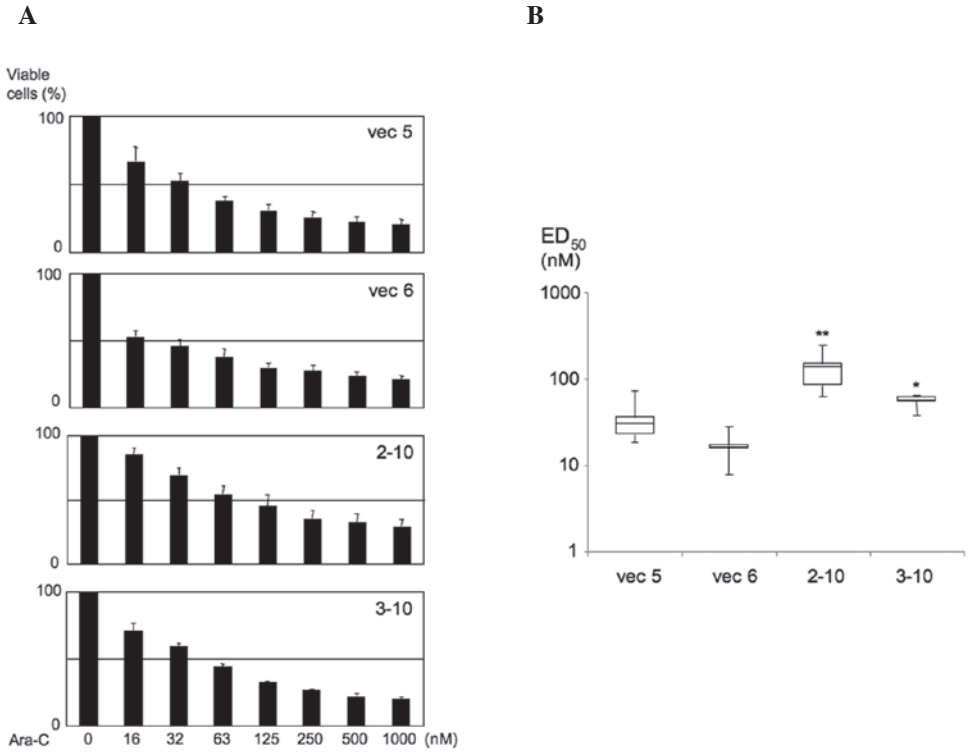

Figure 2. Low-dose cytosine arabinoside (Ara-C) effects on cell viability and effective dose $(ED)_{50}$ values in PU.1-knockdown K562 (K562 PU.1KD) cells. (A) Effects of Ara-C on cell viability. The indicated amounts of Ara-C were added to K562 PU.1KD cells (2-10 and 3-10) and their controls (vec 5 and vec 6). Of note, 2-10 cells are K562 PU.1KD cells with the lowest PU.1 expression (9,10). Data were calculated from 3-4 independent experiments and each experiment was performed in triplicate. (B) ED_{50} values. The ED_{50} values were calculated as described in Materials and methods. Data are presented as boxplots representing the 25th and 75th percentile, median and 5-95 range. P-values less than 0.05 were considered significant, denoted by one asterisk, whereas ** means P<0.01.

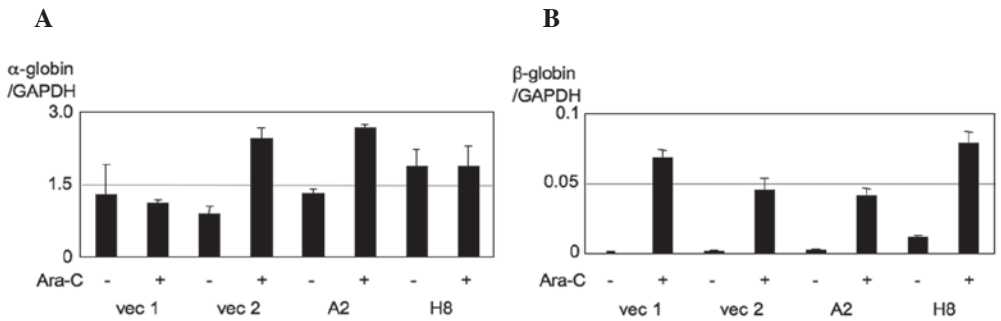

Figure 3. Quantitative polymerase chain reaction (PCR) analyses of (A) α-globin and (B) β-globin using PU.1-overexpressing K562 cells. The α-globin and β-globin gene transcript levels were adjusted by the expression of GAPDH and the relative levels are shown. The data presented were obtained from 3 independent PCR amplifications. Ara-C, cytosine arabinoside.

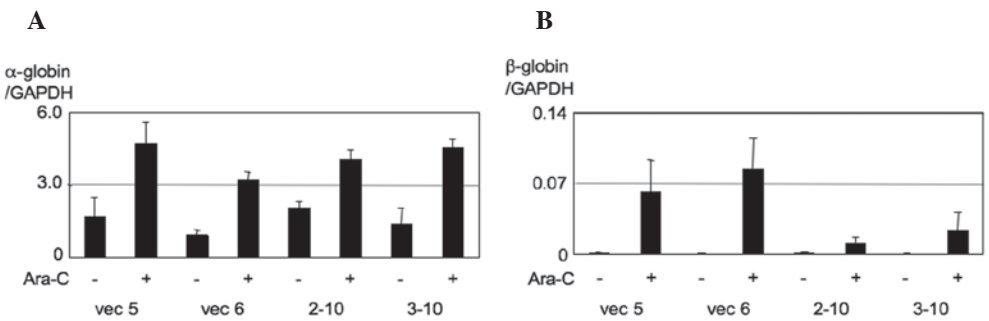

Figure 4. Quantitative polymerase chain reaction (PCR) analyses of (A) α-globin and (B) β-globin using PU.1-knockdown K562 cells. The α-globin and β-globin gene transcript levels were adjusted by the expression of GAPDH and the relative levels are shown. The data presented were obtained from 6 independent PCR amplifications. Ara-C, cytosine arabinoside.

median 16.17 nM). Relatively high ED_{50} values were also observed in K562 PU.1KD 3-10 cells (37.56-65.02 nM, median 56.93 nM; P=0.025), which exhibited the second lowest expression of PU.1 next to 2-10 cells. Collectively, these findings demonstrated that the Ara-C effect on cell viability was suppressed in K562 PU.1KD cells.

β-globin expression is significantly disturbed in K562 PU.1KD cells following treatment with Ara-C. We then investigated whether the indicated dose of Ara-C leads to the differentiation of K562 cells and whether the effects of ED_{50} values are associated with the induction of erythroid differentiation genes. For this purpose, we assessed the expression of α-globin and β-globin by qPCR. In K562 PU.1OE cells, the baseline expression of α-globin is extremely high (α-globin copy number/GAPDH copy number: 1.1-1.8) and moderately affected by the addition of Ara-C (Fig. 3A). By contrast, Ara-C significantly induces β-globin expression, suggesting that measuring β-globin expression may be a good marker to evaluate Ara-C-induced K562 cell differentiation. However, there were no obvious differences between K562 PU.1OE cells and their controls (Fig. 3B). We then investigated these effects using K562 PU.1KD cells. As a result, we observed that β-globin expression was markedly induced by Ara-C and this induction was significantly disturbed in K562 PU.1KD 2-10 and 3-10 cells (Fig. 4). These findings were consistent with ED_{50} values, suggesting that the Ara-C effect on cell viability is associated with the effect on erythroid differentiation of K562 cells and these effects are disturbed by the lack of expression of PU.1.

Discussion

It was previously reported that constitutive upregulation of PU.1 is considered to be the main cause for the inhibition in the differentiation process of murine erythroleukemia (MEL) cells and PU.1 downregulation is required for terminal erythroid differentiation (5,13-15). However, several studies indicated a requirement for PU.1 expression for erythroid differentiation (6,16,17). Back *et al* (16) demonstrated a requirement of PU.1 expression in erythroid differentiation. The authors of that study produced a line of PU.1-deficient mice carrying a green fluorescent protein reporter at this locus and revealed that PU.1-deficient fetal erythroid progenitors lose their self-renewal capacity and undergo proliferation arrest, premature differentiation and apoptosis (16). Wontakal *et al* (17) demonstrated that PU.1 regulates an extensive network of genes that constitute major pathways for controlling the growth and survival of immature myeloid cells. That study further revealed that fetal liver erythroid progenitors, the earliest erythroid-committed cells, are significantly reduced in mice with low PU.1 expression *in vivo* (17). The findings of these studies, including those of the present study, suggest that sufficient expression of PU.1 is indispensable for erythroid differentiation. As previously described (18), one possible explanation for this discrepancy between studies employing MEL cells, is that K562 cells express the endogenous ε-globin and γ-globin genes, but not the adult stage-specific β-globin gene and, therefore, have been considered as a model for embryonic-fetal stages of erythroid development (19,20). Wontakal's and Back's studies employed fetal liver erythroid progenitors from mice, which are used for analyzing the embryonic-fetal stages, whereas MEL cells are considered to be a model for fetal-adult development (20), which are employed in the majority of the previous studies analyzing the functions of PU.1 during erythroid differentiation (6,16,17).

The roles of PU.1 may differ during the different stages of erythroid differentiation. Proper expression of PU.1 is required for the differentiation of immature erythroid cells. We hypothesized that, at least in certain hematological malignancies, measuring the expression of PU.1 may be useful in predicting the efficacy of low-dose chemotherapies in affecting erythroid differentiation.

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