Abstract. Tetraarsenic hexoxide (As4O6) has been used in Korean traditional medicine for the treatment of cancer since the late 1980's, and arsenic trioxide (As2O3) is currently used as a chemotherapeutic agent. Previous studies suggest that the As4O6-induced cell death pathway is different from that of As2O3 and its mechanism of anticancer activity remains unclear. Nuclear factor (NF)-κB is a well-known transcription factor involved in cell proliferation, invasion and metastasis. Hence, in the present study, we investigated the effects of As4O6 on NF-κB activity and NF-κB-regulated gene expression in vitro and in vivo. The cytotoxicity assay revealed that As4O6 inhibited the growth of SW620 cells in a dose-dependent manner, and the half maximal inhibitory concentration (IC50) was ~1 µM after a 48 h treatment. As4O6 suppressed NF-κB activation and suppressed inhibitory κBα (IkBα) phosphorylation stimulated by tumor necrosis factor (TNF). As4O6 also suppressed downstream NF-κB-regulated proteins involved in cancer anti-apoptosis, proliferation, invasion and metastasis. In addition, As4O6 marginally suppressed tumor growth and the anti-NF-κB activity was confirmed using an in vivo xenograft mouse model in which animals were injected with SW620 cells. The present study provides evidence that As4O6 has anticancer properties through suppression of NF-κB activity and NF-κB-mediated cellular responses.

Introduction

Colon cancer is one of the most common cancers in the world (1). Regarding treatment, surgical resection is frequently limited due to metastasis such as in most other cancers. Although several chemotherapeutic drugs are available for the treatment of metastatic lesions, the toxic effects are serious. Recently, with the advancement in science, the life-span has been increasing, and the elderly population with cancer is also increasing. However, these patients cannot tolerate the cytotoxic effects of chemotherapies. Therefore, new treatment strategies are required for elderly patients. Arsenic trioxide (As2O3) had been used in Chinese medicine for cancer treatment, and is now used as a standard treatment for refractory acute promyelocytic leukemia (2,3). Several clinical trials have been performed in certain types of solid cancers (4,5), yet they failed to prove clinical efficacy due to high toxicities (6,7). Tetraarsenic hexoxide (As4O6) has been used as a Korean folk remedy for the management of cancer since the late 1980's and shows no serious toxicities. However, little research regarding the anticancer effects of As4O6 has been conducted even though previous studies have shown that the signaling pathways of As4O6-induced cell death are different from those of As2O3 (8,9). We previously demonstrated that As4O6 has synergistic effects with tumor necrosis factor (TNF). TNF is known as a stimulator of nuclear factor (NF)-κB and NF-κB-mediated cellular responses.

Key words: tetraarsenic hexoxide, nuclear factor-κB, anticancer effects, colon cancer
Materials and methods

Cells and reagents. SW620 human colon cancer cells purchased from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2. As2O3 was provided by the Chonjisan Institute (Seoul, Korea). Antibodies against NF-κB (p65), cyclin D1, Bcl-2, Bcl-xL, XIAP, clAP-1, clAP-2, MMP-2, MMP-9, VEGF, p-NF-κB, transglutaminase 2 (TG-2), Ki-67 and CD34 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An antibody against β-actin was from Sigma (Beverly, MA, USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulins, and an enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL, USA). All other chemicals not specifically cited were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All of these solutions were stored at -20°C. Stock solutions of 4',6-diamidino-2-phenylindole (DAPI) (100 µg/ml) and propidium iodide (PI; 1 mg/ml) were prepared in phosphate-buffered saline (PBS).

Cell viability assay. For the cell viability assay, the cells were seeded onto 24-well plates at a concentration of 5x103 cells/ml, and then treated with the indicated concentration of As2O3 for 24 or 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) plus 0.01 N HCl was added to dissolve the crystals. The incubation, 100 µl of a solution containing 10% SDS (pH 4.8) was subsequently added to each well. After 3 h of additional incubation, 100 µl of a solution containing 10% SDS (pH 4.8) plus 0.01 N HCl was added to dissolve the crystals. The absorption values at 570 nm were determined with an ELISA plus 0.01 N HCl was added to dissolve the crystals. The cells were incubated in buffered saline with a 1:50 dilution of primary antibodies for p65 NF-κB (Santa Cruz Biotechnology, Inc.) for 2 h and then washed in buffered saline three times for 10 min each at room temperature. They were incubated in buffered saline with a 1:250 dilution of biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). Positive staining was visualized with diaminobenzidine, followed by a light hematoxylin counter-staining.

Western blotting. Total cell lysates were obtained using lysis buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5) and protease inhibitors. The concentrations of cell lysate proteins were determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as the standard. To determine the protein expression of NF-κB in the cytoplasm and the nuclei, we prepared separate extracts. The cells were washed with ice-cold PBS (pH 7.4) and lysed in buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 5 µM leupeptin, 2 µM peptatin A, 1 µM aprotinin and 20 µM phenylmethylsulfonyl fluoride] by repeated freezing and thawing. Nuclear and cytoplasmic fractions were separated by centrifugation at 1,000 x g for 20 min. The cytoplasmic extract (supernatant) was obtained. The pellets were washed with buffer A, and resuspended in buffer B [10 mM Tris-Cl (pH 7.5), 0.5% deoxycholate, 1% NP-40, 5 mM EDTA, 0.5 mM DTT, 5 µM leupeptin, 2 µM peptatin A, 1 µM aprotinin and 20 µM phenylmethylsulfonyl fluoride]. The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 x g for 20 min. The supernatant fraction containing nuclear proteins was collected. Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Thirty micrograms of the lysate proteins were resolved by electrophoresis, electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then incubated with primary antibodies followed by a secondary antibody conjugated to peroxidase. Blots were developed with an ECL detection system.

Immunocytochemistry. The cells were placed on coverslips coated with poly-L-lysine (1 mg/ml) in 6-well plates. They were fixed in 4% paraformaldehyde for 10 min followed by 1.0% H2O2/0.1 M PBS treatment for 30 min after washing twice in PBS. Then, cells were treated with 0.3% Triton/0.1 M PBS for 5 min and then washed twice in buffered saline. They were incubated in 5% serum solution for 30 min at room temperature and then serum solution was removed with suction. The cells were incubated in buffered saline with a 1:50 dilution of primary antibodies for p65 NF-κB (Santa Cruz Biotechnology, Inc.) for 2 h and then washed in buffered saline three times for 10 min each at room temperature. They were incubated in buffered saline with a 1:250 dilution of biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). Positive staining was visualized with diaminobenzidine, followed by a light hematoxylin counter-staining.

Luciferase assay. After experimental treatments, the cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the Dual-Luciferase kit (Promega, Madison, WI, USA), and assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) according to the manufacturer's protocol.

Generation of xenograft tumors and immunohistochemical staining. All animal procedures were performed in accordance with a protocol approved by the Ethics Committee for Animal Experimentation, Gyeongsang National University. We followed animal science guidelines for animal experimentation. Xenograft tumors were generated by subcutaneous injection of SW620 cells, as described elsewhere (11). Briefly, nude mice were injected in a single dorsal flank site with 5x10^6 SW620 cells (n=12 mice) in 100 µl of PBS. Injection of these cells into nude mice induced exponentially growing tumors. When tumors reached a volume of 50-100 mm^3 (termed day 0 for our experiments), the mice were treated intraperitoneally with vehicle (1 µl of normal saline) or As2O3 at 5 mg/kg once a day for 12 days. Tumor size was measured every 3-4 days, and tumor growth was quantified by measuring the tumors in two dimensions. Volumes were calculated by the formula: 0.5 x a x b, where a and b are the longest and the greatest perpendicular diameters, respectively. Tumor volumes were expressed as the mean and 95% confidence interval (CI) and expressed as relative change vs. time. Histopathologic evidence of pulmonary toxicity (i.e., edema or inflammation of the bronchial epithelium and alveoli),
inflammation or injury in other organs, such as liver, and kidney were evaluated by a pathologist. Tumors were fixed in 10% buffered formalin, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E) and immunohistochemical staining. Immunohistochemical staining for p-NF-κB, TG, Ki-67 and tumor vessel density was performed as previously described (12).

Statistical analysis. Each experiment was performed in triplicate. The results are expressed as means ± SD. Significant differences were determined using the one-way ANOVA with post-hoc Neuman-Keuls test in the case of at least three treatment groups and Student's t-test for two group comparison. Statistical significance was defined as P<0.05.

Results

As4O6 suppresses cell proliferation of SW620 human colon cancer cells in a dose-dependent manner. To investigate the antitumor activity of As4O6 in SW620 cells, the cells were treated for 24 and 48 h with various concentrations of As4O6 (0.1-5 µM), and the cell growth was assessed by MTT assay. The MTT assay revealed that As4O6 inhibited the growth of SW620 cells in a dose-dependent manner at 24 and 48 h. As4O6 had a strong inhibitory effect after 48 h of treatment and the half maximal inhibitory concentration (IC50) was ~1 µM (Fig. 1A). Next, we assessed the changes in cellular morphology of the As4O6-treated cells under microscopy. The light microscopy results revealed that cell shrinkage and cytoplasmic blebs were observed after 24 and 48 h of incubation (Fig. 1B).

As4O6 suppresses NF-κB activity at least in part through inhibition of IκBa phosphorylation. To determine whether As4O6 inhibits NF-κB activity of SW620 cells, we used western blotting, immunohistochemistry and luciferase assay. Under resting conditions, NF-κB mostly consists of a heterotrimer of p50, p65 and inhibitory κBα (IκBα) in the cytoplasm; when activated, the heterodimer of p50 and p65 is translocated into the nucleus after separating from p-IκBα. Hence, we performed western blot analysis, which revealed that As4O6 reduced both the translocation of NF-κB into the nucleus and the levels of NF-κB in the cytoplasm (Fig. 2A). One advantage of immunohistochemistry is the ability to confirm NF-κB (p65) translocation into the nucleus on activation. As expected, TNF enhanced the NF-κB-mediated translocation of NF-κB into the nucleus and As4O6 inhibited the TNF-induced NF-κB activation (Fig. 2B). To confirm the effects of As4O6 on NF-κB activity, we performed a luciferase assay. As shown in Fig. 2C, the NF-κB gene was successfully transfected into the cells and the NF-κB-luciferase activity was augmented by TNF. The NF-κB-luciferase activity induced by TNF was inhibited by As4O6 (Fig. 2C). As mentioned, NF-κB activation is required for the degradation of IκBα through phosphorylation by kinases. We also tested whether As4O6 suppressed TNF-induced phosphorylation of IκBα. Western blot analysis revealed that As4O6 prevented TNF-induced IκBα phosphorylation (Fig. 2D). This result suggested that As4O6 suppressed NF-κB activity at least in part through inhibition of IκBα phosphorylation.

As4O6 suppresses NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis. NF-κB activation leads to activation of several genes involved in anti-apoptosis, proliferation, invasion and angiogenesis in cancer. NF-κB regulates expression of anti-apoptotic proteins (c-IAP1/2, XIAP and Bcl-xL) (13), cyclin D1 for cell proliferation (14), MMP-2, MMP-9 for invasion and VEGF for angiogenesis of cancer (13,15). Hence, we investigated the effect of As4O6 on these molecules. Western blot analysis revealed that As4O6 suppressed the protein expression of XIAP, Bcl-2, Bcl-xL, cIAP-1, cyclin D1, MMP-2, MMP-9 and VEGF in a dose- and time-dependent manner (Fig. 3). These findings revealed that As4O6 suppressed the NF-κB-mediated cellular responses regarding cancer apoptosis, proliferation, invasion and angiogenesis in the SW620 cells.

As4O6 marginally suppresses the tumor growth of SW620 cells. Next, we evaluated the effect of As4O6 treatment on the growth of SW620 cells (Fig. 4). Tumor growth was marginally suppressed by As4O6 treatment throughout the 12-day treatment regimen, indicating the potent therapeutic efficacy of As4O6 in SW620 cancer cells (Fig. 4A). The volume of the control SW620 xenografts was 798 mm3 and that of the xenografts treated with As4O6 at 5 mg/kg was 115.9 mm3.
Figure 2. Effects of As$_4$O$_6$ on NF-κB and the IκBα phosphorylation. (A) Inhibitory effects of As$_4$O$_6$ on TNF-induced NF-κB translocation into the nucleus. Cells were treated with As$_4$O$_6$ (1 µM) for 48 h at the indicated concentrations. After treatment, nuclear (NE) and cytoplasmic (CE) fractions were extracted from total cell lysates and protein levels were determined by western blot analysis. (B) Immunocytochemical analysis of NF-κB (p65) localization in the SW620 cells. Cells were pretreated with As$_4$O$_6$ (1 µM) or 0.1% DMSO (vehicle control) for 24 h and then treated with TNF (10 ng/ml) for 30 min (x400, magnification; scale bar, 50 µm). (C) Cells were transfected with an empty vector or 1 µg of NF-κB-luciferase (lue). The cells were allowed to recover for 24 h and then treated with 10 ng/ml of TNF with/without a 1-h pretreatment of As$_4$O$_6$ (1 µM). The cells were harvested 1 h post-treatment with TNF-α and luciferase activities are presented as fold-activation relative to that of the untreated control. (D) Inhibitory effects of 1 µM on IκBα phosphorylation. Cells were pretreated with As$_4$O$_6$ (1 µM) for 1 h and then treated with TNF (10 ng/ml) for the indicated times. Each bar graph represents the mean ± SD of three independent experiments.

*P<0.05 between the treated and control group. As$_4$O$_6$, tetraarsenic hexoxide; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; DMSO, dimethylsulfoxide.

Figure 3. Effects of As$_4$O$_6$ on NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis. (A) SW620 cells (5x10$^4$) were either left untreated or pretreated with As$_4$O$_6$ at the indicated doses for 48 h or (B) pretreated with As$_4$O$_6$ at 1 µM for the indicated times and then whole-cell extracts were prepared. Whole-cell lysate (30 µg) was analyzed by western blotting using antibodies against various NF-κB-regulated proteins involved in cancer cell anti-apoptosis, proliferation and invasion and angiogenesis. As$_4$O$_6$, tetraarsenic hexoxide; NF-κB, nuclear factor-κB.
Also, there were no significant difference in body weight between the control and treatment groups (Fig. 4B). 

As4O6 suppresses NF-κB activity and NF-κB-mediated cellular phenotype such as cancer proliferation and angiogenesis in the in vivo xenograft mouse model. We further investigated the in vivo effect of As4O6 treatment on NF-κB activity and NF-κB-regulated proteins in the SW620 xenograft tumors. Immunohistochemical studies revealed that the expression of p-NF-κB in the tumors from the As4O6-treated mice was lower than that in the control tumors from the untreated mice (Fig. 5). Here, we also tested TG-2 since TG-2 expression has a good correlation with NF-κB activity (16), and a difference in p-NF-κB expression is not easily observed. The result indicated that As4O6 significantly suppressed TG-2 expression. In addition As4O6 also clearly suppressed CD34, a protein which is involved in angiogenesis and Ki-67, a nuclear protein that is associated with cellular proliferation. These findings were consistent with p-NF-κB expression and suggest that As4O6 may suppress NF-κB activity and NF-κB-regulated cellular phenotype.

Discussion

The present study was designed to investigate the anticancer effects of As4O6 with special focus on the NF-κB pathway, and NF-κB-regulated gene products, in in vitro and in vivo models. We found that As4O6 inhibited the growth of SW620 cells in a dose-dependent manner at 24 and 48 h. Furthermore, As4O6 inhibited NF-κB activity and NF-κB-regulated proteins involved in anti-apoptosis, cell proliferation, invasion and angiogenesis. Even though this finding is novel for As4O6, there is previous supporting evidence showing that arsenic trioxide (As2O3) suppresses NF-κB-mediated cellular activities (17). NF-κB is a well-known transcription factor involved in cancer proliferation, invasion, metastasis and drug resistance. We found that As4O6 suppressed MMP-2 and MMP-9 activity. MMP-2 and MMP-9 are key molecules in cancer cell invasion (18,19) which have been used as targets.
TETRAARSENIC HEXOXIDE INHIBITS NF-κB ACTIVITY

Figure 6. Schematic representation of the anticancer effects of As$_4$O$_6$ on SW620 human colon cancer cells. As$_4$O$_6$ suppressed the invasive effects of SW620 cells by suppression of NF-κB through inhibition of IκB phosphorylation stimulated by TNF. In addition, TNF participated in induction of NF-κB-regulated proteins involved in cancer cell proliferation (cycin D1), anti-apoptosis (XIAP, IAP1, IAP2, Bcl-xL, Bcl-2, Bax and Bid), and invasion and angiogenesis (MMP-2, MMP-9 and VEGF). Taken together, the present study suggests that As$_4$O$_6$ has anticancer properties through suppression of NF-κB activity and NF-κB-mediated cellular responses. As$_4$O$_6$, tetraarsenic hexoxide; NF-κB, nuclear factor-κB.

In conclusion, the present study demonstrated that As$_4$O$_6$ exerts anticancer effects by suppressing NF-κB and NF-κB-regulated genes involved in anti-apoptosis, proliferation, invasion and angiogenesis in cancer (Fig. 6). The present study provides evidence that As$_4$O$_6$ may have anticancer effects on human colon cancer.

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References


