CELL GROWTH INHIBITION AND CYTOTOXICITY OF 5-AZA-CYTIDINE ON CISPLATIN-RESISTANT NEUROBLASTOMA CELLS

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Abstract - Neuroblastoma (NB) is a malignant tumor of the peripheral sympathetic nervous system in children, accounting for 8% to 10% of all childhood cancers. Cisplatin is a potent anticancer drug highly cytotoxic to NB cells, but clinical use of cisplatin for treatment of NB was limited by side effects and acquired cisplatin-resistance of NB cells. To develop new drugs for treatment of cisplatin-resistant NB, this study aimed to test anticancer activity of 5-Aza-cytidine, a DNA methyltransferase inhibitor, on cisplatin-resistant NB cells. The experimental results of this study demonstrated cell growth inhibition and cytotoxicity of 5'-aza-cytidine on cisplatin-resistant NB cells. Moreover, reduced expression of DNMT1 was detected in the NB cells treated with 5'-aza-cytidine by immunoblot assay. The findings from this study support further study of 5'-aza-cytidine as a promising drug for treatment of cisplatin-resistant NB in children.

Keywords - Neuroblastoma**,** 5-Aza-Cytidine, DNA Methyltransferase, ^{18}F-FDG

I. INTRODUCTION

Neuroblastoma (NB) is a malignant tumor of the peripheral sympathetic nervous system in children, accounting for 8% to 10% of all childhood cancers (Seeger and Reynolds, 1993, Olshan and Bunin, 2000). High risk factors for prognosis of this disease include advanced stage, amplification of N-myc oncogene, and deletion of chromosome 1p36. Acquired resistance to cisplatin accounts for tumor relapse and poor prognosis in the NB patients who had initial good response to chemotherapy with cisplatin (Akiyama et al, 1999). Effective drugs for treatment of NB refractory to chemotherapeutic drugs currently available are urgently needed for improving survival of the patients suffering drug-resistant neuroblastoma. There were efforts to develop anticancer drugs for epigenetic cancer therapy (Egger, G. et al, 2004). DNA methyltransferase (DNMT) mediates hypermethylation of promoters, which results in the silencing of many genes in tumor cells, including those genes involved in regulation of cell proliferation and apoptosis (Bestor, T.H, 2000, Robert, M.F. et al, 2003). Inhibition of DNMT may reactivate the genes silenced by aberrant hypermethylation and inhibit growth of tumor cells (Karpf AR, and Jones DA, 2002). DNMT inhibitors, such as 5'-aza-cytidine, were demonstrated to be effective for treatment of acute leukemia (Kritz AD et al, 1996) and myelodysplastic syndrome (Silverman, L.R. et al. 2002), through reactivation of hypermethylation-silenced genes in the tumor cells. Aberrant methylation of multiple genes was demonstrated in NB, including hypermethylation of the caspase 8 gene associated with N-myc oncogene amplification, and heat shock protein 47 gene (van Noese et al, 2000; Gonzalez-Gomeza et al, 2003; Yang et al, 2004). This study aimed to test anticancer activity of 5'-aza-cytidine on cisplatin-resistant human NB cells for its potential application in epigenetic therapy of neuroblastoma in children.

II. MATERIALS AND METHODS

Cell culture and drug treatment

Colorimetric MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] assay was performed to evaluate growth inhibition activity of 5-aza-cytidine using a MTT assay kit from Chemicon (Temecula, CA), as described previously [Zhang et al, 2007, 2008]. Briefly, SK-N-BE(2)C, a cisplatin-resistant human neuroblastoma cell line from ATCC (Manassas, VA) were cultured in 1:1 EMEM: F12 medium (Biosource International, Camarillo, CA) supplemented with 10% fetal bovine serum, penicillin (100U/ml), streptomycin (100mg/ml) and glutamine (100 mg/ml), at 37°C in an atmosphere of 5% CO2. The cells were seeded in a 96-well plate (1×104 /0.1 ml/well) and were treated with the 5-aza-cytidine or 5-aza-deoxycytidine purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Upon end of drug treatment, MTT solution was added and optical density (OD) was measured at the wavelength of 570 nm with a reference wavelength of 630nm. The results of the MTT assay were recorded as inhibition of cell proliferation (%) calculated from a formula = (OD of the cells in treatment group/ OD of the cells in negative control group) ×100%. Each experiment was repeated at least three times and each point was determined in triplicate.

Cytotoxicity assay by microscopic imaging

To evaluate cytopathological effects, the SK-N-BE(2)C seeded in a 24-well plate were treated with 5-aza-cytidine dissolved in normal saline at noted concentrations for 24 h. The cells of negative control were treated with cell culture medium only. Upon completion of the treatment, the cells were examined

under a microscope and cytopathological effects were recorded using a phase contrast microscope equipped with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). Round up and detachment of the cells indicate cytotoxicity of the drugs.

Immuno-blot assay

Immuno-blot assay was performed to evaluate effects of 5-aza-cytidine on expression of DNMT-1 in the SK-N-BE(2)C cells in a method modified from that described previously [Zhang et al 2008]. Briefly, the SK-N-BE(2)C cells seeded in a Petri dish were treated with 5-aza-cytidine, and the cells were harvested and the cellular proteins extracted from the cells were quantified with a protein quantification assay kit from Bio-Rad (Hercules, CA). The cellular protein samples were separated by SDS polyacryamide gel (SDS-PAGE) and transferred onto a PVDF membrane. After transferring to a PVDF membrane (NEN Life Sciences Products, Boston, MA) and blocking the non-specific binding with 5% milk and 0.1% Tween 20, it was incubated with anti-DNMT1 antibody (clone 60 B1220) purchased from Biocarta (Hamburg, Germany), followed by incubation with the secondary- peroxidase conjugated goat anti-rabbit antibody (1:30000) for 30 min. After washing with TBS×3, the immunoreactivity of DNMT-1 on the membrane was visualized by chemoluminescence using horseradish peroxidase-conjugated rabbit-anti-mouse secondary antibody and an enhanced Immun-StarTM HRP chemoluminescence kit from Bio-Rad. Lamin A/C protein was used as a control for equal loading and protein sample quality.

Cellular 18F-FDG uptake assay

To assess effect of 5-aza-cytidine on cellular uptake of 2-deoxy-2-(^{18}F)fluoro-D-glucose (^{18}F FDG), the cells were seeded into a 24-well plate (5 x 10^4 cells/well) and cultured for 24 h. After treatment of the cells with 5-aza-cytidine, ^{18}F-FDG (74 kBq or 2 mCi/well) were added to the treated cells at 37^{o}C for 1 h, the cells were harvested and washed 3 times with cold phosphate-buffered saline. The radioactivity of the cells was counted with a Packard Cobra II Gamma Counter (Perkin-Elmer).

III. RESULTS

Cell growth inhibition activity and cytotoxicity of 5'-aza-cytidine on NB cells

Proliferation of SK-N-BE(2)C cell was suppressed by treatment with 5-aza-cytidine with an IC50 value of 200 μM. Additionally, cytotoxicity of 5-aza-cytidine on NB cells were demonstrated by detection of cytopathological effects, e.g., shrinkage, spherical morphology and detachment of the cells, of SK-N-BE(2)C cell treated with 5-aza-cytidine at a concentration of 100 μM for 24 h (Fig. 1).

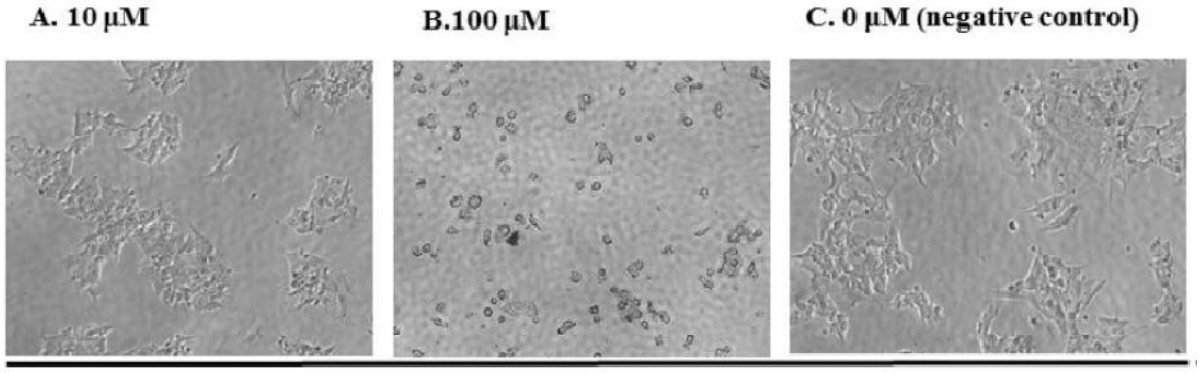


Figure 1. 5'-aza-cytidine exhibit cytotoxicity on NB cells.

Marked cytopathological effects were observed on the SK-N-BE(2)C cell treated with 100 μM of 5-aza-cytidine (B), compared with mild cytopathological effects of the cells treated with a low dose of 5-aza-cytidine at 10 μM (A) or negative control (C). Magnification: 100×.

Reduced expression of DNMT-1 in NB cells treated with 5-aza-cytidine

To study molecular mechanism of cell growth inhibition activity of 5-aza-cytidine, immuno-blot assays were performed to evaluate effects of 5-aza-cytidine on expression of DNA (cytosine-5)-methyltransferase 1 (DNMT1) in the SK-N-BE(2)C cells. Reduced expression of DNMT1 was detected in the SK-N-BE(2)C cells treated with 5-aza-cytidine (Figure 2).

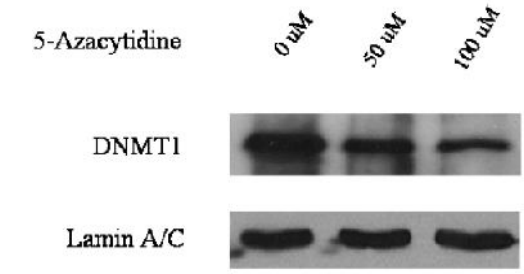


Figure 2. Reduced DNMT1 molecules in the SK-N-BE(2)C cells treated with 5-aza-cytidine for 24 h. No changes of Lamin A/C were detected that were loaded as control.

Suppression of cellular uptake of 18F FDG by 5-aza-cytidine

Malignant transformation of cells is associated with increased glucose metabolism. ^{18}F-FDG is a radiotracer widely used for assessment of tumor glucose metabolism. Cellular uptake of ^{18}F-FDG by SK-N-BE(2)C cells was inhibited following treatment with 5'-aza-cytidine (Figure 3). Cellular uptake of 18F-FDG by SK-N-BE(2)C cells treated with 5 μM of 5'-aza-cytidine was reduced 32% compared to cellular uptake of ^{18}F-FDG of non-treated SK-N-BE(2)C cells. As expected, cellular ^{18}F-FDG uptake was dramatically reduced in SK-N-BE(2)C cells treated with 100 μM of 5'-aza-cytidine.

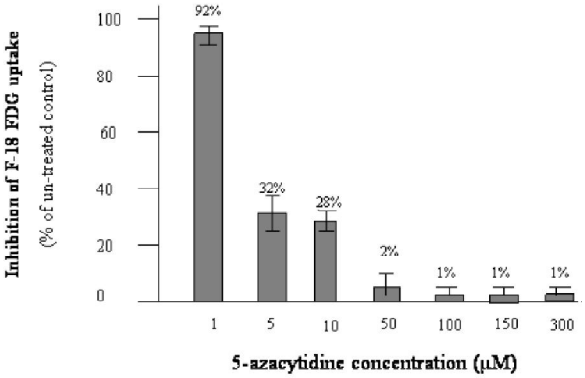


Figure 3. Reduction of cellular uptake of 18F-FDG by SK-N-BE(2)C cells treated with 5'-aza-cytidine.

Following treatment of SK-N-BE(2)C cells cell with 5 μM of 5'-aza-cytidine for 24 h, cellular uptake of ^{18}F-FDG was reduced 32% compared with cellular uptake of 18F-FDG by non-treated SK-N-BE(2)C cells. There was only little ^{18}F-FDG uptake by the SK-N-BE(2)C cells treated with a large dose of 5'-aza-cytidine (> 100 μM).

DISCUSSION

Epigenetic regulation of cancer cell proliferation represents one promising target for development of new drugs for treatment of cancer cells resistant to conventional anticancer drugs currently available. DNMT1 is an enzyme that catalyzes the transfer of methyl groups to specific CpG structures in DNA, a process called DNA methylation. Qiu et al demonstrated that DNMT enzymatic activity was increased in drug resistant murine neuroblastoma cells, contributing towards unregulated cell growth [Qiu YY et al, 2002]. It was demonstrated that 5-aza-2-deoxycytosine, an epigenetic agent, could inhibit NB cell growth in vivo [Yang QW et al, 2003]. In this study, we found that 5-aza-cytosine exhibited cell growth inhibition activity on SK-N-BE(2)C cells. Cytotoxicity of 5-aza-cytosine on SK-N-BE(2)C cells was demonstrated by microscopic imaging of the cytopathological changes of SK-N-BE(2)C cells treated with this epigenetic agent [Figure 1].

In addition to cytotoxicity of 5-aza-cytosine on SK-N-BE(2)C cells, we have demonstrated reduced expression of DNMT1 in the SK-N-BE(2)C cells treated with 5-aza-cytosine (Figure 2). It is necessary to determine effects of 5-aza-cytosine on expression of DNMT1 in other neuroblastoma cell lines. Additional studies of the changes of other gene products in the cell growth signal transduction pathways of neuroblastoma cells treated with 5-aza-cytosine is not only significant for elucidation of molecular mechanisms of anticancer activity of 5-aza-cytosine, but also significant for exploring potential use of other epigenetic agents for treatment of cisplatin-resistant neuroblastoma in children.

Based on increased uptake of 18F-FDG by neuroblastoma xegnografts in mice [Wahl RL, et al, 1991], 18F-FDG positron emission tomography/computed tomography (PET/CT) is widely used for diagnostic imaging and tracking treatment response of NB in children. In this study, we demonstrated reduction of cellular uptake of 18F-FDG by SK-N-BE(2)C cells treated with 5-aza-cytidine, suggesting potential clinical use of 18F-FDG PET/CT to track response to epigenetic therapy in NB patients treated with 5-aza-cytidine and/or other epigenetic anticancer agents.

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