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Full Length Research Paper

Studying arsenic trioxide-induced apoptosis of Colo-16 cells with two-photon and confocal laser scanning microscopy

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With two-photon and confocal laser scanning microscopy in combination with fluorescent probes Hoechst 33342, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and Fluo 3-AM, we simultaneously observed arsenic trioxide (As_2O_3)-induced changes in nuclear morphology, reactive oxygen species (ROS) and intracellular calcium concentration $(Ca^{2+})_i$ within human skin squamous carcinoma cells (Colo-16 cells). Our results indicated that As_2O_3 induced $(Ca^{2+})_i$ elevation and ROS production within Colo-16 cells, and both $(Ca^{2+})_i$ elevation and ROS production were involved in the apoptosis of Colo-16 cells. These results suggested that two-photon and confocal laser scanning microscopy might provide a real-time, sensitive and noninvasive method for simultaneously multi-parameter observation of As_2O_3-induced apoptosis at the single cell level.

Key words: Two-photon laser scanning microscopy, confocal laser scanning microscopy, human skin squamous carcinoma cells (Colo-16 cells), arsenic trioxide, apoptosis

INTRODUCTION

Although arsenic is poisonous and chronic arsenic exposure from industrial or natural sources can cause serious toxicity, arsenic has been used therapeutically for more than 2,400 years (Klaassen, 1996); in traditional Chinese medicine, arsenic trioxide (As_2O_3) is used to treat syphilis, rheumatosis, and psoriasis (Shen et al., 1997). Recent researches demonstrate that As2O3 induces partial cytodifferentiation and triggers apoptosis of the leukemia cells, leading to high clinical and molecular remission rates in patients with relapsed acute promyelocytic leukemia (APL) (Soignet et al, 1998; Muto et al, 2001; Zhu et al, 1997), and As2O3 have been approved by Food and Drug Administration of U.S.A. for use in the treatment of relapsed/refractory APL. Fluorescence microscopy is an essential tool of modern biology and has emerged as a novel and noninvasive method for visualization of living cells and biological tissue (Gustafsson, 1999). The effective sensitivity of fluorescence microscopy measurements is often limited by out-of-focus flare. This limitation is greatly reduced in a confocal laser scanning microscopy, where the out-of-focus background is rejected by a confocal pinhole to produce thin (< 1 μm) and unblerred optical sections from within thick samples (White et al., 1987). The two-photon laser scanning microscopy is a new alternative to confocal microscopy and is also a promising technique to observe biological specimens because of its inherent advantage such as three-dimensional resolution without a

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confocal pinhole, less photobleaching above and below an observing plane and long depth penetration with near-infrared light excitation (Denk et al., 1990, 1995). In two-photon laser scanning microscopy, the fluorophore is excited by a focus beam of light of approximately twice the wavelength of the normal excitation light. For example, a fluorophore that normally absorbs ultraviolet light (-350 nm) can be excited by two red photons (-700 nm) if they reach the fluorophore at the same time (-10^{-18} seconds). Therefore, two-photon laser scanning microscopy in combination with confocal laser scanning microscopy might provide the possibility for simultaneously measurement of multi-labeling biological specimens based on the difference between excitation or emission wavelengths of the fluorescent probes.

Although some Chinese medicines used to cure skin cancer contain As_2O_3 and show good therapeutic efficacy (Gilman et al., 1996), its accurate mechanism is still unknown. In this paper, two-photon and confocal laser scanning microscopy were used to study As_2O_3-induced changes in nuclear morphology, reactive oxygen species (ROS) and intracellular calcium concentration $(Ca^{2+})_I$ within human skin squamous carcinoma cells (Colo-16 cells). Our results indicated that As_2O_3 induced concentration-dependent apoptosis of Colo-16 cells and both As_2O_3 -induced $(Ca^{2+})_i$ elevation and ROS production involved in the apoptosis of Colo-16 cells, suggesting that two-photon and confocal laser scanning microscopy might throw new insight into the anti-skin-cancer mechanism of As_2O_3.

MATERIALS AND METHODS

As_2O_3 was purchased from Sigma (St Louis, Missouri, U.S.A). 1 mM stock solution (in RMPI 1640 medium at 0 to 4°C storage) was prepared and diluted to a working concentration before use. RPMI 1640 and foetal calf serum were bought from GIBCO-BRL (Grand Island, NY, USA). Fluorescent probes Hoechst 33342, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and Fluo 3-AM were purchased from Molecular Probes Inc. (Eugene, Oregon, U.S.A.). Trypsin, ethylenediaminetetra-acetate (EDTA), sodium dodecyl sulphate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT) were obtained from Sigma.

Cell culture

The human skin squamous carcinoma cell lines (Colo-16 cells) were provided by the Department of Biology, Beijing Normal University. All cells were cultured in RPMI 1640 containing 10% fetal bovine serum in an incubator containing 5% CO_2. Exponentially growing cells were used in all the experiments.

Cell viability assays

100 µl of cell suspension (1×10^4 Colo-16 cells) was seeded into 96-well tissue culture plates containing different-concentration of As_2O_3. After treatment for 12, 24, 36, 48 and 60 h separately, 10 µl of MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added into each well. After 4 h of incubation at 37°C, the formazan crystals produced in viable cells were dissolved with 100 µl of 10% SDS. OD_{540} was measured on a spectrophotometer to determine the relative cell viability.

Detection of DNA fragmentation

Colo-16 cells at a density of 10^6 cells/ml were treated with different-concentration of As_2O_3 for 24 h and then were collected by centrifugation at 2500 *g* for 5 min. The resultant cell pellets were resuspended in phosphate-buffered saline (PBS) buffer containing 5 mM $MgCl_2$ and lysed in 500 µl of TE buffer containing 0.1% SDS and 1.5 mg/ml proteinase K overnight at 37°C. After two successive extractions with phenol-chloroform, the aqueous layer was transferred to a new centrifuge tube. The DNA was precipitated with ethanol, resuspended in 100 µl water and treated with 400 µg/ml RNase A for 2 h at 37°C. Electrophoresis was performed in 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After electrophoresis, the gel was stained with 1 mg/ml ethidium bromide, watched and photographed under UV light.

Nuclear morphological observation with two-photon laser scanning microscopy

Following treatment with 0, 4 and 10 As_2O_3 separately for 24 h, cells were loaded with 10 µg/ml Hoechst 33342 in RMPI 1640 for 20 min at 37°C. After rinsing three times with modified Hanks' balanced salt solution (HBSS; in mM: 137 NaCl, 5.37 KCl, 0.81 $MgSO_4$, 0.44 KH_2PO_4, 0.37 Na_2HPO_4, 10 HEPES (pH 7.2), 5.56 glucose and 1.37 $CaCl_2$), cells were imaged with a Bio-Rad MRC 1024MP two-photon laser scanning microscopy at 730 nm excitation and >460 nm emission by using a long-pass emission filter.

Measurement of $(Ca2^+)_i$ with confocal laser scanning microscopy

Cells were preloaded with 5 µM Fluo 3-AM in RMPI 1640 for 1 h at 37°C. After washing with HBSS three times, cells were treated with 10 µM As_2O_3 and images of $(Ca^{2+})_i$ were recorded every 20 s for up to 10 min with confocal laser scanning microscopy at 488 nm excitation and 525 nm emission.

Measurement of reactive oxygen species (ROS) with confocal laser scanning microscopy

Cells were loaded with 20 µM H_2DCF-DA in a HEPES-buffered salt solution (HBSS; in mM: 137 NaCl, 5.37 KCl, 0.81 $MgSO_4$, 0.44 KH_2PO_4, 0.37 Na_2HPO_4, 10 HEPES (pH 7.2) and 5.56 glucose, 1.37 $CaCl_2$) for 15 min at 37°C. After rinsing with HBSS twice, cells were imaged with a Bio-Rad MRC 1024MP confocal laser scanning microscope at 488 nm excitation and 510 nm emission.

Fluorescence from one field of cells per cover slip was typically recorded under different experimental conditions. After acquiring the images of baseline fluorescence levels, 10 µM As_2O_3 was applied and 6 additional images were obtained every 5 min.

RESULTS

Cell viability assays

When Colo-16 cells were cultured for 24 to 60 h with

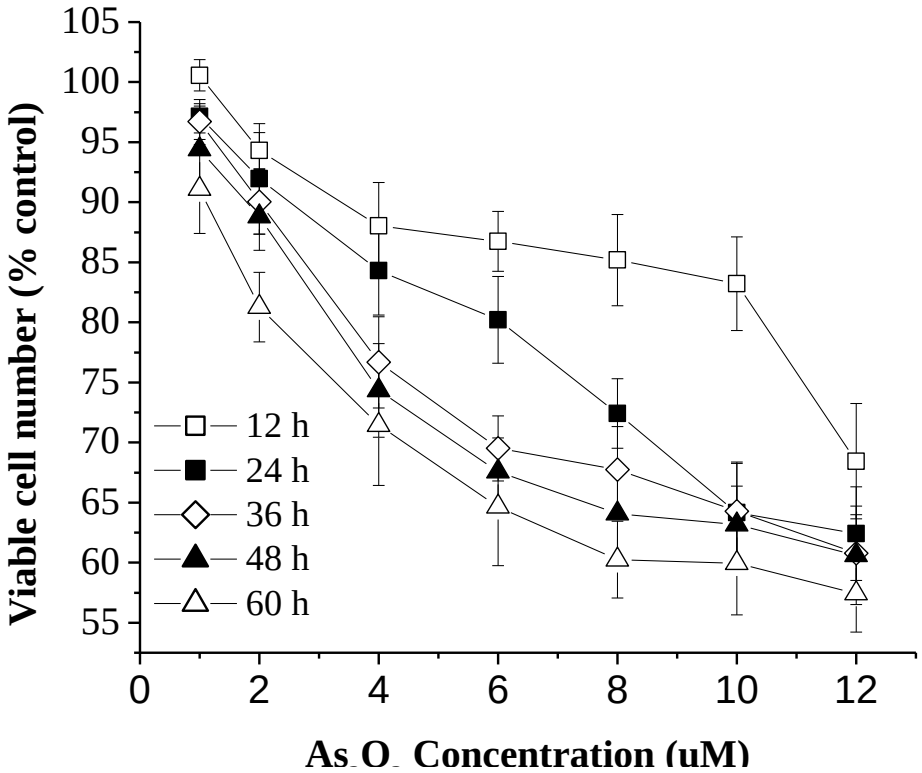

Figure 1. Cytotoxic effect of As_2O_3 on Colo-16 cells. Viable cell number was determined by MTT assay as described in materials and methods. Data shown are expressed as percentage of control values (without As_2O_3 treatment) and represented by the mean ± SEM (n = 5).

As_2O_3 (1 to 12 μM), cell viability were analyzed by MTT and the viable cell number was compared with the untreated control. Figure 1 shows that the viable cell number decreased with both treatment-time and As_2O_3-concentration increased suggesting that the cytotoxic effect of As_2O_3 on Colo-16 cells was both time and concentration dependent.

Detection of DNA fragmentation with electrophoresis and nuclear morphological observation with two-photon laser scanning microscopy

Cells die either following necrosis or apoptosis. Apoptosis (programmed cell death) is an important feature of normal development as well as various disease states, particularly cancer (Kerr et al., 1994). Apoptosis is distinguished from necrosis by characteristic morphological and biochemical changes, including compaction and fragmentation of the chromatin, cell shrinkage and fragmentation into membrane-bound apoptotic bodies, the activation of cysteine proteases such as caspases and internucleosomal fragmentation of DNA by selectively activated DNases (Kerr et al., 1994).

To assess whether Colo-16 cells underwent apoptosis after exposure up to 10 μM As_2O_3, we examined the DNA nucleosomal fragmentation via agarose gel electrophoresis (Figure 2) and nuclear morphology via two-photon laser scanning microscopy (Figure 3).

The biochemical hallmark of apoptosis is the degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180 to 200 bp (characteristic DNA ladder in gel electrophoresis) (Kerr et al., 1994). The results (Figure 2) showed that no DNA ladder appeared with As_2O_3 concentration of less than 8 μM, a faint DNA ladder emerged with 8 μM As_2O_3 and distinct DNA ladder appeared with 10 μM As_2O_3. The result demonstrated that As_2O_3 induced the apoptosis of Colo-16 cells in a concentration-dependent manner.

As shown in Figure 3, the nuclear morphological observation showed that all the examined cells without As_2O_3 treatment had the normal nuclear morphology (Figure 3a). After treatment with 4 μM As_2O_3 for 24 h, some of the examined cells had characteristic chromatin condensation and apoptotic body formation (Figure 3b). Figure 3c shows that after treatment with 10 μM As_2O_3 for 24 h, most of the examined cells had apoptotic body formation. These results further confirmed that As_2O_3 induced apoptosis of Colo-16 cells in a concentration-

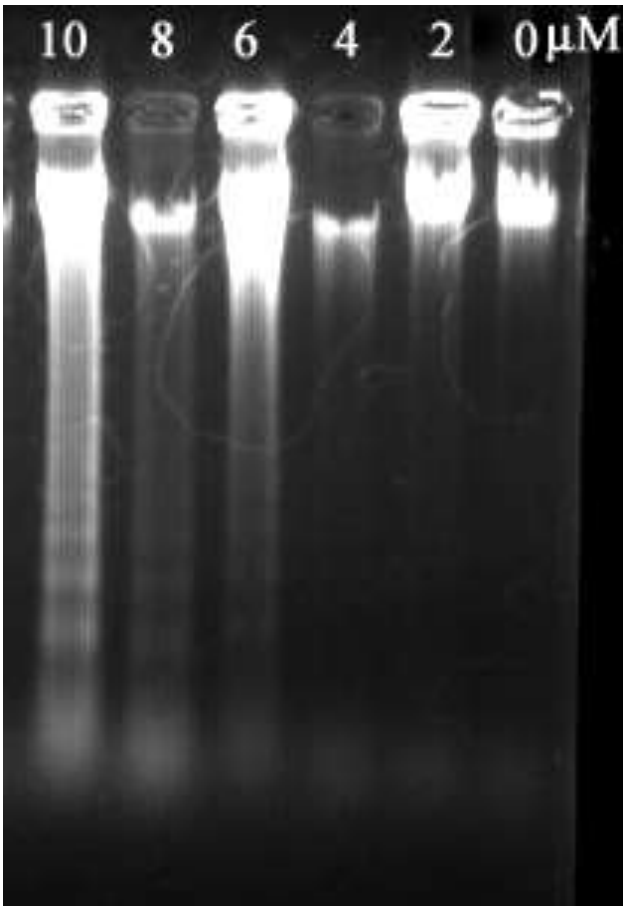

Figure 2. DNA electrophoresis of Colo-16 cells after treatment with As_2O_3 of different concentration for 24 h.

dependent manner.

Measurement of $(Ca2^+)_i$ with confocal laser scanning microscopy

It had been demonstrated that increase in $(Ca^{2+})_i$ played an important role in the induction of Ca^{2+}-dependent endonucleases and in initiation of apoptosis (McConkey et al., 1989, 1988). Simultaneously, observation of nuclear morphological changes via two-photon laser scanning microscopy and $(Ca^{2+})_i$ changes via confocal laser scanning microscopy revealed that the apoptotic cells showed high Fluo-3 fluorescence after 10 μM As_2O_3 treatment for 24 h (Figure 4), and this indicated $(Ca^{2+})_i$ elevation in the apoptotic cells. To confirm whether the $(Ca^{2+})_i$ elevation was induced by As_2O_3 stimulation, we studied As_2O_3-induced changes in $(Ca^{2+})_i$ in a real time with confocal laser scanning microscopy. The results showed that As_2O_3 rapidly increased $(Ca^{2+})_i$ over the treatment time (Figure 5). Both Figures 4 and 5 suggested that As_2O_3-induced $(Ca^{2+})_i$ elevation was involved in the apoptosis of Colo-16 cells.

Measurement of reactive oxygen species (ROS) with confocal laser scanning microscopy

Accumulating evidence pointed to ROS as being second messengers in the activation of enzymes, transcription factors, growth, differentiation and apoptosis (Suzuki et al., 1997; Finkel, 1998; Zhang et al., 2001). In this study, fluorescent probe H_2DCF-DA was used to study As_2O_3-induced ROS production. H_2DCF-DA was not sensitive to the oxidizing agents; when H_2DCF-DA was added to the cells, it diffused across the cell membrane and was hydrolyzed by intracellular esterases to H_2DCF; upon oxidation, H_2DCF yielded highly fluorescent DCF (Zhu et al., 1994). To assess whether ROS was involved in the As_2O_3-induced apoptosis of Colo-16 cells, we simultaneously observed the nuclear morphological changes via two-photon laser scanning microscopy and ROS production via confocal laser scanning microscopy. The results showed that DCF fluorescence increased in the apoptotic cells with chromatin condensation and apoptotic body formation (Figure 6), indicating ROS production in the apoptotic cells. To confirm whether the ROS formation was induced by As_2O_3 stimulation, we

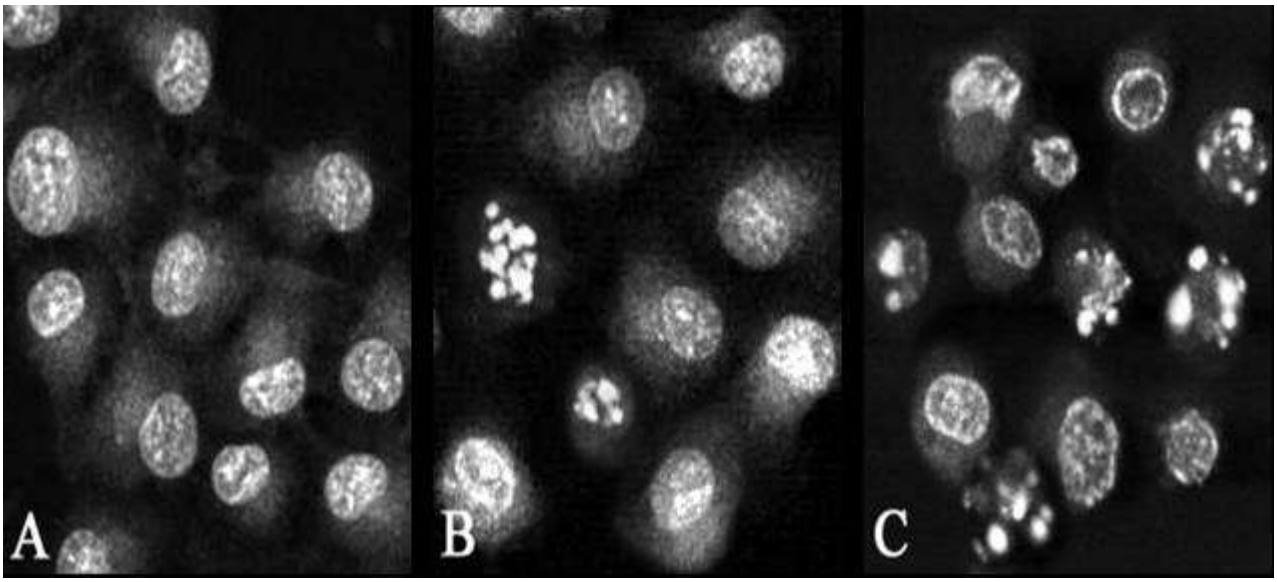

Figure 3. Nuclear morphological changes in Colo-16 cells after treatment with As_2O_3 of different concentration for 24 h. A, 0 μM As_2O_3; B, 4 μM As_2O_3; C, 10 μM As_2O_3. Images of Hoechst 33342 fluorescence was collected by two-photon laser scanning microscopy at 730 nm excitation and >460 nm emission.

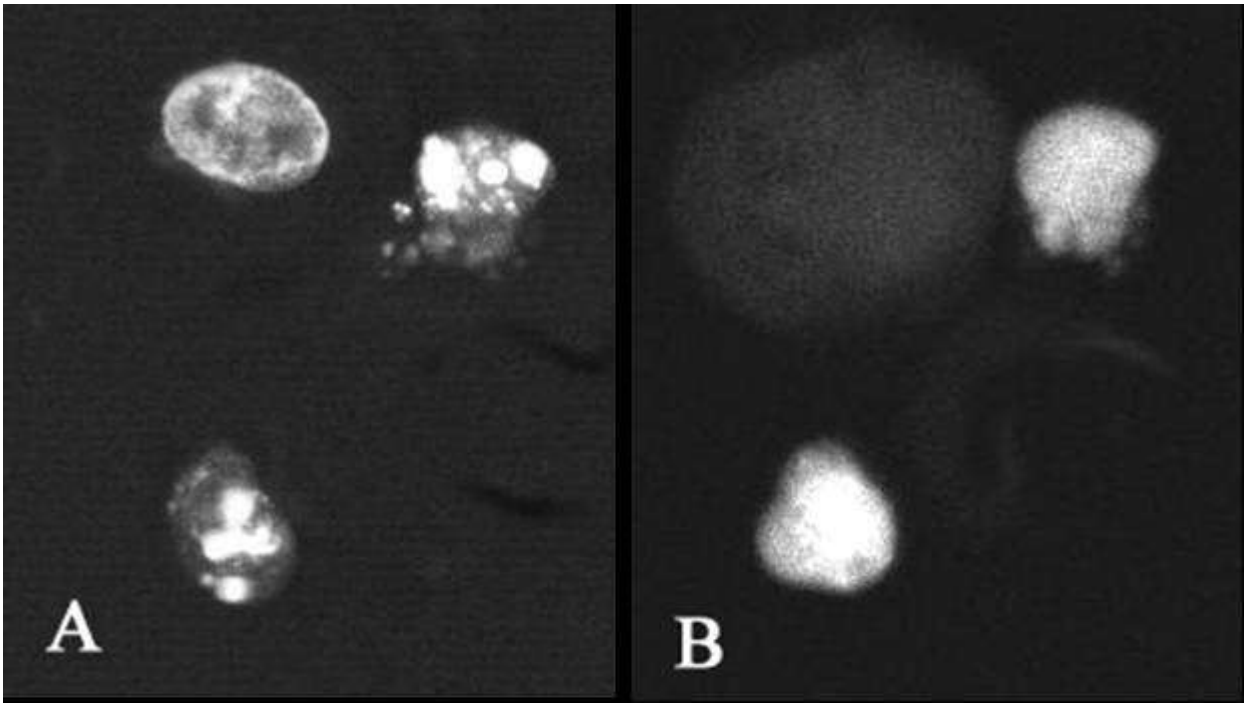

Figure 4. As_2O_3 induced nuclear morphological changes (A) and intracellular calcium changes (B) in Colo-16 cells. Images of Hoechst 33342 fluorescence (A) and Fluo 3 fluorescence (B) were collected simultaneously by two-photon laser scanning microscopy and confocal laser scanning microscopy.

quantitatively investigated As_2O_3-induced ROS production in a real time with confocal laser scanning microscopy. The results showed that the DCF fluorescence increased greatly over time in response to As_2O_3 stimulation; however, DCF fluorescence underwent no dramatic change in the control experiments in which H_2DCF-DA

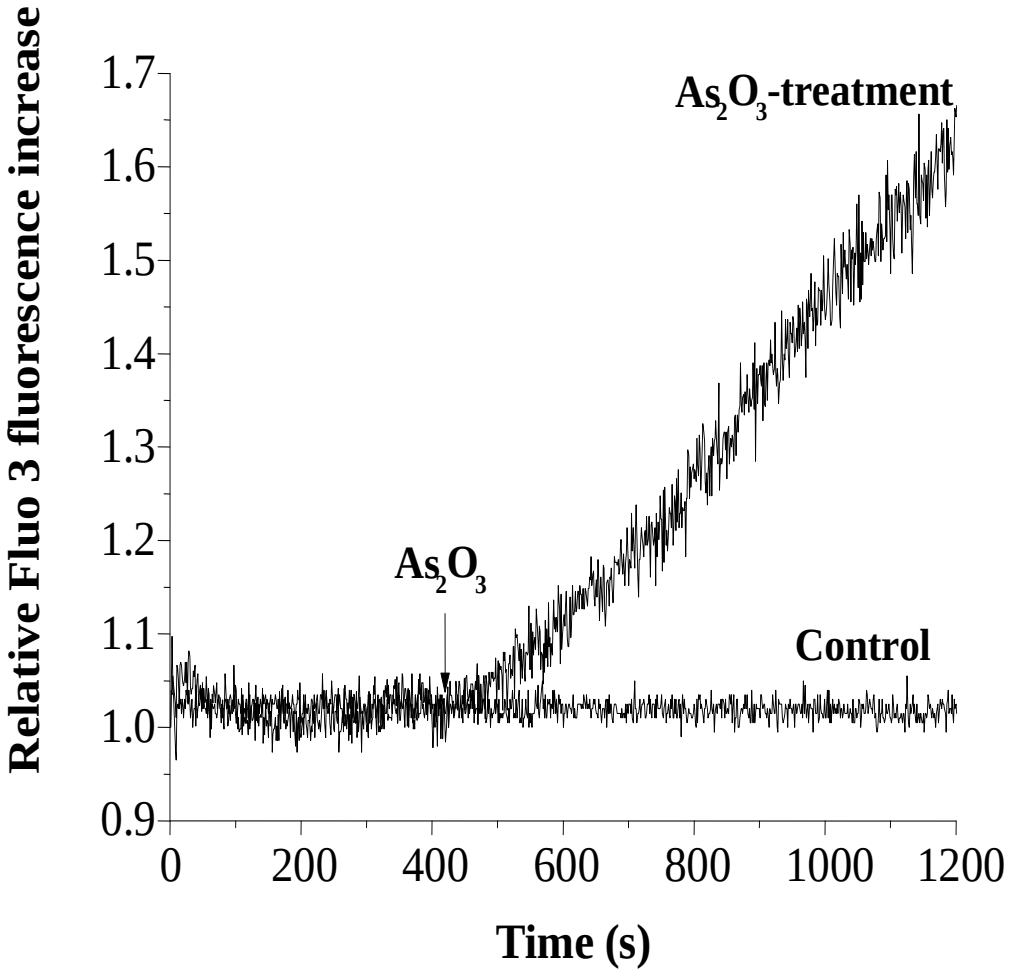

Figure 5. As_2O_3 induced $(Ca^{2+})_i$ elevation within Colo-16 cells. Images of $(Ca^{2+})_i$ were recorded every 10 s for up to 10 min. with confocal microscopy. $(Ca^{2+})_i$ is expressed as emission intensity of Fluo 3 at 525 nm. The PRESENTED data was recorded for a representative single cell and was presented as increase in Fluo 3 fluorescence over time, in multiples of fluorescence at time 0 (= 1).

was loaded without As_2O_3 addition (Figure 7). Both Figures 6 and 7 suggested that As_2O_3 -induced ROS was involved in the apoptosis of Colo-16 cells.

DISCUSSION

Arsenic trioxide (As_2O_3) has been widely used in the clinic treatment of relapsed/refractory acute promyelocytic leukemia (APL); however, exactly how As_2O_3 mediates its clinical efficacy is not fully understood. Two main mechanisms of action of As_2O_3 have been identified from both *in vivo* and *in vitro* studies: promotion of APL cell differentiation (observed at low levels of As_2O_3) and induction of apoptosis (observed at high levels of As_2O_3) (Chen et al., 1997; Shao et al., 1998). The therapeutic potential of As_2O_3 might be extended to malignant diseases beyond APL. As_2O_3 has been reported to have direct cytotoxic effects on different cancer cell lines including leukemic, lymhoma and a variety of solid tumor cell lines in high concentrations (Lee and Ho, 1994). This study showed that As_2O_3 had direct cytotoxic effect on human skin squamous carcinoma cells (Colo-16 cells) in both concentration- and time-dependent manner (Figure 1). Further study with both agarose gel electrophoresis (Figure 2) and two-photon laser scanning microscopy (Figure 3) demonstrated that As_2O_3 induced apoptosis of Colo-16 cells at higher concentration (> 8 μM), suggesting that apoptosis appeared to be the main phenomenon resulting in significant cell death at higher concentrations of As_2O_3. As_2O_3-induced apoptosis occurs via a variety of mechanisms (Miller, 2002). It has been reported that superoxide generation was the main mechanism of apoptosis by As_2O_3 in several experimental

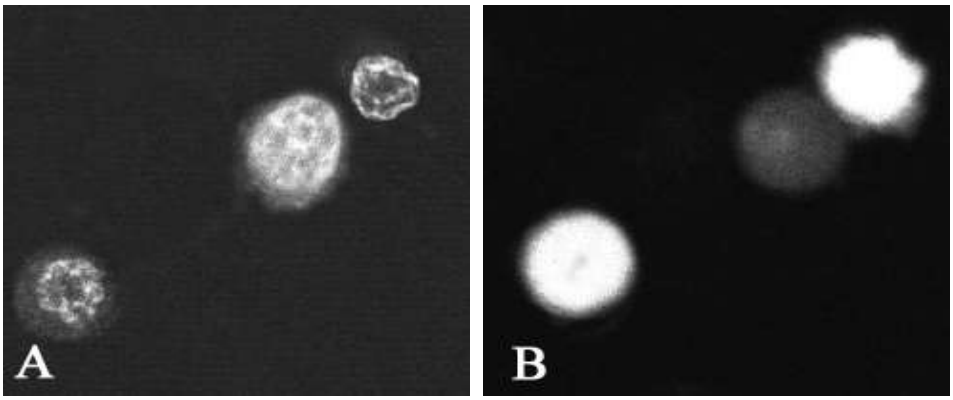

Figure 6. As_2O_3-induced nuclear morphological changes (A) and ROS production (B) in Colo-16 cells. Images of Hoechst 33342 fluorescence (A) and DCF fluorescence (B) were collected simultaneously by two-photon laser scanning microscopy and confocal laser scanning microscopy.

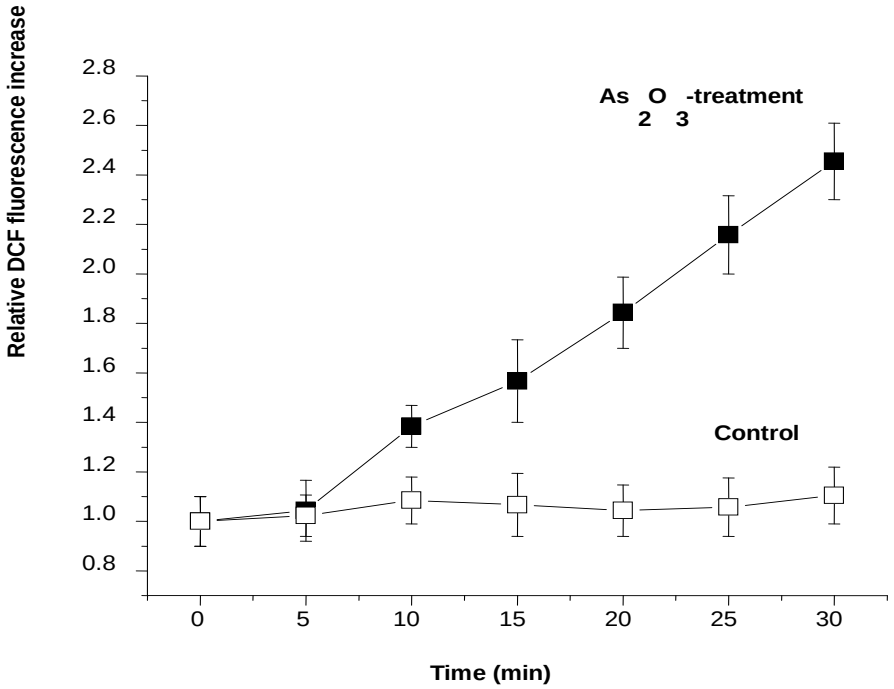

Figure 7. As_2O_3- induced ROS production within Colo-16 cells. ROS production in single cells was expressed as a relative fluorescence increase as compared to the starting fluorescence. The data represents the mean ± SEM of at least 80 cells in each condition.

models (lee and Ho, 1994; Lynn et al., 2000; Jing and Dai, 1999). Generation of reactive oxygen species can cause loss of mitochondrial membrane potential with subsequent changes in membrane permeability and arsenic trioxide can further inhibit glutathione peroxidase and increase cellular hydrogen peroxide content (Jing and Dai, 1999). Our study with the two-photon and confocal laser scanning microscopy (Figure 4) demonstrated that ROS production increased in the apoptotic cells with chromatin condensation and apoptotic body formation and real-time analysis with confocal microscopy (Figure 5) showed that ROS increased greatly over time in response to As_2O_3 stimulation, suggesting that As_2O_3-induced ROS was involved in apoptosis of Colo-16 cells. The increase in $(Ca^{2+})_i$ played an important role in the induction of Ca^{2+}-dependent endonucleases and in the initiation of apoptosis (McConkey et al., 1989, 1988), although in some cases, apoptosis proceeds in the absence of $(Ca^{2+})_i$ changes (Whyte et al., 1993). It has been reported

that intracellular calcium change was involved in As_2O_3-induced apoptosis in three myelocytic leukemia cell lines (Wei et al., 2001). Our study observed with two-photon and confocal laser scanning microscopy (Figure 6) demonstrated that $(Ca^{2+})_i$ was increased in the apoptotic cells with chromatin condensation and apoptotic body formation. Real-time analysis with confocal microscopy (Figure 7) showed that $(Ca^{2+})_i$ increased greatly over time in response to As_2O_3 stimulation, suggesting that As_2O_3-induced $(Ca^{2+})_i$ elevation was involved in the apoptosis of Colo-16 cells.

Apoptosis occurs in many different cell types and in response to diverse stimuli including DNA damage, intracellular damage, toxins and extracellular signals (Kerr et al., 1994). Apoptosis has become a target subject for understanding cellular mechanisms of many diseases, as well as for developing new drugs that interfere with either proapoptotic or antiapoptotic molecular networks. Consequently, it has become important to develop reliable assays to detect apoptosis. Techniques currently available for apoptosis detection are based on the study of morphology of apoptotic cells (light and fluorescence microscopy coupled to nuclear staining with specific dyes and electron microscopy), DNA fragmentation detected by electrophoretic techniques and terminal transferase-mediated dUTP nick-end labeling (TUNEL) and membrane changes detected by annexin V *in vivo* labeling and on immunological assays using antibodies directed to apoptosis-related proteins (Stadelmann and Lassmann, 2000). However, all the earlier mentioned techniques detect only one specific feature of the process of apoptosis. Two-photon and confocal laser scanning microscopies in combination with specific fluorescent dyes provide the possibility for simultaneously multi-parameter observation of apoptosis at the level of single cell. In our study, with combination of two-photon and confocal laser scanning microscopy, we simultaneously observed the changes in nuclear morphology, $(Ca^{2+})_i$ and ROS production during As_2O_3-induced apoptosis of Colo-16 cells, suggesting that two-photon and confocal laser scanning microscopy might provide a real-time, sensitive and noninvasive method for simultaneously multi-parameter observation of drug-induced apoptosis at the single cell level.

Conclusions

With two-photon and confocal laser scanning microscopy in combination with fluorescent probes Hoechst 33342, DCFH-DA and Fluo 3-AM, we simultaneously observed As_2O_3-induced changes in nuclear morphology, ROS and $(Ca^{2+})_i$ within Colo-16 cells. Our results indicated that both As_2O_3 -induced $(Ca^{2+})_i$ elevation and ROS production were involved in the apoptosis of Colo-16 cells. These results suggested that two-photon and confocal laser scanning microscopy might provide a real-time, sensitive and noninvasive method for simultaneously multi-parameter observation of drug-induced apoptosis at the single cell level.

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