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• ESOPHAGEAL CANCER •

Morphological and functional changes of mitochondria in apoptotic esophageal carcinoma cells induced by arsenic trioxide

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Abstract

AIM: **To demonstrate that mitochondrial morphological and functional changes are an important intermediate link in the course of apoptosis in esophageal carcinoma cells induced by As_2O_3.**

METHODS: **The esophageal carcinoma cell line SHEEC1, established in our laboratory, was cultured in 199 growth medium, supplemented with 100mL·L^{-1} calf serum and 3μmol·L-1As_2O_3 (the same below). After 2, 4, 6, 12, 24 h of drug adding, the SHEEC1 cells were collected for light-and electron-microscopic examination. The mitochondria were labeled by Rhodamine fluorescence probe and the fluorescence intensity of the mitochondria was measured by flow cytometer and cytofluorimetric analysis. Further, the mitochondrial transmembrane potential (MTP, ΔΨm) change was also calculated.**

RESULTS: **The mitochondrial morphological change after adding As_2O_3 could be divided into three stages. In the early-stage (2-6 h) after adding As_2O_3, an adaptive proliferation of mitochondria appeared; in the mid-stage (6-12 h) a degenerative change was observed; and in the late-stage (12-24 h) the mitochondria swelled with outer membrane broken down and then cells death with apoptotic changes of nucleus. The functional change of the mitochondria indicated by fluorescent intensity, which reflected the MTP status of mitochondria, was in accordance with morphological change of the mitochondria. The fluorescent intensity increased at early-stage, declined in mid-stage and decreased to the lowest in the late-stage. 24 h after As_2O_3 adding, the cell nucleus showed typical apoptotic changes.**

CONCLUSION: **Under the inducement of As_2O_3, the early apoptotic changes of SHEEC1 cells were the apparent morphological and functional changes of mitochondria, afterwards the nucleus changes followed. It is considered that changes of mitochondria are an important intermediate link in the course of apoptosis of esophageal carcinoma cells induced by As_2O_3.**

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INTRODUCTION

Esophagus cancer is common in China[1-11]. The treatment is still a focus of research[12-17]. Induction of cell apoptosis is a novel therapeutic strategies for cancer[18-25]. In our previous work, we used As_2O_3 to induce apoptosis of esophageal carcinoma cells [26]. The pathomorphological changes evinced that cells became smaller, the cells shrank, the nuclei rounded up, chromatin agglutinated and marginated, nuclear membrane broke down and then followed by the degenerative changes of the cells. All these changes indicated typical morphological changes of apoptosis[27]. The necrotic changes were also found with a large dosage of As_2O_3[28]. We discovered that in the early-stage of cell apoptosis, prior to the obvious change of cell nuclei, the mitochondria showed proliferation. The detailed morphological changes of mitochondria of esophageal carcinoma cells induced by As_2O_3 were firstly described in our paper[29]. We also found that nitric oxide (NO) was released from the cultured esophageal carcinoma cell line after administration of As_2O_3 with increasing amounts at the early apoptotic stage[30]. Furthermore, down regulated expression of bcl-2 and over expression of bax were always found in apoptotic cells induced by As_2O_3[31].

Some authors hold that apoptosis is a programmed cell death (PCD); the death signal originates from the inside of cells; the change chiefly involves the cell nucleus with no apparent changes seen in the cytoplasm and cell organelle[32-33]; making it different from cell necrosis[34]. In our studies, the morphological changes of apoptotic cells induced by As2o3 were different from the programmed cell death in which the latter showed the nuclear changes at first and then cytoplasm, and the former were vice versa[35]. In recent years, it has been explained that apoptosis is related to certain factors, such as Bcl-2/Bax,[36-39] Ca^{2+}[40]and cytochrome c[41-42], which are all located on mitochondria[43]. When they are released from mitochondria, they can inhibit or promote cell apoptosis. Therefore, mitochondria are thought to be the apoptosis regulation center[44]. Mitochondria are also an important organell. They are concerned with cell breathing, oxygen metabolism, enzyme activity and energy supply. All of those functions relate to the permeability of the mitochondria and mitochondrial transmembrane potential(MTP,ΔΨm). When MTP decreases, the mitochondria generate morphological and functional changes[45- 47].

Rhodamine 123 (Rho123), a kind of fluorescent dye, is traditionally used as a mitochondria probe[48]. Rho 123 can quickly gather on living cell mitochondria. The fluorescence intensity of Rho123 represents MTP which reflects the cell in a quiescent or active condition, and in a proliferative or differentiative manner[49]. Flow cytometer and fluorescent microphotometry are the satisfactory

instruments to measure Rho123 fluorescent intensity. The purpose of this paper is to study the mitochondrial morphological and functional changes during the cell apoptosis of esophageal carcinoma cell line induced by As_2O_3, thus demonstrating that mitochondrial changes play an important role in the course of cell apoptosis.

MATERIALS AND METHODS

Cell line and As_2O_3 adding

The esophageal carcinoma cell line SHEEC1 is the human embryonic esophageal epithelial cells malignantly transformed by HPV18 E6 E7 in synergy with TPA[50]. It is cultured in 199 growth medium, supplemented with 100mL·L^{-1} calfserum and antibiotics. In experiments, SHEEC1 cells were cultured separately in culture flasks and on 24-well culture plates (Corning Co.) with the cover slide inside the well, in every well 10^4 SHEEC1 cells were inoculated. As_2O_3 (Sigma, St. Louis, Mo; Lot A 1010) was prepared in concentration of 3μmol·L^{-1} with 199 growth medium. The experimental group and the control group without As_2O_3 administered were examined at definite times. The experiments were repeated once.

Examination under light-and electron-microscope

At 2,4,6,12,24 h after As_2O_3 adding, one culture flask of SHEEC1 cultured cells was taken for examination. The floating cells in the flasks were collected by centrifugation (CytospinIII, Shandon Co.), Giemsa stained and examined by light-microscope. Cells attached to flask were digested with 2.5g·L^{-1} trypsin, centrifuged, the cell pallet was fixed with 25g·L^{-1} glutaraldehyde, and were routinely prepared for electron-microscopic examination.

Rhodamine fluorescent probe labeling and cytofluorimetric analysis (CFA)[51, 52]

SHEEC1 cells were placed on the slide after reacting with As_2O_3 at various times, stained by Rhodamine 123 (Rho123, MW381, Molecular Probe Inc. Eugene) at the concentration of 10mg·L^{-1}, and the cells were incubated in 37℃, 50mL·L-1 CO_2 incubator for 15 min. It was examined by fluorescent microscopy and cytofluorimetry. Using the Nikon fluorescent microscope (Fluophot, Nikon) with Low-cost cooled digital CCD camera system and software STARI (Photometrics LTD. USA), the fluorescent image of mitochondria of SHEEC1 cells labeled by Rho123 were displayed on the screen of monitor, the fluorescent intensity of cells was measured by scanning method, and the average amount of cellular fluorescence was calculated by software.

Flow cytometer (FCM) examination[53]

Following As_2O_3 treatment, SHEEC1 cell cultured in flasks were harvested with trypsinization, washed once with PBS, resuspended in PBS, and incubated with Rho123 (10mg·L^{-1}) at 37℃ for 15 min, stained cells were wash twice with PBS, dispersed, filtered through a 360 mesh nylon net to make single cell suspension. 10^9 cell·L^{-1} were detected by flow cytometer (FACSort, B-D Co. USA) using exciting light 488nm and emission light 515nm to detect Rho123 fluorescent intensity. The histogram managed by the computer was drawn according to the fluorescent intensity value of one cell. Partial of SHEEC1 cells were fixed with 700mL·L^{-1} alcohol, stained with propidium iodide (Sigma) and analyzed with flow cytometer. The cell cycle and apoptotic cell rate were calculated.

Calculation of mitochondrial transmembrane potential (MTP. $\Delta\Psi m$)[46]

Examining 10^4 cells by FCM, the average fluorescent intensity of the cells labeled by Rho 123 before and after As_2O_3 adding were drawn as histograms for comparing. By cytofluorimetric analysis the average fluorescent intensity value (x±s) was calculated from one cell.

RESULTS

Cell apoptosis

Twenty-four hours after As_2O_3 acting on SHEEC1 cells, the apoptotic peak (28% of the cells) before G_1G_0 in DNA histogram of FCM examination appeared (Figure 1). Collecting the floating cells by cytospin and Giesma staining, the cell nuclei showed typical cell apoptotic changes with chromatin agglutinated and marginated (Figure 2).

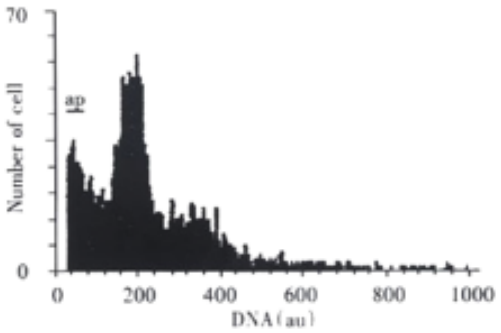

Figure 1 DNA histogram of SHEEC1 cells 24 h after Figure 2 Apoptotic changes 24 h after As_2O_3.

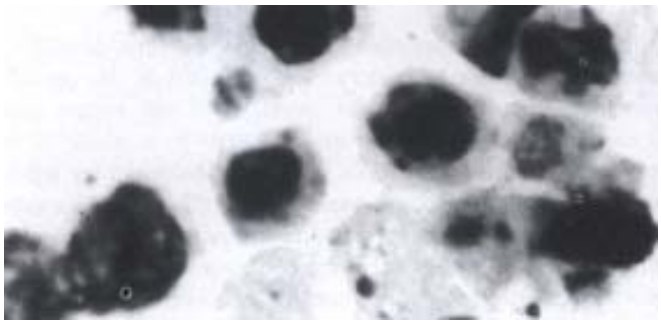

Figure 2 As_2O_3 adding. ap, apoptotic peak. adding, HE×400.

Morphological changes of mitochondria under transmission electron-microscope

Before adding As_2O_3 the mitochondria were located around the nucleus in one or two arrays (Figure 3A). There were fixed intervals between mitochondria, in which other organelles were present. When adding As_2O_3 2-4 h, the mitochondria increased, which showed either concentration in certain areas or in one pole of the cytoplasm or distributed in inner, middle or outer layer of the cytoplasm (Figure 3B). Mitochondria were oval in shape and different in size. The newly proliferated mitochondria were smaller with dense matrix. Some mitochondria were condensed with indistinct ridges and some mitochondria were crowded closely together. After 6 h, the high electron dense and irregular shaped substances precipitated in the mitochondrial matrix, even filled up the whole mitochondria (Figure 3C). The autophagosomes resulting from wrapping of condensed mitochondria by the lysosomes were frequently seen. After 12 h, the mitochondria swelled, its outer membrane broke down, left a single layer of membrane, which were seen like a balloon or a vacuole. After 24 h, the cell nucleus shrank and chromatin agglutinated locating near the nuclear membrane with mitochondria swelling, or becoming vacuole-like or broken down (Figure 3D).

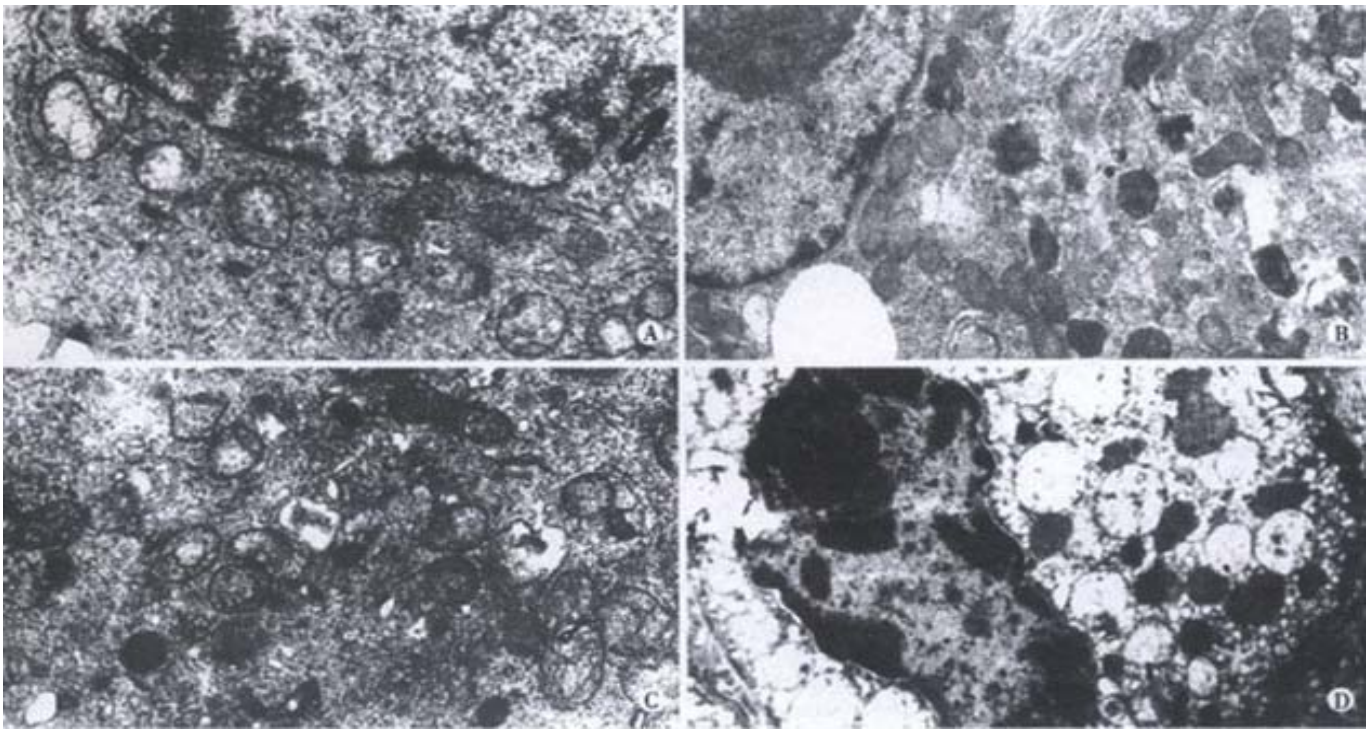

Figure 3 Apoptotic cells (EM x 15000). Mitochondria in 1-2 arrays located around the cell nucleus, not adding As_2O_3; Increment of mitochondria 2-4 h after As_2O_3 adding; Dense substances deposition in mitochondria 4-6h after As_2O_3 adding; Apoptotic cell showed cell nucleus shrank, chromatin agglutinated, mitochondria increased and swelled as balloon-like 24h after As_2O_3 adding.

Functional changes of mitochondria in cell apoptosis: the dynamic changes of MTP (ΔΨm)

Mitochondrial fluorescence intensity detected by FCM After As_2O_3 was added to SHEEC1 cells, the changes of mitochondria fluorescence intensity from different reacting times were seen in histogram (Figure 4 A,B,C,D). A slight increase of mitochondrial fluorescence intensity was observed at 2h after added As_2O_3. With treatment of As_2O_3 for 4-6h, fluorescent intensity of mitochondria was decreased sharply. After 12-24h fluorescent intensity was the lowest.

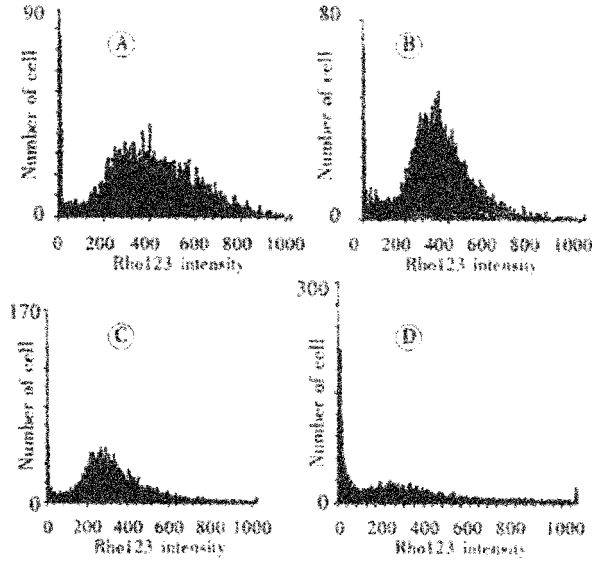

Figure 4 The histogram of mitochondrial fluorescent intensity by FCM after As_2O_3 adding.
A: Control; B: 2-4 h; C: 4-6 h; D: 12-24 h.

Fluorescent intensity by cytofluorimetric analysis Under fluorescent microscope, the number of mitochondria of cells was increased at first (Figure 5) and then decreased. The fluorescent intensity increased in 2h after As_2O_3 added, declined in 4-6h and decreased to the lowest in the 12-24h (Table 1). An increment of fluorescence intensity in partial early-stage apoptotic cells after 2 h of As_2O_3 adding and the intensity decreased hereafter. Following fluorescence associated with the uptake of dye Rho123 allows to evaluate ΔΨm modifications,the results showed the dynamic MTP changes in the apoptotic process induced by As_2O_3.

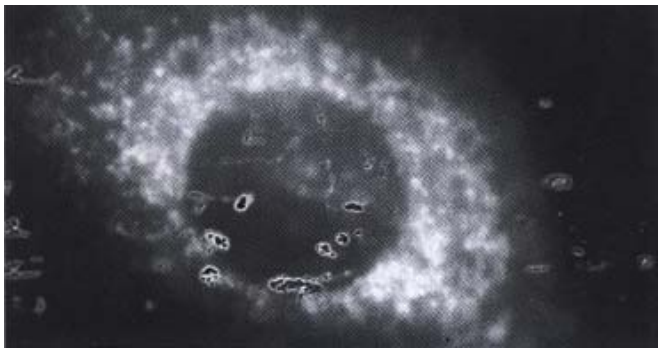

Figure 5 Increment of mitochondria with Rho123 labeled in cytoplasm of SHEEC1 after 2-4 h As_2O_3 adding. ×1000

The Rho123 fluorescence intensity of the labeled mitochondria differed from different reacting times after adding As_2O_3. At first fluorescent intensity increased and then the rapidly declining value of fluorescence intensity was in accordance with both results of FCM and CFA. It taking cell morphology into account, the fluorescence intensity changes may reflect the consequence of As_2O_3 stimulation to mitochondria for different times. 2 h after As_2O_3 was added, the mitochondria proliferated and the fluorescent intensity increased, soon after the

intensity swiftly declined and went to the lowest at 24 h, which indicated that morphological and functional changes of mitochondria induced by As_2O_3 represented the process cell apoptosis.

Table 1 Average fluorescence intensity value of SHEEC1 after As_2O_3 adding (arbitrary unit $x10^{-4}$/cell)

T (after As_2O_3) h	Fluorescence intensity ($\bar{x}\pm s$)
Control	180.3 ± 75.7
2	206.4 ± 93.2
4	170.2 ± 80.3
6	168.2 ± 72.2
12	114.4 ± 70.3
24	90.7 ± 85.6

DISCUSSION

Reports about As_2O_3 inducement of apoptosis of cancer cells have been seen frequently in hemopoietic stem cells and leukemia cells[54-60], but rarely in epithelial tumor cells[61-64]. We have tried to explore the possibility of curing esophageal carcinoma by using As_2O_3 treatment *in vitro*. The experimental results have shown that As_2O_3 can induce cancer cell apoptosis, large doses of As_2O_3 can even induce cell necrosis. Our previous works indicated that at the early-stage of cell apoptosis,morphological changes of the mitochondria might be an important phenomenon in the course of esophageal carcinoma cell apoptosis induced by As_2O_3[29, 31]. Our results showed that morphological and functional changes of mitochondria of SHEEC1 cells were induced by As_2O_3. It could divide into three stages. Two to four h after As_2O_3 administration, the mitochondria proliferated with a lot of new small mitochondria, distributing from the inner layer to the outer layer of cytoplasm. This was the early reaction of mitochondria of SHEEC1 cells to the effect of As_2O_3. 6 h after As_2O_3 inducement, many ridges on mitochondria were seen. The dense substances began to precipitate in the matrix of mitochondria and the condensed or damaged mitochondria were engulfed by lysosomes to form autophagosomes as seen in lymphocytes[65]. Twelve hours after As_2O_3 inducement, the mitochondria were swelling, or vacuolation with mitochondria ridges decreased or disappeared. Twenty-four h after As_2O_3 inducement, apoptotic cells appeared with coagulating chromatin in nucleus and shrinking in the whole cell. The mitochondria swelled like a balloon. During the whole course of cell apoptosis, changes of mitochondria preceded the changes in nuclei.

The fluorescent intensity value detected by CFA and FCM reflects the function of mitochondria[66]. The change of Rho123 fluorescent intensity under As_2O_3 treatment may be divided into 3 time phases: 2-4h after As_2O_3 inducement, mitochondria increased fluorescent intensity, but began to decline after 4-6 h and decreased to the lowest after 12-24 h. These functional changes of mitochondria were in accordance with mitochondrial morphological changes.

The functional changes of mitochondria may be accompanied with decreasing the formation of ATP, reducing the activity of dehydrogenase[67], thus influencing cell respiration, cell metabolism,energy supply and even the cell death. If the mitochondrial release cytochrome c or apoptotic inducement factors (AIF), they may activate the caspases enzyme system, which further act upon cell nucleus and cell keratinoprotein to induce irreversible apoptotic changes[68]. If the mitochondrial changes resulted in lowering of $\Delta\Psi m$, increase of oxygen free radical and blocking up the formation of ATP, the cells will be finally undergo necrosis, because they lose the ability of electron bond transmission. Therefore, the mitochondrial changes may induce cell apoptosis and also cell necrosis[69]. When the inducement factor is strong or highly concentrated it induces cell necrosis. If less in amount and strength, it may give times to activate the caspases enzyme system[70], the cell apoptosis will develop. Mitochondrial fluorescent probe Rho123 is a very useful tool, which may specifically conjugate with mitochondria to indicate cells living state or metabolic state[25]. Detecting Rho123 fluorescence intensity of mitochondria may reveal mitochondrial quantity and function under different kinds of stimuli. The Rho123 fluorescence intensity is stronger in proliferative cells than in quiescent cells, and the intensity decreases in damaged mitochondria caused by harmful stimuli[48]. The amount of Rho123 conjugated with mitochondria differs in different types of cells and in different cell functional status[66]. The mitochondrial changes of SHEEC1 cells induced by As_2O_3 occurred 2-4 h after drug adding. Under the same cultured conditions, mitochondria were supposed to be the firstly targeting site in the course of cell apoptosis. Therefore, under As_2O_3 inducement, the morphological and functional in mitochondria of SHEEC1 cells, which happened prior to cell nuclear DNA change, may be regarded as the important link in cell apoptosis.

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