In Vitro Studies on Cellular and Molecular Mechanisms of Arsenic Trioxide (As$_2$O$_3$) in the Treatment of Acute Promyelocytic Leukemia: As$_2$O$_3$ Induces NB$_4$ Cell Apoptosis With Downregulation of Bcl-2 Expression and Modulation of PML-RAR$_\alpha$/PML Proteins


It has been shown recently in China that arsenic trioxide (As$_2$O$_3$) is a very effective treatment for acute promyelocytic leukemia (APL). APL patients resistant to all-trans retinoic acid (ATRA) and conventional chemotherapy can still respond to As$_2$O$_3$. In this study, we addressed the possible cellular and molecular mechanisms of this treatment by using NB$_4$ cells as a model. The results show that: (1) As$_2$O$_3$ triggers relatively specific NB$_4$ cell apoptosis at micromolar concentration, as proved by morphology, histogramic related nuclear DNA contents, and DNA gel electrophoresis. (2) As$_2$O$_3$ does not influence bax, bcl-x, c-myc, and p53 gene expression, but downregulates bcl-2 gene expression at both mRNA and protein levels. (3) As$_2$O$_3$ induces a significant modulation of the PML staining pattern in NB$_4$, cells and HL-60 cells. The micropunctuates characteristic of PML-RAR$_\alpha$ in NB$_4$ cells disappear after treatment with As$_2$O$_3$, whereas a diffuse PML staining occurs in the perinuclear cytoplasmic region. In addition, a low percentage of untreated NB$_4$ cells exhibits an accumulation of PML positive particles in a compartment of cytoplasm. The percentage of these cells can be significantly increased after As$_2$O$_3$ treatment. A similar PML staining pattern is observed in apoptotic cells. (4) ATRA pretreatment does not influence As$_2$O$_3$-induced apoptosis. These results suggest that induction of cell apoptosis can be one of the mechanisms of the therapeutic effect of As$_2$O$_3$. Moreover, this apoptosis induction occurs independently of the retinoid pathway and may be mediated, at least partly, through the modulation of bcl-2, as well as PML-RAR$_\alpha$ and/or PML proteins.

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A CUTE PROMYELOCYTIC leukemia (APL), a particular subtype of acute myeloid leukemia (AML) with a distinct cytologic morphology (M$_3$ and M$_3$ variant in French-American-British [FAB] classification), is characterized by a specific chromosome translocation t(15;17), which results in the rearrangement of PML (for pogyelocytic leukemia) gene and retinoic acid receptor-$\alpha$ (RAR$_\alpha$) gene and the expression of PML-RAR$_\alpha$ chimeric protein. Recently this protein has been considered to have an important role in APL pathogenesis. Since 1988, it has been widely confirmed that all-trans retinoic acid (ATRA) can induce clinical complete remission (CR) in over 85% of APL patients by a differentiation process, and PML-RAR$_\alpha$ protein may be the direct target of ATRA. However, ATRA treatment has two clinical limitations, namely, retinoic acid syndrome, which causes progressive hypoxemia and multiorgan failure in 5% to 25% of APL patients, and retinoid resistance, which is developed in almost all patients. Therefore, it is of great importance to develop new therapeutic means for APL patients, especially for those resistant to ATRA and conventional chemotherapeutic drugs.

Recently it has been shown that arsenic compounds including arsenic trioxide (As$_2$O$_3$), and arsenic disulfide, two components used in some traditional Chinese remedies, are very effective in APL treatment. For example, a report from the Northeast Region of China showed that As$_2$O$_3$ (10 mg/day, intravenous [IV] infusion for 28 to 60 days) induced clinical CR in 65.6% of APL patients. More interestingly, 28.2% (9 of 32) of patients had a survival of more than 10 years. At the Shanghai Institute of Hematology, a recent clinical trial with As$_2$O$_3$ treatment also showed that CR was achieved in 15 of 16 APL patients who relapsed after ATRA-induced and chemotherapy-maintained CR (Z-X Shen, G-Q Chen, Z-Y Wang, Z Chen, in preparation). It is noteworthy that no significant bone marrow suppression was observed in most patients supervening As$_2$O$_3$ treatment. This situation differs significantly from that observed with conventional cytotoxic chemotherapy.

In this study, we investigated the possible cellular and molecular mechanisms of As$_2$O$_3$ treatment by using NB$_4$ cells, an APL cell line with chromosome translocation t(15;17) from a relapsed APL patient, as an in vitro model. The results show that As$_2$O$_3$ can induce apoptosis in NB$_4$ cells, which is possibly associated with the downregulation of bcl-2 gene expression and the modulation of PML-RAR$_\alpha$/PML proteins.

MATERIALS AND METHODS

Compounds. Drug Antileukemia-1 or Ai-Lin 1 (AL-1, solution 1), a 0.1% As$_2$O$_3$, solution for IV administration, was provided by...
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the Pharmacy of Chinese Traditional Medicine in the First Hospital affiliated to Harbin Medical University (Harbin, P.R. China). Another As2O3 preparation (Sigma, St Louis, MO; Lot A1010, solution II) was also used. Stock solutions were made at the concentration of 1 mmol/L with phosphate-buffered saline (PBS) and diluted to working concentration before use. A total of 1 mmol/L ATRA (Sigma) stock solution was made by ethanol.

Cell culture and cell viability assay. Human leukemia cell lines NB4, HL-60, and U937 cells were cultured by initially seeding 2 × 10^6 cells per milliliter of fresh RPMI 1640 medium (GIBCO-BRL, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 mg/mL streptomycin (GIBCO-BRL), and with or without compounds as described above, in a humidified atmosphere of 95% air/5% CO2 at 37°C. To avoid possible effects of cell density on cell growth and survival, cells were maintained at less than 5 × 10^6 cells/mL with daily adjusting cell concentrations by adding fresh culture medium and corresponding concentrations of compounds when necessary. At assay time, cells were collected, mixed with an equal volume of PBS containing 0.4% trypan blue dye, and manually counted. Actual cell numbers were calculated by multiplying diluted times compared with initial cell numbers. Cell viability % = viable cell numbers/total (viable + dead) cell numbers × 100%. Inhibition % = (control groups - experimental groups)/control groups for viable cell numbers × 100%. Differentiation was evaluated by nitroblue tetrazolium (NBT, Sigma) reduction test.

Cell morphology and flow cytometry assays. A total of 10^6 cells were harvested at different times after As2O3 treatment. For morphological observation, cells were centrifuged onto slides by cytospin (Shandon, 500 rpm, 4 minutes) and stained with Wright’s and hoechst 333258 (10 µmol/L), respectively. For flow cytometry assay, cells were washed twice with cold PBS and injected into cold (-20°C) 100% ethanol, which was then kept overnight at 4°C. Subsequently, cells were rinsed with PBS, treated with Tris-HCl buffer (pH 7.4) containing 1% RNase and stained with propidium iodide (PI) 5 µg/mL. Distribution of cells with different DNA contents was determined on flow cytometry (Becton Dickinson, San Jose, CA). All data were collected, stored, and analyzed by Lysys II software (Becton Dickinson).

DNA fragmentation assay. A total of 10^6 cells with or without As2O3 treatment were washed twice in cold PBS. The pellets were lysed with 2 mL of lysis buffer (50 mmol/L Tris-HCl pH 8, 20 mmol/L EDTA pH 8, 2% sodium dodecyl sulfate [SDS], incubated overnight at 37°C and chilled on ice for 10 minutes. To precipitate proteins, 0.8 mL of a saturated NaCl solution was added and centrifuged twice at 3,000 rpm for 60 minutes. RNAse was then added to supernatants at a final concentration of 20 µg/mL and incubated at 37°C for 15 minutes. DNA was precipitated by adding two volumes of 100% ethanol and electrophoresed overnight on a 1.25% agarose gel. gPEM-7Zf(-) DNA digested with Hae III (marker 1) and λ-DNA digested with Hind III (marker 2) were used as molecular size standards. The stained gel was visualized by transillumination with ultraviolet (UV) light (302 nm) and photographed.

Northern analysis. Total RNA was isolated from 10^6 cells by the guanidinium/CSCl gradient method. A total of 20 µg of RNA was separated on a 1% agarose gel and transferred to nitrocellulose filters. The filters were hybridized with the different probes, including human bax cDNA (229 bp),15 bcl-XL cDNA (0.8 kb),16 bcl-2 genomic DNA (1.5 kb),17 c-myc cDNA, and p53 cDNA. Prehybridization and hybridization were performed in 0.5 mmol/L PBS (pH 7.2), 7% SDS, 1 mmol/L EDTA, and 0.5 mg/mL salmon sperm DNA at 65°C. Filters were washed in 40 mmol/L PBS/1% SDS for 30 minutes at 37°C, dried, and exposed to x-ray film. Ethidium bromide staining of 28S rRNA and rehybridization with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe were used as loading and transfer controls.

Western blot analysis. Protein extracts were prepared from 2 × 10^4 cells injected into 250 µL lysis buffers [0.1 mol/L NaCl, 0.01 mol/L Tris-Cl (pH 7.6), 0.001 mol/L EDTA (pH 8.0), 1 µg/mL aprotinin, and 100 µg/mL phenylmethylsulfonyl fluoride] at 4°C for 30 minutes. Supernatant was recovered after centrifugation, combined with an equivalent volume of 2× SDS loading buffers (0.1 mol/L Tris-Cl, pH 6.8, 0.2 mol/L dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerine). The samples were loaded (30 µL/lane) on a 12% SDS-polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane that was subsequently stained with 0.2% Ponceau red to assure equal protein loading and transfer. After neutralization of the membrane in 1% bovine serum albumin (BSA), the membrane was incubated for 90 minutes with bcl-2 monoclonal antibody (DAKO A/S, Kyoto, Japan) in 1% BSA. The immunocomplex was visualized by colorization with horseradish peroxidase-conjugated second antibody and dianinobenzene substrates. To assure equal protein loading, a similar experiment was performed using anti-actin antibody as internal control.

Immunofluorescence analysis. Cells were pelleted onto slides as described above, and the slides were quickly air-dried. Immunofluorescence staining of the N-terminal region of PML was performed according to a previously described method.18

Intracellular As2O3 content measurement. HL-60 and NB4 cells (10^4) were maintained in culture mediums with 0.5 µmol/L As2O3 for 24 hours, respectively. Cells were washed twice with PBS and pellets were dissolved in 1 mL 0.1% Triton-100. Arsenic contents were measured by gas-phase chromatography on the basis of chemical reaction: tribasic arsenic + dimercapto propanol (British anti-Lewisite, BAL) → pentabasic anular compounds (AS-BAL). Briefly, being mixed in turn with solution A (concentrated nitric acid: concentrated perchloric acid 7:5:1) and solution B (1.5 M/L HCL, 10% KI, 1% sulfocarbamid), 1 mL cell lysates were dried. Then, 0.5 mL 0.2% BAL solution and 1 mL methylenzene was added and centrifuged. A 1-mL supernatant was analyzed for As2O3 content by gas chromatography (GC-900) with the following analytic conditions: two-meter-long column, fixed liquid OV-17, sample-entering column and detector temperature 200°C, with loading gas of pure nitrogen and flow rate 45 mL/minute. Standard curve for peak heights of AS-BAL to concentrations of standard As2O3 was established and a linear curve between 0.1 nmol/L to 10.0 nmol/L was obtained. AS-BAL was well separated (not shown) with gas chromatography. The recovery rate for AS2O3 was 95.2% to 104.3%.

Plasma drug concentration assay after continuous use of As2O3 in relapsed APL patients. The clinical use of As2O3 was recently approved by the Medical Ethics Committee of Rui-Jin Hospital, Shanghai Second Medical University, for relapsed APL patients resistant to ATRA and conventional chemotherapy. The plasma As2O3 concentrations were measured in eight APL patients who were relapsed cases after ATRA-induced and chemotherapy-maintained CR and were included, with informed consent, into a recent clinical trial performed in Shanghai Rui Jin Hospital. As2O3 was given at a dose of 10 mg/day by IV drip (diluted in 500 mL 5% glucose saline and administered within 2 hours). At day 1, middle (days 14 to 16) of the course, and immediately after CR, heparinized blood samples (3 mL each) were obtained before drug administration and 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours thereafter. The measurement of As2O3 concentration was performed as described above. All eight patients achieved CR at day 26 to 52 of the As2O3 treatment.

RESULTS

As2O3 inhibited selectively survival of NB4 cells, but not HL-60 and U937 cells. Plasma As2O3 concentrations deter-
Fig 1. Effects of As$_2$O$_3$ on the growth, survival, and differentiation of leukemic cell lines. NB$_4$ (A, B), HL-60 (E), and U$_{937}$ cells (F) were incubated with culture mediums with different concentrations of As$_2$O$_3$ (solution I). Cells were maintained at less than $5 \times 10^5$ cells/ml. Cell numbers ($\times 10^5$/ml) and cell viability were determined by the method described in Materials and Methods. (C) The inhibitive effects of the treatment for 24 hours with two As$_2$O$_3$ solutions (1 $\mu$mol/L) of different origins on NB$_4$ cell growth were compared. (D) NBT reduction of NB$_4$ cells in response to 1 $\mu$mol/L As$_2$O$_3$ and/or 1 $\mu$mol/L ATRA treatment were measured. Each point represented the mean of the results obtained in at least three independent experiments. The bars extruding from the points or columns represented the standard deviation (SD).

mined in eight APL patients showed that during continuous daily IV infusion, the peak level (reached at 3 to 4 hours) was 4.2 to 6.7 $\mu$mol/L and mean half-lives ($t_1/2$) 0.89 hours, while in most time, the drug concentrations were between 3 and 0.5 $\mu$mol/L. The absence of As$_2$O$_3$ accumulation in the plasma was caused mainly by diffusion into the peripheral tissues, as the accumulation of As$_2$O$_3$ in nail and hair occurred during continuous administration of As$_2$O$_3$, while the daily urine excretion of As$_2$O$_3$ was only 1% to 8% of the total dose of the drug administered (Z-X Shen, G-Q...
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Chen Z-Y Wang, Z Chen, in preparation). Based on these results, the concentration of As2O3 used in in vitro study was between 0.25 and 2 μmol/L, to keep with the in vivo plasma levels. The growth and survival of NB4 cells were markedly inhibited with As2O3 treatment at the concentration of 0.5 ~ 2 μmol/L (Fig 1A and B). With 0.25 μmol/L As2O3 treatment, however, the inhibition was not significant until day 4. Of note, another As2O3 solution (solution II) also showed the same effects as solution I (Fig 1C). In addition, both As2O3 solutions (0.5 ~ 2 μmol/L) had no obvious effects on the growth and survival of HL-60 and U937 cells under the same culture conditions (Fig 1E and F).

As2O3-treated NB4 cells showed morphological changes and DNA fragmentation characteristic of apoptosis. During 6 days of the treatment with 0.25 to 2 μmol/L As2O3, NB4 cells did not show typical differentiation features like those observed in ATRA treatment, such as the increase of NBT reduction rate and obvious morphological maturation (Fig 1D and Fig 2). In contrast, morphological changes typical of apoptosis, including chromatin condensation, fragmentation of the nuclei, and formation of apoptotic bodies were observed in NB4 cells after 24 hours with 2, 1, and 0.5 μmol/L As2O3 treatment (Fig 2). To further study As2O3-induced NB4 cell death, DNA fragmentation reflecting the endonuclease activity characteristic of apoptosis was analyzed. As shown in Fig 3A, examination of histogramic related nuclear DNA contents on flow cytometry showed a distinct, well quantifiable region below G1 phase, which is the typical profile of apoptotic cells in which DNA stainability was reduced due to degradation and subsequent leakage of DNA from cells. In agreement with this result, agarose gel electrophoresis of genomic DNA from cells treated with As2O3 at 0.5 ~ 1 μmol/L for 24 hours showed typical DNA “ladders” corresponding to internucleosomal cleavage (Fig 3B). At the time of gel analysis, more than 88% of cells were still capable of excluding trypan blue dye (Fig 1B), suggesting that DNA fragmentation in these cells did not represent a postlysis phenomenon, but rather was triggered by As2O3 before cell death. It is noteworthy that these changes were not only time-dependent, but also dose-dependent; the most remarkable change was seen with 2 μmol/L and 1 μmol/L, followed by 0.5 μmol/L. With 0.25 μmol/L As2O3, a small apoptotic compartment was visible only after day 4 of continuous treatment (Fig 3C). However, HL-60 and U937 cells did not show similar morphological changes and DNA fragmentation in response to As2O3 treatment, regardless of drug concentration (0.5 ~ 2 μmol/L) and treatment duration (1 ~ 7 days, data not shown). In addition, As2O3 did not affect the cell cycle distribution of HL-60 and U937 cells (data not shown).

Comparison of the intake of As2O3 by NB4 and HL-60 cells. To evaluate whether NB4 cells are different from HL-60 cells in the ability to uptake As2O3, As2O3 contents in both cells after incubation with 0.5 μmol/L As2O3 for 24 hours were measured. The results showed that NB4 and HL-60 cells absorbed As2O3 by similar amounts (0.024 ± 0.06 vs 0.020 ± 0.03 μmol/L/10^7 cells, P > .05 by student’s t-test) and at similar absorbtional rates (0.48% ± 0.11% vs 0.46% ± 0.07%, P > .05 by student’s t-test).

Effects of As2O3 on the expression of apoptosis-associated genes in NB4 cells. We found that NB4 cells constitutively expressed bcl-2 and its associated genes bax, bcl-XL, as well as p53 and c-myc. Treatment with 1 μmol/L As2O3 downregulated progressively the transcriptional expression of bcl-2 gene, especially after 12 hours (data not shown). At the same time, both Western blot and immunocytochemical analysis for bcl-2 protein also showed similar downregulation, and no bcl-2 proteins were detected at 48 hours (Fig 4 and data not shown). However, bax, bcl-X, p53, and c-myc displayed no obvious modification of the transcriptional expression after As2O3 treatment (data not shown).

As2O3 treatment induced a modulation of PML antiserum staining pattern in HL-60 and NB4 cells. Indirect fluorescence staining of PML using antiserum specific to the N-terminal region of PML was performed in HL-60 and NB4 cells before and after different times of 1 μmol/L As2O3 treatment. As a control, similar experiments were also conducted in 1 μmol/L ATRA-treated cells. As shown in Fig 5A, the untreated HL-60 cells displayed the typical normal distribution pattern of PML, ie, about 10 ~ 20 speckles in each nucleus. Strikingly, the number of speckles was significantly reduced after As2O3 treatment for 12 hours (Fig 5B). In untreated NB4 cells, most exhibited hundreds of micropunctates in the nuclei and the cytoplasm (Fig 5C), corresponding to the recently described abnormal staining pattern of PML-RARα and the wild-type PML heterodimerized with PML-RARα. Upon ATRA treatment, the normal PML speckles could be restored in APL cells (Fig 5D). Of note, in a low percentage of cells (4% ~ 5%, when 1,000 cells were counted in three experiments), there was another aspect of anti-PML staining: an accumulation of particles in a cytoplasmic region, often corresponding to the nuclear indentaion. Interestingly, upon As2O3 treatment, there was a diminution of the PML-RARα/PML micropunctates, with concommittant appearance of a diffuse, perinuclear cytoplasmic PML staining pattern, but no durable restoration of PML speckles was seen (Fig 5E). Meanwhile, there was an increased number of cells (11% ~ 12%, when 1,000 cells were counted in three experiments, P < .05 by χ^2 test) with regional cytoplasm accumulation of anti-PML staining particles (Fig 5E, arrow). A phenomenon related to the latter was that apoptotic cells easily observed after As2O3 treatment also contained such striking particles (Fig 5F, arrow). Therefore, As2O3 seems to trigger a modulation of PML/PML-RARα proteins in quite a different way than does ATRA (Fig 5D).

Effects of ATRA pretreatment on As2O3-induced NB4 cell apoptosis. To address the question if ATRA-induced alterations of subcellular localization of PML-RARα protein influences As2O3-induced cell apoptosis, NB4 cells were pretreated with 1 μmol/L ATRA for 0, 24, or 48 hours before exposure to 1 μmol/L As2O3. The results showed that when 1 μmol/L ATRA treatment was used, at least 48 hours were required for ATRA to inhibit NB4 cell growth and viability. This effect was still maintained after ATRA withdrawal (Fig 6A). However, pretreatment of the cells with ATRA for 24 ~ 48 hours did not obviously influence the inhibition of cell growth and the apoptosis triggered by As2O3 (Fig 6).

DISCUSSION

Since the first observation of the relationship between arsenic and skin cancer in the 1820’s, arsenic compounds...
Fig 5. Immunofluorescence analysis of the subcellular localization of PML in HL-60 cells and PML/PML-RARα in NB4 cells. (A) Untreated HL-60 cells. (B) HL-60 cells after exposure to 1 µmol/L As₂O₃ for 12 hours. (C) Untreated NB4 cells. (D) NB4 cells after exposure to 1 µmol/L ATRA for 24 hours. (E) NB4 cells after exposure to 1 µmol/L As₂O₃ for 12 hours. The arrow indicates the cytoplasmic anti-PML staining particles. (F) NB4 cells after exposure to 1 µmol/L As₂O₃ for 24 hours.
have been generally accepted as a potent environmental carcinogen, more likely as a comutagen and cocarcinogen, for human skin and lung, although no animal model has been established.21-22 Biochemically, it is documented that arsenic can inactivate some important enzymes by binding to the sulfhydryl groups. Arsenic can also interfere with the phosphorylation-dephosphorylation process by replacing the phosphate in biochemical reaction. It has also been shown that arsenic can induce chromosome aberrations, sister-chromatid exchanges, DNA-protein cross-links and protein-associated DNA-strand breaks in mammalian cells.23-24 However, a low concentration of some arsenic compounds also have some benefits to human physiology, such as the stimulation of human hematopoiesis. The use of arsenic compounds as a drug has a longer history in Chinese traditional medicine. For instance, it is recorded that arsenic had therapeutic ef-
Apoptosis is a process by which cells undergo programmed cell death. It is a central mechanism for development and homeostasis in multicellular organisms. Recent evidence suggests that the failure of cells to undergo apoptosis is involved in the pathogenesis of many human diseases including cancer. On the other hand, specific therapies are designed to enhance the susceptibility of individual cell types to undergo apoptosis for a variety of human cancers. By which pathways does As$_2$O$_3$ induce NB$_4$ cell apoptosis? Although the precise biochemical mechanisms for apoptosis remain enigmatic, it has been widely accepted that apoptosis is an active gene-directed cellular suicide mechanism and many human genes contribute to its regulation, such as the tumor suppressor gene p53, proto-oncogene c-myc, bcl-2 gene family including bcl-2, bax, bcl-x (including its two subtypes: bcl-X$_L$ and bcl-X$_S$), bad, and bag-1. Among these genes, bcl-2 draws particular attention because it may be one of the key factors of the common final pathways involved in the regulation of cell apoptosis. The interaction between bcl-2 and other genes also plays an important role in the control of apoptosis. It has been recently shown that bcl-2 can form heterodimers with several bcl-2-associated proteins, especially bax protein, which antagonizes bcl-2 function and induces cell apoptosis. Importantly, the anti-apoptosis effect of bcl-2 requires its interaction with bax. In this study, we showed that bcl-2, bax, bcl-X$_L$, c-myc, and p53 genes were significantly downregulated in NB$_4$ cells treated with As$_2$O$_3$. These results suggest that bcl-2, bax, bcl-X$_L$, c-myc, and p53 genes play important roles in the apoptosis induced by As$_2$O$_3$ in NB$_4$ cells.
were all constitutively expressed in NB4 cells. Although \( \text{As}_2\text{O}_3 \) had no influence on the transcriptional expression of bax, bcl-x, p53, and c-myc genes, it potently downregulated bcl-2 gene expression at the mRNA and protein levels (Fig 4). This net result could thus lead to a lower ratio of bcl-2/bax, which may contribute to the As2O3-induced NB4 cell apoptosis. In a recent report, it was found that 9-cis retinoic acid-induced apoptosis of “maturation”-resistant NB4 cells could occur in the presence of high bcl-2 expression, while a full downregulation of bcl-2 in “maturation”-sensitive NB4 cells induced by ATRA did not parallel with the cell death. These seemingly paradoxical results, however, may not contradict the observation of the present work, since apoptosis is regulated by a network of genes and the effects of different apoptotic agents may be mediated by distinct pathways or different factors in the same pathway. To this end, one may suggest that it is not the downregulation of bcl-2 alone, but the modification of relative ratio and interaction between bcl-2 and other apoptosis-related proteins that is more important in determining whether cells undergo apoptosis. Yet to be clarified is whether the downregulation of bcl-2 protein is secondary to the decreased gene transcription and whether downregulation of bcl-2 at both mRNA and protein levels are directly modulated by As2O3. In addition, recent studies found that some cysteine-rich proteases, such as interleukin-1β-converting enzyme (ICE), may belong to the death effector molecules. It is well known that arsenic has a strong affinity with sulfhydryl groups in protein molecules and can affect the activities of many important sulfhydryl-rich enzymes. Therefore, it is essential to address possible relations between As2O3-induced apoptosis and apoptosis-related enzymes.

An unexpected finding in this work was that As2O3 caused a modulation of the subcellular structures containing PML-RARα/PML proteins. PML is a phosphoprotein that has growth suppressive properties similar to the tumor suppressor Rb and displays a cell cycle-specific expression pattern (low level in S, G2, and M phases and significantly higher level in G1 phase). The deregulation of the PML expression pattern was documented during human oncogenesis. Topologically PML is located in a multiprotein nuclear organelle called POD (for PML oncogenic domain) or nuclear body, as seen in HL-60 cells in this work (Fig 5A). In APL cells, the structure of PODs is destroyed, and anti-PML staining is distributed in hundreds of micropunctates (Fig 5C). Interestingly, POD can be reassembled during the maturation induced by ATRA (Fig 5D). The fact that the number of PODs in HL-60 cells was significantly decreased after 1 \( \mu \text{mol/L} \) As2O3 treatment (Fig 5B) suggests that the drug could modulate, through an unknown way, this nuclear ultrastructure. Because no cell cycle distribution changes were found during the As2O3 treatment in HL-60 cells, this modulation seems to be direct. Interestingly, As2O3 treatment could also diminish the anti-PML micropunctate staining in the nuclei of NB4 cells (Fig 5E). Previous work showed that these micropunctates contain both chimeric PML-RARα and wild-type PML. PML is sequestered in such an abnormal structure, probably because it can form heterodimer with the chimera through the “coiled-coil” motif responsible for protein-protein interaction. Of note, PML-RARα can work as a suppressor of apoptosis in myeloid precursor cells. It has also been proposed that PML-RARα affects retinoid signals, which are not only important effectors in inducing maturation of hematopoietic cells, but also major regulators of apoptosis. For these reasons, it deserves consideration that micropunctations containing PML-RARα were modulated during As2O3-induced NB4 cell apoptosis. However, this modulation is different from that caused by ATRA, as no durable restoration of POD structure was observed.

The presence of the regional cytoplasmic anti-PML staining particles in untreated NB4 cells, and the increase of these cells after As2O3 treatment (Fig 5E), were another unexpected observation. Moreover, similar staining patterns exist in As2O3-triggered apoptotic NB4 cells (Fig 5F), suggesting that cells with apparently regional anti-PML staining particles may be on the way of apoptosis. It is noteworthy that HL-60 cells did not have such staining pattern before and after As2O3 treatment. Therefore, it is possible that the intrinsic properties of NB4 cells make themselves more prone to apoptosis and that if PML-RARα/PML modulation was involved in As2O3-induced apoptosis in these cells, the effect on PML-RARα should be more important. Finally, pretreatment for 24 to 48 hours with a high concentration of ATRA (1 \( \mu \text{mol/L} \)), which is sufficient to restore POD structures in NB4 cells, did not affect As2O3-induced apoptosis. This may suggest that this drug acts in a way independent of retinoic acid regulatory pathway.

In brief, this work suggests that As2O3 can directly and specifically induce NB4 cell apoptosis and downregulate bcl-2 gene expression. Interestingly, PML-RARα protein can also be modulated by the drug, suggesting it may represent the common target of the As2O3 and ATRA therapies, although the precise roles and mechanisms of this modulation in As2O3-induced apoptosis needs further investigation. Our findings not only suggest possible mechanisms of As2O3 in the differentiation and apoptosis regulatory pathways in APL cells, but also provide a model for studying the roles that arsenic plays in the regulation of cell life.

**NOTE ADDED IN PROOF**

Recently we have used wider range of concentrations of As2O3 and investigated the in vitro effects of the drug on NB4 cell line and fresh APL cells for longer time (up to 10 days). It was found that at relatively low concentrations (0.1 to 0.25 \( \mu \text{mol/L} \)), As2O3 tended to trigger a partial differentiation of APL cells.

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