Effect of Arsenic Trioxide on Different Cell Lines Derived from Chronic Myeloid Leukemia

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Abstract The objective is to explore the effect and the mechanism of arsenic trioxide (As$_2$O$_3$) on different cell lines of chronic myeloid leukemia (CML). Different concentrations of As$_2$O$_3$(0.2, 2, and 10 μmol/L) were added to CML cell lines KU812 and MEG01 and other leukemia cell lines U937 and PL21, the cell numbers were counted at different times, TUNEL and DNA ladder assays were performed. Different antibodies, such as CD34, CD13, CD33, CD19, CD11b, CD14 and CD7, were added to detect the change of the molecules on cell surface, the change of bcr-abl by RT-PCR and the activity of caspase-3 were assayed. The results showed that different concentrations of As$_2$O$_3$ had different effects on the survival of the 4 cell lines. After culture for 24 hours with As$_2$O$_3$, there was no significant increase in CD11b in all the four cell lines. There were no changes of bcr-abl in the 2 CML cell lines treated and untreated with As$_2$O$_3$ by RT-PCR. Activities of caspase-3 were all increased. It is concluded that As$_2$O$_3$ can induce apoptosis in CML cell lines, the concentration to induce apoptosis is different, CML cell lines are more sensitive than the other 2 leukaemia cell lines. As$_2$O$_3$ induced apoptosis may have some relation with the activation of caspase-3.

Key words arsenic trioxide; chronic myeloid leukemia cell line; apoptosis; bcr-abl mRNA; caspase-3

Introduction

Chronic myeloid leukemia (CML), a particular subtype of leukemia, is a clonal myeloproliferative disorder of a pluripotent hematopoietic stem cell with a specific cytogenetic abnormality, the Philadelphia (φ$^+$) chromosome. This chromosome results from a translocation between the long arms of chromosomes 9 and 22, resulting in the generation of bcr-abl chimeric gene that expresses an abnormal fusion protein with altered tyrosine kinase activity. The median age of presentation is the fifth decade; all age groups are at risk. Although hydroxyurea and IFNα are widely used in the treatment of CML and improved the prognosis, the patients are not expected to have long-term disease-free survival, a chronic phase with a median duration of 3 to 5 years and an accelerated or acute phase of approximately 3 to 6 months' duration, inevitably terminating fatally.

Arsenic - containing compounds have been used as
cancer treatment for hundreds of years. With the advent of X-ray therapy and conventional cytotoxic drugs, the use of arsenicals for cancer treatment in Western countries has been abandoned. Recently it has been shown that arsenic compounds including arsenic trioxide and arsenic disulfide\(^4\), two components used in traditional Chinese remedies, are very effective in acute promyelocytic leukemia (APL) treatment. In vitro studies with NB4 cells derived from APL showed that the apoptosis induced by As\(_2\)O\(_3\) was associated with a downregulation of bcr-2.

In this study we investigated the effect of arsenic trioxide on two CML cell lines with Ph\(^+\) chromosome with various concentrations of As\(_2\)O\(_3\) (0.2 - 10 \(\mu\)mol/L). We examined the effect of As\(_2\)O\(_3\) on the induction of apoptosis and cell differentiation as well as the change of the level of bcr-abl mRNA and the activity of caspase-3.

**Materials and Methods**

**Compounds**

As\(_2\)O\(_3\) (Sigma, Lot A1010) stock solution was made at the concentration of 1 mmol/L with phosphate-buffered saline (PBS) and NaOH, adjust the pH to 7.4, and diluted to the working concentration before use.

**Cell lines and culture**

Two cell lines of CML: KU812 (CML, basophil-like cells; Riken Cell Bank), Meg-01 (CML, megakaryoblast; ATCC, CRL-212) and two cell lines from other leukemias: U937 (monocytic) and PL-21 (promyelocytic) were used. These 4 cell lines were cultured at 1 \(\times\) 10\(^2\) ml in suspension in RPMI 1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 95% air and 5% CO\(_2\) with different concentrations of As\(_2\)O\(_3\) (0.2, 2 and 10 \(\mu\)mol/L). The cell numbers were counted at different times after the treatment. Cell viability was determined using the trypan blue exclusion test.

**Cell morphology**

After culturing with different concentrations of arsenic trioxide the cells were centrifuged on slide by cytospin (Shandon Southern Product, Cheshire, U.K., 800 \(\times\) g, 4 min) and stained with May-Grünwald-Giemsa solution.

**TUNEL assay**

The terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay was used to monitor the extent of DNA fragmentation due to apoptosis. The assay was performed according to the recommendations of the manufacturer (MBL, Japan). Fluorescein-conjugated dUTP incorporated in nucleotide polymers was detected and quantified using flow cytometry. Approximately 10 000 cells per sample were acquired and analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

**DNA ladder**

Apoptosis was proved by DNA ladder using Quick Apoptosis DNA Ladder Detection Kit (MBL). The assay was performed according to the recommendations of the manufacturer.

**Cell surface marker analysis**

After 7 days culture with As\(_2\)O\(_3\), the expression of surface markers on the 4 cell lines were analyzed by fluorescence-activated cell sorting (FACS). The monoclonal antibodies used to recognize the following antigens: CD34, CD13, CD33, CD19, CD11b, CD7 and CD14, which were purchased from Coulter Immunotech.

**Detection of bcr-ABL hybrid mRNA by reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from a constant number of two CML cell lines using the Isogen (Molecular Research Center, Inc., Japan). Reverse transcription reaction was performed with 1 \(\mu\)g RNA and Oligo dT 15 minutes at 42 \(^\circ\)C, 5 minutes at 100 \(^\circ\)C and 5 minutes at 0 \(^\circ\)C, then nested-PCR\(^5\), first step, thirty-five cycles of PCR, with denaturation at 94 \(^\circ\)C for 30 seconds, annealing at 55 \(^\circ\)C for 1 minute and extension at 72 \(^\circ\)C for 1 minute. For the amplification, the antisense primer 5'-TGATTA-TAAGCTTAAAGACCCGGATG-3' and the sense primer 5'-GAAGTGTTCACAGCTTCTCC-3' were used, simultaneously with primers specific for human \(\beta\)-actin 5'-GGGTCAAGAGATGTTCATTTG-3' and 5'-GGGTCAGAAAGATTCTACTTG-3' as an internal control. For the second step of the PCR, thirty-five cycles, with denaturation at 94 \(^\circ\)C for 30 seconds, annealing at 55 \(^\circ\)C for 1 minute and extension at 72 \(^\circ\)C for 1 minute and final extension for 7 minutes, the primers are 5'-TGAGCCTGCAAATGCTGACCG-3' and 5'-ATCTCCACTGGCCACAAAATCATACA. The amplified PCR product was electrophoresed in a 1.8% agarose gel and ethidium bromide stained.

**Caspase-3 activity**

The caspase-3 activity were assayed by the CPP32/caspase-3 colorimetric protease assay kit (MBL, Nakuru Nagoya, Japan) according to the recommendations of the manufacturer.
Results

Effects of different concentration of As$_2$O$_3$ on the growth of the four cell lines

The concentration of 10 $\mu$mol/L As$_2$O$_3$ significantly inhibited the growth of all 4 cell lines, after 7 days culture the viable cells decreased significantly; at the concentration of 2 $\mu$mol/L, the growth inhibition of PL21 and U937 cells was slightly than that of KU812 and Meg-01 cells; of the 2 CML cell lines, the inhibitory effects were different, in KU812 cells more than in Meg-01 cells (Figure 1). At the concentration of 0.2 $\mu$mol/L, all the 4 cell lines grew well without inhibitory effect.

Apoptosis of As$_2$O$_3$ treated cell lines

Typical morphological changes for apoptosis, including chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies, were observed in all 4 cell lines at the 10 $\mu$mol/L As$_2$O$_3$ and the apoptosis was proved by the agarose gel electrophoresis of DNA ladder.

TUNEL assay

The results by TUNEL assay indicated that when the 4 cell lines culture with 0.2 $\mu$mol/L As$_2$O$_3$, there were no significant changes of the number of the apoptotic cells between the treated cell lines and the untreated control; there were some apoptotic cells in the two CML cell lines at 2 $\mu$mol/L, KU812 was a more sensitive cell line than the other CML cell line Meg-01, which is also a sensitive cell line, but the proportion of apoptotic cells was lower than that in KU812 (Figure 2). However, for PL-21 and U937, the overlay of the TUNEL curves almost were the same, that means at this concentration As$_2$O$_3$ can not induce apoptosis. When the As$_2$O$_3$ concentration increased to 10 $\mu$mol/L, it induced apoptosis in all the 4 cell lines.

Cell surface marker

After 7 days culture with 0.2 and 2 $\mu$mol/L As$_2$O$_3$, the expression of surface markers on the 4 cell lines were examined. There was no significant increase of CD11b in all the 4 cell lines.

The change of bcr-abl mRNA

After 24 hours culture with As$_2$O$_3$, the mRNA of the two CML cell lines were extracted for RT-PCR, there were no significant changes of the level in bcr-abl (Figure 3).

Activity of caspase-3

After culture with arsenic trioxide, the activity of caspase-3 was increased in all the cell lines of
Figure 2: TUNEL assay after culture 24 hours with arsenic trioxide overlay of control and experimental group (dark line).

Figure 3: The level of bcr-abl mRNA in KU812 and Meg01 cell lines.
(A) β-actin. Lane M: Marker. Lane 1: Meg01 2 μ mol/L. Lane 2: Meg01 10 μ mol/L. Lane 3: KU812 2 μ mol/L. Lane 4: KU812 10 μ mol/L.
(B) bcr-abl. Lane M: Marker. Lane 1: Meg01 2 μ mol/L. Lane 2: Meg01 10 μ mol/L. Lane 3: KU812 2 μ mol/L. Lane 4: KU812 10 μ mol/L.
Discussion

Recently, As₂O₃ and arsenic disulfide have been applied to the treatment of APL with satisfactory results. *In vitro* studies showed that in NB4 cells, As₂O₃ induced apoptosis with the decrease of bcr-2 gene expression. In the present study, when 2 CML cell lines and 2 other leukemia cell lines were cultured with As₂O₃, all the 4 cell lines growth were inhibited at 10 and 2 μmol/L while at 0.2 μmol/L the cell can grow well without significant growth inhibition. And the 4 cell lines were all induced apoptosis at 10 μmol/L, they showed clear signs of apoptosis of cell shrinkage, chromatin condensation, internuclear DNA cleavage, membrane blebbing, and the formation of apoptotic bodies; but the 4 cell lines showed different sensitivity to this drug. At 2 μmol/L the 2 CML cell lines showed a little sensitive than the other two leukemia cell lines, and among the 2 CML cell lines, KU812 was more sensitive than Meg-01.

Previous studies showed that cells with bcr-abl hybrid gene expression were particularly resistant to apoptosis induced by a number of chemical and biological agents. The bcr-abl fusion transcript, p210bcr/abl protein, confers a growth advantage to CML cells over normal precursors and also suppresses the apoptotic machinery of CML cells. Other cell types without bcr-abl expression, are easily and massively triggered to apoptosis. The 2 CML cell lines with bcr-abl can be induced to apoptosis and a little more sensitive than other two leukemia cell lines. Therefore, the effects before and after treatment with arsenic trioxide on the expression of the bcr-abl fusion gene in KU812 and Meg-01 were studied. Our experiment showed that the mRNA level of the three CML cell lines showed no significant decrease after treatment with As₂O₃. It indicates that the apoptosis induced by arsenic trioxide has nothing to do with bcr-abl. Perkins et al. reported that after treatment with arsenic trioxide the level of BCR-ABL protein deceased, indicating that the modulation of BCR-ABL protein might be posttranscriptional.

Including caspase-3 (CPP32, Yama, apopain), at least 10 caspases have been identified and this family of proteases plays a critical role in the biochemical events governing apoptosis. Among them, caspase-3 (CPP32(Yama, apopain)-like proteases are responsible for the cleavage of some substrates at the onset of apoptosis. It now appears that many inducers of cell death ultimately converge on the activation of caspase-3 (like) proteases, which then appear to launch the terminal and execution stages of apoptosis. Chen et al. have shown that in arsenic-induced apoptosis, reactive oxygen species and caspase-3 activation are involved. Akao et al. showed that As₂O₃ can induce apoptosis in neuroblastoma cells and the apoptosis was shown to occur through the activation of caspase-3 by means of Western blot analysis and apoptosis inhibition assay. It seemed that the sensitivity of neuroblastoma cells to As₂O₃ was inversely proportional to their intracellular level of reduced glutathione. Laroche et al. have shown that arsenite induces apoptosis via a direct effect on the mitochondria permeability transition pore, accompanied by a loss of mitochondrial transmembrane potential. But Zhu et al. showed that caspase-3 activation, usually resulting from collapse, was not always associated with As₂O₃-induced apoptosis. Bcr-abl fusion gene exerts antiapoptotic effect against diverse apoptotic stimuli; the mechanism of its antiapoptotic effect is the blockage of mitochondrial release of cytochrom C and activation of caspase-3. To determine the mechanism of apoptosis by As₂O₃ in CML, we detected the activity of caspase-3. The results showed that the activity of caspase-3 in all the cell lines of KU812, PL21, Meg-01 and U937 increases by 1.3 to 3.3 fold; it may suggest that the apoptosis induced by arsenic trioxide has some relation with the activation of caspase-3. Interestingly, the caspase-3 activity is lower in the CML cell lines, which are easier to induce apoptosis in clinical attaching concentration of...
2 μmol/L; for U937, which is more resistant to As2O3 at 2 μmol/L, the caspase activity is more higher. There may be some other mechanism on the induction of apoptosis, that should be done further.

Our results indicate that As2O3 can induce apoptosis at 2μmol/L in some CML cell lines, that is a clinically achievable concentration and seems to be a promising candidate of therapeutic agent for some CML patients.

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