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A preliminary study on the effect of myeloperoxidase on etoposide-induced HPRT mutants in HL-60 cells

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ABSTRACT

Topoisomerase II (Topo II)-directed antitumor drug, etoposide (VP-16)-related leukemia (T-cell acute myelogenous leukemia, t-AML) occurs in about 10% of drug-treated patients. Understanding the mechanisms underlying leukemia induced by VP-16 is important for the development of safer drugs against leukemia. It has been postulated that redox cycling of VP-16 initiated by myeloperoxidase (MPO) produces reactive oxygen species responsible for the genotoxicity of this agent in myeloid precursors, leading to chromosomal translocations responsible for t-AML. To test this hypothesis, MPO activity of HL-60 cells were down-regulated by treating the cells with succinyl acetone (SA). The genotoxicity of VP-16 in these cells was then determined by analyzing the frequencies of mutations in the hypoxanthine phosphoribosyl transferase (HPRT) gene and compared with that in cells without prior SA-treatment. The results showed that the VP-16-induced HPRT mutant frequency in cells whose MPO activity was inhibited by prior SA treatment was significantly decreased by 78.8%, as compared with cells without prior SA-treatment. These results support our hypothesis that VP-16 induces genotoxicity and carcinogenicity through MPO-mediated oxidative activation of VP-16. *Ryukyu Med. J.*, 23(3) 85~91, 2004

Key words: etoposide, myeloperoxidase, hypoxanthine phosphoribosyl transferase gene, HL-60 cells, mutants.

INTRODUCTION

Etoposide (VP-16) is a potent and extensively used anti-tumor drug with inhibitory effect on topoisomerase II^{1,2)}. However, many reports have revealed that the same treatment regimen that is associated with the impressive clinical efficacy of VP-16 is also related to an increased risk of secondary treatment-related acute myeloid leukemia (t-AML)³⁻⁵⁾ which is characterized by distinct translocations involving the chromosome 11q23 region⁶⁾. The mechanism underlying t-AML caused by VP-16 is still obscure. Evidences have shown that many antitumor drugs are cytotoxic due to their ability to generate free radicals, characterizing specific metabolic pathways which may regulate the pro-oxidant

action of antitumor drugs⁷⁾.

VP-16 has been reported to induce the formation of lipid peroxy radicals and to increase lipid peroxidation in different clones derived from Chinese hamster ovary cells⁸⁾. We hypothesized that redox cycling of VP-16 initiated by myeloperoxidase (MPO) amplifies the genotoxicity and carcinogenicity of this agent in myeloid precursors which contain MPO activity. It was postulated that MPO converts VP-16 to free radical species and oxidized metabolites, which cause oxidative DNA damage, resulting in the induction of chromosomal translocations some of which may be responsible for the development of t-AML.

HL-60 cells contains high MPO activity^{9,10} and have been widely used in biochemical studies of phenolic compound-induced cytotoxicity and genotoxicity. It has been reported that MPO activity of HL-60 cells can be down-regulated by inhibitors of heme synthesis such as succinyl acetone⁹, or through specific inhibition by benzoic acid hydrazide¹¹.

The gene coding for the purine scavenger enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT) has been used as a selective marker for the detection of environmental mutagens or carcinogens in mammalian cell cultures¹². The mutations at the single functional HPRT locus directly affect cellular phenotype, and the presence or absence of the enzyme can be efficiently drug selected. HPRT enzyme-deficient cells can be efficiently selected and scored by using cytotoxic purine analog, such as 6-thioguanine (6-TG). This selective procedure has been successfully used for the determination of HPRT mutations for a variety of chemical and physical agents and drugs¹³.

To test the hypothesis that MPO activity mediates VP-16-induced mutagenicity, in this study we evaluated the genotoxicity of VP-16 in HL-60 cells by determining mutation frequencies in the HPRT gene locus of HL-60 cells in which MPO activity was up or down regulated. VP-16 treated HL-60 cells with regulated MPO activity were cultured for phenotypic expression, then treated with 6-thioguanine (6-TG), and cloned to select mutant cells deficient at the HPRT gene locus. The mutant frequencies were then determined for each treated culture and compared.

MATERIALS and METHODS

Cell culture

Human myeloid leukemia cells (HL-60) were cultured in DMEM (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD) and 100 units/ml penicillin, 100 μ g/ml streptomycin, and 1% L-glutamine at 37 °C in a 5% CO₂ humidified incubator. Cell viability and numbers were routinely determined microscopically on a haemocytometer, using the trypan blue exclusion method.

Succinyl acetone treatment

To down-regulate the MPO activity of HL-60 cells, succinyl acetone (SA) was added to the culture medium. SA was purchased from Sigma (St. Louis, MO) and freshly dissolved in distilled water, then diluted into aliquots. Cells with a concentration of 2×10^5 cells/ml in a complete medium were supplemented with 500 μ M SA, and the control cells were added with an equivalent amount of phosphate buffer solution (PBS). Control and SA-treated cells were incubated for 72 h, then harvested for MPO assay.

MPO assay

MPO activity of HL-60 cells was determined as described by Kagan *et al.*¹⁴. Briefly, cells were harvested and washed twice with buffer A containing 25 mM HEPES, 10 mM glucose, 115 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 5 mM NaH₂PO₄, then the samples for measuring MPO activities were prepared by homogenizing cells. A spectrophotometric assay (Shimadzu UV 160 U spectrophotometer, Kyoto, Japan) of MPO activity was used in which guaiacol oxidation was monitored by changes of absorbance at 470 nm. Cell homogenate (0.5×10^6 cells) was added to 100 mM disodium phosphate buffer (pH, 7.3) containing 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 13 mM guaiacol, 0.02% cetylmethylammonium bromide, and 3.75 mM 3-AT. The reaction was started by the addition of 670 μ M H₂O₂. Activity of MPO was calculated in nmol tetraguaiacol formed/min/ 10^6 cells at initial and 1 min MPO. The data were obtained using Shimadzu PC 160 software version 1.2.

Etoposide treatment

Etoposide (VP-16) (Sigma, St. Louis, MO) was freshly prepared in dimethylsulfoxide (DMSO) and diluted into aliquots in a complete medium. The final concentration of DMSO was lower than 0.1% which had no effect on cell growth. Both control and SA-treated cells were treated with 0 and 0.5 μ M of VP-16, at 37 °C for 4 hours. The cells were then harvested and washed twice with PBS, resuspended with a complete medium, and the cell concentration was adjusted to 2×10^5 cells/ml. The cells were cultured and passaged as necessary for 6 days for recovery and expression of the mutant phenotype. The cells were enumerated at 24 h, 48 h, and 72 h after VP-16 treatment.

Table 1 HPRT mutant frequencies in HL-60 cells

Concentration of VP-16	Mutant frequencies ($\times 10^{-6}$)	
	SA treatment (-)	SA treatment (+)
0	1.66 \pm 0.36*	1.50 \pm 0.29 [#]
0.5 μ M	7.67 \pm 1.07	1.62 \pm 0.70 ^{##}

Values are means \pm SD. * is background mutant frequency of HL-60 cells. The rests of mutant frequencies are already reduced by subtracting the background mutant frequency, and ^{##} is further subtracted with the value of[#].

6-TG selection of HPRT mutants

The fresh 6-thioguanine (6-TG) (Sigma, St. Louis, MO) stock solution was prepared by dissolving in 1 N NaOH and diluted into aliquots, the final concentration of NaOH was lower than 0.01 N which did not affect the pH of the medium. After a 4 h incubation period, as mentioned above, cells were harvested and resuspended in a fresh medium, and adjusted to a concentration of 4×10^5 cells/ml, then supplemented with 20 μ M 6-TG. A 100 μ l aliquot of this cell solution containing 4×10^4 cells was plated into each well of eight 96-well flat microplates (a total of 768 wells) and cultured for 3 weeks. The resulting 6-TG resistant cell colonies were determined visually and confirmed using microscope examination. The numbers of HPRT mutant colonies from each treatment were counted. To determine the viability of the control cells, SA-treated cells, VP-16 treated cells or SA and VP-16 treated cells in the absence of 6-TG, samples of each culture were diluted to a concentration of 10 cells/ml and plated out at 2 cells/well of two 96-well flat microplates and cultured until the clones were visible and confirmed microscopically. The HPRT mutant frequency was then calculated as cloning efficiency in the presence of 6-TG versus cloning efficiency in the absence of 6-TG, using the equation proposed by Kennelly, *et al.*¹⁵⁾.

Three replicates of independent experiments as described above were carried out separately.

Statistical analysis

The value of HPRT mutant frequency was logarithmically transformed for statistical analysis. Student's t-test was used to determine the significant difference of MPO activities and HPRT mutant frequencies between the groups, and χ^2 -test was used

to determine the difference of the changes of HL-60 cell concentrations after VP-16 treatment between control and SA-treated groups. The statistical significance of differences was set at $p < 0.05$.

RESULTS

MPO activity in SA-treated HL-60 cells

After incubating HL-60 cells for 72 h in the presence of 500 μ M SA, a marked decrease of MPO activity was observed. As shown in Fig.1, the MPO activities measured at time 0 and 1 minute in cells from the control cultures were 37.83 and 30.1 nmol tetraguaiacol/min/ 10^6 cells, respectively. For comparison, in SA-treated HL-60 cells, the MPO activities significantly decreased by 93.23% and 85.25% ($p < 0.01$, Student's t-test), respectively. These results are consistent with those reported previously¹⁰⁾.

The effect of VP-16 on HL-60 cell growth

To investigate the cytotoxic effect of VP-16 on HL-60 cells, a time course study of cell growth following 4 h of VP-16 treatment was carried out. As shown in Fig.2, the proportion of viable cells treated with 0.5 μ M VP-16 dropped by 31.6% at 24 h, 32.6% at 48 h, and 15.7% at 72 h, compared with control cells without VP-16 treatment, implying that VP-16 treatment was capable of inhibiting HL-60 cells. In contrast, as shown in Fig.3, in cells with prior SA treatment, a 0.5 μ M VP-16-treatment resulted in 8.1%, 13.2%, and 8.1% reduction in viable cells' number, at 24 h, 48 h and 72 h, respectively, compared with the SA-treated cells without VP-16 treatment. These reductions were significantly lower than those caused by VP-16 in the cells without SA-treatment (8.1% versus 31.6% at 24 h, 13.2% versus 32.6% at 48 h, and 8.1% versus 15.7% at 72 h; $p < 0.01$,

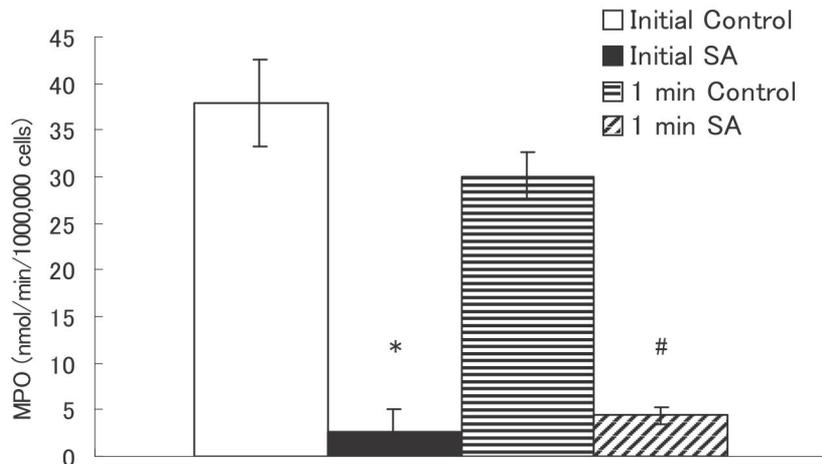


Fig. 1 MPO activity in HL-60 cells with or without SA treatment. Values are means \pm SD. * and # significantly different from initial control and 1 min control values at $p < 0.01$, respectively.

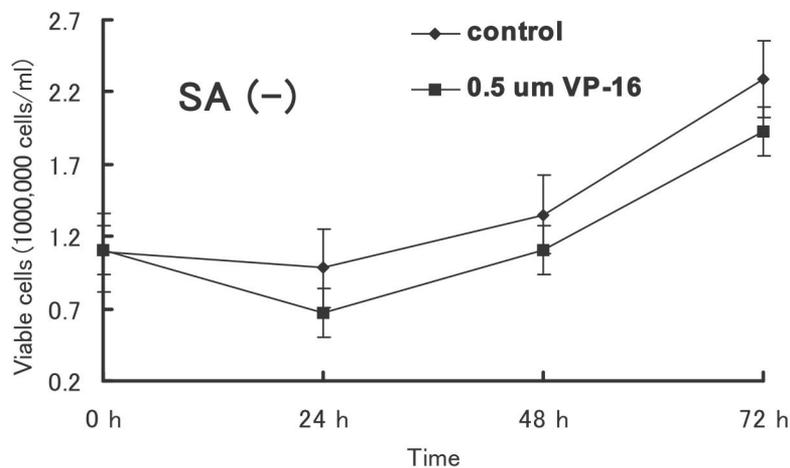


Fig. 2 The concentrations of control HL-60 cells after VP-16 treatment. Values are means \pm SD.

χ^2 -test). These data show a decreased in VP-16-induced cytotoxicity in HL-60 cells whose MPO activity was down-regulated by SA-treatment.

HPRT mutant frequency

Table 1 shows the average frequencies of HPRT mutants obtained from the three independent experiments mentioned above. The background HPRT mutant frequency for control HL-60 cells without prior SA-treatment was 1.66×10^{-6} . A treatment of the cells with 0.5μ M VP-16 resulted in an HPRT mutant frequency of 9.33×10^{-6} , or 7.67×10^{-6} after subtracting the background mutant frequency, demonstrating the mutagenicity of VP-16 at the HPRT locus in HL-60 cells. In SA-treated

cultures, the HPRT mutant frequency without VP-16-treatment was 3.16×10^{-6} , or 1.50×10^{-6} after subtracting the background mutant frequency. The HPRT mutant frequency in these cells after treatment with 0.5μ M VP-16 was 4.78×10^{-6} , or 1.62×10^{-6} after subtracting the background and SA-induced mutant frequency. Therefore, there was a significantly decreased VP-16-induced HPRT mutant frequency of 78.8% (7.67×10^{-6} to 1.62×10^{-6} , $p < 0.05$) in HL-60 cells previously treated with SA, compared with HL-60 cells without prior SA-treatment. Moreover, the decreased VP-16-induced HPRT mutant frequency in SA-treated cells was repeatedly observed in the three independent experiments. These results showed a trend that down-

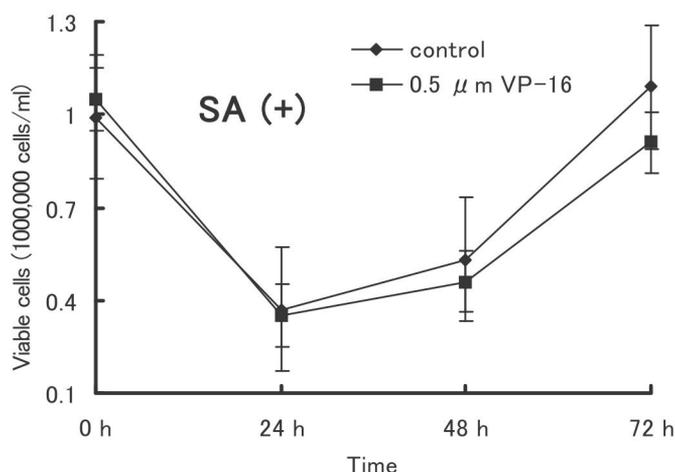


Fig. 3 The concentrations of SA-treated HL-60 cells after VP-16 treatment. Values are means \pm SD.

regulation of MPO activity by prior SA treatment might inhibit VP-16 induced mutation of HPRT locus in HL-60 cells.

DISCUSSION

As an efficacious antitumor drug, etoposide (VP-16) has been extensively used not only as a single agent but also in combination chemotherapy with other antitumor drugs¹⁶, as well as in concurrent chemoradiotherapy¹⁷. There have been reports that chemotherapy-induced apoptosis and subsequent phagocytosis of cancer cells rely on the redox status and the intracellular balance between pro- and antioxidants¹⁸, and that chemotherapy regimen can cause the depletion of antioxidants in the plasma of patients^{19, 20}. VP-16 possesses a hindered ring phenol which is a critical determinant of drug-induced Topo II inhibition²¹ and antitumor activity. The formation of phenoxy and semiquinone radicals and orthoquinones caused by peroxidative activation of the VP-16 E-ring phenol results in increased DNA strand breakage. Evidences have shown that oxidative activation of VP-16 by cytochrome P 450 monooxygenases, MPO, prostaglandin synthetase, and tyrosinase may contribute to its cytotoxicity²²⁻²⁵. The carcinogenicity of VP-16 has been revealed by the fact that its treatment schedule is also associated with an increased risk of secondary treatment-related acute myeloid leukemia. It has been suggested that VP-16 induces genotoxicity and carcinogenicity through the MPO-catalyzed production of VP-16 phenoxy radicals (VP-16-O) in

myeloid progenitors and their subsequent prooxidant effects¹⁴. Based on these evidences, we proposed that MPO-mediated oxidative activation of VP-16 in myeloid precursor cells results in the DNA damage and alterations responsible for t-AML.

In this study, the constitutive levels of MPO activity in HL-60 cells were down-regulated by using SA treatment and determined by the measurement of the levels of guaiacol oxidation. Our results demonstrated that VP-16 was cytotoxic and mutagenic to HL-60 cells. This is in agreement with previous reports^{26, 27}. However, both the cytotoxicity and genotoxicity of VP-16 were markedly decreased in HL-60 cells whose MPO activity was down-regulated by prior SA-treatment, as compared with non SA-treated cells. These data suggest that MPO activity mediates the process of cytotoxicity and genotoxicity in HL-60 cell caused by VP-16, through a mechanism(s) to be yet elucidated.

A study by Kagan's group¹⁴ indicated that the formation of MPO-dependent phenoxy radical of VP-16 occurs in HL-60 cells and that there are potentially important biological consequences related to VP-16 oxidative activation. The idea that VP-16 phenoxy radicals can induce oxidative stress causing both cytotoxicity and genotoxicity was supported by the evidence that they can be produced in cells that contain oxidizing enzymes capable of one-electron transfer reactions such as peroxidases and tyrosinase. These contribute not only to the antitumor activity of VP-16 in cancer cells

producing phenoxy radicals, but also to the carcinogenic action of VP-16 in myeloid stem cells that contain MPO.

Previous studies demonstrated that VP-16 induced mutations at the HPRT locus frequently involve gene deletions and/or rearrangements²⁸⁻³⁰. The mutation spectra of HPRT locus in HL-60 cells caused by VP-16 were not analyzed in the present study. To elucidate the mechanism underlying HPRT gene mutation induced by VP-16, the mutant colonies from the control untreated cells representing background mutations and from each treatment group representing drug-induced mutations should be further investigated by using Southern blotting or multiple PCR analysis.

In conclusion, our data showed that MPO-mediated oxidative activation of VP-16 may be responsible for cytotoxicity and genotoxicity caused by VP-16 in HL-60 cells. These results may shed a light on the mechanisms underlying VP-16 related secondary leukemia (t-AML).

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