The cell cycle related apoptotic susceptibility to arsenic trioxide is associated with the level of reactive oxygen species

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ABSTRACT

Double staining flow cytometry was performed using 7-amino actinomycin D and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, to detect the level fluctuation of reactive oxygen species (ROS) during the cell cycle of normal NB4 cells. Our results showed that NB4 cells possessed higher level of ROS in G2/M phase than in G1 and S phases. Double staining flow cytometry, with TdT mediated dUTP nick end labeling (Tunel) and propidium iodide (PI), indicated that As₂O₃ (2 μM) could induce apoptosis in NB4 cells prevailing from G2/M phase, and this efficacy was enhanced upon co-administration of 2, 3-dimethoxy-1, 4-naphthoquinone (DMNQ) (2.5 μM) which could produce the endogenous ROS. These results suggested that different ROS level in different cell cycle phases of NB4 cells might determine the selective induction of G2/M apoptosis and the cells' susceptibility to apoptosis by As₂O₃.

Keywords: arsenic trioxide, apoptosis, cell cycle, reactive oxygen species (ROS).

INTRODUCTION

Arsenic trioxide (As₂O₃) has been successfully introduced into clinic trial and thus approved by US Food and Drug Administration (FDA) for treatment of acute promyelocytic leukemia (APL) recently [1-6]. Although it has been widely accepted that, like As₂O₃, many chemotherapeutic drugs function as pro-apoptotic signals which can trigger apoptosis in tumor cells, the heterogeneity of cellular susceptibility to these cytotoxic agents limited their usage on more types of tumors in clinic [7-13].

Reactive oxygen species (ROS), including superoxide radical (O₂^{·-}), hydrogen peroxide (H₂O₂), hydroxyl radical (OH) and singlet oxygen (¹O₂), continuously generated from mitochondrial respiratory chain, have powerful oxidative potential, and are known to be capable of resulting in the oxidative damages. Cells possess an efficient antioxidant defense system, composed of mainly the enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, which can scavenge the excessive ROS and keep its level relatively stable under physiological conditions [14-16]. Recent data showed that many pro-apoptotic signals could raise the cellular ROS level, and cellular inherent ROS level may be associated with the susceptibility of tumor cells to pro-apoptotic signals [10-13, 17-29].

Some researchers reported that low concentration (1-2 mM) of As₂O₃ preferentially induced apoptosis in NB4 cells

of G2/M phase [30]. Our recent work indicated that different susceptibility of cell lines to As₂O₃-induced apoptosis may possess different ROS levels, and thus suggested that inherent ROS level might be determinative in cellular apoptotic susceptibility to As₂O₃ [11]. We therefore questioned whether there was a ROS level fluctuation in cell cycle duration, and cell cycle-related apoptosis of NB4 cells induced by As₂O₃ was associated with such a ROS level variation in cell cycle phases. We herein, first, using a flow cytometric double-staining method, detected the ROS levels in the respective phases of the normal cell cycle, and then investigated the relationship between the variation of ROS level during cell cycle and the apoptosis induced by As₂O₃ in NB4 cells.

MATERIALS AND METHODS

Reagents

As₂O₃, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 7-amino actinomycin D (7AAD), propidium iodide (PI) and N-Acetyl-cystein (NAC) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) was purchased from Calbiochem (San Diego, CA, USA). Catalase was purchased from Huamei Company (Luoyang, Henan, China). As₂O₃ was dissolved in small amounts of 1.0 M NaOH, then diluted to 10.0 mM with phosphate-buffered saline (PBS) as stock solution.

Cell culture

Acute promyelocytic leukemia cell line NB4, kindly supplied by Shanghai institute of hematology, was cultured in RPMI-1640 me-

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dium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 100 IU/mL penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS; GibcoBRL) in a fully humidified atmosphere with 5% CO₂ at 37°C. Cell culture was daily split and maintained at a density less than 5×10⁵ cells/ml.

ROS detection

DCFH-DA was used for ROS detection. DCFH-DA is cleaved intracellularly by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound DCF [31]. DCFH-DA working solution was added directly to the medium to reach 10 μM, and then incubated at 37°C for 15 min. Cells were then washed once, resuspended in PBS and kept on ice for an immediate detection by FACScan (Becton Dickinson, USA). When determining the effects of drugs and antioxidants on ROS level, cells were exposed to antioxidants catalase or NAC for 2 h before drug treatment, and DCFH-DA staining was applied simultaneously with the drug exposure for 15 min.

Double staining flow cytometry was performed, using 7AAD to display DNA content, and DCFH-DA to detect ROS, thus showing the level fluctuation of ROS during a normal cell cycle of NB4 cells free of pro-apoptotic treatment. 7AAD was applied to measure DNA content in order to identify the cell cycle distribution [32]. NB4 cells were treated with 10 μM DCFH-DA for 10 min in normal cultured conditions. After washing in PBS, cells were resuspended in 7AAD labeling buffer (0.1 M phosphate-citrate buffer containing 0.15 M NaCl, 5 mM EDTA, 0.5% BSA and 0.004% saponin, pH 6.0) before 7AAD was added in a final concentration of 20 μM [32]. Cells were analyzed by FACScan (Becton Dickinson, USA) following 7AAD incubation for 5–7 min.

Apoptosis and cell cycle analysis

In order to visualize the apoptosis, NB4 cells were exposed to either 2 μM As₂O₃ alone or in combination with some ROS producer and inhibitors for 16 hours.

TdT-mediated dUTP nick-end labeling (Tunel) / propidium iodide (PI) double staining flow cytometry was performed, to assess the apoptotic rate and to know from which phase these apoptotic cells came. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing twice with PBS, cells were resuspended with 70% pre-cold ethanol and immediately transferred into freezer. The samples were stored at -20°C overnight. Cells were then rehydrated by washing twice with PBS before resuspended in 50 mL Tunel reaction mixture (In Situ Cell death Detection Kit, Roche, Germany) and incubated for 60 min at 37°C in a humidified atmosphere in dark. After washed twice with PBS, cells were treated with 100 U/ml RNase containing 0.002% TritonX 100 for 15 min, and stained with PI (the final concentration 50 μg/ml) for 20 min before analyzed by FACScan [30, 33].

RESULTS

The ROS level varied during the normal cell cycle of NB4 cells

During the single cell cycle, intracellular ROS levels of the intact NB4 cells increased significantly from G1, S phase, to G2/M phase (Fig 1).

As₂O₃ selectively induced apoptosis of NB4 cells phases from G2/M phase

Upon 2 μM As₂O₃ administration for 16 h, NB4 cells were selectively induced apoptosis from G2/M phase (Fig 2 A,B).

DMNQ enhanced the efficacy of As₂O₃ in ROS-dependant manner

2 μM As₂O₃ in combination with 2.5 μM DMNQ (endogenous ROS producing reagent [34]) induced more cells to apoptosis from G2/M phase than As₂O₃ alone (Fig 2C).

To know whether the effect of DMNQ on promoting As₂O₃-induced apoptosis was ROS-dependant, we investigated cellular ROS level upon DMNQ treatment alone or together with two types of antioxidants, or ROS inhibitors and scavengers, catalase and NAC. The data showed that DMNQ did elevate the cellular ROS level and the treatment of catalase or NAC could reverse it totally. Exposure to As₂O₃ alone also resulted in a ROS elevation, and this efficacy was strikingly enhanced by DMNQ cotreatment. Catalase or NAC had the capacity to reverse ROS level elevated by As₂O₃/DMNQ combination (Fig 3).

Correspondingly, the proportion of apoptotic cells was closely associated with the cellular ROS level. DMNQ dramatically enhanced the efficacy of As₂O₃ on apoptosis induction of NB4 cells. Upon administration of 2 μM As₂O₃ together with DMNQ and catalase (or NAC) treatment, the proportion of apoptotic cells was similar to that with As₂O₃ treatment alone (Fig 4). This showed that the apoptosis-enhancing efficacy of DMNQ was reversed by catalase or NAC. Thus we came into a conclusion that the effect of DMNQ on promoting As₂O₃-induced apoptosis from G2/M phase was ROS-dependant.

DISCUSSION

The previous studies indicated that the changes of ROS level might play a role in apoptosis induced by As₂O₃ [12, 13]. Our recent work indicated that cell lines differently susceptible to As₂O₃-induced apoptosis possessed different ROS levels which might be determinative in their apoptotic susceptibility to As₂O₃. For instance, we manifested that, there was an inherently existed difference in whole ROS quantity among four kinds of leukemia cell lines NB4, HL60, K562 and U937, and a positive correlation between the inherent ROS level and their apoptotic sensitivity to As₂O₃. Furthermore, by interference using a ROS producer DMNQ, we demonstrated that an elevation of ROS level would sensitize the cells to As₂O₃-induced apoptosis [11].

In the present study our results showed that a low dose (2 μM) of As₂O₃ induced the apoptosis of NB4 cells pre-

Fig 1. 7AAD/DCFHDA double labeling flow cytometry displaying ROS level during a normal cell cycle. A. DNA histogram. B. 7AAD/DCFHDA bivariate dot-blots, R1: G1 phase, R2: S phase, R3: G2/M phase.

Fig 3. Effect of DMNQ on elevating ROS level determined by flow cytometry and represented by DCF fluorescence intensity (mean±SD, n=3). NB4 cells were treated with DMNQ alone or together with As₂O₃, catalase or NAC for 15min. (Catalase and NAC were pre-incubated with the cells for 2 h.)

vailing from G2/M phase. This was coincident with other researcher's results[30].

To our knowledge, methodology applying 7AAD and DCFH-DA double staining flow cytometry had not been documented, even if 7AAD used in DNA measurement and DCFH-DA in ROS detection were separately established[31,32]. The feasibility of this novel approach was demonstrated in the present work. Our data showed that during a normal cell cycle in NB4 cells, ROS levels gradually increased from G1, S to G2/M phases, or alternatively speaking, G2/M phase cells possessed inherently greater content of ROS than G1 and S phase cells. The higher level of ROS in G2/M phase might be thus logically assumed to account for that G2/M cells were more predisposed to apoptosis induction by 2 μM As₂O₃.

The cell cycle related differences in susceptibility to apoptosis induced by various antitumor agents have been noticed for long in HL-60 cells[35], in which cells in G2/M phase were largely apoptotic when treated with a serine/threonine kinase inhibitor H7 or with gamma-irradiation

Fig 2. TUNEL/PI bivariate dot-blots displaying prevailling apoptotic cells from G2/M phase of NB4 cells upon administration of As₂O₃ at 2 μM alone or in combination with 2.5 μM DMNQ for 16 h. A: control; B: As₂O₃ alone; C: As₂O₃ in combination with DMNQ.

Fig 4. Apoptotic rates analyzed by TUNEL/PI double labeling flow cytometry (mean±SD, n=3). NB4 cells were treated with As₂O₃ alone or together with DMNQ, catalase or NAC for 16 h.

whereas some other agents induced G1 or S phase cell apoptosis selectively. Though the clinic relevance of this mode of cell death was discussed, the mechanisms underlying cell cycle related differences in apoptosis susceptibility were virtually obscure[35]. B lymphocytes' growth arrest and apoptosis induced by activation displayed G1 phase specificity[36], and this was partially explained by the delicately regulated expression or posttranslation modification of cyclin-dependent kinase 2 and other cell cycle proteins. In the present study we found the link of ROS level variance in the cell cycle with the cycle-related apoptosis induced by As₂O₃. Since ROS has been considered as one of mediators of cell apoptosis induced by UV exposure and gamma-irradiation[37], and also by arsenic trioxide[10-13] in varied types of cell, the higher apoptosis susceptibility in G2/M phase to gamma-irradiation might be presumed to reflect a mechanism similar to one described here, in which ROS played a crucial role in determining the cell cycle related apoptosis susceptibility.

The link of ROS variance during the cell cycle with the

apoptosis susceptibility might imply that there existed a “threshold level” for ROS, under which cell could survive its oxidative damages and apoptosis triggering. Thinking of that As_2O_3 could elevate the ROS level of NB4 cells, we deduce that, upon treatment with As_2O_3 , the ROS level of G2/M phase cells exceeded that cell could endure. Hence the cells in G2/M phase were induced to apoptosis after a transitory replication arrest. The ROS levels of G1 and S phases cells were still under the “threshold level” after treatment with As_2O_3 . These cells could progress to next stage of cell cycle, but ultimately be arrested in G2/M phase and triggered to apoptosis as well.

To affirm the association of ROS variance in the cell cycle with apoptosis susceptibility, we use DMNQ in combination with As_2O_3 for apoptosis induction. DMNQ facilitated the efficacy of As_2O_3 on selective induction of apoptosis of NB4 cells from G2/M phase. This efficacy of DMNQ could be reversed by catalase and NAC. DMNQ, one type of quinones that underwent redox cycling and was widely used to investigate oxidative stress, has been proved to generate endogenous ROS and carried into execution of apoptosis through ROS-dependent pathway [34]. Since DMNQ produced ROS whereas catalase and NAC removed ROS from cells, our results further argued that ROS level difference in the respective cell cycle phases of NB4 cells should be pivotal in cycle-related cellular sensitivity to As_2O_3 -induced apoptosis.

As some other investigators demonstrated that a high dose of As_2O_3 (8 μM) induced apoptosis of NB4 cells from each phase of cell cycle [30], and a very low dose of As_2O_3 (less than 0.5 μM) didn't induce apoptosis at all [38], we made a systematic observation on the efficacy caused by different concentration of As_2O_3 (0.5-8 μM) and got the similar findings (data not shown). This phenomenon, as we assumed, might be attributed to ROS threshold hypothesis. ROS levels in each phase of NB4 cell cycle, upon 8 μM As_2O_3 administration, might surpass the threshold and elicit an intensive apoptosis of cells in all phases, while ROS levels keep safe for the cells in all phases after administration with less than 0.5 μM As_2O_3 .

The results in the present study and the ROS threshold hypothesis we proposed based on them seemed to shed more lights on the mechanisms by which As_2O_3 induce apoptosis in certain types of tumor cells. Not only As_2O_3 induced apoptosis be ROS dependent, also ROS likely be of determination in cellular susceptibility to apoptosis induction by As_2O_3 . As it has been clear that mitochondria is a central executor in apoptosis [39], it is conceivable to recognize the unique and pivotal role of ROS. Identification in detail how ROS mediates and signals As_2O_3 induce apoptosis will certainly help to elucidate the mechanisms of apoptosis induced by As_2O_3 and other chemotherapeutic

agents.

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