Effect of arsenic trioxide on Epstein-Barr virus latent membrane protein 1-mediated E-cadherin silencing in nasopharyngeal carcinoma

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Abstract

Epstein–Barr virus latent membrane protein 1 (LMP1) activates cellular DNA methyltransferases, which results in hypermethylation, and thus, silencing of E-cadherin. We previously demonstrated that arsenic trioxide (As2O3) can reduce LMP1 expression in human nasopharyngeal carcinoma (NPC) cells in vitro. The purpose of this study was to determine whether As2O3 can rescue the expression of E-cadherin in NPC cells through repressing LMP1. The stable LMP1 (HNE1-LMP1) and its parental (HNE1) cell lines were treated with various concentrations of As2O3 for 48 h. DNA methylation of the E-cadherin gene promoter of survival cells was investigated using methylation-specific polymerase chain reaction. E-cadherin mRNA and protein expression levels in these cells were analysed by quantitative RT-PCR and Western blot, respectively. Transwell assays were used to measure the invasive properties of these cells after exposure to As2O3. The characteristic hypermethylation within silenced E-cadherin was found in HNE1-LMP1 cells when compared with HNE1 cells. As2O3-induced demethylation restored the activity of the E-cadherin gene promoter in HNE1-LMP1 cells. E-cadherin mRNA and protein expression levels were upregulated after As2O3 treatment. Accordingly, the invasive ability of HNE1-LMP1 cells also reduced after exposure to As2O3. To conclude, As2O3 could reverse LMP1-mediated methylation and silencing of the E-cadherin gene in NPC, restore the gene’s promoter activity and expression and indicate a potential therapeutic strategy for NPC.

Introduction

Nasopharyngeal carcinoma (NPC), a human squamous cell cancer prevalent in southeastern China, is notorious for its highly aggressive and metastatic nature. NPC presents with Epstein–Barr virus (EBV) infection. Accumulating evidence has indicated that EBV latent membrane protein 1 (LMP1), a transmembrane protein encoded by the LMP1 gene, plays a critical role in NPC pathogenesis1,2. LMP1 expression is positively associated with NPC metastasis3. One of the mechanisms is that LMP1 downregulates the expression of E-cadherin4, which is a calcium-dependent cell-cell adhesion glycoprotein that mediates cell-cell contact and acts as a tumour suppressor. Cooperating with cytoplasmic catenins (α-, β- and γ-catenins), E-cadherin plays a major role in the maintenance of intercellular junctions in normal epithelial cells by forming an E-cadherin–catenin complex5. The loss of function of E-cadherin contributes to cancer progression by increasing proliferation, invasion and metastasis. E-cadherin repression is associated with decreased adhesion and enhanced migration of epithelial tumour cells. Inactivation of the E-cadherin gene, which results from LMP1-mediated transcriptional silencing by CpG islands methylation6, is central to the development of NPC. LMP1 has been shown to induce hypermethylation of the E-cadherin promoter and downregulation of E-cadherin gene expression through activation of cellular DNA methyltransferases8.

Arsenic trioxide (As2O3) is a potent drug in the treatment of acute promyelocytic leukemia9. As a single agent, it induces complete remissions, causing few adverse effects and minimal myelosuppression. As2O3 can influence numerous signal transduction pathways, which results in a vast range of cellular effects, including apoptosis induction, growth inhibition, differentiation induction and angiogenesis inhibition10. At present, As2O3 is a broad-spectrum anti-cancer drug used for treating a variety of cancers. We previously found that As2O3 reduced LMP1 expression in NPC cells in vitro, indicating that As2O3 may be a potential anti-cancer drug in the treatment of NPC11. However, it is yet unknown whether As2O3 can restore the expression of LMP1-mediated methylation and silencing of the E-cadherin gene, and subsequently exert its anti-tumour effect on NPC cells. In the current study, we used cell-based assays to investigate whether As2O3 could rescue the expression of E-cadherin in NPC cells through repressing LMP1, and...
Introduction (cont.)

thus, reverse the malignant behaviour, including cell invasion and migration of NPC cells.

Materials and methods

Cell lines and cell culture

HNE1-LMP1 stable cell line with constitutional expression of LMP1 was established by transfecting the LMP1 cDNA into HNE1 cells. HNE1-LMP1, the parental cell line without expression of LMP1 and which was derived from poorly differentiated NPC, was used as a negative control. Both cell lines, which were established at the Cancer Research Institute of Hunan Medical University, were kindly provided by Professor Y. Cao. Cells were cultured in RPMI 1640 medium with 10% foetal bovine serum, under a humidified atmosphere of 95% air and 5% CO2 at 37°C.

As2O3 or 5-aza-dC treatment

Both cell lines, at a density of 1×10⁶/mL, were treated with As2O3 (Harbin-Eda Pharmaceutical Co. Ltd., China) at various concentrations (0, 0.5, 1 and 2 μM/L) or 20 μM/L of 5-aza-2′-deoxycytidine (5-aza-dC, Sigma) for 48 h. Floating cells were then discarded, while the residual cells were cultured and collected when they reached confluence. Cells that were not treated with As2O3 and normal nasopharyngeal epithelial cells from three healthy volunteers were used as controls.

RNA extraction and reverse transcription polymerase chain reaction

Total cellular RNA was extracted from cultured cells and frozen stored tissues using TRizol reagent (Invitrogen). Reverse transcription of RNA (3 μg) from each sample was performed with random hexamers and avian myeloblastosis virus reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The E-cadherin cDNA was subjected to PCR amplification with 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. The amplification products were separated on 2% agarose gels and visualized by ethidium bromide staining. The housekeeping gene β-actin was amplified as an internal control. The gene-specific primer sequences were F: 5′-TTAAGGGGCTGCTATGGGAAGT-3′ and R: 5′-GTGTAACCGATGCGCCATTGTGA-3′ for E-cadherin and F: 5′-GAACCCCAAGGCCAACGGGAGA-3′ and R: 5′-TGACCCCGTACCGGAGTCCATC-3′ for β-actin.

Western blot analysis

Approximately 30 μg of total protein from each sample was separated on a 12% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) and incubated overnight in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA, Sigma) at 4°C. The membrane was briefly washed with 0.1% Tween 20 (Sigma) in PBS and incubated with rabbit polyclonal E-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or LMP1 antibody (Dako Cytomation, Glostrup, Denmark) at a dilution of 1:1000. The membrane was washed again and incubated in goat anti-rabbit horseradish peroxidase-conjugated IgG (Amersham, Arlington Heights, IL) at a dilution of 1:5000. The expression of each target protein was detected using the enhanced ECL kit (Amersham). To verify equal loading of the samples, the same membrane was stripped using 0.2 M NaOH for 5 min and incubated with mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham).

DNA extraction and sodium bisulphite treatment

DNA was extracted from cell lines using QIAamp DNA mini kit (Qiagen, Hilden, Germany), and 1 μg of genomic DNA from each sample was modified by sodium bisulphite using the EpiTect Bisulfite Kit (Qiagen), according to the manufacturer’s instructions.

Methylation analysis

The methylation status of the E-cadherin gene was investigated by subjecting the modified DNA to methylation-specific PCR (MSP) and bisulphite genomic sequencing (BGS). MSP was performed with 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s after an initial denaturation at 94°C for 3 min. MSP primer sequences were F: 5′-TAATTTTAGGTTAGGTTATCGC-3′ and R: 5′-CTCACAAATACTTTAACAATCCGC-3′ for methylated E-cadherin and F: 5′-ATTTCAGGTAGGGTTATTTG-3′ and R: 5′-CAACATCACAATATTCTTAACAATCCA-3′ for unmethylated E-cadherin. For BGS, the region within the CpG islands of the E-cadherin gene ranging from –416 to +15 bp with respect to the ATG site was amplified from bisulphite-converted DNA. Amplification was carried out with 35 cycles of 94°C denaturation for 1 min, 51.5°C of annealing for 30 s, and 72°C of extension for 30 s, followed by a final 72°C extension for 7 min. Primer sequences for BGS were F: 5′-AGTGTAAGGTTTTTTTGGTTTTTA-3′ and R: 5′-ACTCCAAAAACCCATAACTAAACCACCC-3′. The purified PCR product was cloned into a pGEM-T vector using a TA cloning kit (Invitrogen). The sequence of the E-cadherin promoter was determined in four independent colonies. The average methylation level was examined by determining the number of methylated cytosine residues.

In vitro invasion assay

The cells were treated with 1 μM/L of As2O3 or 20 μM/L of 5-aza-dC for 48 h. The invasion assays were performed using Falcon cell culture inserts (pore size, 8.0 μm; Becton Dickinson). The chambers were set in a 6-well plate. The upper layer of the culture insert was then coated with 750 μg Matrigel, a reconstituted extracellular matrix (Becton Dickinson). Cells were

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Materials and methods (Cont.)

seeded at a density of 2 × 10⁴ cells/well into the upper layer of the culture insert and cultured with serum-free RPMI 1640. Then, 1 mL of culture medium supplemented with 0.1% BSA and 250 μg solubilized Matrigel was placed in the lower layer of the culture insert as a chemoattractant. After the cells were incubated for 24 h, the remaining cells in the upper layer were swabbed with cotton, while the penetrating cells in the lower layer were fixed with 95% ethanol and removed for haematoxylin staining. Cells passing through the Matrigel matrix and each 8-μm pore of the culture insert were counted using light microscopy. Ten fields per well were randomly selected and counted. The inhibition rate (IR) was calculated as a formula: IR (%) = (number of penetrating cells in the negative control group – number of penetrating cells in the test group)/number of cells in the penetrating negative control group × 100%.

Statistical analysis

Results were expressed as the means ± standard deviations. Data were analysed using the Student’s t-test. A P value of less than 0.05 was considered statistically significant.

Results

Reduced E-cadherin expression in LMP1-transfected cells

Loss of E-cadherin expression has been associated with cancer metastasis. We examined whether the ectopic transfection of the LMP1 gene contributes to the decreased expression of E-cadherin. Using Western blot analysis, we observed that the E-cadherin protein was significantly downregulated in stably transfected HNE1-LMP1 cells in comparison with that in the parental HNE1 cells (Figure 1). These cell lines with differential expression levels of E-cadherin were employed for subsequent analyses to define the mechanism underlying the inactivation of E-cadherin.

Downregulation of E-cadherin correlates with promoter methylation in NPC cell lines

Because DNA methylation is a major mechanism in the regulation of E-cadherin expression, MSP analysis was performed to determine whether the promoter methylation status is associated with reduced E-cadherin expression in HNE1-LMP1 cells. We observed that both methylated and unmethylated alleles occurred in HNE-1 cells, but only methylated allele was detected in HNE1-LMP1 cells (Figure 2).

Detailed profile of E-cadherin promoter methylation status

To acquire accurate information of E-cadherin methylation, we performed a high-resolution analysis of methylation status in the 5′ CpG islands of its promoter region. The CpG islands located on the 5′ end of the E-cadherin gene harbour 33 CpG sites (Figure 3A), in keeping with the published sequence from NCBI. A 431 bp region surrounding E-cadherin CpG islands was amplified with bisulphite-modified DNA from NPC cells, and the desired fragments were sequenced. Indeed, in comparison with HNE1 cells, a majority of the CpG dinucleotides within the examined region was hypermethylated in HEN1-LMP1 cells (Figure 3B).

5-Aza-dC restored E-cadherin expression in NPC cell lines

To examine whether methylation of the promoter was directly responsible for E-cadherin downregulation, HNE1 and HNE1-LMP1 cells were treated with 20 μM/L of 5-aza-dC, a demethylating agent. Unmethylated E-cadherin alleles increased after 5-aza-dC treatment, as determined by MSP (Figure 4). Moreover, the explicit increase of E-cadherin mRNA level was also observed in HNE1-LMP1 cells after 5-aza-dC treatment. These data suggest that downregulation of E-cadherin expression is due to methylation of the gene promoter in HNE1-LMP1 cells.

Figure 1: Western blot analysis of E-cadherin in HNE1-LMP1 and HNE1 cells. E-cadherin protein was significantly downregulated in stably transfected HNE1-LMP1 cells compared with that in the parental HNE1 cells.

Figure 2: MSP results of E-cadherin promoter methylation in HNE1 and HNE1-LMP1 cells. M, methylated; U, unmethylated.

Figure 3: E-cadherin promoter methylation status. (A) Scheme of the E-cadherin promoter region. Thirty-three CpG sites within the E-cadherin gene promoter were included for analysis using bisulphite sequencing. Vertical lines indicate the relative locations of CpG sites to the start codon ATG, which is indicated as ‘+1’. (B) Bisulphite sequencing of CpG islands in the E-cadherin promoter in HNE1 and HNE1-LMP1 cell lines. PCR products were subsequently subcloned into a sequencing vector, and 4–5 clones were sequenced for each sample. Every row represents a single clone. Open circle, unmethylated CpG; solid circle, methylated CpG.

Figure 4: E-cadherin mRNA level in HNE1-LMP1 cells treated with 5-aza-dC. Data are expressed as the mean ± standard deviation. *P < 0.05 compared with HNE1-LMP1 cells without 5-aza-dC treatment.

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Results (cont.)
Arsenic trioxide restored E-cadherin expression in HNE1-LMP1 cells
We then determined whether altered E-cadherin expression occurs after As$_2$O$_3$ treatment. There was no difference in E-cadherin mRNA and protein expression levels before and after As$_2$O$_3$ treatment, as demonstrated by RT-PCR in HNE1 cells (Figures 5A and B). However, E-cadherin mRNA and protein expression levels were significantly upregulated in stably transfected HNE1-LMP1 cells, especially when exposed to As$_2$O$_3$ at a concentration of 1 μM/L (Figures 6A and B).

Figure 4: 5-Aza-dC restored E-cadherin expression. (A) MSP analysis of E-cadherin methylation status in HNE1-LMP1 and control HNE1 cells without 5-aza-dC treatment. M, methylated; U, unmethylated. (B) MSP analysis of E-cadherin methylation status in HNE1-LMP1 and control HNE1 cells with 5-aza-dC treatment. (C) Recovered expression of E-cadherin mRNA by RT-PCR after treatment with 5-aza-dC. HNE1 and HNE1-LMP1 cells were treated with 5-aza-dC for 48 h.

Figure 5: Expression of E-cadherin in HNE1 cells after As$_2$O$_3$ treatment at various concentrations for 48 h. Normal nasopharyngeal tissue was used as a positive control and β-actin as an internal loading control. (A) mRNA level of E-cadherin detected using RT-PCR. (B) Protein level of E-cadherin detected using Western blot analysis.

Restoration of E-cadherin by As$_2$O$_3$ treatment through repressing LMP1 expression
Because stable LMP1 expression is associated with reduced E-cadherin, we next investigated whether As$_2$O$_3$ treatment could restore E-cadherin expression through demethylation of the CpG islands within the gene promoter region. Notably, LMP1 protein was significantly reduced in HNE1-LMP1 cells after As$_2$O$_3$ treatment at 1 μM/L for 48 h (Figure 7A). To confirm the demethylating function of As$_2$O$_3$ in the recovering E-cadherin expression, we performed MSP analysis using bisulphite-modified DNA extracted from HNE1-LMP1 cells treated by As$_2$O$_3$. We observed the occurrence of unmethylated E-cadherin alleles after As$_2$O$_3$ treatment (Figure 7B). Our results suggest that As$_2$O$_3$ similar to 5-aza-dC, may demethylate and reactivate the E-cadherin gene.

Metastasis-suppressing capability of As$_2$O$_3$
The anti-metastatic role of As$_2$O$_3$ was studied using invasion assays in vitro. The drug-treated cells were placed on a Matrigel layer to evaluate their invasion capability. The results revealed that As$_2$O$_3$, at a concentration of 1 μM/L, could reduce cell invasion of the NPC cell lines (Figure 8). However, the metastasis-suppressing capability of As$_2$O$_3$ was stronger in HNE1-LMP1 cells than in the HNE1 cells, with an IR of 49.50% ± 1.6% and 26.11% ± 1.2%, respectively ($P < 0.0001$). The data indicated that NPC cells expressing ectopic LMP1 protein are more susceptible to suppression by As$_2$O$_3$ treatment.

Figure 6: Expression of E-cadherin in HNE1-LMP1 cells after As$_2$O$_3$ treatment at various concentrations for 48 h. Normal nasopharyngeal tissue was used as a positive control and β-actin as an internal loading control. (A) mRNA level of E-cadherin detected using RT-PCR. (B) Protein level of E-cadherin detected using Western blot analysis.

Figure 7: Expression of E-cadherin in HNE1-LMP1 cells after As$_2$O$_3$ treatment at various concentrations for 48 h. Normal nasopharyngeal tissue was used as a positive control and β-actin as an internal loading control. (A) Western blot analysis of LMP1 in HNE1-LMP1 cells treated with As$_2$O$_3$ at 1 μM/L for 48 h. (B) MSP analysis of E-cadherin methylation status in HNE1-LMP1 cells and HNE1 cells treated with As$_2$O$_3$ at 1 μM/L for 48 h. M, methylated; U, unmethylated.

Figure 8: Effect of 1 μM/L As$_2$O$_3$ treatment on the invasive potential of cells. The assay was performed using Matrigel as reconstituted extracellular matrix in Falcon cell culture inserts in vitro. The small rings are 8-μm membrane pores of the Falcon cell culture inserts indicated by a solid arrow. The invasive cells indicated by an open
Results (cont.)
arrow were randomly selected and counted at least in 10 fields per insert. (A) Penetrating HNE1-LMP1 cells in the negative control. (B) Penetrating HNE1-LMP1 cells in the As$_2$O$_3$ treated group. (C) Penetrating HNE1 cells in the negative control. (D) Penetrating HNE1 cells in the As$_2$O$_3$-treated group.

Discussion
We found that As$_2$O$_3$ reverses LMP1-mediated methylation and silencing of E-cadherin in LMP1-expressing NPC cells, as well as inhibits cell invasion and migration in vitro. The underlying mechanism by which As$_2$O$_3$ restores promoter activity and protein expression of the E-cadherin gene in human NPC cells involves initiation of the cellular DNA demethylation machinery by As$_2$O$_3$. NPC has highly invasive and metastatic properties compared with other types of head and neck cancers. Approximately 90% of NPC patients show cervical lymph node metastases at the time of diagnosis. However, the molecular events leading to high invasiveness and metastasis of NPC are yet to be well understood. It is well accepted that EBV is a major aetiological agent for cancer formation and progression. LMP1 is an EBV latent gene-encoded oncoprotein that transforms rodent cells and enhances cell migration, which in turn represses E-cadherin expression. We have reported that single doses of 5 mg/kg As$_2$O$_3$ cause apoptosis and differentiation in NPC xenografts. Moreover, As$_2$O$_3$ can inhibit LMP1 expression. In the current study, we observed that As$_2$O$_3$ can restore the expression of the E-cadherin gene, which is silenced by LMP1-induced methylation. We also found that the invasive behaviour of LMP1-overexpressing NPC cells can be inhibited by As$_2$O$_3$. Therefore, the current study has extended our previous findings of the inhibitory effects of As$_2$O$_3$ on NPC by demonstrating the role of As$_2$O$_3$ in the re-expression of the E-cadherin gene and its relationship with the changes in the metastasis potential of NPC cells. As detected by MSP, downregulation of E-cadherin due to methylation was detected only in LMP1-expressing cells, but not in the parental cells. A high resolution analysis of the methylation status by sequencing the 5′ CpG islands of the E-cadherin gene promoter region provided more accurate information. BGS results clearly revealed a detailed map of the distribution of CpG methylation within the E-cadherin promoter region. The CpG islands were significantly heavily methylated in LMP1-transfected cells, in comparison with those in the parental cells, which only showed minor existence of methylated CpG sites. The application of 5-aza-dC treatment in LMP1-expressing cells resulted in demethylation and reactivation of methylation-silenced E-cadherin, confirming that LMP1-mediated methylation downregulates E-cadherin expression. Arsenic has been shown to induce DNA hypomethylation by continuous methyl depletion through consuming S-adenosylmethionine. A recent study showed that low concentrations of As$_2$O$_3$ could restore the expression of p16INK4a, RASSF1A, E-cadherin and GSTP1 in hepatocellular carcinoma cell lines. Sodium arsenite, another form of arsenic, suppresses DAPK gene expression by promoter hypermethylation. Collectively, the biological effects of arsenic on gene methylation are complicated. Here, we have shown that methylation-mediated silencing of E-cadherin could be reactivated by As$_2$O$_3$ treatment, concomitantly with reduced LMP1 expression. We also administrated 5-aza-dC, a demethylating agent, to LMP1-expressing cells and compared its effects with those of As$_2$O$_3$. The results obtained imply that As$_2$O$_3$ and 5-aza-dC have similar effects on demethylation and restoration of the E-cadherin gene. Because LMP1 downregulates E-cadherin gene expression and induces cell migration via DNA methylation machinery, we infer that As$_2$O$_3$ treatment induces upregulation of E-cadherin expression through repressing LMP1 in NPC cells. Taken together, our data suggests that the anti-metastasis activity of As$_2$O$_3$ restores the functional expression of E-cadherin. This finding indicates a novel molecular mechanism underlying the anti-cancer effects of As$_2$O$_3$, which could be employed as a potential demethylating agent for NPC therapy.

Abbreviations list

As$_2$O$_3$, arsenic trioxide; EBV, Epstein–Barr virus; IR, inhibition rate; LMP-1, latent membrane protein 1; MSP, methylation-specific polymerase chain reaction; NPC, nasopharyngeal carcinoma.

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