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Induction of apoptosis by arsenic trioxide and hydroxy camptothecin in gastriccancer cells in vitro

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Subject headings gastric cancer; apoptosis; arsenic trioxide; hydroxycamptothecin

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Abstract

AIM **To study the effects of arsenic trioxide and HCPT on dif ferent degrees of differentiated gastric cancer cells (SGC-7901, MKN-45, MKN-28) with respect to both cytotoxicity and induction of apoptosis *in vitro*.**

METHODS **The cytotoxicity of As_2O_3 and HCPT on gastric cancer cells was determined by MTT assay. Morphologic changes of apoptosis of gastric cancer cells were observed by light microscopy and transmission electron microscopy. Apoptosis and cell cycle changes of gastric cancer cells induced by HCPT and As_2O_3 were investi gated by TUNEL method and flow cytometry.**

RESULTS **As_2O_3 and HCPT had remarkable cytotoxic effects on different degrees of diff e rentiated gastric cancer cells. The IC^{50} of As_2O_3 on well differentiat ed gastric cancer cell MKN-28, moderately differentiated gastric cancer cell SG C-7901, and poorly differentiated gastric cancer cell MKN-28 were 8.91 μmol/L, 10.57 μmol/L, and 11.65 μmol/L, respectively. The IC^{50} of HCPT on MKN-28, SGC-7901, and MKN-45 were 9.35 mg/L, 10.21 mg/L, and 12.63 mg/L respectiv ely after 48 h treatment. After 12 h of exposure to both drugs, gastric cancer cells exhibited morphologic features of apoptosis, including cell shrinkage, nuclear condensation, and formation of apoptotic bodies. A typical subdiploid pe ak before G_0/G_1 phase was observed by flow cytometry. The apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 13.84%, 22.52%, and 9.68%, respectively after 48 h exposure to 10 μmol/L As_2O_3. The apoptotic rates of SGC-7901, MKN-4 5, and MKN-28 were 21.88%, 12.35%, and 30.26%, respectively after 48 h expo sure to 10 mg/L HCPT. The apoptotic indice were 7%-15% as assessed by TUNEL method. The effect of As_2O_3 on SGC-7901 showed remarkable cell cycle specificity, which induced cell death in G_1 phase, and blocked G_2/M phase. HCPT also showed a remarkable cell cycle specificity, by inducing cell death and apoptosis in G_1 phase and arrest of proliferation at Sphase.**

CONCLUSION **As_2O_3 and HCPT exhibit significant cytotoxicity on gastric cancer cells by induction of apoptosis. As_2O_3 and HCPT might have a promising prospect in the treatment of gastric cancer, which needs to be further studied.**

INTRODUCTION

Gastric cancer is one of the most common malignant tumors in China. Evidences ha ve demonstrated that stomach cancer is a disease caused not only by excessive cellular proliferation and poor differentiation, but also by decrease in apoptosis of the gastric cells[1]. Though the disease in its early stage can be treated by surgical resection, in advanced stage its response to conventional che motherapy or radiotherapy is usually not satisfactory. Therefore, we think, that indu ction of apoptosis of gastric cancer cells might be a new means in the treatment of gastric cancer. Arsenic trioxide (As_2O_3) and hydroxycamptothecin (HCPT) have long been used in China. The former was first reported by investigators in Harbin and Shanghai to be an effective drug in the treatment of patients with a cute promyelocytic leukemia (APL)[2,3]. It induces apoptosis of the leuk emic cells at a concentration achieved in the plasma of treated patients (0.5×10^{-6} mol/L-1×10^{-6} mol/L), as demonstrated by studies on all -trans-retinoic acid (ATRA)-susceptible or resistant APL cell lines, on primary APL cell culture ,and on patients' blood samples

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obtained during treatment with arsenic[4]. It is not known whether As_2O_3 is effective in the treatment of solid tumors such as gastric cancer or not.

HCPT is a unique antitumor drug that has been extracted and synthesized by Chine se scientists from *Camptotheca accuminata* which is a native plant in China[5]. HCPT can act directly on topoisomerase I inhibiting its activity. HCP T possesses stronger cytotoxicity to tumor cells with less side effects, as com pared to camptothecin[6]. More recent studies have shown that camptothec in have strong apoptosis induction effects in human leukemia cell lines[7]. However, there is yet no report about HCPT-induced apoptosis in stomach cells. Therefore, we studied the effects of HCPT and As_2O_3 on induction of apoptosis in gastric cancer cells.

MATERIALS AND METHODS

Cell culture and chemicals

Human moderately differentiated gastric adenocarcinoma cell line SGC-7901 was o btained from Shanghai Sixth People's Hospital, human poorly differentiated sto mach adenocarcinoma cells line MKN-45 and well differentiated stomach adenocarc inoma cell line MKN-28 were kindly provided by Japanese Cancer Research Resourc es Bank Corp (Tokyo, Japan). SGC-7901, MKN-45 and MKN-28 were maintained in a humidified, 5% CO_2 atmosphere and cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 2 μmol/L L-glutamine, 100 units/mL penicillin, and streptomycin. HCPT 1 g/L and 0.1% As_2O_3 preparation for iv administr ation were kindly provided by Hubei Huangshi Second Pharmaceutical Company,and Shanghai Institute o f Hematology, respectively.

MTT method and cytotoxicity

One hundred μL cancer cells in exponential growth at 1×10^4/mL w ere added into flat-bottomed 96-well plates (NUNC) 24 h prior to drug treatment. Cells w ere treated with 1 mg/L-100 mg/L HCPT, (0.5-10) μmol/L As_2O_3 an d with no drugs (control) in triplicate for 24 h and 48 h, respectively. After washing th e medium was replaced by 100 μL RPMI 1640 (GIBCO) medium containing 1 g/L3(4,5dimethylthiazol)-2,5diphenyltetra-zolium(MTT, MERCH). After 4 h , plat es were centrifuged at 800 × g for 5 min, the MTT medium was removed, and th e blue dye was dissolved in 200 μL of warm dimethylsulfoxide (DMSO). Absorban ce was measured at 570 nm. The cytotoxicity rates were measured by the formula:

$$\text{The cytotoxicity vate} = (1-\frac{OD_{570}\ \text{test}}{OD_{570}\ \text{control}} \times 100\%$$

The IC_{50} was evaluated from the cytotoxicity curve.

Apoptosis cell morphology

One hundred μL cancer cells at concentration of 5×10^6/mL were in cubated into 6-well plates with previously placed glass slides. After 24 h, the medium was replaced with the drug containing medium. After incubation with drugs for 12 h, 24 h, 48 h, and 72 h, glass slides with cancer cell growth were fixed with 4% p olyformalin, and stained with hematoxylin-eosin. Cell morphology was examined under light microscopy.

Transmission electron microscopy

Culture cells were fixed in 2% glutaraldehyde in 0.1 mol/L, pH 7.4 PBS at 4 ℃ and postfixed with 1% osmium tetroxide for 2 h, then the cells were embedded wi th Ep pon 812 and ultrathin sections were cut. Cells were observed under transmission electron microscope (H-500, Japan).

TUNEL assay

Apoptosis was assessed by dUTP labeling of DNA nicks with terminal deoxynucleoti dyl transferase (TUNEL). One hundred μL cancer cells at concentr ation of 5×10^6/mL were inoculated into 6-well plates with previously placed glass slides. After 24 h, the medium was replaced with the drug containing medium. After incubation w i th drugs for 24 h and 48 h, glass slides with cancer cells growth were fixed wit h 4% polyformalin. The TUNEL assay was performed according to the instructions in the *In Situ* Cell Death Detection Kit (Boehringer-Mannheim, Germany). Briefly, after washing twice with pH 7.4 PBS, 50 μL TUNEL reaction soluti on was add ed to the well, then incubated at 37℃ for 2 h. After substrate reaction, s tained cells were examined under light microscopy. Apoptotic cells were scored a nd expressed as the number of positively stained cells per 500 cells (n = 4-5) .

Flow cytometry

Apoptotic cells were also detected by flow cytometry which was performed as des cribed previously. About 1×10^6 cells were treated with drugs for 24 h and 48 h. After trypsin digestion, the cells were collected by centrifugation, then fix ed in 70% ethanol /phosphate buffered saline for at least 12 h at 4℃. After 100 μL (1 g/L) RNase treatment, cells were stained with 50 mg/L propidium iodide. Cells were examined by flow cytometry using a FACScan (Becton-Dickinson , USA). The results were analyzed with Lysis II software (Becton-Dickinson).

RESULTS

As_2O_3 and HCPT cytotoxicity

HCPT and As_2O_3 had strong cytotoxic effect on gastric cancer cells (Figure 1). The IC_{50} of As_2O_3 on MKN-28, SGC-7901, and MKN-45 was 8.91 μmol/L, 10.57 μmol/L, and 11.65 μmol/L, respecti vely.The IC_{50} of HCPT on MKN-28, SGC-7901, MKN-45 was 9.35 mg/L, 1 0.21 mg/L, and 12.63 mg/L, respectively.

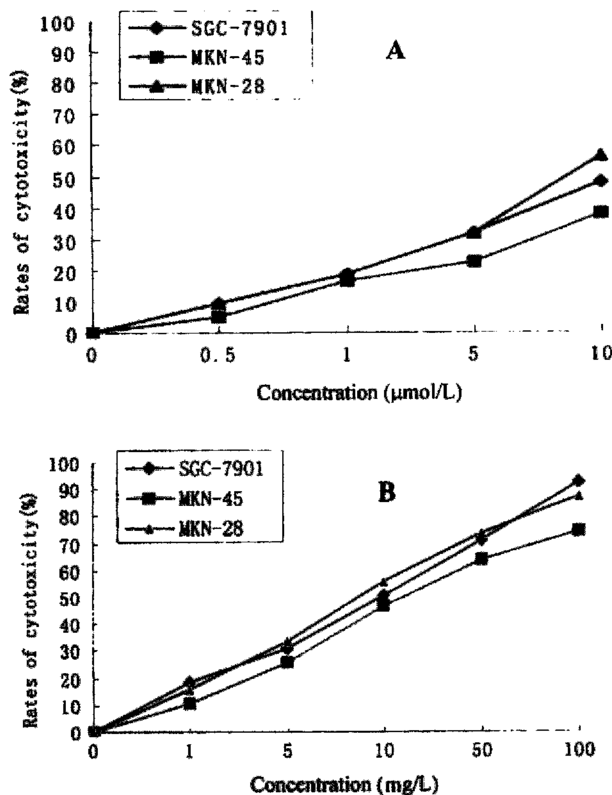

Figure 1 Cytotoxicity of As_2O_3 (A), and HCPT (B), on gastric cancer cells.

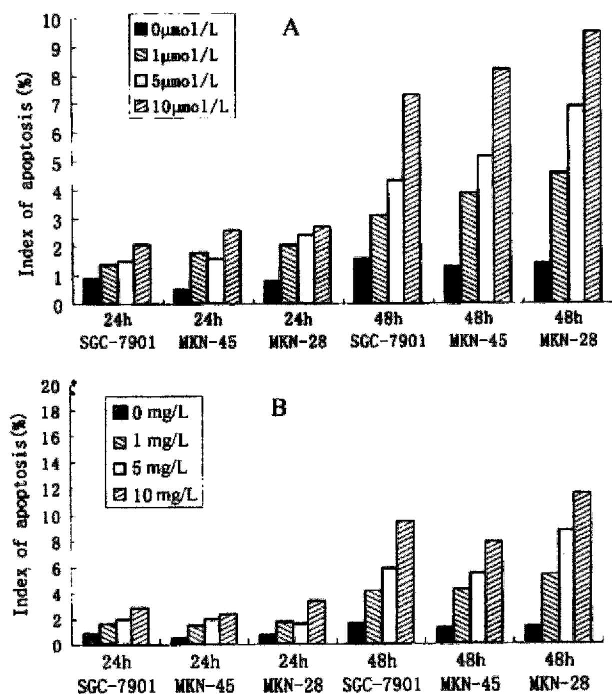

Figure 3 The index of apoptosis of gastric cancer cells induce d by As_2O_3 (A) and by HCPT (B).

Induction of apopotosis by As_2O_3 and HCPT

After 12 h of exposure to As_2O_3 and HCPT, gastric cancer cells MKN-28 a nd SGC-7901, began to show morphologic features of apoptosis. The apoptotic cells increased on prolongation of exposure time to drugs. The ultrastructural feat ures of apoptosis was observed in MKN-45 and SGC-7901 by transmission electron microscopy, including cell shrinkage, cytoplasmic blebs, condensation of chromatin, nuclear condensation, fragmentation of nucleus, and formation of apoptotic bodies, and so on. The results are presented in Figure 2.

As_2O_3 and HCPT induced time- and dose-dependent apoptosis in three strains of gastric cancer cells. The indice of apoptosis were measured by TUNEL. The results are presented in Figure 3A and 3B.

To further study the inducing effect of As_2O_3 and HCPT on gastric cancer cells, we analysed the DNA fragment reflecting the endonuclease activity during apo ptosis. (Figure 4). Examination of histogramic related nuclear DNA contents on FACS showed a distinct region below G_1 phase, which is the typical profile of apop totic cells in which DNA stainability is reduced due to degradation and subseque nt leakage of DNA from cells. After 48 h exposure to 10 mg/L HCPT, the apoptot ic rates of SGC-7901, MKN-45, and MkN-28 were 21.88%, 12.35%, and 30.26%, res pe ctively. After 48 h of exposure to 10 μmol/L As_2O_3, the apoptotic rates of SG C-7901, MKN-45, and MKN-28 were 13.84%, 22.52%, and 9.68%, respectively (F igure 4).

Effect of As_2O_3 and HCPT on cell cycle of SGC-7901 cells

The effect of As_2O_3 and HCPT on SGC-7901 cells show remarkable cell cycle specificity. There was no significant change in cell cycle after 10 μmol/L A s_2O_3 treatment for 24 h. The fraction of G_0/G_1 was decreased from 54.2% to 17.7%, while the fraction of G_2/M was significantly increased from 20.2% t o 63.4% with 10 ¦Ìmol/L As_2O_3 treatment in SGC-7901 cells for 48 h. The re sults showed that As_2O_3 induced gastric cancer cell death was in G_1 phase , blocked at G_2/M phase. The fraction of G_0/G_1 phase was decreased from 54 .2% to 37.6%, while the fraction of S phase was increased from 25.6% to 38.6% with 10 mg/L HCPT treatment in SGC-7901 cells for 48 h. The results showed that HCPT-induced apoptosis was at G_1 phase, and arrested at S phase.

DISCUSSION

Since the first observation of the relationship between arsenic and skin canc er in 1820s, arsenic compounds have been generally recognized as potent environ mental carcinogens, more likely as a co-mutagen and co-carcinogen for human sk in and lungs[8], although no animal model had been established[9]. Biochemically, it is documented that arsenic can inactivate some important enzymes by binding their sulfhydryl groups. Arsenic can also interfere with the p hosphorylation-dephosphorylation process by replacing the phosphorylation reaction. It has

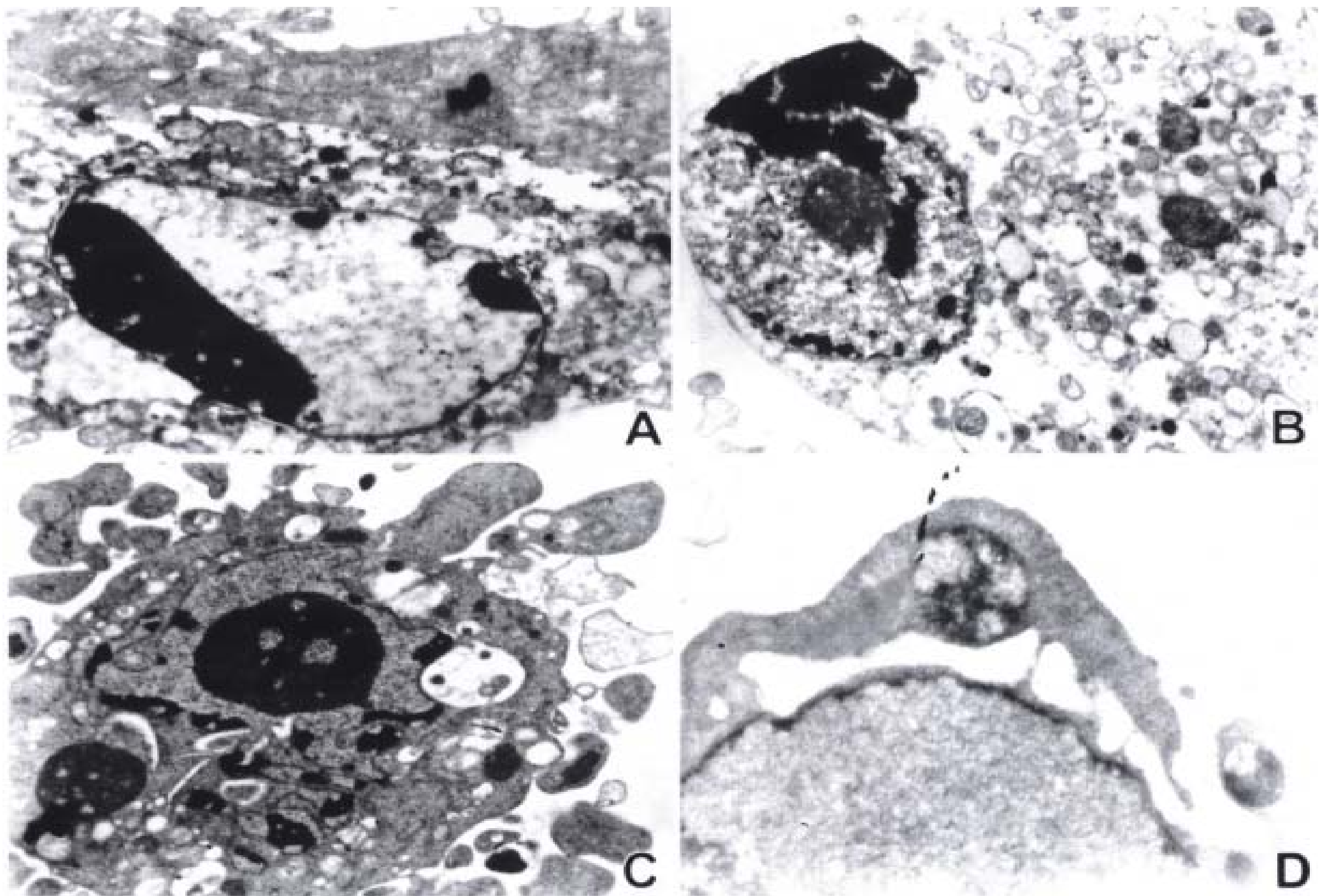

Figure 2 Electron microscopic observation of ultrastructural changes in gastric cancer ce lls treated with 10 μmol/L As_2O_3 for 48 h. The early change of apoptosis, nuclear chromatin condensation, looks like a new moon subjacent to the nuclear membranes (A). The intermediate stage of apoptosis presents cytoplasmic blebs, nu clear condensation, overflow of nuclear chromatin, and like-sprout (B). The late stage of apoptosis is characterized by splitting of nuclear membrane, nuclear chr omatin condensation, overflow of some parts of nuclear chromatin, and fragmentation of nucleus (C), and formation of apoptotic bodies (C) (D).

also been shown that arsenic can induce chromosome aberrations, sister-chromatin exchanges, DNA-protein cross links and protein-associated DNA-strand breaks in mammalian cells[10,11]. However, low concentration of some arsenic compounds also had some benefits to human physiologically,such as stimulation of human hematopoiesis. The use of arsenic compounds as drugs has a long history in Chinese traditional medicine. For example, it was recorded that ar senic had therapeutic effects on some human diseases such as psoriasis, syphilis, and rheumatosis. Recently, it has been shown that two arsenic compounds As_2O_3[2] and arsenic disulfide[12], which were used in some tradit ional Chinese prescriptions, are very effective in APL treatment. For instance, a report from the northeastern region of China showed that As_2O_3 (10 g/L v ia intravenous infusion for 28 to 60 days) induced clinical complete remission (CR) in 65.6% of APL patients. More interestingly, 28.2% (9/32) of patients survived more than 10 years. A more recent clinical trial with As_2O_3 trea tment also demonstrated that CR was achieved in 15 of 16 APL patients who relapsed after ATRA-induced and chemotherapy-maintained CR[4].

It has been suggested that As_2O_3 might induce apoptosis selectively in APL cells[13]. Furthermore, at pharmacological concentrations, it has no eff ect on the growth and survival of the leukemia myeloid cells U937 and HL60[3]. However, preliminary reports suggest that the apoptotic effect of As_2O_3 is not specific for APL cells but can be observed in various lines of either myeloid or lymphoid origin[14-18]and in blast cells from patients wi th non-M3 acute myeloid leukemia [19]. Another arsenic-containing comp ound, the melaminyl-phenyl-arsenoxide melarsoprol, which is used in the treatm ent of human African trypanosomiasis, has a broad efficacy against leukemia cells of both lymphoid and myeloid lineage[20]. Both As_2O_3 and melarsoprol also markedly induce apoptosis in plasma cell lines and in plasma cells from multiple myeloma[21]. Recent reports have demonstrated that As_2O_3 can induce apoptosis in various cell lines of solid tumor. As_2O_3 inhibited the growth and survival of solid tumor cell

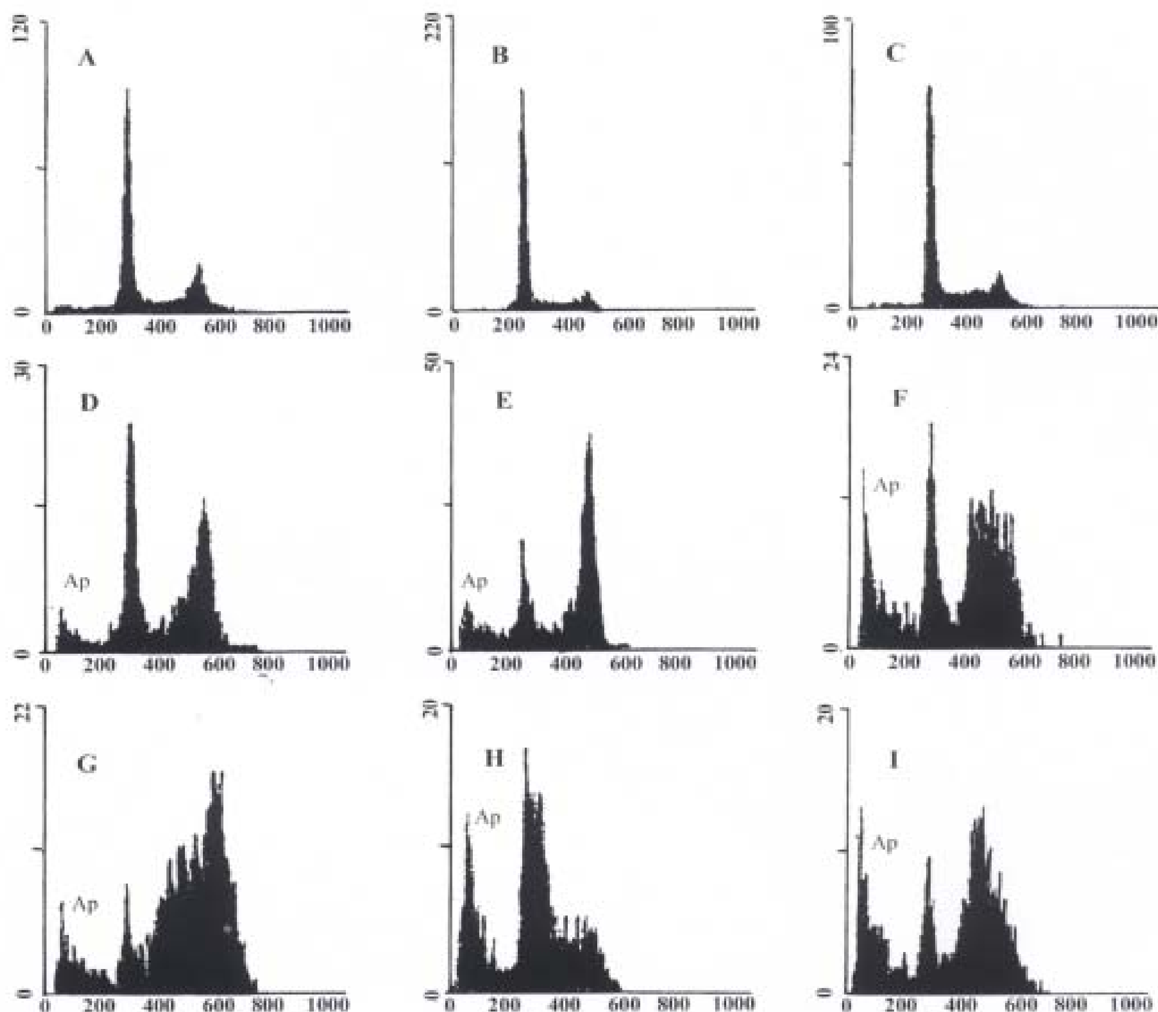

Figure 4 Nuclear DNA contents measured by flow cytometry in As_2O_3 and HCPT-induced apoptosis in gastric cancer cells at 48 h. Ap represents apoptotic cells. A: Untreated MKN-45 cells; B: Untreated SGC-7901 cells; C: Untreate d MKN-28 cells; D: MKN-45 cells treated with 10 μmol/L As_2O_3; E: SGC-79 01 cells treated with 10 μmol/L As_2O_3; F: MKN-28 cells treated with 10 μmol/L As_2O_3; G: MKN-45 cells treated with 10 mg/L HCPT; H: SGC-7901 c ells treated with 10 mg/L HCPT; I: MKN-28 cells treated with 10 mg/L HCPT.

lines in gastric[22], esop hageal[23,24], lung[24], cervix [25], liver[26] tumor and neuroblastoma[27] by triggering programmed cell death. These re sults suggest that As_2O_3 may be an effective drug in treatment of solid tum ors. As_2O_3 has been intensively investigated for the treatment of cancer.

There is growing evidence indicating that apoptosis plays a crucial role in both carcinogenesis and developement of stomach tumors[1,28-30]. Induct ion of apoptosis in gastric cancer cells might be a new means in the treatment of gastric cancer. It was reported that both radiation and chemotherapy (5-fluorouracil, cisplatinum, mitomycin and trichosanthin) can induce apoptosis in gast ric cancer[31-33]. Our previous study showed that As_2O_3 had an effect on inhibiting proliferation and inducing apoptosis in gastric cancer cell SG C-7901[22]. In this study, we found that As_2O_3 exhibited a dose- and time-dependent cytotoxicity on gastric cancer cells at different degrees of differentiation. The IC_{50} of As_2O_3 on MKN-28, SGC-7901, and MKN-45 were 8.9 μmol/L, 10.5 μmol/L, and 11.6 μmol/L, respectively. Toxicity of As_2O_3 was not significantly different among gastric cancer cells at different de grees of differentiation. As_2O_3-treated gastric cancer cells presented char acteristic morphological changes of apoptotic cells. Results showed DNA degrada tion into oligonucleosomal fragments and these changes occurred in a time-and dose-dependent manner. The apoptotic index was 7%-15% as assessed by TUNEL. A typical subdiploid peak before G_0/G_1 phase was observed by flow cytometry. Af ter 48 h of exposure to As_2O_3, the apoptotic rates of SGC-7901, MKN-45 , and MKN-28 were 13.8%, 22.5%, and 9.68%,respectively. We concluded th at one of the main effects of As_2O_3 on gastric cancer cells at the concentrations u sed is to induce cell death by apoptosis.

It was recently shown[45] that the cell cycle time was prolonged in As_2O_3 treated malignant lymphocytes

Namalwa and Raji cells, and that no substan tial increase in cell cycle time was found in Jurkat cells treated with As_2O_3, as compared with untreated cells. This result suggests that As_2O_3 can inhibit proliferation of some malignant lymphocyte cell lines by prolonging the cell cycle instead of arresting cells in a specific phase. In the present study, we demonstrated that the effect of As_2O_3 on SGC-7901 showed remarkable cell cycle specificity in inducing cell death at G_1 phase, and blocking prolifer ation at G_2/M phase. This result is consistent with the report of Deng *et al*[46], in which As_2O_3 induced apoptosis in HeLa cells by arresting G_2/M phase of the cell cycle.

Camptothecin and its analogues are agents with a unique spectrum of antitumor activity mediated by a selective inhibition of eukaryotic DNA topoisomerase I (Topo I). The cytotoxicity of these compounds is pr edominantly exerted during S phase, and is associated with an inhibition of DNA replication. This inhibition is generally thought to be mediated by stablization of the CPT-Topo I-DNA cleavable complex[34]. Recent stud ies have shown that camptothecin and its analogues can strongly induce apoptosis in human leukemic cells[35-36], prostate[36-37], colon[38]and breast[39-40]cancer cells as well as glioma cells[41].

HCPT has been used in the treatment of gastrointestinal tumor[42,43], but its mechanism of anticancer action is still not completely understood. Recently, there were reports that HCPT can induce apoptosis of cancer cells of colon[47], pancreas[48], and bladder[49]. Our previous study showed HCPT can induce apoptosis in gastric cancer cell SGC-701 [44]. In this study, we found that HCPT had a strong dose- and time-dependent cytotoxicity to gastric cancer cells at different degrees of differentiation. The IC_{50} of HCPT on MKN-28, SGC-7901, and MKN-45 was 9.35 mg/L, 10.21 mg/L, and 12.62 mg/L, respectively. There was no significant difference in toxicity of HCPT on gastric cancer cells at different degrees of differentiatio n. Gastric cancer cells treated with HCPT presented characteristic morphological changes of apoptosis. The effects of inducing apoptosis in gastric cancer cells could be co rrelated with time and dosage of HCPT treatment. A typical subdiploid peak before G_0/G_1 phase was observed by flow cytometry. After 48 h of exposure to 10 mg/L HCPT, the apoptotic rates of SGC-7901, MKN-45, and MKN -28 were 13.8%, 22.5%, and 9.68%, respectively. In this study, the fraction of G_0/G_1 p hase was decreased from 54. 2% to 37.6%, while the fraction of S phase was in creased from 25.6% to 38.6% with 10 mg/L HCPT treatment of SGC-7901 c ells for 48 h. The result suggested that HCPT also showed a remarkable cell cycle specificit y, induced cell death and apoptosis at G_1 phase, and arrested proliferation at Sphase.

The molecular mechanism of As_2O_3 on APL cells showed that the inhibition of cell proliferation was due to a direct induction of apoptosis through downreg ulation of bcl-2 expression and modulation of PML/RARα/PML pro tein *in vitro* studies[3]. Furthermore, the activation of caspases was also involved in As_2O_3-induced apoptosis in APL cells [50]. Akao *et al* also reported that As_2O_3 induced apoptosis through the down-regulation of Bcl-2 protein and activation of caspases in B-cell leukemia cell lines[51]. It was also reported that As_2O_3 induced apoptosis in neuroblastoma cell line s through the activation of caspase-3[27]. Litvak *et al* reported that inhibition of gastric cancer by camptothecin involves apoptosis and multiple cellular pathways, and induction of apoptosis of gastric cancer cell was mediated by up-regulation of p53, p21Waf1/Cip1, and p27Kip1 and the down-regulation of Bcl-2 and Bcl-XL[52]. Camptothecin induced apoptosis thr ough activation of caspase in U-937 cells[53]. Our results showed that HCPT and As_2O_3 exerted significant cytotoxicity on gastric cancer cells and induction of apoptosis *in vitro*. The molecular mechanism of apoptotic effect of HCPT and As_2O_3 on gastric cancer cells the remains to be further investi gated, and the effect of apoptosis must be confirmed by *in vivo* studies. However , the results of the present study suggest that HCPT and As_2O_3 might be cand idate drugs to be used in the treatment of gastric cancer, thus needing further stud ies.

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