Experimental Hematology

Experimental Hematology 2015;43:207–214

Melatonin overcomes resistance to clofarabine in two leukemic cell lines by increased expression of deoxycytidine kinase

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(Received 2 July 2014; revised 16 October 2014; accepted 3 November 2014)

Drug resistance remains a serious problem in leukemia therapy. Among newly developed nucleoside antimetabolites, clofarabine has broad cytotoxic activity showing therapeutic promise and is currently approved for relapsed acute lymphoblastic leukemia. To investigate the mechanisms responsible for clofarabine resistance, we established two clofarabine-resistant lymphoblastic leukemia cell lines from parental lines. To elucidate the mechanisms against clofarabine resistance in two newly established clofarabine-resistant cell lines, we measured the expression of export pumps multidrug resistance protein 1, multidrug resistance–associated protein 1, and ATP-binding cassette subfamily G member 2. There were no differences in the expression between clofarabine-sensitive and -resistant cell lines. Next, we determined expression of *deoxycytidine kinase (dCK)*, which phosphorylates clofarabine to exert cytotoxicity, in clofarabine-sensitive and -resistant cells. Clofarabine-resistant cells showed significantly decreased expression of *dCK* RNA when compared with sensitive cells. To elucidate the mechanisms of decreased *dCK* expression in clofarabine-resistant cells, we analyzed the methylation status of CpG islands of the *dCK* promoter and found no differences in methylation status between clofarabine-sensitive and -resistant cells. Next, we measured the acetylation status of histone and found that total histone acetylation, and histone H3 and H4 acetylation on chromatin immunoprecipitation assay were significantly decreased in resistant cells. Melatonin is an indolamine that functions in the regulation of chronobiological rhythms to exert cytotoxic effects. We examined the effects of melatonin in clofarabine-resistant cells and found that melatonin treatment led to significantly increased cytotoxicity with clofarabine in resistant cells via increased acetylation. Melatonin may be a useful candidate for overcoming clofarabine resistance in two newly established clofarabine resistant leukemia cell lines.

Drug resistance remains a serious problem in leukemia therapy, and children with leukemia cells exhibiting in vitro resistance to antileukemic agents have substantially worse prognosis than children whose leukemia cells are drug-sensitive [1]. Nucleoside antimetabolites are the most widely used and effective types of anticancer drugs [2]. Recently, several improved purine nucleoside analogues derived from adenine have been developed; one such compound, clofarabine (2-chloro-2-arabino-fluro-2-deoxyadenosine), has broad cytotoxic activity, showing therapeutic promise, and is currently approved for relapsed acute lymphoblastic leukemia [3]. Clofarabine is readily phosphorylated by deoxycytidine kinase (dCK) and exerts cytotoxicity against both proliferating and nonproliferating cells [4–6].

Resistance to nucleoside derivatives reportedly involves either impaired cellular uptake or reduced conversion to nucleoside monophosphates. By reducing the concentration of nucleoside monophosphate by either increased export pump function or decreased phosphorylation function, a reduction in higher phosphorylated nucleosides leads to reduced cytotoxicity. The increased export pump function of ATP-binding cassette subfamily G member 2 (ABCG2) has been reported as a novel mechanism of clofarabine resistance [7]. On the other hand, resistance to these nucleoside antimetabolites has been linked to deficiency or decreased expression of dCK [8–10].

Aberrant methylation has been shown to play a potent role in tumorigenesis, where genome-wide hypomethylation and regional hypermethylation of tumor suppressor

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0301-472X/
http://dx.doi.org/10.1016/j.exphem.2014.11.001

gene promoters are characteristic hallmarks in many cancers [11]. DNA methylation occurs in eukaryote DNA at CpG sites, usually enriched in the promoters of genes. Increasing evidence has shown that epigenetic changes can be a crucial driving force behind the acquisition of drug resistance, with changes in gene expression occurring after chemotherapy without gene mutations [12].

Histones also control gene expression by modulating the structure of chromatin, and the accessibility of regulatory DNA sequences to transcriptional activators and repressors [13,14]. Acetylation of histone increases gene expression by relaxing chromatin structure, allowing access of transcription factors to DNA.

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine that functions in the regulation of chronobiological rhythms and endocrine function [15]. In addition, melatonin has antiproliferative and cytotoxic effects on brain tumors [16]. Melatonin also increases the methylation of the *ABCG2* promoter and decreases ABCG2 expression and function [16], and it has been reported to significantly increase histone H3 and histone H4 acetylation in the hippocampus [17].

Here, we found that melatonin reduces clofarabine resistance by epigenetic mechanisms related to *dCK* regulation in two newly established clofarabine-resistant leukemia cell lines.

Materials and methods

Drugs and chemicals

Clofarabine, melatonin, and dimethyl sulfoxide were obtained from Wako Pure Chemical Industries (Osaka, Japan). Phosphate-buffered saline without metal salt solution was from Nissui (Tokyo, Japan). Roswell Park Memorial Institute medium 1640, Hanks' Balanced Salt Solution without Ca^{2+} or Mg^{2+}, fetal calf serum and gentamicin were purchased from Life Technologies (Gaithersburg, MD). Vorinostat (N-Hydroxy-N'-phenyloctanediamide) was purchased from TOKYO Chemical Industry Company (Tokyo, Japan).

Cell lines

NALM6/P, a parental cell line of human B-cell lymphoblastic leukemia, and SKW3/P, a parental cell line of human T-cell leukemia, were purchased from RIKEN (Tsukuba, Japan). Clofarabine-resistant cells (NALM6/Clo, SKW3/Clo) were selected by stepwise and continuous exposure to clofarabine using the limiting dilution method [12]. Before use in each experiment, clofarabine-resistant cells were cultured without clofarabine for 2 weeks. All cell lines were free from mycoplasma organisms, as confirmed by the MycoAlert mycoplasma detection kit (Lonza Japan, Tokyo, Japan).

Cytotoxicity assay

Cytotoxicity was assessed by trypan blue dye exclusion assay, as described previously [12]. Briefly, 1×10^5 cells/mL were incubated with various concentrations of anticancer drugs, including clofarabine, and melatonin for 48 hours. Viable cells were then counted after trypan blue staining. The synergistic effect of melatonin with clofarabine was analyzed by combination index method [18].

Genomic DNA isolation and quality assessment

DNA extractions from fresh cells were performed using the QIAamp DNA Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Genomic DNA quality was assessed by low agarose gel (0.5%) electrophoresis under low voltage. Thresholds for genomic DNA quality check were: (a) showing a high molecular band (<40 kbp) in 0.6% agarose gel low-voltage electrophoresis (3 hours) and no strong bands of low molecular weight (<2.0 kbp) and (b) OD260/280 within a range of 1.8–2.0.

Bisulfite conversion

Bisulfite conversion of genomic DNA was performed with the methylSEQr bisulfite conversion kit (Applied Biosystems, Tokyo, Japan), in accordance with the manufacturer's instructions. Bisulfite-converted samples were immediately subject to methylation-specific polymerase chain reaction (MSP) analysis, as described below.

RNA extraction

Total RNA from each sample was isolated individually using the QIAGEN RNA Mini kit (QIAGEN) according to the manufacturer's protocol, and RNA integrity was confirmed using 1% agarose gel electrophoresis.

Flow cytometric analysis

Flow cytometric analysis was performed using MACSQuant Analyzer (Miltenyli Biotech, Tokyo, Japan) with FlowJo (Treestar, Tokyo, Japan), in accordance with the manufacturer's instructions. Antihuman multidrug resistance protein 1 (MDR1), multidrug resistance–associated protein 1 (MRP1) and ABCG2 antibodies were purchased from R&D (Minneapolis, MN).

Quantitative real-time polymerase chain reaction

To evaluate mRNA levels of dCK, quantitative real-time polymerase chain reaction (qPCR) was performed using the ABI Prism 7500 sequence detection system (Applied Biosystems). All primers for dCK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were purchased from Applied Biosystems. We normalized dCK expression levels against GAPDH expression levels.

Methylation-specific polymerase chain reaction analysis

For MSP analysis, genomic DNA was obtained and 300 ng of DNA per sample was treated as described above. Primer pairs for MSP of *dCK* promoter were designed based on methylated and unmethylated DNA sequences in the promoter region, as follows [19]: dCK1 methylation-specific primers (dCK1M); *dCK* promoter 233–243 (5′-TTTATTTTTTTTTTTTATTCG ATTTCG-3′, 5′-ATCACGTAAAAACCGAAACG-3′); dCK1 unmethylation-specific primers (dCK1U); *dCK* promoter 233–243 (5′-GGTTTATTTTTTTTTTTTATTTGATTTTG-3′, 5′-ACA AATCACATAAAAACCAAAACA-3′); dCK2M; *dCK* promoter 292–299 (5′-GTTGGAGGCGGGCG-3′, 5′-AATACGCACACT AAAACTCGC-3′); dCK2U; *dCK* promoter 292–299 (5′-AATTTGTGTTGGAGGTGGGTG-3′, 5′-ACCACAAATACACA CACTAAAACTCACA-3′); dCK3M; *dCK* promoter 333-345