Arsenic Targets Tubulins to Induce Apoptosis in Myeloid Leukemia Cells

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Abstract

Arsenic exhibits a differential toxicity to cancer cells. At a high concentration (>5 μM), As2O3 causes acute necrosis in various cell lines. At a lower concentration (0.5–5 μM), it induces myeloid cell maturation and an arrest in metaphase, leading to apoptosis. As2O3-treated cells have features found with both tubulin-assembling enhancers (Taxol) and inhibitors (colchicine). Prior treatment of monomeric tubulin with As2O3 markedly inhibits GTP-induced polymerization and microtubule formation in vitro but does not destabilize GTP-induced tubulin polymers. Cross-inhibition experiments indicate that As2O3 is a noncompetitive inhibitor of GTP binding to tubulin. These observations correlate with the three-dimensional structure of β-tubulin and suggest that the cross-linking of two vicinal cysteine residues (Cys-12 and Cys-213) by trivalent arsenic inactivates the GTP binding site. Furthermore, exogenous GTP can prevent As2O3-induced mitotic arrest.

Introduction

Arsenic is the oldest and also the newest form of treatment for leukemia. Forkner (1) noted the 1865 observation of Lissauer, who administered Fowler’s solution (potassium arsenite and an extract of lavender) to patients suffering from leukemia. He restudied the effect of arsenic and demonstrated a remarkable regression in chronic myelogenous leukemia. Recently, arsenic compounds have become a subject of renewed attention from clinical observations of their effect on APL (2–5). This was a rediscovery of a traditional Chinese remedy. For more than 1000 years, the Chinese medications pi shuang and xiong huang (Realgar), which are now known to contain As2O3 and As2S2, respectively, have been used for the treatment of cancer and other conditions (6).

The molecular mechanism of arsenic therapy is not well understood. It has been proposed that arsenic targets PML fusion proteins in APL with a t(15;17) translocation (3, 7), but recent studies have shown that PML fusion protein is not required for the arsenic effect (8). The consensus of several reports is that arsenic can induce apoptosis in leukemia cells by activating apoptotic genes (3, 5). However, the primary cellular target of arsenic in inducing apoptosis has remained unknown.

Trivalent arsenic causes toxicity by binding and inactivating a number of sulfhydryl-containing proteins and enzyme systems. Arsenic affects mitochondrial enzymes and impairs tissue respiration, inhibits succinic dehydrogenase activity, and uncouples oxidative phosphorylation (9). The cytoskeleton is another cellular target for arsenic. Several lines of evidence suggest that arsenic has a particular affinity for the cytoskeleton, which contains proteins with a higher sulfhydryl content than the soluble fraction (10). Subunits of the microtubule, tubulin α and β, have been isolated by arsenic affinity chromatography (11). The functional ability of centrosomes to nucleate microtubule assembly is inhibited by arsenite (12). Disruption of microtubule assembly and spindle formation by sodium arsenite can induce aneuploidy (13). Because the microtubule is a primary target for many antileukemia drugs, we have investigated cell cycle changes in arsenic trioxide (As2O3)-treated leukemia cells and provide evidence for a mechanism of arsenic-induced apoptosis through microtubule inhibition.

Materials and Methods

Cell Culture and Staining. All cells (ATCC) were cultured in RPMI 1640 with 10% fetal bovine serum at 37°C with 5% CO2. Mitotic analysis was performed by cytoxin preparation with Wright-Giemsa stain (Baxter) using the Sakura hematology stainer. The slides were viewed and photographed under a light microscope.

Tubulin Polymerization. Bovine tubulin (Sigma; 5 mg/ml) was kept on ice to maintain monomeric conditions. The tubulin assembly assay was performed following the manufacturer’s protocol (Sigma). For pretreatment, tubulin was incubated with the indicated reagents or control buffer for 30 min at room temperature and then depolymerized by freezing and thawing three times. Monomeric tubulin was purified by centrifugation at 14,000 × g for 20 min at 4°C. Tubulin polymerization was determined by measuring A350 nm with the spectrophotometer.

Electrophoresis of Tubulin. The degree of polymerization of tubulin was determined by SDS-PAGE. Tubulin (5 mg/ml) was incubated with the indicated reagents at 37°C for 1 h. Monomeric and polymerized tubulin were separated by centrifugation. Both supernatants (monomers) and pellets (polymers) were subjected to electrophoresis under denaturing conditions. The amount of tubulin in different states was estimated after staining the gel with Brilliant Blue stain (Sigma).

Results and Discussion

The differential cytotoxicity of arsenic compounds can be taken advantage of in therapeutics. Clinical study showed that the effective level of arsenic in vivo was 0.5–3 μM in plasma, and a similar concentration can induce apoptosis in APL cells in vitro (3). We have reported previously that arsenic shows differential cytotoxicity in various types of leukemia cells and causes significant morphological changes in certain myeloid cells (14). Further study shows that at
concentrations of 5–10 μM or higher, As₂O₃ causes acute necrosis in 20 different cancer cell lines including leukemia cells and cells from solid tumors. This acute cytotoxicity appears within 6–12 h, and no apoptotic features are observed.

Effects of Arsenic on Mitosis. At concentrations between 0.5 and 5 μM, As₂O₃ blocks the proliferation of leukemia cell lines representing various stages and lineages, including K562 (erythroid myeloid cells), U937 (macrophage-like cells), HL60 (promyelocytic cells), and NB4 (APL cells). A striking similarity of the effect of arsenic on these cells is mitotic arrest, resembling the effect of antitubulin drugs. As demonstrated in K562 cells, most untreated cells are normally in interphase (Fig. 1A), and only 1–2% of cells are in different stages of mitosis (Fig. 1, B–E). When treated by microtubule-disrupting drugs such as Taxol and Colcemid (a colchicine derivative), cells exhibit distinct types of mitotic arrest. The chromosome pattern of Taxol-treated cells resembles prophase (Fig. 1F), whereas the colchicine-treated cells show condensed nuclei due to the loss of the microtubule (Fig. 1G). At 2.5 μM, As₂O₃ induces chromosomal morphological changes with features of both Taxol (Fig. 1H) and colchicine (Fig. 1I). These morphological changes were also observed in other leukemia cell lines such as APL NB4 cells. However, PMA-differentiated K562 cells remain unchanged with As₂O₃ treatment at this level (Fig. 1J).

The time course of the arsenic effect at a low dose is shown in Fig. 2. These cells first stop proliferation, and the percentage of mitotic figures increases significantly by 12 h. As at 48 h, the majority of cells are in mitosis (Fig. 2A). Quantitative analysis showed that As₂O₃, Taxol, and Colcemid have very similar effects, arresting cell proliferation in mitosis and preventing the cells from entering telophase (Fig. 2B). A specificity study showed that the arsenic effect is restricted to the trivalent inorganic form. BAL, a diethyglycerol with a...
high affinity to arsenic, can abrogate this effect, and arsanilic acid (pentavalent organic arsenic) proved to be nontoxic to the cells (Fig. 2B). As anticipated, other apoptotic inducers such as actinomycin D stopped cell proliferation but did not cause mitotic arrest. Furthermore, PMA-differentiated cell lines did not show mitotic arrest with the same dose of arsenic, suggesting that arsenic acts only on rapidly proliferating cells.

Arsenic-induced Maturation and Apoptosis. A characteristic feature in myeloid cells treated with a low dose of arsenic is an increase in the expression of maturation markers. At 6 h, K562 cells expressed an increased level of glycophorin A, HLA-DR, CD33, and CD34 on the cell surface, indicating maturation (Fig. 3A). Similar changes were observed in Taxol- and Colcemid-treated cells. After 12 h, arsenic-treated cells showed features consistent with the apoptotic changes. Fluorescence staining showed that arsenic-treated cells expressed a higher level of the apoptotic marker annexin V together with an increase in PI-positive cells, indicating a loss of viability (Fig. 3B, 1 and 2). The apoptotic change was also confirmed by morphological observation under Wright-Giemsa and fluorescent stains (Fig. 3B, 5 and 6). In contrast, a B-cell line treated with As$_2$O$_3$ (5 μM) did not express apoptotic markers or mitotic arrest, although the viability decreased as indicated by PI positivity (Fig. 3B, 3 and 4).

The downstream pathway of arsenic-induced apoptosis has been investigated by several groups who have shown that arsenic-treated APL cells had an altered expression of apoptosis-related genes including Bcl-2, caspase-2, and caspase-3 (3, 5). Interestingly, it is known that both Taxol and colchicine can induce myeloid cell maturation (15), alter the expression of these apoptotic genes (16), and induce apoptosis (16, 17). This raises the possibility that arsenic shares the same downstream signal pathway with other microtubule inhibitors for the induction of apoptosis in leukemia cells.

Effects of Arsenic on Tubulin. Consistent with the cellular effect, the in vitro microtubule assembly assay showed that prior treatment of monomeric tubulin with As$_2$O$_3$ markedly inhibits GTP-induced polymerization and microtubule formation (Fig. 4A). The addition of Taxol did not enhance GTP-dependent polymerization in the presence of arsenic. However, when tubulin is preincubated with GTP, the addition of arsenic did not change the state of tubulin polymerization (Fig. 4B). Arsenic slightly decreased background tubulin polymerization in the absence of added GTP. These cross-inhibition experiments suggest that As$_2$O$_3$ is a noncompetitive inhibitor of GTP binding to tubulin. Analysis of monomeric tubulin by electrophoresis showed that arsenic, like Colcemid, could prevent tubulin polymerization, whereas Taxol enhanced tubulin polymerization (Fig. 4C). These results indicate that arsenic targets free tubulins in a manner similar to colchicine. This does not rule out the possibility that arsenic may bind to polymerized tubulins and stabilize microtubules.

As shown above, the morphology of the mitotic figure of arsenic-treated cells had features found with both Taxol and Colcemid. These observations correlate with recent findings on the three-dimensional structure of tubulin (18) that show that when the GTP binding site is unoccupied, two cysteine residues, Cys-12 and Cys-213, are in close proximity in the three-dimensional structure, separating after GTP binding occurs. Reaction of trivalent arsenic with these vicinal cys-

[Fig. 3. Arsenic induces the expression of myeloid maturation and apoptotic markers. A, expression of glycophorin A, HLA-DR, CD33, and CD34. K562 cells were treated with As$_2$O$_3$ (2.5 μM), Taxol (50 ng/ml), and Colcemid (50 ng/ml) for 6 h. The expression of myeloid or erythroid markers was determined by flow cytometry. Data are expressed in overlays of control (blank) and treated (colored) histograms. B, expression of apoptotic markers. K562 cells and transformed B cells were treated with As$_2$O$_3$ as described above for 12 h. Cells were stained with FITC-conjugated annexin V (1 and 3) or PI (2 and 4). Data are expressed as the overlays of control (solid) and treated (blank) histograms. Micrographs of As$_2$O$_3$-treated cells stained with Wright-Giemsa (5) and FITC-conjugated annexin V (6) are also shown.]
teine residues would inactivate the GTP binding site. This is further supported by an earlier finding that two cysteines in tubulin can be cross-linked after the removal of GTP (19). These features provide a biochemical basis for the action of arsenic as a noncompetitive inhibitor of GTP binding to tubulin. Confirmation of this mechanism is provided by the preventive effect of exogenous GTP (250–750 μM) on myeloid cell cultures (Fig. 2B). \(\text{As}_2\text{O}_3\)-induced mitotic arrest in K562 cells was completely blocked in the presence of exogenous GTP, but no effect was produced on the acute cytotoxicity of \(\text{As}_2\text{O}_3\) at a higher concentration. In contrast, ATP did not show such an effect. A schematic model of arsenic interaction with tubulin and GTP is presented in Fig. 4D.

A unique target site of arsenic action on tubulin may account for the observation that the mitotic arrest of arsenic-treated cells resembles the effects of both the tubulin polymerization inhibitor and promoter. Because arsenic prevents tubulin polymerization but does not depolymerize the GTP-stabilized microtubule (Fig. 4B), it is possible that arsenic binds both tubulin monomer and polymer and stabilizes them. The relatively narrow range of the effective concentration of arsenic could be determined by the availability of arsenic binding sites, which would depend on the intracellular GTP level. If sensitive myeloid cells have limited GTP production during mitosis, arsenic might bind to tubulin more readily. Leukemic cells apparently have an increased level of tubulin and a higher ratio of monomeric tubulin:polymerized tubulin, which will provide a large number of targets for arsenic (20).

In summary, data presented here suggest that in myeloid cells, trivalent arsenic binds two cysteine residues in tubulin, blocks the binding site for GTP, and disrupts the normal dynamic of microtubules during mitosis. As seen with other microtubule inhibitors, this activates a cascade of genes for programmed cell death and leads to apoptosis. These results suggest that tubulin is the molecular target for arsenic in leukemia therapy. Consistent with clinical observations, these results also suggest that arsenic can be used at a low concentration that selectively targets the microtubules of rapidly dividing tumor cells, thus minimizing general toxicity. Evidence that the cross-linking of vicinal dithiols in tubulin has an important role in leukemia therapy raises the possibility of developing rationally designed arsenic-based antimitotic agents.

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References


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