Simultaneously Multi-parameter Determination of Hematonosis Cell Apoptosis by Two-Photon and Confocal Laser Scanning Microscopy

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Flow cytometry (FCM), TdT-mediated dUTP Nick End Labeling (TUNEL), and DNA ladder are conventional methods to detect apoptosis of drug-treated cells. However, the assumption of cell number restricts their applications in clinics. In this paper, we report a cell-saving imaging method for quick identification of the hematonosis cell apoptosis induced by chemotherapeutic drugs. By the combination of two-photon and confocal microscopy, three main apoptosis parameters

(the change of nuclear morphology, collapse of mitochondrial membrane potential, and increase of intracellular calcium) were recorded simultaneously for single As₂O₃-induced Molt-4 cells. The results are highly in accordance with those produced by classical flow cytometry. This work suggests that this new imaging method would be promising in the quick identification of hematonosis cell apoptosis. *J. Clin. Lab. Anal.* 18:271–275, 2004. © 2004 Wiley-Liss, Inc.

Key words: arsenic trioxide; apoptosis; two-photon laser scanning microscopy; confocal laser scanning microscopy

INTRODUCTION

Inducing apoptosis of tumor cells is one of the main functions of most chemotherapeutic drugs (1). However, the effects of those chemical agents on different patients are very different. After the treatment, it is important to check if the drugs are truly effective. At the present, popular methods for such a purpose include DNA gel electrophoresis, FCM, and TUNEL. However, these methods often require a large number of cells, which prevent them from being widely applied in clinical practice. Confocal and two-photon fluorescence microscopy are techniques for 3D imaging of living cells at submicron resolution. They are particularly useful in the studies of dynamic problems in biological systems. In two-photon microscopy, molecules absorb two infrared photons at the same time and reach energy levels that are accessible by visible or ultraviolet photons only in confocal microscopy. Two-photon excitation results in reduced photo damage, better imaging quality, and deeper penetration into a thick specimen (2,3). With suitable fluorescent probes as contrast agents, confocal and two-photon microscopy can become powerful tools for identifying apoptosis. One could image a small group of cells and observe changes separately in nuclear morpho-

logy, mitochondrial membrane potential, and [Ca²⁺]_i (4,5). However, it is not credible to reach a conclusion of apoptosis by any single parameter. Recently, our laboratory developed a new method with which we can simultaneously record these apoptosis parameters by the combination of two-photon and confocal microscopy (6). In this paper, we demonstrate this method by identifying As₂O₃-induced apoptosis in Molt-4 cell and highlight the application of this method in evaluating the effect of chemotherapeutic agents on hematonosis treatment.

MATERIALS AND METHODS

Chemical Reagents

Arsenious acid injection (1 g/L) was provided by Harbin YiDa Pharmaceutical Co., Ltd., Harbin, China.

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Cell Culture

Human Molt-4 cell line was provided by the Department of Dermatology, Sino-Japanese Friendship Hospital (Beijing, China). Cells were cultured in an RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Carlsbad, CA), in a humidified atmosphere of 95% air/5% CO₂ at 37°C.

Flow Cytometric Analysis of Apoptosis

In viable cells, phosphatidylserine (PS) locates almost exclusively on the inner side of the plasma membrane. Annexin V, a small Ca²⁺ and phospholipid-binding protein, has a high and selective affinity for PS. Using a DNA binding dye such as propidium iodide (PI) with fluorescein-5-isothiocyanate (FITC)-conjugated annexin V, apoptotic cells can be identified and discriminated from necrotic cells.

Molt-4 cells (3×10^6 /well) in 6-well plates were treated with 1, 2, 4, 6, 8, 10, and 12 μ M As₂O₃ for 24 hr, washed twice with phosphate buffered saline (PBS), and resuspended in 200 μ l binding buffer. A total of 15 μ l Annexin V-FITC and 5 μ l PI (Molecular Probes Co., Eugene, OR) was added, left for 15 min at room

temperature in the dark, and then 300 μ l binding buffer was added for flow cytometric analysis.

Observation of Nuclear Morphology Using Two-Photon Laser Scanning Microscopy

Following treating with As₂O₃ for 24 hr, cells were loaded with 10 mg/L Hoechst 33342 (Molecular Probes Inc., Eugene, OR) in RPMI-1640 (Invitrogen Corp., Carlsbad, CA) for 20 min at 37°C. After rinsing three times with PBS, cells were imaged with a Bio-Rad MRC 1024MP two-photon laser scanning microscope (Bio-Rad Laboratories Inc., Hercules, CA) at 730 nm excitation and >460 nm emission using a long-pass emission filter.

Simultaneous Observation of As₂O₃-Induced Changes in Nuclear Morphology, Mitochondrial Membrane Potential, and [Ca²⁺]_i in Molt-4 Cells

After treating with 1 μ M As₂O₃ for 24 hr, cells were loaded with Hoechst 33342 (10 mg/L), Fluo3-AM (10 μ M), and TMRE (5 μ M) (Molecular Probes Inc., Eugene, OR) for 20 min. Then, simultaneous observations of nuclear morphological changes using two-

Fig. 1. Flow cytometry dot plots showing the simultaneous binding of annexin V-FITC and PI uptake by Molt-4 cells after treating with 0, 4, 6, and 10 μ M As₂O₃ for 24 hr. This experiment is representative of three others that show almost identical results.

photon laser scanning microscopy and changes in mitochondrial membrane potential and $[Ca^{2+}]_i$ using confocal laser scanning microscopy were carried out.

Fluorescence intensity of $[Ca^{2+}]_i$ and mitochondrial membrane potential were analyzed by a commercial software, Confocal Assistant, that came with the microscope.

Fig. 2. Variance of apoptotic ratio with As₂O₃ concentration. Apoptosis was assessed by annexin V-FITC and PI binding. Each bar represents the mean SEM of four experiments.

RESULTS

Flow Cytometric Analysis of Apoptosis

When tumor cells were cultured for 24 hr in As₂O₃ (1~12 μM), apoptosis cells were analyzed by FACS and the apoptotic rate was compared with the untreated control. The apoptosis rate of Molt-4 cells increased significantly in a dose-dependent manner as shown in Figs. 1 and 2.

Effect of As₂O₃ on Nuclear Morphological Changes in Molt-4 Cells Detected by Two-Photon Microscopy

In Molt-4 cells treated with As₂O₃ 4 μM and 6 μM, the chromatin began to condense around the nuclear periphery, bleb off from the nuclear surface, and became apoptotic bodies (Fig. 3B,C) while the untreated cells presented intact nuclear morphology (Fig. 3A). After being treated with As₂O₃ 8, 10, and 12 μM, more characteristic apoptotic bodies appeared (Fig. 3D-F).

Fig. 3. Nuclear morphological changes in Molt-4 cells after treating with As₂O₃ of different concentrations for 24 hr. **A:** As₂O₃ 0 μM; **B:** As₂O₃ 4 μM; **C:** As₂O₃ 6 μM; **D:** As₂O₃ 8 μM; **E:** As₂O₃ 10 μM; **F:** As₂O₃ 12 μM. After being treated with As₂O₃ for 24 hr, cells were loaded with 10 mg/L Hoechst 33342 in RPMI-1640 for 20 min at 37°C. After rinsing with Hank's balanced salt solution (HBSS) twice, cells were imaged with a Bio-Rad MRC 1024 MP two-photon laser scanning microscope at 730 nm excitation and >460 nm emissions by using a long-pass emission filter.

Fig. 4. Nuclear morphological changes (A and D), mitochondrial membrane potential changes (B and E), and intracellular calcium concentration changes (C and F) in the same group of Molt-4 cells after treating with 10 μM As_2O_3 for 24 hr. Cultured Molt-4 cells were triple labeled with Hoechst 33342, TMRE, and Fluo3-AM as described in Materials and Methods. Image of Hoechst 33342 fluorescence (A and D) was obtained via two-photon laser scanning microscopy and image of Fluo3-AM and TMRE were obtained simultaneously with confocal laser scanning microscopy. Apoptotic cells (marked by arrow) showed chromatin condensation and apoptotic body formation (D), mitochondrial membrane potential collapse (E), and high Fluo3-AM fluorescence (F).

These results indicated that As_2O_3 -induced apoptosis could be detected from the morphology of nuclei.

Simultaneous Observation of As_2O_3 -Induced Changes in Nuclear Morphology, Mitochondrial Membrane Potential, and $[\text{Ca}^{2+}]_i$ in Molt-4 Cells

Molt-4 cells were triple labeled with Hoechst 33342, Fluo3-AM, and TMRE. Nuclear morphological changes were measured with two-photon laser scanning microscopy. At the same time, changes in mitochondrial membrane potential and $[\text{Ca}^{2+}]_i$ were measured with confocal laser scanning microscopy. Figure 4A–C showed the nuclear morphology, $[\text{Ca}^{2+}]_i$, and mitochondrial membrane potential in the normal Molt-4 cells without As_2O_3 treatment. After treating with 10 μM As_2O_3 for 24 hr, apoptotic Molt-4 cells exhibited chromatin condensation. The same cells also exhibited apoptotic body formation (see arrow in Fig. 4D), mitochondrial membrane potential collapse (see arrow in Fig. 4E), and $[\text{Ca}^{2+}]_i$ increase (see arrow in Fig. 4F), suggesting that As_2O_3 -induced increase in $[\text{Ca}^{2+}]_i$ and

Fig. 5. The fluorescence intensity of intracellular calcium and mitochondrial membrane potential.

decrease in mitochondrial membrane potential were involved in the apoptosis of Molt-4 cells.

The fluorescence intensity of intracellular calcium concentration and mitochondrial membrane potential of each group were measured and listed in Fig. 5. As shown in this figure, more and more cells underwent apoptosis

with increasing concentrations of As_2O_3 when compared with the untreated cells. Fluorescence intensity of the intracellular calcium increased, and mitochondrial membrane potential decreased drastically for apoptotic cells.

DISCUSSION

In the past decades, arsenic trioxide has been recognized in Chinese clinical practice as an effective drug for patients suffering from hematonosis. It triggers apoptosis in acute promyelocytic leukemia (APL) and non-APL (7).

However, As_2O_3 is also well known for its toxic effects (8). As_2O_3 -treated patients can develop skin rashes, musculoskeletal pain, headaches, and peripheral sensorimotor neuropathy. In order to avoid side effects, it is necessary to check if this drug can be truly effective for a patient before the treatment is performed.

For this purpose, conventional methods such as DNA gel electrophoresis, FCM, and TUNEL have been used in the clinical laboratory. However, these methods need at least $0.5\text{--}2 \times 10^6/\text{mL}$ blood cells. Moreover, gel electrophoresis does not allow the detection of apoptosis in individual cells.

Using two-photon and confocal scanning microscopy in combination with three fluorescent probes, Hoechst 33342, TMRE, and Fluo3-AM, we studied As_2O_3 -induced apoptosis by observing changes in nuclear morphology, mitochondrial membrane potential, and intracellular calcium concentration for single Molt-4 cells. Our results showed that the new method was as reliable as other conventional methods, such as flow cytometry, but was faster and required much less sample.

Although we used As_2O_3 as the agent and Molt-4 as the target cell, this method may be extended to other apoptotic agents and other hematonosis. This means

that we may not only predict the therapeutic result of agents at the early stage of treatment, but also monitor the therapeutic process with a single drop of peripheral blood.

In conclusion, simultaneous observation of nuclear morphology, $[\text{Ca}^{2+}]_i$, and mitochondrial membrane potential using two-photon and confocal fluorescence microscopy provide a simple and sensitive technique for the fast detection of apoptosis cells. It is potentially a powerful tool for evaluating the effects of chemotherapeutic drugs.

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