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Methylation of the DPH1 promoter causes immunotoxin resistance in acute lymphoblastic leukemia cell line KOPN-8

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Abstract

Moxetumomab pasudotox (HA22) is an immunotoxin with an anti-CD22 Fv fused to a portion of Pseudomonas exotoxin A that kills CD22 expressing ALL cells. HA22 produced significant responses in some cases of ALL. To understand how to increase response rate, we isolated HA22-resistant KOPN-8 cells and found that HA22 cannot inactivate elongation factor-2 (EF2) due to low levels of DPH1 RNA and protein. Resistance was associated with methylation of the CpG island in the DPH1 promoter. 5-azacytidine prevented resistance and methylation of the CpG residues and merits evaluation to determine if it can increase the efficacy of HA22 in ALL.

Keywords

DNA methylation; Drug resistance; ADP-ribosylation; Diphthamide synthesis; Epigenetic regulation

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Conflict of interest statement

Ira Pastan is an inventor on patents of immunotoxins that are assigned to the NIH. Alan S. Wayne is a co-inventor on patents assigned to the NIH. The authors otherwise declare no Conflict of Interest.

Authors' Contributions

Conception and design - ASW, TKB and IP

Acquisition of data - XH, HW, LX and OC

Analysis and interpretation of data - ASW, TKB and IP

Writing, review and/or revision of manuscript - XH, HW, ASW, TKB and IP

Study supervision - TKB and IP

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1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy of children accounting for approximately 25% of childhood cancers. Five-year survival rates have improved steadily and currently exceed 90% in patients receiving protocol-based therapies [1, 2]. Because many children still die from this disease and others have side effects from the intensive therapy used to treat ALL, there is a need to develop novel therapies.

Our laboratory has focused on the development of recombinant immunotoxins (RIT) for cancer treatment [3]. These proteins are composed of a variable fragment (Fv) of a monoclonal antibody fused to a 38-kD portion of *Pseudomonas* exotoxin A (PE38) [4]. The antibody fragment binds to an antigen on the cancer cell and the toxin is then internalized and kills the cell. The B-lineage differentiation antigen CD22 is a useful target for precursor-B cell ALL because it is expressed on cells from all patients with this disease [5]. Moxetumomab pasudotox (HA22) is a RIT, containing an anti-CD22 Fv fused to PE38. The Fv binds to CD22, and the immunotoxin is then internalized via receptor-mediated endocytosis. After processing by furin, which separates the Fv from the toxin, the toxin portion is carried to the endoplasmic reticulum, where it then escapes into the cytosol [6–9] and catalyses the ADP-ribosylation and inactivation of elongation factor (EF2). Arrest of protein synthesis by PE38 is due to inactivation of EF2 by ADP-ribosylation of a diphthamide residue located on histidine 715. There are 6 proteins known to be required for the post-translational modification of His 715 to diphthamide. These are DPH1-5 and WDR85 [10–15].

In an ongoing clinical trial in children with chemotherapy-refractory ALL, HA22 has produced complete and partial remissions in several patients, but others showed no response or minor responses [16]. To investigate possible reasons for the lack of a response we have selected ALL cell lines for resistance to HA22. We previously reported on a HA22 resistant HAL-01 cell line in which resistance was due to silencing of the *DPH4* gene due to specific methylation of a CpG island in the DPH4 promoter [17]. In this paper we report on mechanism of resistance of a KOPN-8 cell line in which the *DPH1* gene is silenced.

2. Materials and methods

2.1 Reagents

HA22 was produced in our laboratory as previously described [18]. Azacytidine (AZA) (Sigma) was dissolved in RPMI-1640 medium. Antibodies to actin and EF2 were from Abcam and anti-DPH1 from Sigma. Other reagents were from the same source as described in Wei et al. [17].

2.2 Establishment of resistant cell line

The ALL cell line KOPN-8 was maintained in RPMI-1640 medium with 10% FBS. Its identity was confirmed by Short Tandem Repeat analysis at the Frederick National Laboratory for Cancer Research. To isolate resistant cells, 2×10^7 cells were seeded in 10 mL RPMI-1640 medium with HA22 at 1 ng/mL and incubated for 72 hours. Residual living cells were expanded over 7 days in normal medium. A second and third round of selection was similarly performed with HA22 at 10 ng/mL and 100 ng/mL of concentration respectively.

2.3 Antigen expression and internalization of HA22

Measurements of CD22 surface expression and HA22 internalization were performed as previously described [19, 20].

2.4 Cell viability assay

Survival of cells treated with HA22 was measured by WST-8 assay using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) as previously described [21]. Briefly, 10^4 cells/well were incubated with various concentrations of HA22 in a 96-well plate for 72 hours, after which 10 μ L WST-8 was added to each well, and the cells were incubated for 4 hours at 37°C. The absorbance of the sample at 450 nm was measured with a reference wavelength of 650 nm. Cycloheximide (10 μ g/mL final concentration) was used as a control for 100% cell death. Cytotoxicity is defined by IC₅₀, concentration that gave a 50% decrease on cell viability, which is midway between the level of viability in the absence of toxin and that in the presence of 10 μ g/mL cycloheximide (as a positive control). All experiments were performed in triplicate wells. Protein synthesis inhibition was performed as previously described [22].

2.5 Toxin-induced ADP ribosylation of EF2

KOPN-8 (3×10^6) or KOPN-8-R cells were lysed in 0.3 mL radioimmunoprecipitation assay buffer [50mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100] with protease inhibitors, and 0.01 mL of cell lysate (30 μ g) was incubated with 100 ng of HA22 in ADP ribosylation buffer [20 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 50 mM DTT] with 5 mM 6-Biotin-17-NAD (Trevigen, Gaithersburg, MD) for 60 minutes at 25°C. Samples were subjected to SDS/PAGE followed by western blotting with streptavidin HRP conjugate (Invitrogen) to detect biotin-ADP ribose-EF2.

2.6 RT and real-time PCR

Measurement of mRNA levels was performed as previously described [23].

2.7 Immunoblots

Cells were collected, washed with cold Dulbecco's PBS twice, and solubilized in lysis buffer [25 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1.0% Nonidet P-40 (vol/vol), and 0.5% sodium deoxycholate (wt/vol)] with protease inhibitor mixture (Sigma). Detection of proteins was performed as previously described [24].

2.8 DNA methylation analysis

DNA methylation analysis of the DPH1 promoter was performed by EpigenDx (<http://www.epigenDx.com/>).

2.9 Determination of the extent of EF2-Diphthamide modification

Measurement of EF2-Diphthamide modification was performed as described [17].

2.10 Electroporation of KOPN-8-R Cells

KOPN-8-R cells were transfected with the pCMV6-DPH1-Myc or pCMV6-GFP plasmid using the Amaxa Cell Line Optimization Nucleofector Kit and Amaxa Nucleofector (Lonza). First, 5×10^6 KOPN-8-R cells were harvested and suspended in 100 μ L of Nucleofector Solution L, and the cell suspension was then mixed with 10 μ g of plasmid DNA. The mixture was electroporated using Nucleofector Program A-020 in the Amaxa Nucleofector. The transfected cells were cultured in RPMI-1640 medium, two days later these cells were used for analysis.

2.11 Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical analysis was performed using the Student t test for comparison between two groups. All experiments were repeated at least twice.

3. Results

3.1 Establishment and Characterization of HA22 Resistant Cell Line

We established a HA22 resistant cell line by exposing KOPN-8 cells to HA22 as described in Materials and Methods. The parental cell line is very sensitive to HA22 with an IC_{50} of 0.08 ng/ml, whereas the KOPN-8-R cells are resistant to HA22 over 100 ng/ml (Fig. 1A). We examined the stability of the resistant cells by growing them for 4 months without HA22 and found that they were still resistant to HA22 over 100 ng/ml (Fig. 1B). Despite the resistance of the cells there was no significant difference in the growth rate of the two cell lines (data not shown).

To elucidate the mechanism of resistance, we began by measuring the expression of CD22 on the cell surface of KOPN-8 and KOPN-8-R cells by FACS. Figure 1C shows that CD22 expression in resistant cells is not decreased and is actually increased from a geometric mean of 189 to 320. We then measured the internalization rate of HA22 using HA22 labeled with Alexa-647 and did not find a significant difference in the rate or amount internalized over 2 hours (Fig. 1D). This indicates HA22 is undergoing endocytosis at a normal rate.

We next measured the ability of HA22 to inhibit 3H -leucine incorporation, which occurs due to inactivation of EF2, and found as shown in Figure 1E that HA22 did not inhibit 3H -leucine incorporation into protein in the mutant cells. Failure to inhibit 3H -leucine incorporation suggested that EF2 was not ADP-ribosylated and inactivated by HA22.

To measure the ability of HA22 to catalyze ADP-ribosylation, we used an assay in which cell-free extracts are incubated with 6-biotin-17-NAD with or without HA22. The ADP-ribosylation of EF2 was assessed by a western blot in which the amount of biotin-NAD incorporated into EF2 is determined with Streptavidin-peroxidase. Figure 1F shows that HA22 could catalyze the ADP-ribosylation of EF2 in extracts of KOPN-8 cells but not in KOPN-8-R cells (Fig. 1F). This failure was not due to a loss of EF2 because a western blot with an antibody to EF2 showed no differences in EF2 levels. These results indicate that EF2 cannot be inactivated by ADP-ribosylation, probably because the His 715 residue on EF2 is not modified by diphthamide.

To determine whether His-715 of EF2 contained the diphthamide modification in the KOPN-8-R cells, EF2 was isolated from normal and resistant cells by DEAE chromatography and gel electrophoresis. The peptides were analyzed by MALDI-MS after digestion of the EF2 band by trypsin. Figure 2 shows the results for selected peptides from cells. MS/MS analysis of m/z 1745.835 from KOPN-8-R cells identifies a peptide (FDVHDVTLHADAIHR) that contains an unmodified His-715 residue. We did not find the corresponding peptide in KOPN-8 cells. Instead, analysis of m/z 1828.812 and 1836.186 from KOPN-8 cells demonstrated that both ions correspond to a diphthamide-modified peptide but with loss of a trimethylamino group that could have occurred because of an elimination reaction [25] during protein isolation. Also m/z 1887.913 corresponds to an intact diphthamide-modified peptide. In summary sequence analysis of EF2 in KOPN-8-R cells shows it is not modified to diphthamide.

3.2 Promoter hyper-methylation of DPH1 gene in KOPN-8-R

To investigate if the diphthamide biosynthesis is impaired in KOPN-8-R cells, we used RT-PCR to measure the mRNA levels for the 6 proteins in this pathway. As shown in Figure 3A, DPH1 expression was decreased by 93%, whereas the levels of the other 5 mRNAs did not change in such a dramatic manner. Two RNAs were slightly elevated and 2 others slightly reduced. To determine if the DPH1 protein was also changed, we measured the

levels of the DPH1 protein by western blot and found a profound decrease in its level (Fig. 3B).

To determine whether the HA22 resistant phenotype of KOPN-8-R cells is solely contributed by reduced expression of DPH1 and could be restored by expression of DPH1, we introduced an expression plasmid encoding a DPH1-Myc fusion protein by electrotransfection into the KOPN-8-R cells. Transfection efficiency of KOPN-8-R cells was 20–25% as measured by fluorescence positive cells transfected with a control GFP expressing plasmid (data not shown). Figure 3C and 3D shows that the transient expression of DPH1 was about 25% and 40% as compared with control vector transfected cells at the RNA and protein level, respectively. As shown in Figure 3E the sensitivity to HA22 was restored to about 50% in KOPN-8-R cells transfected with DPH1 indicating the resistant phenotype of KOPN-8-R cells is solely contributed by reduced expression of DPH1.

To explore why DPH1 gene expression was decreased, we amplified and sequenced the DPH1 promoter region (–625 to +655 relative to the ATG) to determine if there were any changes in the DNA sequence and did not detect any changes (data not shown). The *DPH1* promoter contains a CpG island that could regulate transcription of this gene, therefore we measured its methylation status by bisulfite sequencing. We found that *DPH1* promoter of KOPN-8-R cells is hyper-methylated while that of KOPN-8 is not (Fig. 3F). To determine if DPH1 promoter hyper-methylation reflected a specific or global specific change, we tested the methylation status of 24 other gene promoters using an array of genes whose expression is often altered in ALL [26–30] and found that their methylation status did not correlate with the status of the *DPH1* gene (Fig. 3G) except for the LMNA and HOXB5 genes. Both LMNA and the HOXB5 promoters are hypomethylated in KOPN-8-R cells as compared with KOPN-8 cells, but the extent of changes are not as large as the DPH1. The relevance of these changes in methylation for these genes in KOPN-8-R cells is not clear.

3.3 AZA can prevent and reverse resistance to HA22

AZA is a drug that inhibits methyl transferase activity, which is required for methylation of CpG islands. To determine whether AZA could prevent drug resistance through inhibiting methylation of CpG islands in the *DPH1* promoter, we grew 5×10^6 KOPN-8 cells for 27 days with either HA22 at 1 ng/mL, or AZA at 300 nM, or the combination of HA22 and AZA (Fig. 4A). We found that HA22 killed 82% of cells, but some resistant KOPN-8 cells survived and eventually re-grew. When AZA was present with HA22, no viable cells could be detected. AZA by itself slowed growth by only 24%. This experiment shows that AZA can prevent the emergence of resistant cells.

In another series of experiments we examined if AZA could reverse resistance to HA22. To do this, KOPN-8-R cells were treated with 100 nM AZA. AZA was removed after 2 days, and the cells were cultured in normal medium for 2 days. Then cells were treated in a similar manner four more times. Finally the cells were cultured in normal medium for 1 week before testing. The data in Figure 4B shows that the sensitivity of the KOPN-8-R cells was partially restored by AZA treatment to an IC_{50} of 0.7 ng/ml from an IC_{50} of >100 ng/ml. Based on the shape of the cytotoxicity curve, it appears that AZA treatment restored the sensitivity of 70–80% of the cells, since even at 100 ng/ml 20% of the cells remained HA22 resistant (Fig. 4B). To determine if a change in the levels of DPH1 RNA or protein accompanied the reversion of the cells to HA22 sensitivity, we measured DPH1 RNA and protein and found both were substantially increased in the revertant cell population (about 60%) (Fig. 4C and 4D). We also analyzed the ability of HA22 to catalyze the incorporation of Biotin-NAD into EF2 and found this was also corrected (Fig. 4E). Finally to determine if AZA treatment altered the methylation of the *DPH1* promoter we analyzed the promoter of the revertant cells and found that methylation was decreased by about 50% (Fig. 3F). This is

consistent with the cytotoxicity data that showed the majority of cells were killed by HA22 but the IC₅₀ was shifted from 0.15 to 0.7 ng/ml. In addition about 20% of the cells were still resistant to HA22 (Fig. 4B).

4. Discussion

We describe here the isolation and characterization of an ALL cell line with resistance to immunotoxin HA22 through an epigenetic mechanism. We have previously reported on the properties of an HA22 resistant HAL-01 cell line in which resistance was due to hyper-methylation of the DPH4 promoter [17]. Since the mutations found among ALL cells differ and can affect their response to therapy, we decided to determine if there is a common mechanism of resistance for many cell lines and chose to study KOPN-8 which has different mutations than HAL-01. HAL-01 has the t(17;19)(q22;p13) translocation leading to a TCF3/E2A-HLF fusion [31], whereas KOPN-8 has a t(11;19)(q23;p13) translocation leading to the MLL-ENL fusion [32]. Whether these differences are responsible for the different *DPH1* genes being altered in the resistant cells is a subject of further study.

In both cell lines we found that the resistance was due to hyper-methylation of a CpG island in the promoter resulting in low RNA levels, but the genes involved differ. For HAL-01 it is DPH4 that is inactivated, whereas for KOPN-8 it is DPH1. Both are needed for diphthamide synthesis. Another difference is that resistance in the HAL-01-R cells is unstable and the cells revert to HA22 sensitivity over several months, whereas the resistance in KOPN-8-R cells is stable over several months.

Aberrant methylation of CpG islands located at or near gene promoters is associated with inactivation of gene expression [33]. There are many reports showing that CpG methylation can cause drug resistance and drug resistance can be reversed by the treatment of cells or tumors with the demethylating agents AZA or 5-aza-2'-deoxycytidine (decitabine) [34]. Strathdee et al. [35] reported that A2780, a human ovarian cancer cell line resistant to cisplatin due to loss of expression of the mismatch repair protein hMLH1 exhibits hyper-methylation of the promoters of both hMLH1 alleles. Treatment of resistant cell lines with AZA, results in re-expression of hMLH1 and increased sensitivity to cisplatin. Fulda et al. [36] reported that the decreased expression of caspase-8 and hyper-methylation are correlated with drug resistance in Ewings tumors and that the resistance can be reversed by AZA. Therefore, aberrant DNA hyper-methylation can be a critical phenomenon in the control of gene expression.

From a therapeutic viewpoint, prevention of methylation or promotion of demethylation could be a useful anticancer therapy because epigenetic events are reversible, and quiescent genes may be reactivated by the demethylating agents [37]. Previous studies have shown the efficacy of combination therapy that used demethylating agents and chemotherapeutic agents [38, 39]. We plan to examine cells from ALL patients on our clinical trial to determine if methylation of genes involved in diphthamide synthesis occurs in ALL patients.

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Figure 1.

Cytotoxic effects of HA22 on KOPN-8 and KOPN-8-R cells maintained without HA22 for (A) 7 days, (B) 120 days. (C) CD22 expression. KOPN-8 and KOPN-8-R cells were incubated on ice with RFB4 (mouse anti-CD22 antibody) or control mouse IgG1, followed by goat anti-mouse antibody-phycoerythrin and analyzed by FACS. (D) Rate of HA22 internalization. KOPN-8 and KOPN-8-R cells were incubated with HA22-Alexa 647 for 10, 60, 120, 180 and 360 minutes. Internalized immunotoxin is shown as the median fluorescence intensity (MFI). (E) Protein Synthesis Inhibition by HA22. Cells were treated with HA22 for 20 hours; protein synthesis was measured by incorporation of ^3H -leucine. (F) ADP-ribosylation of EF2. Cell lysates (30 µg) were incubated with or without 100 ng of

HA22 for 60 minutes at 25°C in the presence of biotin-labeled NAD. Samples were analyzed by western blot with streptavidin HRP conjugate to detect biotin-ADP ribose-EF2.

Figure 2. MALDI-TOF MS of tryptic peptides of EF2 purified from KOPN-8 and KOPN-8-R cells. In KOPN-8-R, a peptide with m/z 1745.835 is FDVHDVTLHADAIHR with un-modified His-715. This un-modified peptide is not present in KOPN-8 cells.

Figure 3.

(A) DPH1–5 and WDR85 mRNA levels were analyzed by quantitative RT-PCR. (B) Western blot showing DPH1 levels. (C) RT-PCR analysis showing the level of DPH1 RNA expression in KOPN-8R cells after transient transfection of pCMV-DPH1-Myc plasmid. (D) Graph showing level of DPH1 protein expression in KOPN8-R cells after transient tranfection of pCMV-DPH1-Myc plasmid. (E) Sensitivity of HA22 of KOPN-8-R cells after electrotransfection was assessed by WST-8 assays. Data presented as % cell survival at 1 ng/ml of HA22 treatment. (F) Levels of methylation of the CpG island in the DPH1 promoter in sensitive, resistant and revertant cells. (G) Analysis of methylation status of promoters of genes expressed in ALL cells.

Figure 4.

(A) AZA prevents resistance of KOPN-8 cells. KOPN-8 cells were treated with azacytidine or HA22 (1 ng/ml) or both for 27 days. Fresh HA22 was added every 3 days, and azacytidine was added every day. The live cell number was determined by Trypan blue exclusion. (B) Viability of KOPN-8 cells treated with 4 cycles of AZA. KOPN-8-R cells were treated with 100 nM AZA. AZA was removed after 2 days, and cells were cultured in normal medium for 2 days. Cells were treated in a similar manner four more times and then cultured in normal medium for 1 week before testing. Sensitivity to HA22 was assessed by tetrazolium (WST) assays. (C) DPH1 mRNA levels in sensitive resistant and revertant cells. (D) DPH1 protein levels. (E) ADP ribosylation of EF2.