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Original Paper

Induction of Apoptosis and Inhibition of Human Gastric Cancer MGC-803 Cell Growth by Arsenic Trioxide

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Arsenic trioxide (As_2O_3), used to treat human diseases for centuries in traditional Chinese medicine, has been identified as a very effective antileukaemic agent, but its effect on solid tumours which could be more suitable for clinical treatment with arsenic compounds is still unknown. In this study, we investigated the *in vitro* effect of As_2O_3 at concentrations of 0.01–1 μM against six human malignant cell lines, MGC-803, HIC, MCF-7, HeLa, BEL-7402 and A549 cells. As_2O_3 inhibited growth and induced apoptosis in these malignant cells at varying degrees, in a time dose-dependent manner. The most marked effects were seen in the gastric cancer cell line, MGC-803. In contrast, minimal growth inhibition and induction of apoptosis occurred in human embryonic pulmonary cells following treatment with As_2O_3 found at the same concentrations. Changes in intracellular Ca^{2+}, following As_2O_3 treatment were measured by Ca^{2+} sensitive fluorescent probe Indo-1/AM in flow cytometric assays. The increase in intracellular Ca^{2+} correlated with the sensitivity of these cells to As_2O_3, possibly indicating that a critical intracellular Ca^{2+} signal transduction pathway could be involved in As_2O_3-mediated cell-death and its selectivity. The marked sensitivity of MGC-803 cells *in vitro* suggests that As_2O_3 may be a potential antigastric cancer agent.

Key words: As_2O_3, MGC-803 cell, apoptosis, intracellular Ca^{2+}
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INTRODUCTION

FOR CENTURIES, arsenic trioxide (As_2O_3), present in numerous traditional Chinese medicines, has been used to treat several human diseases, such as anaemia, dyspepsia and some tumours [1]. Recently, As_2O_3 was identified as a very potent antileukaemic agent [2–4]. In China As_2O_3 is an effective treatment for acute promyelocytic leukaemia (APL) [2, 3] and studies *in vitro* have demonstrated that it relatively selectively inhibits growth and induces apoptosis of the APL cell line NB_4 at low-doses [4]. However, these observations not only indicate that ancient Chinese traditional remedies can provide new alternatives for modern treatment of tumours, but also raises the question of whether other tumours could also be sensitive to arsenic. Indeed, its effects on other tumours, in particular solid tumours and the sensitivity of different tissue cells including normal and malignant cells to As_2O_3 are still unknown. Arsenic has sustained a mysterious and quixotic public image for centuries. It is beneficial and harmful to humans, which depends on doses or concentrations of exposure [5]. Thus, it is an important issue to determine which tumours are sensitive to arsenic, especially to lower levels, and its mechanisms of action in order to reduce its toxicity and increase its anticancer effect.

It has been shown that tumours develop not only from abnormal cell proliferation and inhibition of differentiation, but also from reduced cell-death due to inhibition of apoptosis [6]. The possibility of modulating apoptosis of tumour cells suggests new strategies for improving chemotherapy, but actual cause and the precise mechanism of apoptotic death is not clear yet [7]. Recent evidence from a number of independent laboratories suggested intracellular Ca^{2+} could play an important role in the process of apoptosis [8]. Apoptosis also features in cytotoxicity of many chemical toxicants. We, therefore, hypothesise that As_2O_3 at lower levels may influence the induction of apoptosis of some solid tumour cells and homeostasis of intracellular Ca^{2+}.

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To investigate the effect of As_2O_3 on different tumours, we tested the effectiveness of As_2O_3 in several solid malignant cell lines, including MGC-803, BEL-7402, A549, MCF-7, HIC and HeLa, and examined the change in intracellular free Ca^{2+} in arsenic-induced apoptotic cells.

MATERIALS AND METHODS

Compounds

As_2O_3 was purchased from Sigma (St Louis, Missouri, U.S.A.; Lot A1010). 1 mM stock solution (in RMPI 1640 medium at 0–4°C storage) was prepared and diluted to a working concentration before use. All fluorescent probes were purchased from Molecular Probes Inc. (Eugene, Oregon, U.S.A.) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT) was obtained from Sigma.

Cell culture and viability assays

The following human cell lines were used: gastric cancer MGC-803, intestinal cancer HIC, breast carcinoma MCF-7, cervical carcinoma HeLa, liver carcinoma BEL-7402, lung cancer A549 and human embryonic pulmonary cells (HEP). These were obtained from Beijing Medical University and the Institute of Cancer Research Medical Academia Sinica (Beijing, China). The cells were maintained in RMPI 1640 medium (GIBCO-BRL, Grand Island, New York, U.S.A.), supplemented with 10% heat-inactived fetal bovine serum, 0.01% penicillin/streptomycin, and in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After seeding and culture for 24 h, final concentrations of 0.01–1 μM As_2O_3 were added to the culture medium. Cell viability after treatment with As_2O_3 for 12–48 h was assessed by the trypan blue exclusion test and the MTT assay. In As_2O_3-treated cultures, the number of excluded trypan blue cells was greater than 95%. Cell growth was measured using the MTT calorimetric dye reduction method [9]. In brief, eight replicate wells per time point were plated at a density of 1×10^4 cells in 0.2 ml of medium and data were expressed as the change in absorbance values at 560 nm over time relative to that obtained from the control group. Assays were repeated at least three times.

DNA gel electrophoresis

A total of 10^6 cells with or without As_2O_3 treatment were gently scraped from the dishes and washed twice in cold PBS. The pellets were collected by centrifugation and resuspended in 1 ml of buffer containing 150 nM NaCl, 10 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0 and 0.5% SDS. After thorough mixing, 20 μl of proteinase K (10 mg/ml) was added and incubated at 50°C for 2 h and cooled to 0°C, 2 ml of ethanol (at −20°C) was added to precipitate DNA, which was collected by centrifugation (1200*g*, 20 min), dried in air and dissolved in 50 μl of 10 mM Tris, 1 mM EDTA at pH 8.0(TE buffer). Each DNA preparation was mixed with 2 ml RNAase (10 mg/ml) and 6 μl of loading buffer (30% glycerol, 0.1% bromophenol blue) and incubated for 15 min at 37°C. Samples were then loaded on to 1.5% agarose gels and electrophoresed for approximately 3.5 h (2 V/cm gradient). A marker was obtained from Boehringer Mannhiem, Penzberg, Gemany. The gels were stained with ethidium bromide, and the DNA bands were visualised under ultraviolet light and photographed.

Cell morphology

Cell morphological changes with or without As_2O_3 treatment were observed by phase-contrast microscopy and photographed every 6 h. Furthermore, transmission electronic microscopy was also employed to detect morphological changes of the cells. Glutaraldehyde fixation was performed as previously described [10], followed by dehydration in graded series of ethanol. Embedding was performed in Epon and thin sections were double-stained with uranyl acetate and lead citrate.

Flow cytometric analysis of apoptosis

The cells treated with different concentrations of As_2O_3 and for a variety of time periods and controls were collected, centrifuged, washed in PBS, and then resuspended in 1 ml PBS. Hoechst33342 (Ho342, at a final concentration of 5 μg/ml) was added, then after 30 min at the room temperature (22°C), propidium iodide (PI) (at a final concentration of 5 μg/ml) was then added for 30 min. Samples (10^5 cells/ml) were directly analysed by flow cytometry (EPICS, ELITE; Coulter Inc., Miami, U.S.A.) with ultraviolet laser excitation (60 mW) at 340 nm and blue fluorescence (Ho342, 480 nm) and red fluorescence (PI, 600 nm) and the fluorescent ratios of 480/600 nm were measured. All data were collected, stored and analysed by the instrument software.

Flow cytometric analysis of intracellular Ca^{2+} concentration ($[Ca_i^{2+}]$)

$[Ca_i^{2+}]$ was analysed with the probe Indo-1/AM (cell permeates). The cells with or without As_2O_3-treatment were scraped, centrifuged and resuspended in PBS buffer (2×10^6 cells/ml). The probe was added to the cell suspension to obtain a final concentration of 10 μl (DMSO, final concentration = 0.002%, had no detectable effect on the cell). The cells were incubated using an orbital shaker at 50 rpm at room temperature (22°C) for 30 min. Then the cells were washed twice with buffer, and resuspended in PBS buffer. The suspension was directly analysed by flow cytometry. $[Ca_i^{2+}]$ was measured in terms of the fluorescence intensity at 405 ± 10 and 480 ± 10 nm bandpass filter (by dividing the fluorescence at 405 nm by that at 480 nm), while excitation was at 365 ± 10 nm. For Indo-1/AM each cytometric parameter between 9000–10 000 cells were analysed in each experiment. The mean fluorescence for any given population or subpopulation was provide by the instrument software. For each experiment, data for $[Ca_i^{2+}]$ were normalised to one for the control cells. Each experiment was repeated three times.

Statistics

All data are expressed as means ± S.D. (standard deviation); statistical significance was assessed by the Student's *t*-test.

RESULTS

The effect of different concentrations of As_2O_3

All cell lines examined varied in their sensitivity to 0.01–1 μM As_2O_3 following exposure for 24 h (Figure 1a) The inhibitory effect of As_2O_3 on MGC-803 cells was the most significant of all the six malignant cells tested, while there was no effect on the human embryonic pulmonary cells (HEP), even at the higher concentration of 1 μM As_2O_3 with 48 h exposure (Figure 1). For the other five cell lines longer exposure times resulted in greater inhibition (Figure 1b) As_2O_3-induced growth suppression was time- and dose-dependent and relatively selective at lower concentrations. The order of sensitivity to As_2O_3 was MGC-803 > HIC > MCF-7 ≥ HeLa > BEL-7402 ≥ A549 > HEP.

The effect of As_2O_3 on apoptosis

Induction of apoptosis in the malignant cell lines by As_2O_3 also appeared to be time- and dose-dependent. 1 μM As_2O_3 induced apoptosis of all six malignant cell lines; 0.1 μM induced apoptosis in all malignant cell lines except Bel-7402 and A549 cells; and 0.01 μM As_2O_3 only induced apoptosis in MGC-803 and HIC cells. Figure 2 shows typical changes in cell morphology, including blebbing of the plasma membrane, chromatin condensation and formation of apoptotic bodies in MGC-803 cells, following exposure to 0.1 μM As_2O_3 for 24 h. A typical DNA 'ladder' corresponding to internucleosomal cleavage of genomic DNA was observed in

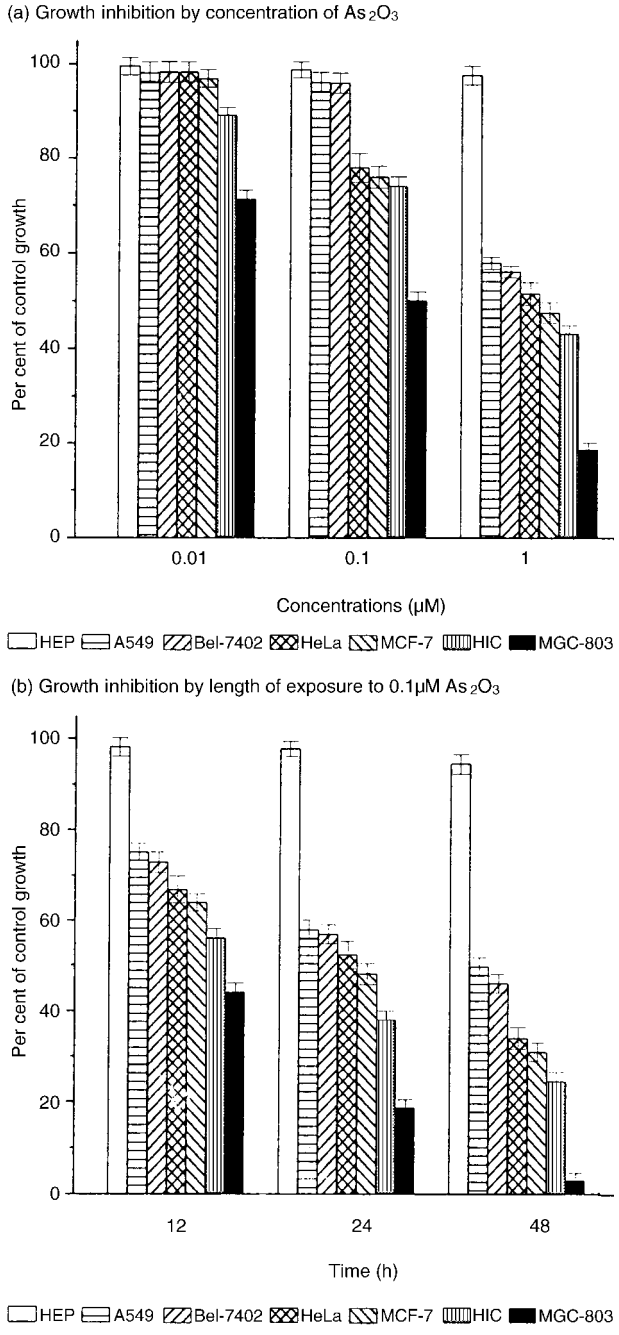

Figure 1. The inhibitory effects of As_2O_3 on seven cell lines tested. (a) Seven cell lines were incubated in serum-containing medium for 24 h in the presence of increasing concentrations of As_2O_3 and growth was measured by the MTT assay. The data were expressed as the percentage growth at the indicated As_2O_3 concentration relative to the control. (b) Percentage grown of seven cell lines exposed to 0.1 μM As_2O_3 for 12–48 h relative to the control, measured by the MTT assay. Results are expressed as mean of three different experiments ± standard deviation (S.D).

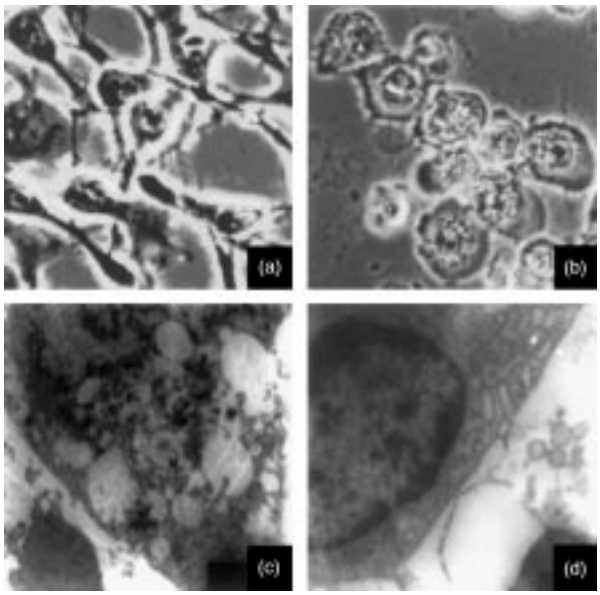

Figure 2. Morphological changes of As_2O_3-treated MGC-803 cells. (a–b) phase-contrast microscopic changes. (a) Control, (b) After 24 h treatment with 0.1 μM As_2O_3. Note blebbing of the plasma membrane and chromatin condensation. (c–d) Electronic microscopic changes. (c) Control. (d) After 24 h treatment with 0.1 μM As_2O_3. Note chromatin condensation and formation of apoptotic bodies.

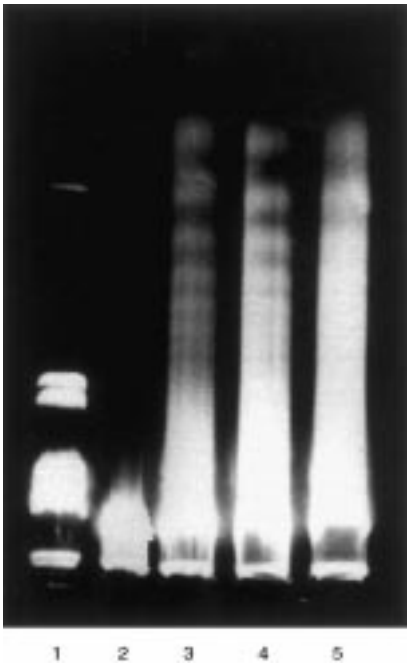

Figure 3. Agarose gel electrophoresis of genomic DNA from MGC-803 cells treated with As_2O_3 for 24 h. lane 1 – marker; lane 2, control; lanes 3–5 1, 0.1 and 0.01 μM As_2O_3 treated group, respectively.

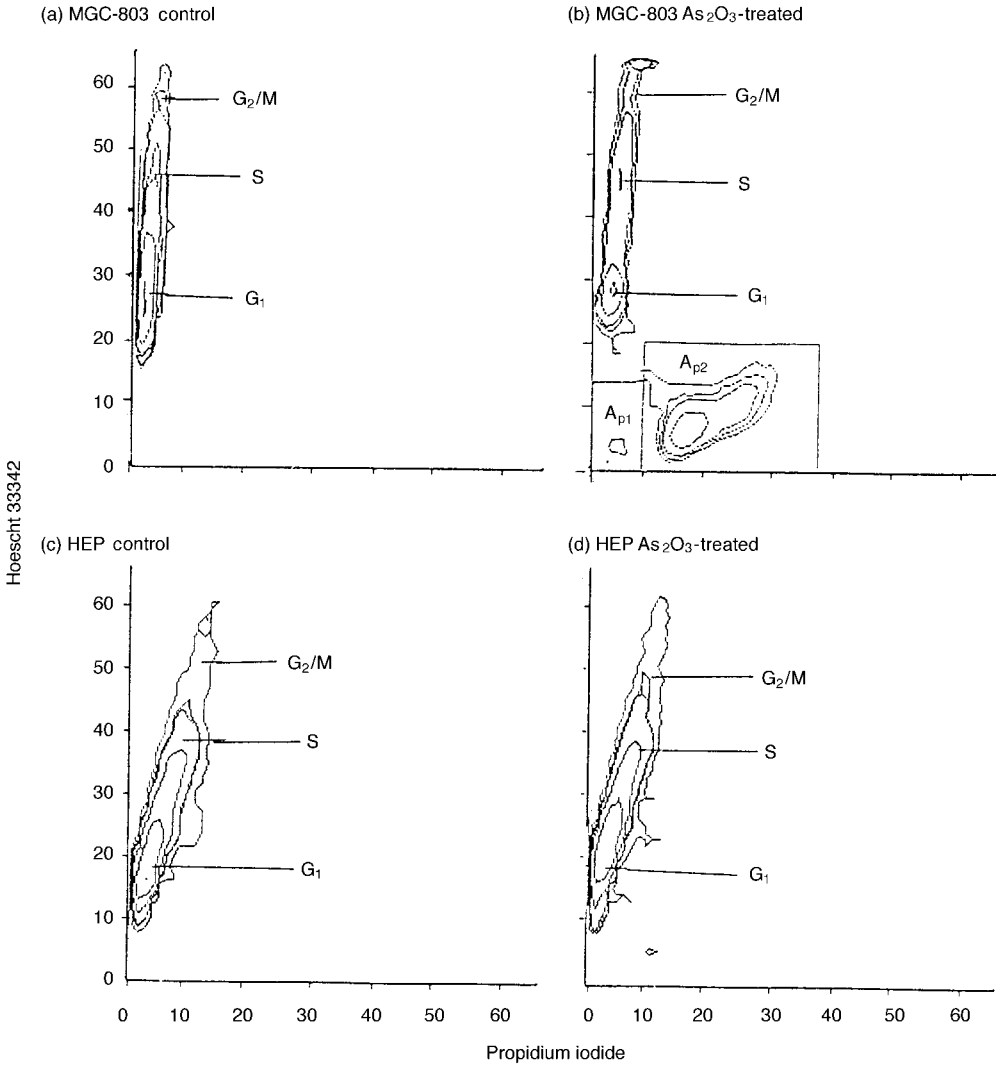

Figure 4. Flow cytometry profile of apoptotic cells of MGC-803 (0.1 μM As_2O_3) and embryonic pulmonary cells (1 μM As_2O_3) treated with As_2O_3 for 24 h.

MGC-803 cells treated with 0.01–1 μM As_2O_3 for 24 h (Figure 3). In addition, flow cytometry also showed a distinct, quantifiable population of apoptotic cells below G_1 phase. This is due to degradation and subsequent leakage of small molecular DNA from cells and a decrease in DNA stainability with H0342 in apoptotic cells, leading to an increase in red/blue fluorescence ratio (PI/H0342; 600/480 nm). Figure 4 shows the profile of apoptotic cells of MGC-803 and HEP cells following 24 h treatment with 0.1 and 1 μM As_2O_3, respectively, indicating that As_2O_3 could not induce apoptosis in HEP cells. The change in the apoptotic ratio of MGC-803 cells following As_2O_3 treatment is shown in Figure 5, indicating that the process was time- and dose-dependent. These results indicate that the effect of As_2O_3 on apoptosis was similar to that on growth inhibition.

The effect of As_2O_3 on intracellular $[Ca_i^{2+}]$ in MGC-803 cells

Intracellular Ca^{2+} mobilisation in cells subjected to different concentrations of As_2O_3 was studied. The changes of intracellular Ca^{2+} from MGC-803 cells are shown in Figure 6. 0.01–1 μM As_2O_3 caused a significant increase in the 405/480 nm ratio value of Indo-1/AM emission, reflecting an

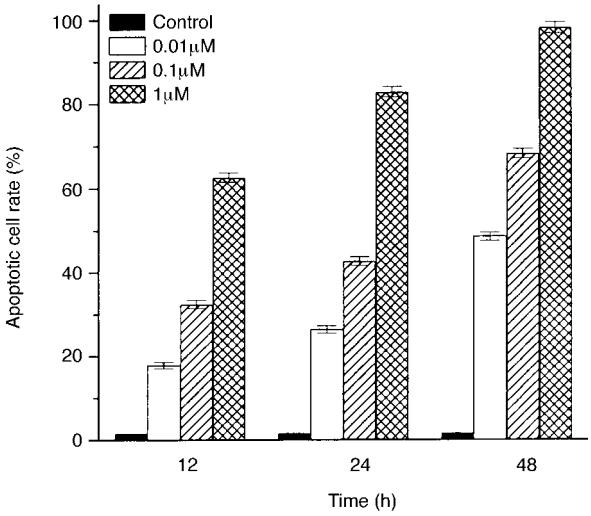

Figure 5. The percentage of apoptotic MGC-803 cells analysed by flow cytometry following 0.01–1 μM As_2O_3 treatment for 12–48 h. The data are means ± standard deviation (S.D.) for three experiments.

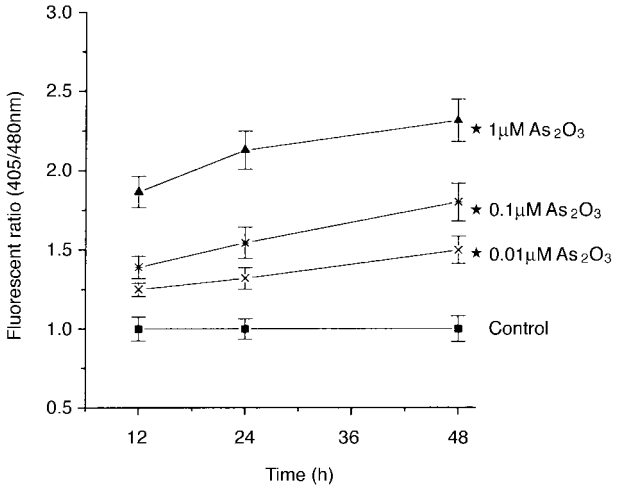

Fig 6. Cytosolic free Ca^{2+} in MGC-803 cells after As_2O_3 treatment measured by Indo-1/AM and flow cytometry. The data on Ca_i^{2+} were normalised to 1 for untreated control cells. The data are means ± standard deviation (S.D.) for three experiments. *$P<0.05$.

increase in $[Ca_i^{2+}]$. The increase of $[Ca_i^{2+}]$ in MGC-803 cells parallels the effect on apoptosis. An increase in $[Ca_i^{2+}]$ of other malignant cells was also detected (data not shown). In human embryonic pulmonary cells resistant to As_2O_3 few changes of $[Ca_i^{2+}]$ were found (data not shown). These results indicate that As_2O_3-mediated apoptosis is associated with changes in intracellular Ca^{2+} triggered by it, which could be the basis of its selective activity in different cells.

DISCUSSION

The use of arsenic compounds as pharmaceuticals has a long history in traditional Chinese medicine. Recently, its effect against tumours has been highlighted by the finding of the remarkable effectiveness of As_2O_3 in leukaemia [2, 3, 11], although the reports are limited only to leukaemia. The toxicity of arsenic to humans is a concern if it is to be used for clinical application. As such arsenic may be better used to treat sensitive solid tumours rather than haemopoietic cancers as for the latter it needs to be administered intravenously, while for the former it could be administered to the local region of the tumour, which would greatly reduce the dose of arsenic required and its toxicity. Therefore, it is important to determine the effects of arsenic on solid tumours.

In this study, As_2O_3 influenced the growth of some solid tumour cell lines, although there was great variation in sensitivity (Figure 1). Time- and dose-dependent significant growth suppression and induction of apoptosis were particularly observed in MGC-803 cells (>80% as compared with controls after treatment for 48 h) (Figures 1 and 5). In contrast human embryonic pulmonary cells were relatively resistant to the same concentrations of As_2O_3. Previous studies have only demonstrated the effects of arsenic on APL cells *in vitro* and in patients [2, 4, 11, 12], with few effects observed in $\leq 0.1\,\mu M$ As_2O_3-treated NB_4 cells. Our data indicate that MGC-803 is another tumour cell line sensitive to As_2O_3, more so than NB_4 cells, since doses of As_2O_3 as low as $0.01\,\mu M$ inhibited MGC-803 cell growth by inducing apoptosis. The effectiveness of this lower concentration of As_2O_3 on MGC-803 cells is important because As_2O_3 is a well-known poison and this is the critical factor which limits its utility as an anticancer agent.

Other important findings are that As_2O_3 at lower concentrations can induce apoptosis of MGC-803 cells and As_2O_3-induced apoptosis is associated with increased cytosolic Ca^{2+}. Recently, apoptosis has become a focus of interest in oncology because of disruption of apoptotic cell death in tumours [13, 14]. Specific therapies are being designed to enhance the susceptibility of individual cell types to undergo apoptosis for a variety of human cancers. In addition, accumulating evidence indicates that Ca^{2+} might play a central role in regulating apoptosis in many tissues [8]. As a consequence of increased $[Ca_i^{2+}]$, various Ca^{2+}-dependent degradative enzymes (e.g. phospholipases, proteases and endonucleases) that may contribute to apoptosis can be activated [15]. Our data showed that exposure to lower concentrations of As_2O_3 induced a sustained increase in $[Ca_i^{2+}]$ in MGC-803 cells. The increase was dose- and time-dependent and correlated with the increase in apoptotic cells (Figures 5 and 6). These results suggest that an increase in $[Ca_i^{2+}]$ is associated with As_2O_3-induced apoptosis of MGC-803 cells and that a critical intracellular Ca^{2+} signal transduction pathway is involved in As_2O_3-mediated cell-death.

Although possible cellular mechanisms underlying arsenic treatment have been addressed [4, 10, 16], its precise mechanism of action is still unknown. Arsenic can inhibit some enzyme activities and induce DNA strand breaks in mammalian cells, affecting some gene responses, including *bcl-2*, c-*myc* and P53 [4, 17, 18–20]. These factors and the increase in intracellular Ca^{2+}, described above, play various regulatory roles in cell growth and apoptosis, thus partly explaining the induction of apoptosis following As_2O_3 exposure. The mechanism for arsenic's relative selectivity is not yet clear. Probably, it is related to the biological property and phenotype of the cells, arsenic's metabolism by the cells and target differences in different cells. Results from this study showed that the change in $[Ca_i^{2+}]$ in the malignant cells treated with As_2O_3 paralleled change in the ratio of apoptotic cells and that the greatest changes in $[Ca_i^{2+}]$ occurred in the most sensitive MGC-803 cells, whereas no change in $[Ca_i^{2+}]$ occurred in the resistant human embryonic pulmonary cells. These results perhaps indicate that the effect of As_2O_3 on $[Ca_i^{2+}]$ could be one of the mechanisms for its selective effects.

The data presented here not only provide preliminary evidence that As_2O_3 can inhibit some solid tumour cells *in vitro*, but also demonstrate that at lower concentrations the effects are relatively selective or specific, with MGC-803 cells the most sensitive of those tested, possibly indicating that As_2O_3 may be a potential compound for the treatment of human gastric cancer, especially by intervention therapy. Further investigations in other tumours are warranted.

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