Arsenic trioxide induces cervical cancer apoptosis, but specifically targets human papillomavirus-infected cell populations

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Objectives **Human papillomavirus (HPV) is directly associated with the occurrence and development of cervical cancer. Targeting HPV infection has become the priority in treatment and prevention. Some treatment strategies have shown a limited therapeutic potential in suppressing and reversing the oncogenic effects of HPVs, but are compromised by the toxicity, immune suppression and the expense. Arsenic trioxide (As_2O_3) has shown therapeutic efficacy in the treatment of haematological and solid cancer and has been demonstrated to effectively induce apoptosis of HPV-infected cervical cancer cells *in vitro*. Here, we examined the effects and possible molecular pathway of As_2O_3-induced apoptosis in HPV-infected and noninfected cervical cancer cells.**

Methods **As_2O_3 was added to HPV-infected cell lines HeLa and CaSki and the HPV-negative cell line C33A at concentrations from 1 to 10 μmol/l. Cell proliferation rates were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays after exposure. Expressions of tumour suppressor gene p53, activated caspase-3 and cell cycle distribution were evaluated in relation to HPV-E6 protein expression by confocal microscopy immunofluorescent staining and flow cytometry.**

Results **As_2O_3 reduced cell populations by 16% in C33A but by 48–60% in HPV-infected cell lines CaSki and HeLa. The expression of HPV-E6 proteins was drastically down-regulated in a dose-dependent manner, whereas p53 and activated caspase-3 expressions increased in the HPV-infected cell lines. Flow cytometry demonstrated cell cycle arrest in S-G2/M phases, and increasing apoptotic bodies were seen in HPV-infected lines only.**

Conclusion **As_2O_3, at low concentrations, inhibited HPV-E6 protein expression, leading to up-regulated p53 levels, induced S to G2/M arrest and apoptosis. *Anti-Cancer Drugs* 23:280–287 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: arsenic trioxide, caspase-3, cell cycle arrest, flow cytometry, human papillomavirus-E6, p53

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Introduction

Cervical cancer is one of most common cancers affecting women worldwide [1]. Human papillomavirus (HPV) infection has now been established as the key causative agent for the development of cervical cancer. Furthermore, studies have also suggested that HPV infection may play a leading role in the pathogenesis of other carcinomas, such as urogenital tumours [2], upper respiratory–digestive tract cancers [3] and different human organ sites [4].

HPV are a group of small nonenveloped DNA viruses that usually cause benign lesions, but are now clearly associated with malignancy, especially cervical cancers. HPV replicates and assembles within the nucleus and infects the basal layer of the stratified squamous epithelium. After infection, viral gene expression is led by six nonstructural viral regulatory proteins (early expression region of the HPV genome), E1, E2, E4, E5, E6, E7, and two structural regulatory proteins from the late region of the viral genome, L1 and L2 [1]. Each genome protein has different roles in the infected cells, but the expression of E6 and E7 can induce cell immortalization and transformation. In particular, they inactivate the tumour suppressor cell cycle regulator proteins, p53 and pRb [5]. Many subtypes of HPV have been recognized and further categorized into high-risk and low-risk types according to their relationship to involvement with tumours. There are now 15 high-risk HPVs, which have been described and which accounted for 100% of HPV-attributable cancers [6]. Among them, HPV 16 and HPV 18 are the subtypes most frequently associated with cancers, responsible for more than 80% of the total HPV-related cancers [6].

Thus, targeting HPV infection has become the priority goal in treating and managing many cancers. Treatment

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strategies include introducing RNA interference nucleotide analogues, antioxidants and herbal derivatives, which have shown some effective therapeutic potential, but there have been considerable disadvantages such as toxicity, immune system suppression and high cost [6].

Arsenic agents have long been known to act as a carcinogen at high levels, leading to skin, lung, kidney and urinary tract cancers [7]. Conversely, at low levels, they have also been demonstrated to have anticancer activity. In traditional Chinese medicine, arsenic trioxide (As_2O_3) was recorded in the Compendium of Materia Medica, by Mr Li Shi-Zhen, as of therapeutic benefit in 1518. Later, arsenous oxide (Fowler's solution) was also used as the treatment choice of chronic myeloid leukaemia in the 19th century. Because of the toxic side effects and the introduction of modern radiotherapy and chemotherapy, the use of arsenic as a treatment for chronic myeloid leukaemia was abandoned by Western medicine [8]. However, recently, its therapeutic effect on acute promyelocytic leukaemia [9] has initiated a re-awakening of interest in arsenic compound in medicines. The actions of As_2O_3 on other cancers have now been investigated extensively at both molecular/cellular and clinical levels [10–13]. As_2O_3 has now been reported to induce tumour cell apoptosis, to inhibit tumour cell proliferation and be an antiangiogenic agent. A study by Zheng *et al.* [10] indicated that As_2O_3 can induce apoptosis in HPV-16-infected cervical epithelial cells by down-regulating HPV-E6 expression.

Our current in-vitro study set out to address whether As_2O_3 has any therapeutic effect on cervical cancer cells and whether these effects are associated with the HPV status of these cancer cells.

Materials and methods

Cell lines and cultured conditions

Three cervical cancer cell lines, CaSki (HPV 16 positive = more than 200 copies per cell), HeLa (HPV 18 positive = 10 copies per cell) and C33A (HPV negative), were purchased from the American Type Culture Collection (UK). These were cultured routinely in RPMI 1640 medium (Sigma, Dorset, UK) supplemented with 10% foetal calf serum (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma), at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Media were changed regularly every 2 days, and cell samples were collected when the cells were at the logarithmic growth phase.

MTT assays

As_2O_3-containing media were prepared freshly at concentrations ranging from 1 to 50 µmol/l As_2O_3 (Sigma – stock solution 50 mmol/l As_2O_3 in sterile PBS). Confluent cell cultures were washed with PBS, and then trypsinized, counted and seeded into 96-well plates (Costa, New York, New York, USA) at 5×10^3/100 µl per well in triplicates. After 24 h settlement, spent media were replaced by complete medium containing As_2O_3, at concentrations of 0, 1, 2, 5 and 10 µmol/l in triplicates. Cells were observed every 12 h; cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Promega, Madison, Wisconsin, USA) assay after 48 h.

The absorbance of each well was read at 490 nm using a microplate reader (BMG Labtech, Ortenberg, Germany). The MTT assays were repeated three times, and intra-correlation of variance (CV) and inter-CV of the absorbance were at 6.8 and 8.5%, respectively.

In order to render repeat experiments from different plates and different days comparable, all MTT absorbances were normalized as a percentage of the absorbance reading averaged for the nontreated control wells on the 96-well assay plate. Thus, all data are expressed as a percentage absorbance at optical density 490 nm of the control, and SDs are the percentage of control variance when experiments were repeated ($n \geq 5$).

Double fluorescent staining

Cyanine 5 labelling HPV-E6 protein

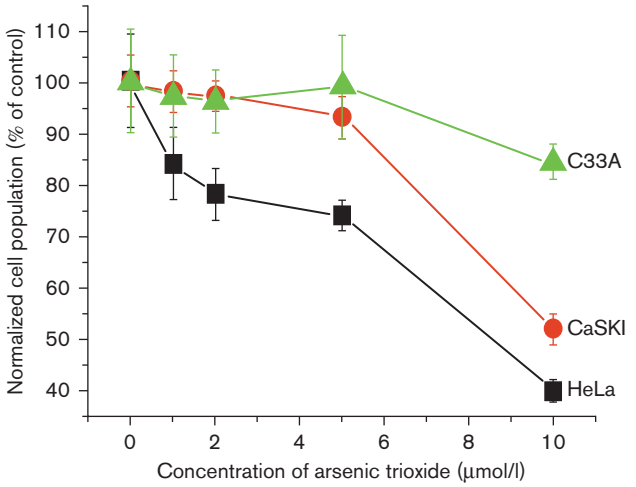
The three cervical cancer cell lines were seeded at 5×10^4/ml on cover slips in six-well plates and grown for 24 h before As_2O_3 treatment. As_2O_3-containing media were prepared as described and filtered through a 0.22-µm-diameter filter. The cells were exposed to different concentrations of As_2O_3 solution (0, 1, 2, 5 µmol/l) for up to 48 h after 24 h cell settlement. Cells were then fixed with 4% paraformaldehyde (Sigma) in PBS for 10 min before staining.

After fixation, cells were permeablized by 0.2% Triton-100 (Sigma), followed by 50% horse serum (Sigma) to block unspecific binding. The first primary antibody, monoclonal anti-HPV16 E6/HPV18 E6 (Santa Cruz Biotechnology, Heidelberg, Germany), at one in 100 dilution was then applied for 90 min at room temperature, followed by 45 and 30 min of second and third antibodies using ABC universal Kit (Vector Lab, Peterborough, UK). Afterwards, the tyramide signal amplification reagent conjugated by the fluorophore cyanine 5 (PerkinElmer, Waltham, Massachusetts, USA) was applied to detect any horseradish peroxidase-conjugated antibody binding to HPV-E6 protein.

Fluorescein isothiocyanate labelling p53 and active caspase-3 staining

After Cys5 labelling/detection of HPV-E6 protein, as above, goat serum (Sigma,) was applied to block unspecific binding sites; a polyclonal rabbit antibody anti-p53 (Invitrogen, Paisley, UK) was applied at 1 in 100 dilution with PBS and incubated overnight in dark at 4°C. FITC-labelled HRP-conjugated anti-rabbit IgG (Sigma) was added on the next day. Finally, 300 nmol/l 4′,6-diamidino-2-phenylindole

Fig. 1



MTT cell proliferation assay: cell numbers after incubation with increasing concentrations of As_2O_3 (0–10 μmol/l) were determined by the MTT assay, whereby the optical density of the reduced tetrazolium salt for test samples was expressed as a percentage of that obtained for untreated controls (normalized) for cell lines C33A (green), CaSki (red) and HeLa (black). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

(Sigma) solution were applied before mounting in Immunomount (Shandon, Pittsburgh, Pennsylvania, USA).

A similar approach was carried out by labelling HPV-E6 using tyramide signal amplification reagent conjugated by fluorophore cyanine 5 reagent, as described above; however, instead of an anti-p53 antibody, a monoclonal rabbit anti-human active caspase-3 (Abcam, Cambridge, UK).

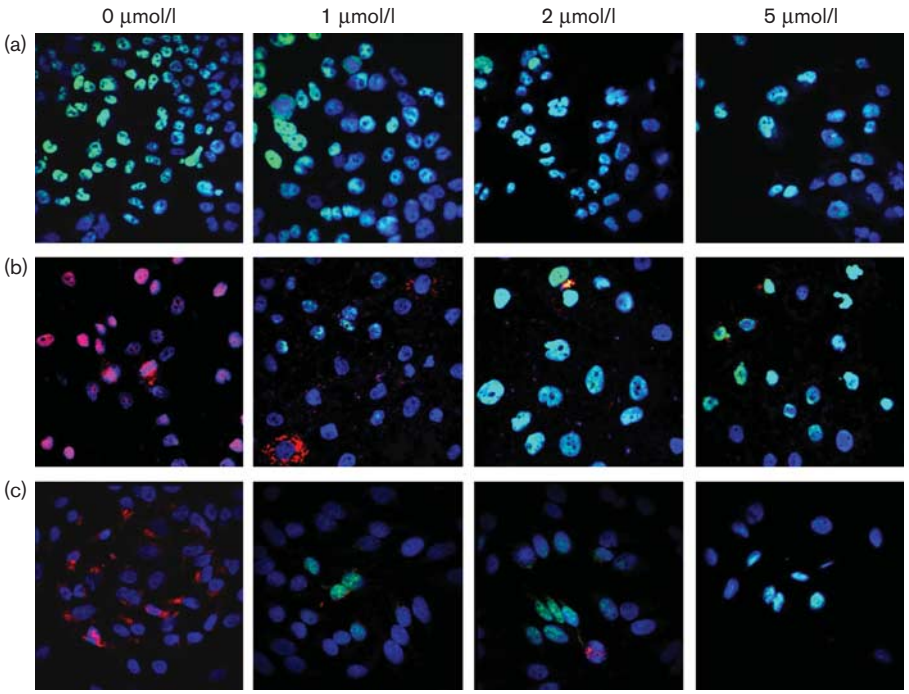
Confocal fluorescence microscopy examination

A confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to capture the images of double fluorescent staining: HPV-E6 (in red) and caspase-3 (in green), or p53 (in green) and counter-stained by 6-diamidino-2-phenylindole (blue nuclei). The images were taken within 2 h after the staining, and combined images are shown in Figs 1 and 2 (double-stained HPV/p53 and double-stained HPV/caspase-3 for C33A, CaSki and HeLa cells, respectively).

p53-fluorescein isothiocyanate assay by flow cytometry analysis

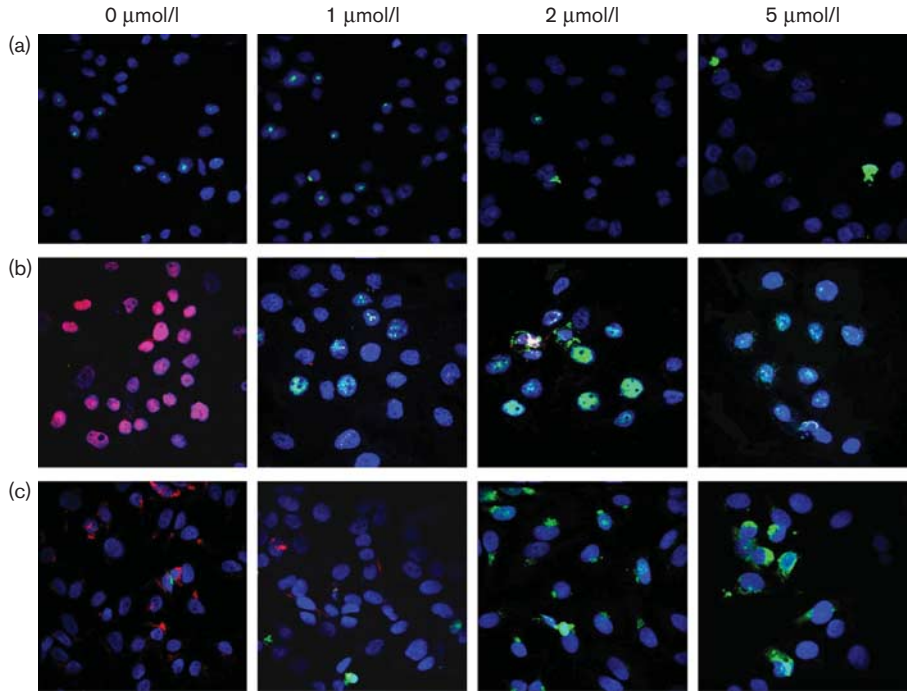
Cell lines were seeded at 1×10^5 cells/ml/well in six-well culture plates. After overnight settlement, As_2O_3 (0, 1, 2,

Fig. 2



Confocal microscopic examination of human papillomavirus-E6 (red) and p53 (green) for cell lines C33A (a), CaSki (b) and HeLa (c) in response to 48 h exposure to As_2O_3 at 0, 1, 2 and 5 μmol/l in culture media. Cells were counter-stained with DAPI (blue) to reveal the nuclear/DNA location. DAPI, 6-diamidino-2-phenylindole.

Fig. 3



Confocal microscopic examination of HPV-E6 (red) and caspase-3 (green) by cell lines C33A (a), CaSki (b) and HeLa (c) in response to 48 h exposure to As_2O_3 at 0, 1, 2 and 5 μmol/l in culture media. Cells were counter-stained with DAPI (blue) to reveal the nuclear/DNA location. DAPI, 6-diamidino-2-phenylindole.

5 μmol/l) was added in fresh complete media and cultured for a further 48 h.

Trypsinized cells were harvested and centrifuged at 4°C at 1500 rpm for 5 min. The supernatant was discarded and washed in cold PBS twice. Cells were then incubated with 20 μl of FITC-labelled anti-p53, or 20 μl mouse IgG1 isotype control (BD Pharmingen, San Diego, California, USA) and 480 μl staining solution (BD Pharmingen) for 30 min at 25°C avoiding light. 2 μmol/l propidium iodide (PI) was added (Sigma) before analysing the samples by a fluorescence-activated cell-sorting system (FACS calibur; BD Biosciences, Oxford, UK) within 1 h. The Cellquest 7.0 software (BD Biosciences, San Jose, California, USA) was applied and means were calculated by statistical analysis provided by the software.

Cell cycle distribution by flow cytometry

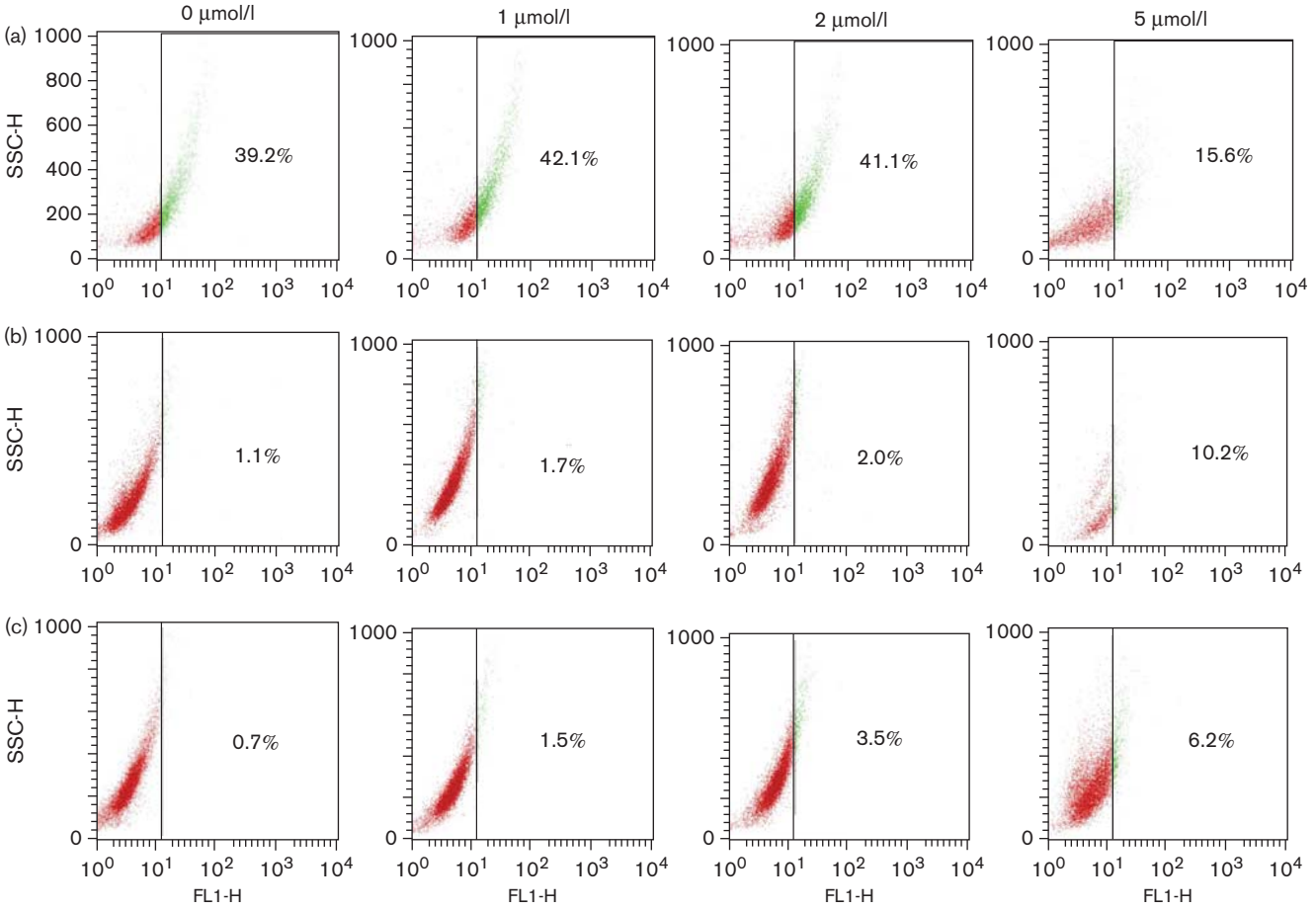
Different stages of cell cycle distribution were determined by staining the DNA with PI. In brief, 1×10^6 cells were pelleted, washed in PBS and resuspended in ice-cold 70% ethanol. After overnight fixation at 4°C in 70% ethnol, they were washed in cold PBS, and then treated with 1 mg/ml RNase A (Sigma) for 30 min at 37°C. One millilitre PI (10 μg/ml) was then added to each tube for 30 min at room temperature. The DNA content was then analysed by flow cytometry (FACS Calibur; BD Biosciences). The distribution of cell cycle was analysed by Cellquest 7.0 software, which was used to determine the percentage of cells in each phase of the cell cycle, sub-G1, G0/G1, S and G2/M. CVs and means were calculated by statistical analysis provided by the software.

Results

A cell viability assay (MTT assay, see Fig. 1) indicated that As_2O_3 was particularly inhibitory to the HPV-infected cell lines CaSki and HeLa, reducing the cell population by 48 and 60%, respectively in a dose-dependent manner. The effect on the non-HPV-infected cervical cancer cell line C33A was much less pronounced, with only a 16% reduction in cell numbers at the highest concentration used (10 μmol/l).

Double fluorescent staining of HPV-E6 protein with p53 protein demonstrated that we could not detect HPV-E6 protein in the non-HPV-infected cell line C33A cells. However, p53 (known to be mutated) was expressed at a high level and, essentially, did not change with increasing concentration of As_2O_3, except at 5 μmol/l, at which point levels appeared to decrease (Figs 2a and 3a). In both HPV-infected cell lines (CaSki and HeLa), HPV-E6 protein was detected and at high levels. Upon exposure to As_2O_3, the levels reduced drastically and appeared to become extranuclear before disappearing from cells (Figs 2b and 3b). Concomitant with the reduction of

Fig. 4



Flow cytometry analysis to show p53 positively expressed cell populations (labelled by FITC shown in FL1) for C33A (a), CaSki (b) and HeLa (c) cells. The percentages of gated p53 positively expressed cell populations after exposure to As_2O_3 at 0, 1, 2 and 5 μmol/l in culture media are indicated. FITC, fluorescein isothiocyanate.

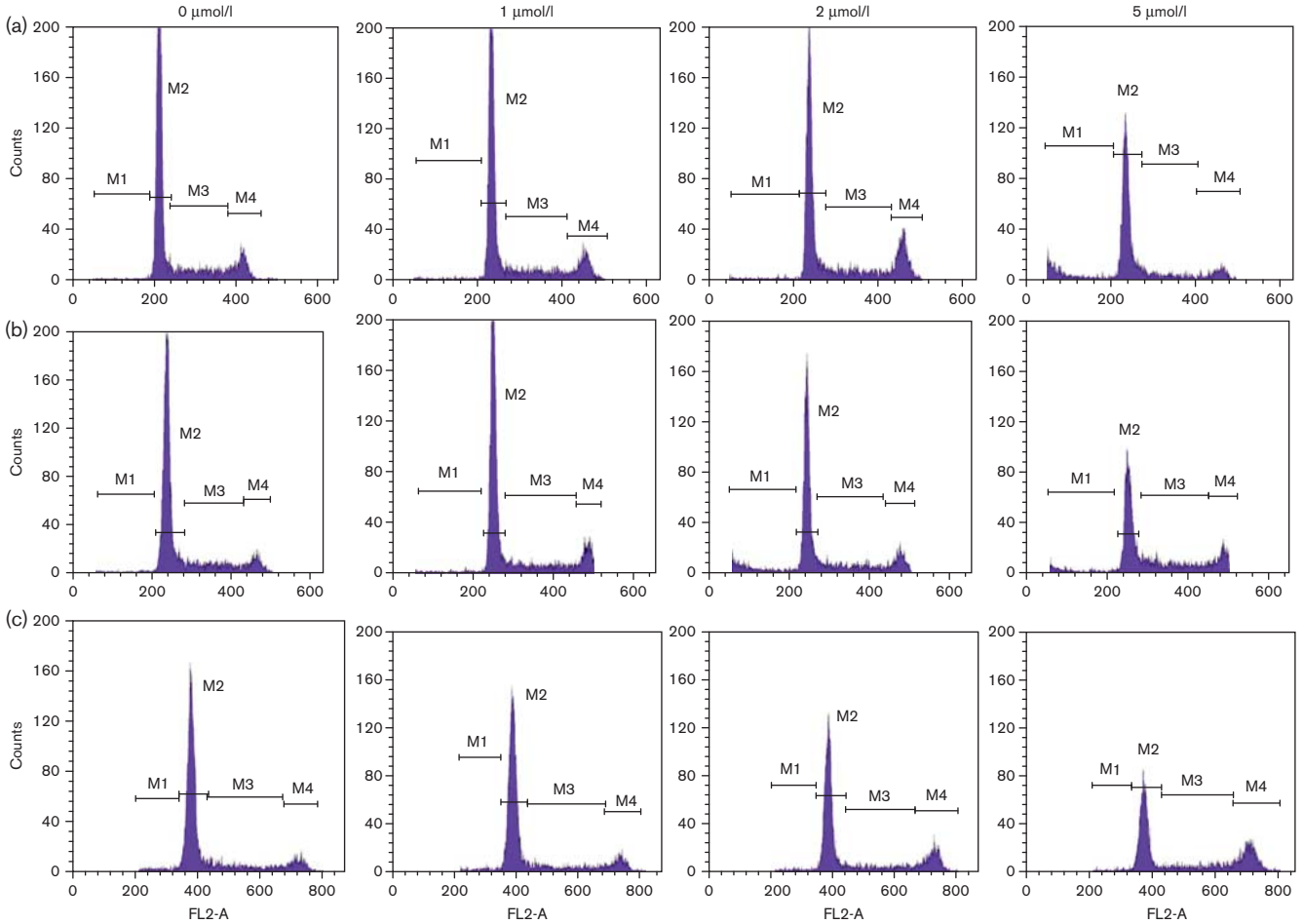
HPV-E6 detection, a visible and drastic increase in p53 protein was evident on confocal microscopic examination (Fig. 2b and c) and FACS analysis (Fig. 4b and c). Similarly, although C33A displayed little active caspase-3 expression and no induction of this protein after exposure to As_2O_3 (Fig. 3a), As_2O_3 increased the expression of active caspase-3 in both CaSki and HeLa cells in a dose-dependent manner, which paralleled a reciprocal decrease in HPV-E6 protein expression (see Fig. 3b and c).

Cell cycle analysis revealed that As_2O_3 had no effect on the passage through the cell cycle for the HPV-negative (p53 mutant) cell line C33A, only increasing the proportion of apoptotic bodies at higher concentrations (Fig. 5a). However, both CaSki and HeLa demonstrated a dose-dependent reduction in G0/G1 proportion and an increased cell proportion found in S and G2/M phases, which were concomitant with an increased proportion of apoptotic cells (see Fig. 5b and c and Table 1).

Discussion

HPV have been confirmed as a major cause of cervical cancer, especially high-risk types (HPV 16, 18, 31, 45) [14,15]. Both E6 and E7 are early genes of HPVs that play vital roles in viral replication and host cells immortalization and transformation [16,17]. E6 exerts its effects by binding, inactivating and then degrading the tumour suppressor gene p53, whereas E7 binds to and inactivates gene pRb [18–22]. Although designed to prevent the arrest of DNA synthesis and apoptosis, these HPV proteins are key potentiators of the oncogenic process in anogenital cancer: inactivating p53, inhibiting apoptosis permitting unchecked cell proliferation and loss of epithelial cell adhesion [23]. E6 is particularly important in this respect and along with binding p53, it also binds efficiently to double-stranded DNA and might function as a transcriptional regulator of other key oncogenic regulators [24,25]. Any strategies that can control or inhibit E6 protein expression would be desirable for use

Fig. 5



Analysis of DNA cell cycle phase distribution by flow cytometry: As_2O_3 at 0, 1, 2 and 5 μmol/l in culture media was added to C33A (a), CaSki (b) and HeLa (c), fixed and stained with propidium iodide before being subjected to flow cytometry and cell cycle determined. The gated proportion was assigned as M1-sub-G1, M2-G0/G1, M3-S phase and M4-G2/M.

as a therapeutic tool to control HPV-associated cancers, such as cervical cancers.

Although clinically the introduction of anti-HPV vaccination is considered to be a major preventative measure in the management of cervical carcinoma, developed countries still have to fully realize the benefit of this in the reduction of cases [26]. In other developing and third-world countries, cervical cancer will remain a major cause of female cancer-related death for many years [27]. Furthermore, posttreatment relapse in cervical cancers is common and is harder to deal with because of a paucity of second-line therapies. Conventional strategies to treat cervical cancers (surgery, radiotherapy and conventional toxic chemotherapy) have now been supplemented with more targeted molecular therapies such as humanized antibodies, RNA interference nucleotide analogues and viral oncogene antisense constructs [6]. In combination, there is still a need to develop new chemotherapeutic strategies. These can be either second-line or first-line, low-toxicity and low-morbidity therapies, which allow for the conventional therapies to be rendered as the treatment of last resort. Much cheaper antioxidants and herbal derivatives have only achieved some success as adjuvants to conventional therapy by virtue of their low toxicity and nonimmune system suppression [6]. In this scenario, there is still a need to develop more effective and less destructive alternative remedies for HPV-associated cervical, and other, cancers.

As_2O_3 has been known as a carcinogen for many years; conversely, at low levels, it has also been recognized to have an anticancer effect. Its beneficial properties were first described in the treatment of haematological malignancies [28], and subsequently solid cancers [10,29–32]. It has been reported that As_2O_3 suppresses

Table 1 Frequency distribution of the stages of cell cycle estimated by the profile of the fluorescence activated cell sorter at different dose treatments of arsenic trioxide: percent of each stage was estimated by the analysis of 10 000 cells of C33A, CaSki and HeLa cell lines

ATO dosage	Concentration	1 μmol/l	2 μmol/l	5 μmol/l
Sub-G1 (%)				
C33A	1	2	3	16
CaSki	2	2	10	7
HeLa	3	4	4	3
G1 (%)				
C33A	68	67	60	62
CaSki	73	68	60	50
HeLa	72	70	61	49
S (%)				
C33A	18	18	18	13
CaSki	18	18	19	30
HeLa	16	16	17	21
G2/M (%)				
C33A	13	14	19	10
CaSki	9	11	12	15
HeLa	9	11	19	28

The data analysis used the Cellquest software.
Sub-G1 (%): the percentage of apoptotic cell population.
G1 (%): the percentage of cell population in the G1 phase.
S (%): the percentage of cell population in the DNA synthetic phase.
G2/M (%): the percentage of diploid population in the G2/M phase.
ATO, arsenic trioxide.

the growth of tumour cells by cell cycle arrest, induction of cyclin-dependent kinase inhibitors and apoptosis. This has been demonstrated for the myeloma cell line MC/CAR [33], the human leukaemia cell line NB4 [34], the cervical carcinoma cell line HeLa [35], and human pancreatic cancer cell lines HPAF, MiaPaCa-2 and PANC-1 [36]. However, most previous works have shown that As_2O_3 can induce various cancer cell apoptosis or cause G1 or G2/M arrest, the exact mechanism(s) of action is not clear.

We examined the effect of As_2O_3 on HPV-infected (HeLa-HPV18; CaSki-HPV16) and noninfected (C33A) cell lines. Although toxic to all three cell lines at low concentrations (< 10 μmol/l), the HPV-infected cell lines were much more sensitive. Most of the cancer cells were dead at 3 days' drug exposure. Previous work by Nakagawa *et al.* [37] also showed that low-dosage As_2O_3 (1–2 μmol/l) reduced cell growth and promoted cell apoptosis in colon cancer cells. In clinical studies, concentrations ranging from 1 to 5 μmol/l in blood demonstrate cancer cell-selective killing [38–40] without any severe clinical side effects in clinical trials [38,39].

The difference in sensitivity to As_2O_3 was investigated with respect to the detection of HPV-E6 protein and p53 protein. At ascending concentrations of 1, 2 and 5 μmol/l (48 h), HPV-E6 protein was visibly reduced if not eliminated completely in the infected cell lines HeLa and CaSki. This was paralleled by an increase in p53 protein expression (Fig. 2b and c) and active caspase-3 (Fig. 3b and c). This was matched in cell cycle studies with a reduction in the proportion of cells in G0/G1, an increase in S to G2/M phases and an increased fraction of sub G0/G1 apoptotic bodies (Fig. 5b and c).

These findings are consistent with the expected consequences of specific inhibition of HPV early gene (or specifically E6) expression: after viral integration, the HPV-E6 protein of high-risk types of HPV binds to and stimulates p53 degradation [20,22]. The degradation of the active and functional p53 contributes to the immortalized transformation of cervical cells overcoming its growth arrest and apoptosis-inducing functions [41,42]. By inhibiting constituently expressed E6, p53 accumulates, causing cell cycle arrest during S and G2/M phases and subsequently apoptosis, as seen in highly activated caspase-3 (Fig. 3b and c) and sub-G0/G1 apoptotic bodies on flow cytometry (Fig. 5b and c).

In the non-HPV-infected cervical cancer cell line C33A, p53 is still a core component target in the oncogenic profile, but has been mutated and accumulates as a nonfunctional protein. Upon exposure to low levels of As_2O_3 (1–2 μmol/l), we see no effects on the level of p53 (mutant) until higher concentration at 5 μmol/l, which showed a slight reduction (Fig. 4a). This may be attributable to a gross toxicity rather than any specific mechanism. Indeed we see no change in activated caspase-3 levels (Fig. 3a) or a change in the proportions of cells in G0/G1, S or G2/M phases, but a gradual increase in the sub-G0/G1 apoptotic portion (Fig. 5a).

Conclusion

Our studies here and previously published work have shown that As_2O_3 can induce apoptosis and inhibit the proliferation of various human cancer cells [10–13]. However, this study demonstrates that for cervical cancers, the mechanism by which As_2O_3 acts may depend on the HPV status of the tumour cell. Only one other study has suggested that As_2O_3 can target HPV-E6 protein and cause cell apoptosis [10], but unfortunately, they did not include a HPV-negative cell line for control.

The different responses between HPV-positive and HPV-negative cervical cell lines after As_2O_3 treatment suggest that there are two different therapeutic pathways of As_2O_3 action. In HPV-infected cells, this would appear to be through specific inhibition of E6 and possible other early expression HPV genes. The molecular consequences are a rapid resumption in active p53 and specific HPV-infected cell apoptosis. This may indicate a high degree of selectivity and sensitivity of HPV to low-concentration As_2O_3 therapies. Further investigation of the molecular mechanisms of As_2O_3 on other HPV infectious diseases and other HPV-attributable cancers is warranted.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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