

Arsenic trioxide induces apoptosis of human gastrointestinal cancer cells

Zhi-Bin Ma, Hong-Yu Xu, Miao Jiang, You-Lin Yang, Lian-Xin Liu, Ying-Hua Li

Zhi-Bin Ma, Hong-Yu Xu, Miao Jiang, You-Lin Yang, Lian-Xin Liu, Department of Gastroenterology, First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China

Ying-Hua Li, Department of Hematology, First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China

Author contributions: Ma ZB and Xu HY contributed equally to this study, and are co-first authors; Ma ZB and Xu HY were responsible for statistical analysis of the data; Jiang M was responsible for the immunohistochemistry analysis; Yang YL was responsible for pre-treatment sample collection and specimen collection; Liu LX was responsible for clinical use; Li YH was the main designer of this study.

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Correspondence to: Ying-Hua Li, MD, PhD, Department of Hematology, First Affiliated Hospital of Harbin Medical University, No. 23 Youzheng Street, Nangang District, Harbin 150001, Heilongjiang Province, China. mazhibin1973117@126.com

Telephone: +86-451-85555119 Fax: +86-451-85555119

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As₂O₃ was administered intravenously at a dose of 0.01 g/d diluted with 5% glucose in normal saline for 2-3 h for 3 consecutive days before surgery. Morphological changes associated with apoptosis of gastrointestinal cancer cells were observed by light microscopy. Changes in the apoptotic index induced by As₂O₃ were investigated using the terminal deoxynucleotidyl transferase dUTP nick end labelling method. Expression levels of p53 and Bcl-2 proteins in gastrointestinal cancer tissues were determined by immunohistochemistry.

RESULTS: The apoptotic index of human gastrointestinal cancer cells was higher in cells from patients treated with As₂O₃ than in those not treated ($P < 0.05$). p53 protein expression in gastrointestinal tissues was unchanged by As₂O₃ ($P > 0.05$). However, Bcl-2 protein expression in gastrointestinal tissues was down-regulated by As₂O₃ ($P < 0.01$).

CONCLUSION: These results demonstrate that As₂O₃ treatment in patients with gastrointestinal cancers can induce apoptosis in gastrointestinal cancer cells and down-regulate Bcl-2 protein expression.

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Abstract

AIM: To investigate the changes in apoptosis in gastrointestinal cancer cells from patients with gastrointestinal cancers treated with arsenic trioxide (As₂O₃); and to study the possible molecular mechanisms of such changes by detecting the expression levels of p53 and Bcl-2.

METHODS: Twenty patients with gastrointestinal adenocarcinoma based on endoscopic and biopsy findings (ten patients with gastric cancer and ten patients with colorectal cancer) who received treatment in our hospital between August 2007 and December 2008 were included in this study. None of the patients had received anti-tumour agents prior to As₂O₃ treatment.

Key words: Gastrointestinal cancer; Arsenic trioxide; Apoptosis; p53; Bcl-2

Core tip: In this study, patients with gastrointestinal cancer were treated with arsenic trioxide for the first time. The use of arsenic trioxide in neoadjuvant chemotherapy prior to surgery improved surgical results.

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INTRODUCTION

Gastrointestinal cancer (GIC) is one of the most common malignant tumours in China. Although early GIC can be treated by surgical resection, the response of advanced-stage GIC to conventional chemotherapy or radiotherapy is usually unsatisfactory. Following studies in Harbin and Shanghai, arsenic trioxide (As₂O₃) was reported to be an effective drug for the treatment of patients with acute promyelocytic leukaemia (APL), by selectively inducing apoptosis in APL cells^[1-3]; the effect of As₂O₃ on solid tumours has since been demonstrated in GIC cells *in vitro* and *in vivo*^[4-7]. However, the clinical efficacy of As₂O₃ for the treatment of GIC remains unknown. Based on the above studies, we treated GIC patients with As₂O₃, observed its ability to induce apoptosis in GIC cells, and explored the possible molecular mechanism underlying this effect by detecting the expression levels of p53 and Bcl-2 in As₂O₃-treated GIC cells.

MATERIALS AND METHODS

As₂O₃

As₂O₃ for injection (0.01 g/10 mL) was produced by Harbin Yida Pharmaceutical Co., Ltd. (Harbin, China).

Drug treatment

Twenty patients with gastrointestinal adenocarcinoma based on endoscopic and biopsy findings, including ten patients with gastric cancer and ten patients with colorectal cancer who received treatment in our hospital between August 2007 and December 2008, were included in this study. None of the patients had received anti-tumour agents. As₂O₃ was administered as an intravenous infusion at a dose of 0.01 g/d diluted with 5% glucose in normal saline for 2-3 h for 3 consecutive days before surgery.

Human tissue collection

Human gastrointestinal adenocarcinoma tissue specimens were obtained endoscopically from the same patients before and after As₂O₃ treatment and surgery.

Apoptotic cell morphology

The apoptotic cell morphology of GIC cells was examined by light microscopy.

Terminal deoxynucleotidyl transferase dUTP nick end labelling assays

Apoptosis was assessed *via* transferase dUTP nick end labelling (TUNEL) assay according to the instructions of the *In Situ* Cell Death Detection Kit (Boehringer-Mannheim, Germany). Briefly, after three washes with phosphate-buffered saline (PBS), 400 µL TUNEL reaction solution was added to the well, followed by incubation at 37 °C for 1 h. Stained cells were examined under the light microscope. Apoptotic cells were scored as the

number of positively stained cells per 1000 cells ($n = 5$). The apoptotic index (AI) was calculated as follows: $AI = (\text{number of apoptotic cells} / \text{total number of cells}) \times 100\%$.

Immunohistochemistry

The streptavidin-peroxidase (S-P) method was used to detect the expression levels of p53 and Bcl-2 proteins in human gastrointestinal adenocarcinoma tissues. Monoclonal rabbit anti-human p53 and rabbit anti-human Bcl-2 antibodies (antiserum titre, 1:50) were provided by Zhongshan Bio-Tech Co., Ltd. (Beijing, China). The expression levels of p53 and Bcl-2 proteins were determined using an UltraSensitive S-P (Mouse/Rabbit) kit (Zhongshan Bio-Tech Co., Ltd.) according to the manufacturer's protocol. The percentage of positive cells was calculated by examination of the images under the light microscope.

Statistical analysis

Results were analysed using the SPSS13.0 statistical software package. All values were expressed as mean \pm SD. Comparisons of values before and after As₂O₃ treatment were performed using one-sample *t* tests, and correlation analysis was performed using linear correlation. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Morphological observation of apoptotic human GIC cells by light microscopy

The number of apoptotic cells increased after As₂O₃ treatment as determined by light microscopy. Typical apoptotic morphological features were detected in GIC cells after treatment, including cellular shrinkage, cell nuclear fragmentation, chromatin condensation, and the production of apoptotic bodies (Figure 1).

Apoptosis induction in human GIC cells by As₂O₃

As shown in Figure 2, the number of apoptotic GIC cells as indicated by brown staining was increased after 3 days of As₂O₃ treatment. Correspondingly, the AI of GIC cells increased after As₂O₃ treatment relative to that before treatment ($P < 0.05$) (Table 1).

Effect of As₂O₃ on p53 protein expression in human GIC cells

There was no significant change in p53 protein expression in either gastric or colon cancer tissues after As₂O₃ treatment, suggesting that the anti-apoptotic *p53* gene was not sensitive to As₂O₃ at a dose of 0.01 g/d for 3 d (Table 1, Figure 3).

Effect of As₂O₃ on Bcl-2 protein expression in human GIC cells

As shown in Figure 4, Bcl-2 protein expression was reduced in gastric cancer tissues after As₂O₃ treatment, and similar results were seen in colon cancer tissues, suggest-

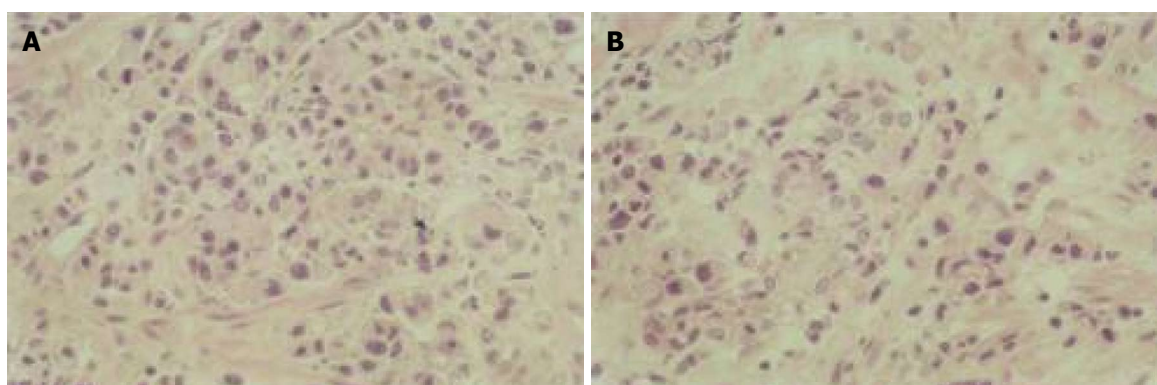


Figure 1 Morphology of apoptotic cells in gastrointestinal cancer seen under light microscopy. The number of apoptotic cells increased following a 3-d period of As₂O₃ treatment. Typical apoptotic changes were seen in GIC cells after As₂O₃ treatment, including cellular shrinkage, cell nucleus fragmentation, chromatin condensation, and the appearance of apoptotic bodies. A: Before As₂O₃ treatment; B: After As₂O₃ treatment (HE × 40).

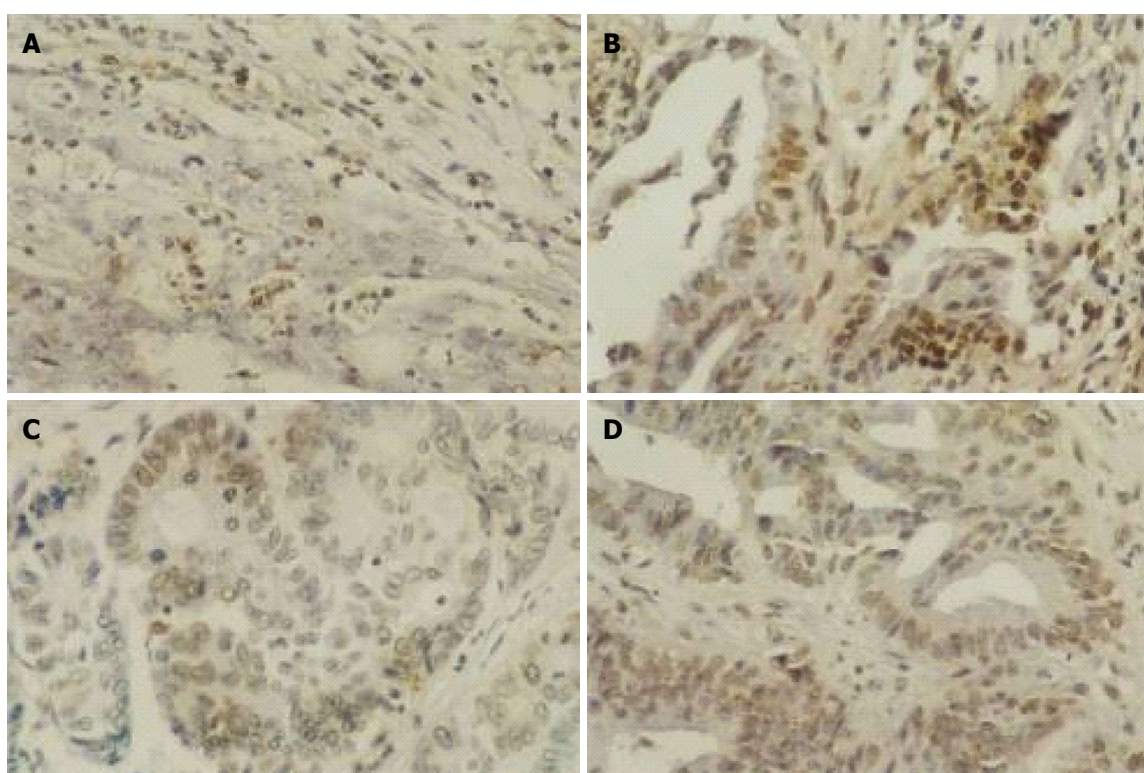


Figure 2 Human gastrointestinal cancer cells were observed by transferase dUTP nick end labelling assay, and identified by brown-stained nuclei. The number of apoptotic cells was counted in order to calculate the apoptotic index. A: Gastric cancer before As₂O₃ treatment; B: Gastric cancer after As₂O₃ treatment; C: Colon cancer before As₂O₃ treatment; D: Colon cancer after As₂O₃ treatment (× 400).

ing that As₂O₃ had down-regulated the expression of Bcl-2 protein (Table 1).

Correlation between apoptosis and Bcl-2 expression

According to the above observations, the AI of GIC cells increased following the down-regulation of Bcl-2 protein expression. The correlation between apoptosis and Bcl-2 expression was examined to determine whether changes in Bcl-2 expression represented the molecular mechanism responsible for As₂O₃-induced apoptosis of GIC cells. The results showed no significant correlation between these measures in either gastric or colon cancer

cells (Figure 5).

Clinical safety of As₂O₃ treatment

Two patients experienced vomiting after As₂O₃ treatment, which resolved after symptomatic treatment. Second-degree atrioventricular block occurred during As₂O₃ treatment in one patient, who recovered after treatment. There were no other secondary effects in any other patients.

DISCUSSION

The use of arsenic compounds as drugs has a long his-

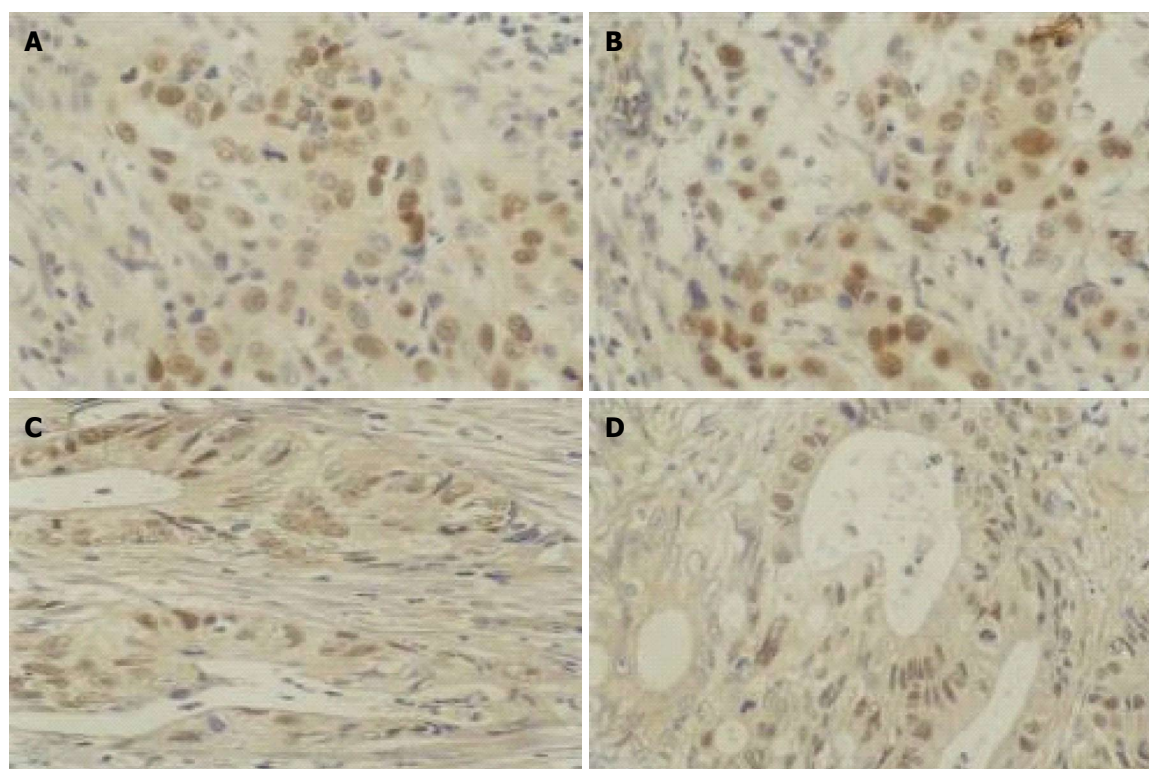


Figure 3 p53 protein expression in human gastrointestinal cancer cells as indicated by brown-stained nuclei. A: Gastric cancer before As₂O₃ treatment; B: Gastric cancer after As₂O₃ treatment; C: Colon cancer before As₂O₃ treatment; D: Colon cancer after As₂O₃ treatment (× 400).

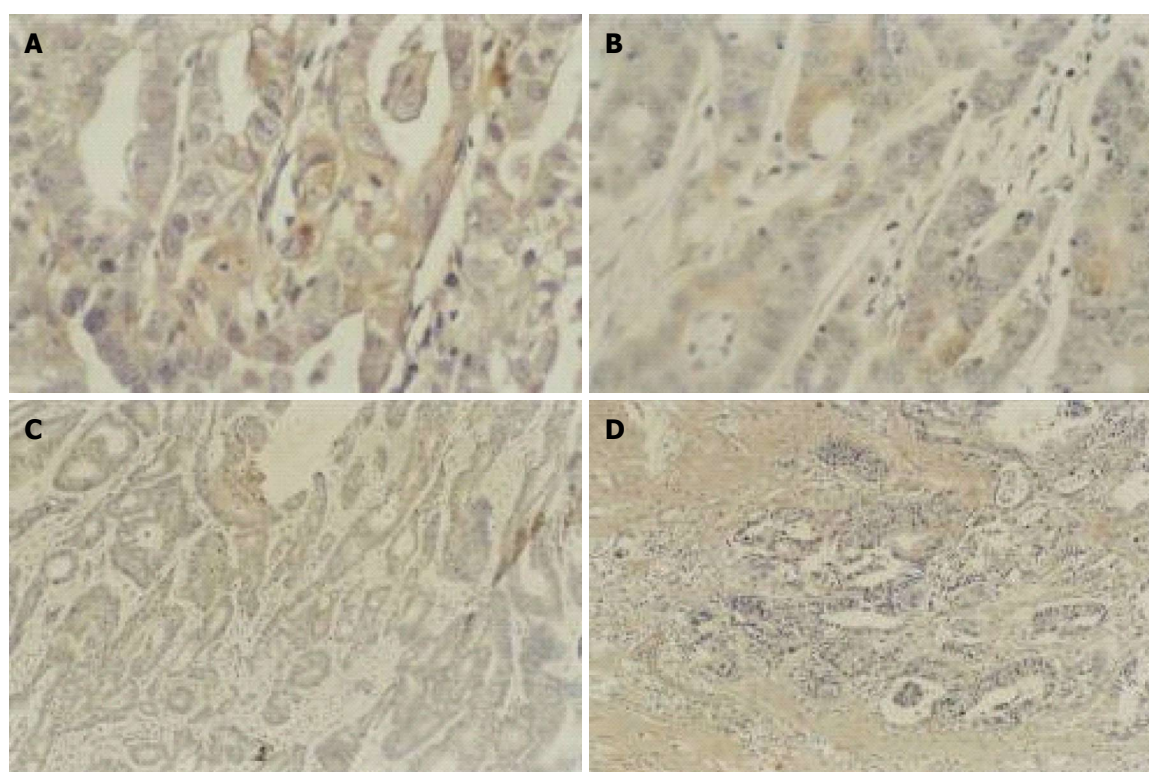


Figure 4 Bcl-2 protein expression in human gastrointestinal cancer cells as indicated by brown-stained cytoplasm. A: Gastric cancer before As₂O₃ treatment; B: Gastric cancer after As₂O₃ treatment; C: Colon cancer before As₂O₃ treatment; D: Colon cancer after As₂O₃ treatment (× 400).

tory in traditional Chinese medicine. As₂O₃ has been identified as an effective drug for the treatment of APL,

with induction of apoptosis representing the main mechanism of action. Numerous clinical trials on As₂O₃ are

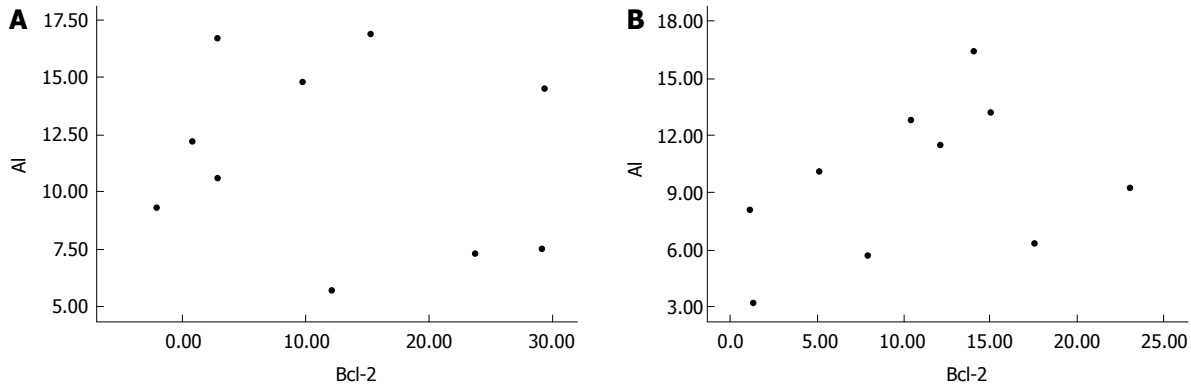


Figure 5 Linear correlation analyses between apoptotic index and Bcl-2 expression in gastric cancer (A) and colon cancer (B) (%). Data are given as mean \pm SD. No significant correlation was detected.

Table 1 Apoptotic index, p53 protein expression and Bcl-2 protein expression in human gastrointestinal cancer cells

Group	Index	Before As ₂ O ₃ treatment	After As ₂ O ₃ treatment	P value
Gastric cancer	Apoptotic index (%)	6.88 \pm 1.80	15.53 \pm 4.51	< 0.05
Colon cancer	p53 protein (%)	7.11 \pm 1.48	16.76 \pm 4.16	< 0.05
Gastric cancer	p53 protein (%)	38.08 \pm 18.52	35.42 \pm 17.19	> 0.05
Colon cancer	Bcl-2 protein (%)	31.58 \pm 14.55	30.83 \pm 19.52	> 0.05
Gastric cancer	Bcl-2 protein (%)	37.16 \pm 18.94	23.80 \pm 3.30	< 0.05
Colon cancer	Bcl-2 protein (%)	28.69 \pm 13.00	16.90 \pm 5.74	< 0.05

Data are presented as mean \pm SD of the percentage of apoptotic cells.

therefore underway for the treatment of haematopoietic malignancies and solid tumours^[8-10].

Recent studies^[11-15] reported that As₂O₃ could induce apoptosis in various solid tumour cell lines, including GIC cell lines, as well as in animal models of GIC, especially gastric cancer. However, no clinical trial on As₂O₃ in patients with gastric cancer has been reported.

In the present study, we treated GIC patients with As₂O₃ and examined its effect on GIC cells. Light microscopy showed morphological changes characteristic of apoptosis in GIC cells in As₂O₃-treated patients. TUNEL assay showed that AI was also significantly increased in GIC cells of treated patients. These results suggest that As₂O₃ induces apoptosis of GIC cells not only in cell lines and animal models, but also in the clinical setting. The induction of apoptosis in GIC cells from GIC patients may indicate a new potential strategy for the treatment of these tumours. Gu *et al*^[16] found that the induction of apoptosis was time- and concentration-dependent. In line with previous clinical reports, we chose a relatively safe dose of As₂O₃ in this study. However, the optimal dose required to produce the maximal clinical effect with minimal toxicity remains to be determined.

Although the effect of As₂O₃ on the induction of GIC cell apoptosis has been addressed, its precise mechanism remains unanswered. In APL cells, As₂O₃ was shown to inhibit cell proliferation by direct induction of apoptosis through down-regulation of Bcl-2 expression and modulation of the PML/RAR α protein in *in vitro* studies^[2]. Liu *et al*^[17] reported that the PIG11 gene may be involved in

As₂O₃-induced apoptosis in HepG2 cells, and suggested that the adaptive response of PIG11 expression was an important factor in enhancing cell sensitivity to As₂O₃-induced apoptosis. As₂O₃ has been shown to induce apoptosis in different GCC lines through up-regulation of p53 expression and down-regulation of Bcl-2 expression^[18]. Many chemotherapy agents can induce apoptosis in gastric cancer and colon cancer cells by modulating the p53 gene^[19]. Jiang *et al*^[20] found that As₂O₃ induced apoptosis in the GCC lines AGS and MKN-28 through up-regulation of p53. Akao *et al*^[21] also reported that As₂O₃ induced apoptosis through the down-regulation of Bcl-2 protein and activation of caspases in B-cell leukaemia cell lines, while As₂O₃-induced apoptosis was related to p53 and Bcl-2 gene expression in studies of colon cancer cell lines and in an animal model^[6,7].

To further confirm the possible mechanism of As₂O₃-induced apoptosis, we determined the effect of As₂O₃ on p53 and Bcl-2 expression. In contrast to the previous reports, our immunohistochemical analysis found no significant change in p53 protein expression in either gastric or colon cancer tissues following As₂O₃ treatment. Possible reasons for this discrepancy are as follows: (1) As₂O₃ treatment was only administered for a short period (3 d); (2) the dose of As₂O₃ used in the current study may have been too low; (3) the effect of As₂O₃ on p53 expression may be related to the stage of GIC; or (4) the internal environment in GIC patients may be more dynamic than that in cell lines. Further studies are needed to clarify the effect of As₂O₃ on p53 protein expression in human GIC cells.

Bcl-2 is considered a survival gene that plays an important role in inhibiting tumour cell apoptosis^[22]. Our experimental results revealed that As₂O₃ down-regulated the expression of Bcl-2 protein in human GIC tissues. However, the change in Bcl-2 protein expression was not correlated with AI in either gastric or colon cancers, suggesting that other genes may be involved in the process of As₂O₃-induced apoptosis in human GIC.

In conclusion, the results of this study indicate that clinical treatment with As₂O₃ induced apoptosis of GIC cells without causing serious adverse effects. In addition, although Bcl-2 gene expression was down-regulated by

As₂O₃, this did not seem to be the mechanism responsible for As₂O₃-induced apoptosis. Similar to *P53* and *Bcl-2* genes, *SIRT6* is a new tumor suppressor gene in colorectal cancer as shown in recent studies, and it is possible that the glycolytic pathway limits the growth of tumors. We also used SIRT6 antibody on the tissue samples before and after As₂O₃ treatment, and SIRT6 was found to be higher after treatment than before treatment. Further comprehensive clinical studies are needed to clarify the significance and precise mechanism of action of As₂O₃ in the treatment of GIC patients.

COMMENTS

Background

Gastrointestinal cancer is a common clinical malignancy, and is often diagnosed when it is unresectable. Therapeutic options are required to improve patient condition prior to surgery and this is a research hotspot.

Research frontiers

This study assessed the effects of arsenic trioxide on gastrointestinal cancer cell apoptosis.

Innovations and breakthroughs

Arsenic trioxide was administered preoperatively in gastrointestinal cancer patients and its efficacy as neoadjuvant chemotherapy was assessed.

Applications

The use of arsenic trioxide as neoadjuvant chemotherapy may be extended to other types of tumors.

Peer review

The experimental design is reasonable and practical, with patients giving fully informed consent, and complies with legal requirements, with satisfactory results.

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