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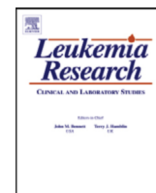

Clofarabine induces hypomethylation of DNA and expression of Cancer-Testis antigens

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ABSTRACT

In this study, treatment of lymphoid tumor cells with low dose clofarabine upregulated the expression of Sp17 and SPAN-Xb. This was associated with an increase in hypomethylated CpG dinucleotides and a decrease in global DNA methylation, as demonstrated by decreases in the percent of methylated *Alu* repeats. The most optimal concentration of clofarabine to induce DNA hypomethylation and CT antigen expression was between 1×10^{-9} and 1×10^{-8} M. Above this, clofarabine resulted in tumor cell growth inhibition and apoptosis. Our results provide the first evidence for the CT antigen-inducing and DNA hypomethylating property of low concentration clofarabine.

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1. Introduction

Cancer-Testis (CT) antigens are a group of normal testicular proteins aberrantly expressed in tumor cells. They are potential targets for tumor vaccine development because of their limited expression in normal tissues and their *in vivo* immunogenicity in cancer-bearing patients [1–3]. We previously identified Sperm protein 17 (Sp17) [4] and SPAN-Xb [5] as CT antigens in hematologic malignancies. Both are nuclear/cytoplasmic proteins. We also demonstrated that the expression of these two genes was regulated through promoter methylation [6,7]. Treatment of tumor cells with the DNA hypomethylating agent, 5-azacytidine, resulted in the upregulation of Sp17 and SPAN-Xb expression. CT antigens are highly immunogenic auto-antigens and are particularly suited as targets for immunotherapy of hematologic malignancies such as chronic lymphocytic leukemia or multiple myeloma since these patients are usually severely immunosuppressed, due to either the disease process or therapy for the disease, and will not respond optimally to immunotherapy targeting a weak antigen. However, a problem commonly observed with CT antigens is that CT antigens tend to be expressed heterogeneously, even within individual patients. The heterogeneity of antigen expression provides the opportunity for tumor escape from immunotherapy, with the preferential growth of antigen-negative variant tumor cells. Investigations examining the different strategies to upregulate antigen expression within individual patients are, therefore, much needed.

Clofarabine is a second generation purine nucleoside analog. It is a hybrid molecule of fludarabine and cladribine, although structurally it is more closely related to cladribine. Clofarabine is active in non-dividing cells and in cells with a low proliferation rate. Although clofarabine has been used successfully in patients with refractory acute myeloid leukemia, acute lymphoblastic leukemia and myelodysplastic syndrome [8,9], the precise mechanism of action of clofarabine on dividing and non-dividing cells is unclear. *In vitro* data suggests that clofarabine induce cytotoxicity through inhibition of DNA synthesis and repair [10]. Clofarabine 5′-triphosphate inhibits both DNA polymerase α and ribonucleotide reductase [11] and causes a depletion of the intracellular deoxynucleotide triphosphate pools and inhibition of elongation of DNA strands during synthesis [12]. Clofarabine also induces apoptosis and disrupts the integrity of mitochondria [13].

Since clofarabine is a nucleoside analog, we set out to determine whether or not clofarabine treatment of tumor cells result in the expression of Sp17 and SPAN-Xb, changes in the methylation status of the Sp17 promoter gene and a decrease in the global methylation of the long interspersed nucleotide elements (LINE-1).

2. Materials and methods

2.1. Materials

Three human lymphoid tumor cell lines were used: JVM-2 cells are EBV-transformed tumor cells derived from a prolymphocytic leukemia patient, RL cells are EBV-negative B-cell non-Hodgkin's lymphoma cells, and Granta-519 cells are EBV-transformed tumor cells derived from a mantle cell lymphoma patient in

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leukemic phase. All cells were maintained at liquid culture in 10% FCS prior to being used for the experiments. Clofarabine was a kind gift from Genzyme Oncology, USA.

2.2. *In vitro treatment of tumor cells with clofarabine*

Tumor cells were treated with varying concentrations of clofarabine in 6-well tissue culture plates. The culture medium, containing 10% FCS, was replaced daily with fresh culture medium containing clofarabine. The tumor cells were harvested at 96 h and used for in the experiments. Control cultures were grown under identical conditions but without clofarabine.

2.3. *RT-PCR amplification of Sp17 and SPAN-Xb mRNA*

RT-PCR was carried out to amplify Sp17 and SPAN-Xb cDNA. Briefly, all RNA specimens were first treated with DNAse I (Ambion Inc., Austin, TX) to remove genomic DNA contamination. First strand cDNA was synthesized from 1 μg of total RNA using a random hexamer primer. For qualitative PCR, the primers for Sp17 were 5′-*CAG CAG AAT GGG GGA GTA AG*-3′ and 5′-*CAG CTT GGA TTT TGA CAG CA*-3′. They amplified a Sp17 gene segment of 200 bp. The primers for qualitative SPAN-Xb PCR were 5′-*AAT GAG GCC AAC AAG ACG AT*-3′ and 5′-*TCG GGG TTG ATT CTG TTC TC*-5′. They amplified a SPAN-Xb gene segment of 185 bp. PCR was performed using 35 amplification cycles at an annealing temperature of 60 °C. Negative controls in all the PCR reactions contained the PCR reaction mixture except for cDNA, which was substituted with water. RNA integrity in each sample was checked by amplification of a β-actin gene segment. Successful removal of genomic DNA contamination was confirmed in each sample by amplification of the RNA without prior RT reaction. PCR products were visualized on an ethidium bromide agarose gel. All results were confirmed on two independent RT-PCRs.

For quantitative PCR analysis of Sp17 and SPAN-Xb mRNA copy numbers, we used the SYBR-Green-based real time PCR approach (Bio-Rad, USA). Reverse transcription was carried out using random hexamer primers on 250 ng total RNA. All PCR reactions were carried out in triplicates. The primers for Sp17 were: 5′-*CAG CAG AAT GGG GGA GTA AG*-3′ and 5′-*CAG CTT GGA TTT TGA CAG CA*-3′. The primers for SPAN-Xb were: 5′-*AAT GAG GCC AAC AAG ACG AT*-3′ and 5′-*TCG GGG TTG ATT CTG TTC TC*-3′. Thermal cycle conditions were as follows: 50 °C for 2 min, 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. All results were normalized to 18S rRNA amplification (Applied Biosystem, CA). Plasmids containing Sp17 and SPAN-Xb cDNA were used for constructing the standard curves. The plasmids were serially diluted, starting 10^5 plasmid copies and analyzed in triplicate. A standard curve was constructed by plotting the C_T and the known copy number on a logarithmic scale.

2.4. *Immunohistochemistry*

Cytospin specimens of the tumor cells were prepared and air-dried. The fixed cells were treated with 0.4% Triton X solution before the addition of diluted Sp17 MoAb [14] or SPAN-Xb MoAb for 2 h. Following washing, the slides were overlaid with a peroxidase-conjugated rabbit anti-mouse secondary antibody for 30 min. The slides were washed again in PBS and overlaid with diaminobenzidine reagent. Following washing in PBS, the slides were counterstained in hematoxylin.

2.5. *Sodium bisulphite genomic DNA modification*

Genomic DNA was first digested with *EcoRI*, then denatured with 0.3 M NaOH for 15 min. The denatured DNA was reacted with 3.6 M sodium bisulphite and 1 mM hydroquinone (55 °C for 14 h). The DNA was desalted using a DNA clean up kit (Wizard DNA Clean Up, Promega) and precipitated for PCR. PCR was carried out using the oligonucleotide primers that amplify the Sp17 promoter gene from −480 to −290: 5′-*GGT TGG AGA TGT GAG TTT TTA*-3′ and 5′- *ATA CTC AAA CTA ATA CTA CCA*-3′. The PCR products were cloned into the TA-cloning system. Up to ten recombinant clones were randomly picked from each transfection for nucleotide sequence analysis to determine the proportion of hypomethylated promoter sequence.

2.6. *Methylation-sensitive PCR for Sp17 promoter gene*

A panel of oligonucleotide primers was used to amplify overlapping gene segments on the Sp17 exon 1 that contained eight *Hpa* II sites. The nucleotide sequences of the panel of primers are as described previously [6]. Genomic DNA was extracted from tumor cells using a commercially available DNA extraction kit. The DNA was digested for 3 h at 37 °C with 10 U/μg methylation-sensitive restriction enzyme *Hpa* II, extracted with phenol and chloroform–isoamyl alcohol (25:24:1), and recovered by ethanol precipitation. To ensure completion of digestion, the above process was repeated. This restriction digest protocol has been used successfully in previous works [15,16] and has been found to consistently result in complete digestion of the genomic DNA by *Hpa* II restriction enzyme. Following ethanol precipitation, 100 ng of the digested DNA was used for amplification in a final volume of 50 μl PCR mixture. PCR was carried out for 30 cycles in a thermal cycler. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. All results were confirmed in two independent PCR.

2.7. *LINE-1 PCR analysis*

Methylation changes at LINE-1 sequences were determined using the COBRA assay as described previously [17]. Briefly, genomic DNA prepared from tumor cells before and after clofarabine treatment was first subjected to bisulfite modification as described above. The bisulfite-treated DNA was isolated, following purification, was precipitated and resuspended in water before being used in PCR. Low stringency PCR was used to amplify the *Alu* repeats of the long interspersed nucleotide elements. The primers used were: 5′-*TTG AGT TGT GGT GGG TTT TAT TTA G*-3′ and 5′-*TCA TCT CAC TAA AAA ATA CCA AAC A*-3′. PCR cycling conditions were 95 °C for 5 min, followed 40 cycles at an annealing temperature of 56 °C. The final PCR product was digested overnight at 37 °C with 50 U of *Hinf* I. The digested PCR products, consisted of one methylated DNA band and three unmethylated DNA bands, were fractionated in 12% polyacrylamide gel. Gel bands representing methylated DNA were quantified using Quantity One 1-D analysis Software (Bio-Rad, USA) and calculated as % of undigested PCR DNA fragments run on the same gel. All experiments were carried out in triplicates.

3. Results

3.1. *Upregulation of Sp17 and SPAN-Xb expression by clofarabine*

Treatment of tumor cells with clofarabine induced the upregulation of the CT antigens, Sp17 and SPAN-Xb. Before treatment with clofarabine, JVM-2 and Granta-519 cells expressed a very low level of Sp17 gene and undetectable Sp17 protein, and RL cells did not express any Sp17 gene or protein. In all three tumor cells, Sp17 expression was either induced or upregulated at the transcript (Fig. 1a) and protein levels (Fig. 1b) following treatment of the tumor cells with clofarabine. Using real time PCR, we also found a dose response of the mRNA levels encoding Sp17 with increasing concentrations of clofarabine used in the cell culture, up to a clofarabine concentration of 1×10^{-8} M (Fig. 1c). However, above this concentration, the levels of mRNA encoding Sp17 declined progressively.

Clofarabine treatment also upregulated SPAN-Xb expression in tumor cells. Before clofarabine treatment, transcripts encoding SPAN-Xb were already detected in all three cell lines by RT-PCR (data not shown). At the protein level, RL cells and Granta-519 cells expressed high level and JVM-2 cells low level of SPAN-Xb protein by immunocytochemistry. Following treatment with clofarabine, SPAN-Xb protein expression was greatly upregulated in all three tumor cell lines (Fig. 2a). These results, therefore, provide the first evidence showing the ability of clofarabine to induce the expression of Sp17 and SPAN-Xb and support the notion that low concentration clofarabine induces DNA hypomethylation. However, not unlike in Sp17, we also found a dose response of the mRNA levels encoding SPAN-Xb with increasing concentrations of clofarabine used in the cell culture, up to concentrations between 1×10^{-9} and 1×10^{-8} M (Fig. 2b). Above this optimal concentration, SPAN-Xb gene expression declined.

3.2. *Association between Sp17 expression and hypomethylation of Sp17 promoter gene*

We previously found a correlation between hypomethylation of the Sp17 promoter gene and Sp17 expression [6]. To determine whether the clofarabine-induced CT antigen expression was also associated with promoter hypomethylation, we determined changes in the methylation status of the CpG dinucleotides within the Sp17 promoter gene following treatment of the tumor cells with 1×10^{-9} M clofarabine. There are a total of 14 CpG dinucleotides within the Sp17 promoter gene. Following bisulfite conversion of DNA derived from tumor cells before and after clofarabine treatment, Sp17 promoter gene was amplified and the PCR products subjected to nucleotide sequence analysis. In all three tumor cells, the CpG dinucleotides within the Sp17 promoter were consistently less methylated after treatment with clofarabine ($p < 0.05$) (Fig. 3a).

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