Biochemical Pharmacology, Vol. 60, pp. 771–780, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00397-X

Induction of Mitosis-Mediated Apoptosis by Sodium Arsenite in HeLa S3 Cells

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ABSTRACT. Arsenic has been used effectively as a chemotherapeutic drug for the treatment of acute promyelocytic leukemia patients. Numerous studies have demonstrated that arsenic induces apoptosis in various cell types. In the present study, we showed that ~35% of arsenite-treated HeLa S3 cells arrested in mitosis. After release from arsenite treatment, more than 80% of arsenite-arrested mitotic cells subsequently underwent apoptosis, as indicated by anachronistic nuclear envelope reformation, DNA ladder occurrence, chromatin condensation, and activation of caspases 3 and 9. In exploring how these cells entered apoptosis mechanistically, we found an inverse correlation between mitotic indexes and apoptotic frequencies. As shown by using Percoll density gradient fractionation and flow cytometric analysis, the mitosis-mediated apoptosis induced by arsenite was accompanied by delayed cyclin B degradation and altered mitotic exit. Furthermore, treatment of arsenite-arrested mitotic cells with staurosporine or 2-aminopurine resulted in a rapid degradation of cyclin B, moved these cells forward to interphase without cell division, and abrogated apoptosis. These results suggest that apoptosis occurs in arsenite-arrested mitotic cells that exit mitosis abnormally. BIOCHEM PHARMACOL **60**;6: 771–780, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 2-aminopurine; apoptosis; arsenite; cyclin B degradation; caspase 3; caspase 9; mitotic arrest; staurosporine

Although arsenic is a widely distributed environmental carcinogen [1, 2], arsenic compounds have been shown recently to be very effective in the treatment of patients with APL,§ particularly in patients showing resistance to therapy with all-*trans*-retinoic acid or other chemotherapeutic drugs [3–5]. Numerous studies have demonstrated that various arsenic compounds induce apoptosis in APL cells and other cell types [6–8]. Among different cell types and arsenic compounds, mechanisms for the induction of apoptosis include down-regulation of Bcl-2 expression [6, 8, 9] or generation of reactive oxygen intermediates [10, 11]. In addition to killing the tumor cells by apoptosis, arsenic also exerts its therapeutic activity by promoting the degradation of PML/RARα protein and hence inducing differentiation [5, 7, 9, 12].

Previously, we have shown that arsenite, a trivalent inorganic arsenic compound, induces mitotic arrest in a variety of human cancer cell lines [13]. We found that treatment of a cervical cancer cell line, HeLa S3, with 5 μM arsenite for 24 hr results in 35% of the cells being arrested in the mitotic stage [13]. Our studies and other reports have demonstrated that arsenite disturbs the assembly of spindle microtubules in a variety of cell systems [13–16]. Therapeutic agents such as taxol or colchicine that interrupt spindle fibers and cause mitotic arrest have been shown to induce apoptosis in various cancer cell lines [17–19]. Accumulated evidence has shown that cell cycle arrest may result in apoptosis due to the existence of cell cycle checkpoints and feedback control [20]. Therefore, understanding the consequences of arsenite-arrested mitotic cells would be helpful in the clinical application of arsenic compounds as therapeutic agents.

In the present study, we examined the effects of reincubating arsenite-arrested mitotic cells in arsenite-free medium. We demonstrated that mitosis-mediated apoptosis was accompanied by atypical mitotic exit and delayed cyclin B degradation as well as a decrease in $p34^{cdc2}$/cyclin B kinase activity. We also showed that accelerating cyclin B degradation, which enforced mitotic exit, rescued the arsenite-arrested mitotic cells from apoptosis.

MATERIALS AND METHODS

Cell Culture and Arsenite Treatment

HeLa S3 cells were subcultured twice weekly in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cultures were maintained in a humidified

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§ *Abbreviations*: APL, acute promyelocytic leukemia; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein-5-isothiocyanate; α-MEM, α-minimum essential medium; M.I., mitotic index; and PML/RARα, promyelocytic leukemia gene/retinoic acid receptor-α.

Received 13 December 1999; accepted 28 February 2000.

incubator at 37° with 5% CO_2 and 95% air. Logarithmically growing cells were replated at a density of 2.5×10^4 cells/cm^2 1 day prior to treatment. Throughout this study, we treated HeLa S3 cells with 5 μM sodium arsenite for 24 hr. Sodium arsenite was prepared freshly by dissolving in double-distilled water. Since arsenite treatment resulted in cell arrest in the mitotic stage and rounding up [13], the rounded-up mitotic cells were harvested by a mechanical shake-off technique, and the remaining attached population was obtained by trypsinization.

M.I. Analysis

For M.I. analysis, the cells harvested by shake-off or trypsinization techniques were treated with 0.5% KCl for 12 min at room temperature. After centrifugation, the cells were fixed with a methanol:acetic acid solution (3:1, v/v). An aliquot of the cell suspension was dropped onto a clean slide, air-dried, and stained with 3% Giemsa solution in Sörensen buffer. The mitotic figures then were examined under a microscope, and at least 200 cells were scored for M.I. determination. For each treatment, M.I. was determined by three independent experiments.

Colony-Forming Assay

The survival rates of the arsenite-treated cells were assayed by a colony formation method as described previously [21]. In brief, the logarithmically growing cells were treated with 5 μM sodium arsenite for 24 hr and were separated into mitotic and attached populations by the shake-off technique. They were replated at 200–1000 cells per 60 mm dish in triplicates and incubated in fresh medium for 10 days. Then the cultures were fixed with methanol and stained with a 10% Giemsa solution. The colony number was counted under a dissecting microscope. The colony formation efficiency of untreated HeLa S3 cells was 58 ± 16%.

DNA Fragmentation Analysis

Apoptotic DNA fragmentation was assayed according to the method described by Herrmann *et al.* [22]. In brief, after treatment with 5 μM sodium arsenite for 24 hr, the arrested mitotic cells and the attached cells were harvested separately and re-incubated in arsenite-free medium for 0–24 hr. At the time indicated, fragmented DNA from an aliquot of 2×10^6 cells was extracted with 100 μL of lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5) for 10 sec. The clear extracts were brought to 1% SDS, treated with RNase A (1.5 mg/mL) for 2 hr at 56°, and then digested with proteinase K (1 mg/mL) for at least 2 hr at 37°. DNA fragments were precipitated with 2.5 vol. of cold ethanol after addition of 1/2 vol. of 10 M ammonium acetate. The DNA pellets were dissolved in gel-loading buffer and then separated electrophoretically on a 1% agarose gel.

Ultrastructural Examination of Apoptotic Cells

After re-incubation of arsenite-arrested mitotic cells in drug-free medium for 2 hr, the cells were harvested by trypsinization, washed twice with PBS, fixed with 2% glutaraldehyde for 40 min and 1% OsO_4 for 1 hr, and then embedded into EPON-812. Ultrathin sections were examined with a Jeol 1200EX electron microscope.

Caspase Activity Assay

Arsenite-arrested mitotic cells were re-incubated with drug-free medium for 4 hr. Cells were extracted in lysis buffer as described previously [23]. Activities of caspases 3, 6, 8, and 9 were analyzed by adding their own fluorogenic substrate (Calbiochem) into 80 μg cell extracts, incubating at 37° for 1 hr, and reading in a fluorospectrophotometer with excitation at 400 nm and emission at 505 nm.

Apoptosis Analysis

The apoptotic cells were determined by using an ApoAlert™ Annexin V apoptosis kit (Clontech). The cells were trypsinized, washed once with PBS, and resuspended in 100 μL of binding buffer containing 5 μL of Annexin V–FITC and 5 μL of propidium iodide solutions. After a 10-min incubation at room temperature, the cells were examined under a fluorescence microscope. At least 200 cells were scored in each sample, and three independent experiments were performed to determine the apoptotic frequencies.

Percoll Gradient Fractionation

Percoll gradient fractionation was used to separate apoptotic and nonapoptotic cells [24, 25]. Nine parts of Percoll solution mixed with one part of 10x α-MEM was designated as a 100% Percoll solution. A stepwise gradient was prepared by sequentially filling the centrifuge tube with 60% (3 mL), 40% (4 mL), and 10% (2 mL) Percoll solution in 1x α-MEM. Arsenite-arrested mitotic cells were incubated with arsenite-free medium for 4 hr. Afterward, cells were harvested, resuspended in 2 mL of serum-free α-MEM, layered onto the top of Percoll gradients, and centrifuged in swing buckets at 2000 *g* for 10 min. Cells located at the interfaces of 40–10% and 60–40% Percoll gradients were designated as upper and lower fractions, respectively. These two cell fractions were harvested separately and subjected to M.I. and apoptosis analysis.

DNA Content Analysis

The DNA content was analyzed by flow cytometry as previously described [26]. In brief, after fixation with cold 70% ethanol at 4° overnight, cells were centrifuged and resuspended in PBS containing 4 μg/mL of propidium iodide, 0.1 mg/mL of RNase A, and 0.1% Triton X-100.

The DNA content of 10,000 cells was analyzed individually with a flow cytometer (FACStar, Becton Dickinson Immunocytometry Systems).

Cyclin B Analysis

The cellular cyclin B levels were analyzed by either a flow cytometric or an immunoblotting technique. For flow cytometric analysis, cells were fixed with 3.7% formaldehyde and 0.1% Triton X-100 in PBS. Then, cells were incubated with cyclin B1 antibody (Oncogene Research Products) and FITC-conjugated secondary antibody. The cyclin B levels in individual cells were analyzed by a flow cytometer as described by Sherwood *et al.* [17]. For immunoblotting analysis, cells were lysed in 2x SDS sample buffer [27] and boiled for 5 min. Twenty micrograms of cellular proteins was separated electrophoretically by 10% SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were immunoblotted with cyclin B1 antibody or actin antibody and horseradish peroxidase-conjugated secondary antibody, and visualized with a chemiluminescent substrate system (Pierce). The relative density of cyclin B1 was estimated with a densitometer (Molecular Dynamics). Protein concentrations were determined with a Bio-Rad assay kit (Bio-Rad).

Assay of p34^{cdc2}/Cyclin B Kinase Activity

Preparation of cell extracts and assay of p34^{cdc2}/cyclin B kinase activity were carried out essentially as described by Juan and Wu [28]. An aliquot of cell extracts (40 μg of cellular proteins) was reacted with 1 μg of cyclin B1 antibody. The immunocomplexes were trapped on protein A-conjugated agarose and pelleted by centrifugation. p34^{cdc2}/Cyclin B kinase activity was assayed in a reaction mixture containing 10 μL reaction buffer, 2.5 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and 10 μg histone H1. After incubation at 37° for 30 min, the reaction was stopped by adding 10 μL of 2x SDS sample buffer and boiling for 5 min, and then the sample was loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, phosphorylated histone H1 was visualized by autoradiography.

RESULTS

Apoptosis of the Arsenite-Arrested Mitotic Cells

Treatment of HeLa S3 cells with 5 μM sodium arsenite for 24 hr resulted in 35% of the cells becoming rounded up and arrested at the mitotic stage [13]. The survival rate of these arsenite-treated HeLa S3 cells was 61% as determined by the colony-forming assay. To understand the correlation between arsenite-induced mitotic arrest and its cytotoxicity, the rounded-up mitotic cells and the remaining attached cells were fractionated by a shake-off technique and subjected to a cell survival assay, respectively. Using the colony-forming assay, the survival rates of mitotic and attached cells were 14 and 97%, respectively (average of 3 experiments). These results implied that arsenite-induced cytotoxicity was attributable mainly to mitotic arrest. To explore how arsenite-arrested mitotic cells died, the cellular morphology was examined by electron microscopy. When arsenite-arrested mitotic cells were re-incubated in arsen-

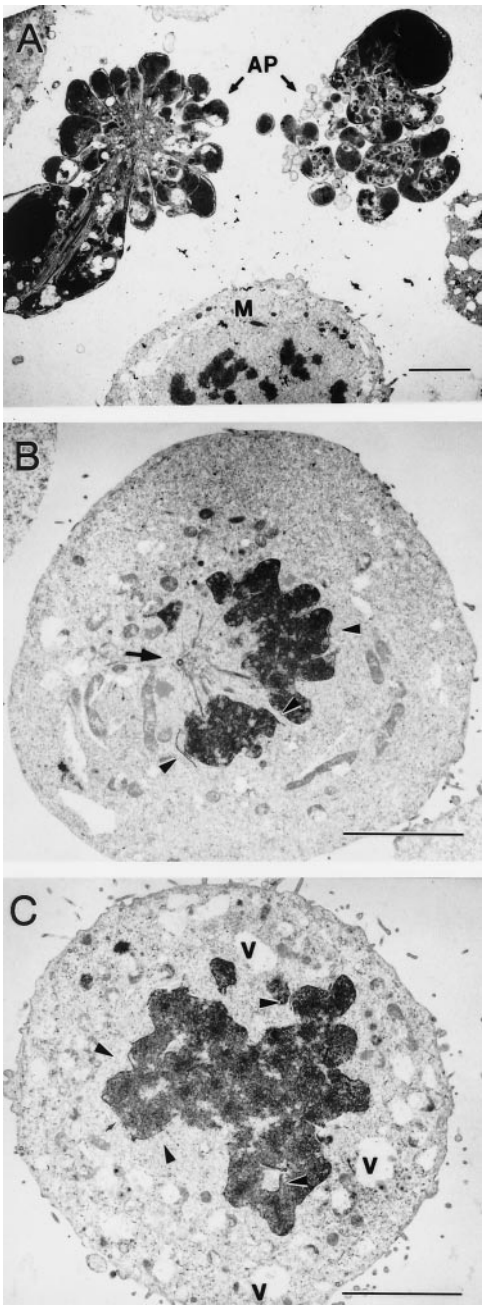

FIG. 1. Ultrastructure of arsenite-induced mitosis-mediated apoptotic cells. Logarithmically growing cells were treated with 5 μM arsenite for 24 hr. Arsenite-arrested mitotic cells were shaken off and re-incubated with arsenite-free medium for 2 hr. The cells were fixed and processed for electron microscopic examination. (A) Apoptotic cells with chromatin condensation and blebbing. (B and C) Cells with nuclear envelope reformation. AP, typical apoptotic cells; M, mitotic cell, V, vacuoles; arrow, spindle apparatus; arrowhead, nuclear envelope. Bars represent 4 μm.

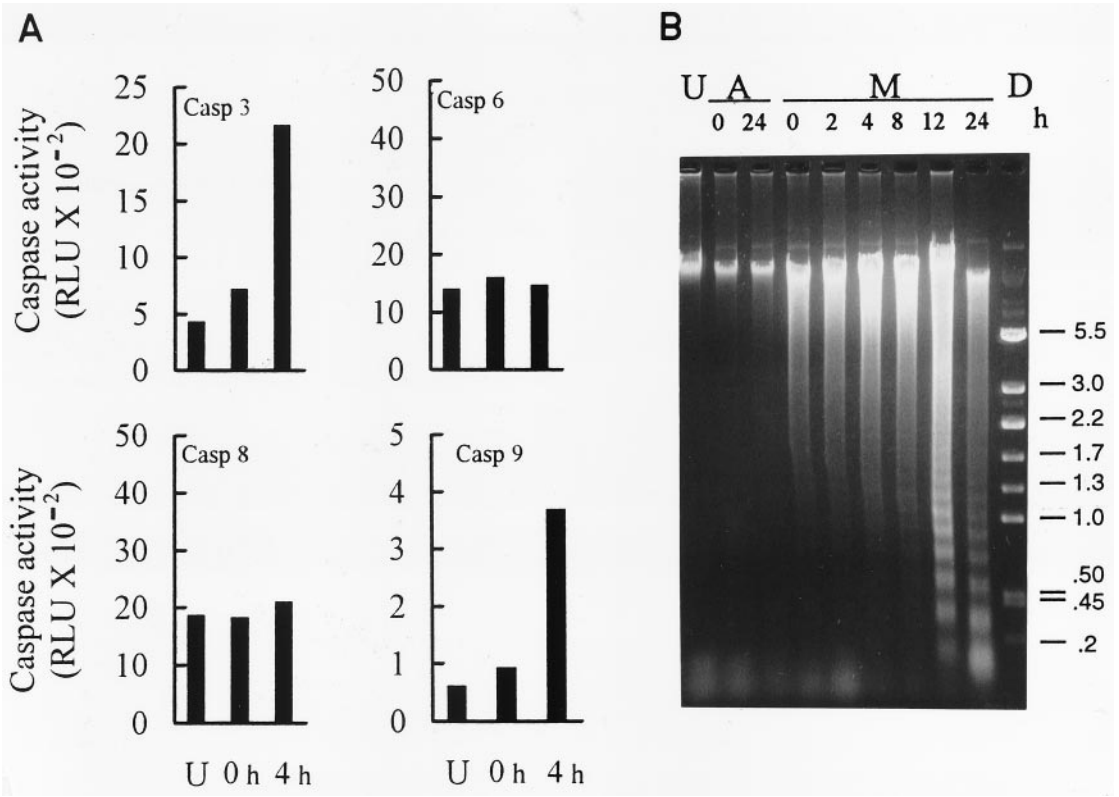

FIG. 2. Caspase activation and DNA fragmentation in arsenite-arrested mitotic cells. (A) Activities of caspases 3, 6, 8, and 9 of untreated control cells (U) or arsenite-arrested mitotic cells, which were re-incubated with arsenite-free medium for 0 and 4 hr, were analyzed as described in Materials and Methods. RLU = relative light units. Representative data of two experiments are shown. (B) DNA fragmentation of arsenite-arrested mitotic cells and the remaining attached cells, which were separated by a shake-off technique and re-incubated with arsenite-free medium for 0–24 hr as indicated. The cells were harvested by trypsinization, and their fragmented DNA was extracted and electrophoretically analyzed on a 1% agarose gel as described in Materials and Methods. Lanes: (U) untreated control; (A) the remaining attached cells after arsenite treatment; (M) arsenite-arrested mitotic cells; and (D) DNA size markers.

ite-free medium for 2 hr, cell blebbing and chromatin condensation commonly were observed (Fig. 1A).

As determined by using fluorogenic substrates, the activities of caspases 3 and 9 were increased 3- and 4-fold 4 hr after incubation of arsenite-arrested mitotic cells in drug-free medium (Fig. 2A). The activities of caspases 6 and 8 were not changed. In addition to morphological alterations and caspase activation, DNA laddering, a hallmark of apoptosis, appeared when arsenite-arrested mitotic cells were re-incubated in arsenite-free medium for 12 hr (Fig. 2B). No DNA laddering was observed in the attached cell population even after a 24-hr re-incubation (Fig. 2B). These observations clearly demonstrated that arsenite-arrested mitotic cells, but not the remaining attached cells, died by apoptosis.

Altered Mitotic Exit

In addition to the appearance of apoptotic cells, chromosome patches surrounded by double nuclear membranes were observed frequently when arsenite-arrested mitotic cells were re-incubated in arsenite-free medium for 2 hr. Two examples are shown in Fig. 1, panels B and C, respectively, indicating the reformation of the nuclear envelope. The nuclear envelope generally is reformed during telophase, in which the mitotic apparatus is dissolving and cytokinesis is taking place. However, in these nuclear envelope-reforming cells, the mitotic apparatus including centrioles and spindles remained intact (Fig. 1B), and no plasma membrane invagination, a manifestation of cytokinesis, could be found (Fig. 1, B and C). Furthermore, an abundance of vacuoles appeared in the cytoplasm (Fig. 1C). The appearance of vacuoles was also a characteristic sign of apoptosis. These results implied that, after removal of arsenite, the arsenite-arrested mitotic cells did not follow the normal process to exit mitosis, i.e. the arsenite-arrested mitotic cells may directly jump to the step of nuclear envelope reformation without dissolving the mitotic spindles and undergoing cytokinesis. Apparently, mitotic exit of arsenite-arrested mitotic cells did not follow the normal mitotic processes.

Delayed Cyclin B Degradation and $p34^{cdc2}$/Cyclin B Kinase Down-Regulation

The accumulation of cyclin B is necessary for cells to enter mitosis, whereas its prompt degradation is essential for cells to move forward to anaphase and complete a cell cycle [29].

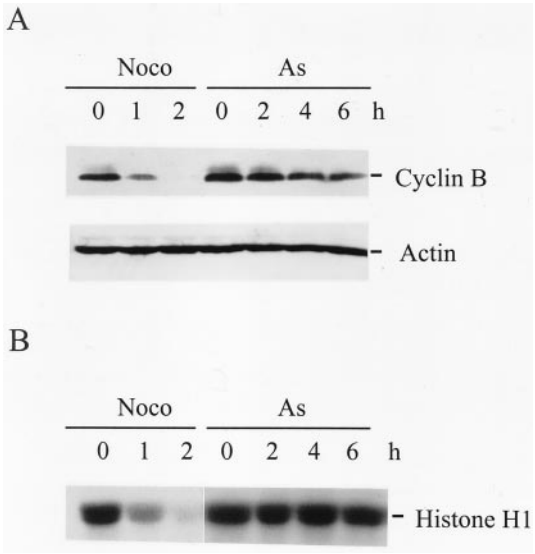

FIG. 3. Delayed cyclin B degradation and p34^{cdc2}/cyclin B kinase down-regulation in arsenite-arrested mitotic cells. Arsenite (5 μM, 24 hr)- or nocodazole (0.1 μM, 4 hr)-arrested mitotic cells were re-incubated with drug-free medium for various times as indicated. (A) Immunoblot analysis of cellular cyclin B and actin levels; (B) p34^{cdc2}/cyclin B kinase activity of arsenite- or nocodazole-arrested mitotic cells as described in panel A. The assay to determine p34^{cdc2}/cyclin B immunocomplex kinase activity was as described in Materials and Methods. As, arsenite-arrested mitotic cells; Noco, nocodazole-arrested mitotic cells.

Therefore, the cellular cyclin B levels were monitored during the re-incubation period. In these experiments, nocodazole-arrested mitotic cells, which were obtained by shaking off the rounded-up cells from nocodazole-treated cultures, were used as a control for comparison. As shown in Fig. 3A, the cellular cyclin B in nocodazole-induced mitotic cells was degraded completely within a 2-hr incubation in drug-free medium. Accompanying the cyclin B degradation, these mitotic cells divided into two daughter cells (data not shown). However, the cyclin B degradation was delayed in arsenite-arrested mitotic cells that were re-incubated in arsenite-free medium. A significant amount of cyclin B remained detectable even when the cells were re-incubated for 6 hr (Fig. 3A). The relative amounts of cyclin B were determined by the aid of a densitometer. The $T_{1/2}$ values of cyclin B degradation were 40 min and 4 hr in nocodazole- and arsenite-arrested mitotic cells, respectively.

Cyclin B couples with p34^{cdc2} to form the key mitotic regulatory kinase. To determine whether delayed cyclin B degradation results in persistent activation of p34^{cdc2}/cyclin B kinase, the p34^{cdc2}/cyclin B kinase activities were analyzed by cyclin B immunoprecipitation coupled with a solid-phase kinase assay technique. As shown in Fig. 3B, the p34^{cdc2}/cyclin B kinase activity in nocodazole-induced mitotic cells was degraded promptly within 2 hr, whereas that in arsenite-arrested mitotic cells remained at very high levels after being incubated in drug-free medium for 6 hr (Fig. 3B). These results indicated that delayed cyclin B degradation kept p34^{cdc2}/cyclin B kinase in a functionally activated state.

Occurrence of Apoptosis Accompanying Altered Mitotic Exit

To understand the relationship between the altered mitotic exit and apoptosis, we traced the M.I. and the apoptotic frequencies of arsenite-arrested mitotic cells. As demonstrated in our previous study [13], 80% of nocodazole-arrested mitotic cells completed their division in 1 hr, and almost all mitotic divisions were finished in 2 hr after release from nocodazole. In contrast, the decrease of M.I. of arsenite-arrested mitotic cells was slow when they were re-incubated in arsenite-free medium (Fig. 4). The M.I. decreased from 81% to 68 and 55% after 2 and 4 hr of re-incubation, respectively (Fig. 4). Apparently, a delayed mitotic exit occurred in arsenite-arrested mitotic cells. Accompanied with decreased M.I., the apoptotic frequencies were increased from 14% to 28 and 44%, respectively (Fig. 4). These results showed an inverse correlation between the appearance of apoptosis and the decrease in M.I.

To confirm the relationship between mitosis and apoptosis further, arsenite-induced rounded-up cells were shaken off. These cells were mainly mitotic cells (Fig. 4) with 4C DNA content (Fig. 5C). After a 4-hr re-incubation in drug-free medium, these shaken-off cells were loaded onto a Percoll gradient and separated into lower and upper fractions. On a Percoll density gradient, apoptotic cells with a higher density locating at the lower fraction could be separated from non-apoptotic cells with a lower density locating at the upper fraction [24, 25]. As shown in Fig. 5A, the lower fraction was composed of 90% apoptotic cells and 5% mitotic cells, whereas the upper fraction was composed of 85% mitotic cells and 15% apoptotic cells.

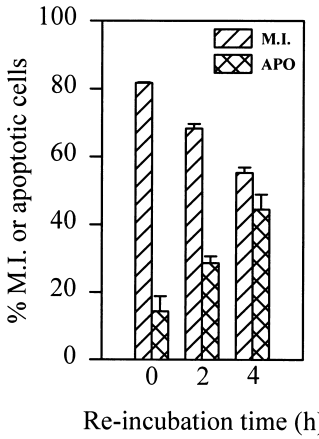

FIG. 4. Inverse correlation of M.I. and apoptotic frequencies. The arsenite-arrested mitotic cells were re-incubated with arsenite-free medium for 0, 2, and 4 hr and then harvested for M.I. and apoptotic frequency (APO) assays as described in Materials and Methods. Bars represent SD of 3 independent experiments.

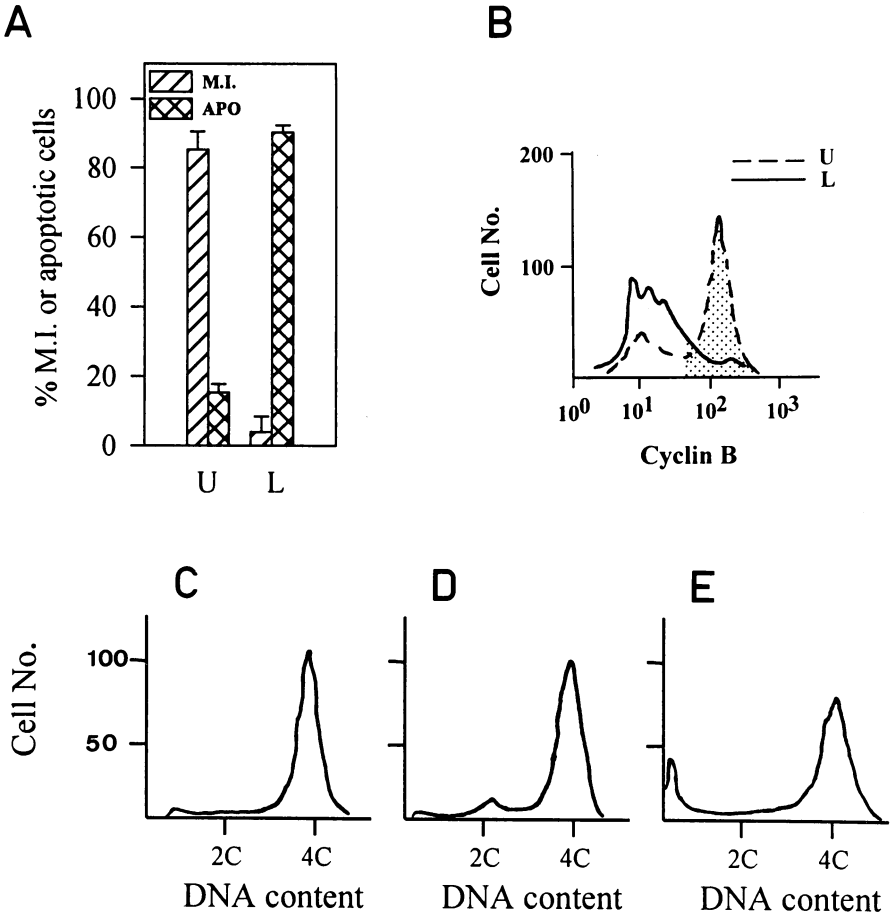

FIG. 5. Occurrence of apoptosis concomitantly with altered mitotic exit. Arsenite-arrested mitotic cells were re-incubated with arsenite-free medium for 4 hr, and then fractionated into upper (U) and lower (L) fractions on a Percoll density gradient as described in Materials and Methods. (A) Separated U and L fractions were subjected to M.I. and apoptotic frequency (APO) assays. Bars represent SD of 3 independent experiments. (B) Cellular cyclin B levels in cells of the U and L fractions were analyzed by a flow cytometric technique. The shaded area indicates the population with high cellular cyclin B content. (C, D, and E) Cellular DNA contents in arsenite-arrested mitotic cells without re-incubation in arsenite-free medium (C) and fractions U (D) and L (E) as described in panel A, respectively, were analyzed by a flow cytometric technique.

Then, the cellular cyclin B levels and DNA contents of the fractionated cells were analyzed by a flow cytometric technique. Cells located in the upper fraction showed a high level of cyclin B (Fig. 5B, shaded area) and 4C DNA content (Fig. 5D), whereas cells in the lower fraction lost their cyclin B (Fig. 5B) but retained their DNA content at 4C (Fig. 5E). Since the lower fraction on the Percoll gradient was mainly apoptotic cells showing a low M.I., low cyclin B levels, and 4C DNA content, we therefore inferred that apoptosis occurred in cells accompanying altered mitotic exit but without a division.

Abrogation of Apoptosis by Accelerating Cyclin B Degradation

By re-incubation of arsenite-arrested cells in arsenite-free medium, apoptosis was triggered, accompanied by delayed cyclin B degradation and alteration of mitotic exit. Whether accelerating cyclin B degradation in arsenite-arrested mitotic cells could prevent apoptosis was next examined. Staurosporine and 2-aminopurine, known as potent kinase inhibitors to override mitotic blockade [30, 31], were adopted to facilitate cyclin B degradation and mitotic exit. As shown in Fig. 6A, the cellular levels of cyclin B remained unchanged when arsenite-arrested mitotic cells were re-incubated in fresh medium for 1 hr. However, cyclin B became negligible within 1 hr in response to supplementation with 44 nM staurosporine or 10 mM 2-aminopurine in arsenite-free medium (Fig. 6A). In addition, the M.I. were down to zero within 2 hr when cells were treated with 44 nM staurosporine or 10 mM 2-aminopurine. These results confirmed that staurosporine and 2-aminopurine effectively facilitated cyclin B degradation and mitotic exit. By the same treatment protocol, the apoptotic cells were examined after a 6- or 24-hr re-incubation in drug-free medium. As shown in Fig. 6B, the apoptotic frequencies of the arsenite-arrested mitotic cells were reduced by a 2-hr treatment with staurosporine or 2-aminopurine. However, genistein (100 μM), a protein tyrosine kinase inhibitor, neither accelerated cyclin B degradation (Fig. 6A) nor prevented the cells from undergoing apoptosis (Fig. 6B). By morphological examination, after a 24-hr incubation in drug-free medium, the attached interphase cells of arsenite-treated cultures continued to grow healthily (Fig. 7A), whereas the arrested mitotic cells showed severe disintegration (Fig. 7B). In contrast, 2-aminopurine treatment prevented the arsenite-arrested mitotic cells from disintegration (Fig. 7C). Flow cytometric DNA analysis confirmed the morphological observation. After a 24-hr incubation, the attached cells showed a normal DNA histogram without G_1 arrest (Fig. 7D), whereas numerous disintegrated cells were derived from the arsenite-arrested mitotic cells (Fig. 7E). 2-Aminopurine treatment inhibited

cell disintegration and resulted in an increase in cell populations with 4C and 8C DNA (Fig. 7F), indicating that 2-aminopurine moved the mitotic cells toward interphase without cell division. Similar results were observed with staurosporine treatment (data not shown). These results suggest that prompt cyclin B degradation enforces the exit of arsenite-arrested mitotic cells from mitosis and prevents them from undergoing apoptosis.

We also treated the arsenite-induced mitotic cells with cycloheximide, emetine, or α-amanitin during the re-incubation in arsenite-free medium. These translation or transcription inhibitors did not prevent the occurrence of apoptosis (data not shown). Thus, no newly synthesized protein was required for triggering arsenite-induced mitosis-mediated apoptosis.

DISCUSSION

Apoptosis is an active process of cell death. In response to a variety of environmental toxic agents, endogenous factors, or chemotherapeutic drugs, the exposed cells activate a preprogrammed cascade of metabolic events that culminate in cell disintegration. Apoptosis shares a number of morphological features with mitosis, including cell rounding-up, lamin disassembly, and chromatin condensation [32]. Therefore, mitosis and apoptosis may also share overlapping biochemical pathways.

In HeLa S3 cells, arsenite apparently mimics chemotherapeutic agents such as taxol or colchicine to induce mitotic arrest [13]. These therapeutic agent-induced mitotic cells subsequently die by apoptosis [17–19, 33]. In the present study, we demonstrated that (i) arsenite-arrested mitotic cells died by apoptosis, (ii) the occurrence of mitosis-mediated apoptosis was associated with delayed cyclin B degradation and altered mitotic exit, and (iii) staurosporine or 2-aminopurine accelerated the degradation of cyclin B, enforced mitotic exit, and hence abrogated apoptosis in arsenite-arrested mitotic cells. Taken together, these observations suggest that apoptosis occurs in arsenite-arrested mitotic cells that exit mitosis abnormally.

The $p34^{cdc2}$/cyclin B kinase is the key regulator controlling the cells going in and out of mitosis, and cyclin B should be degraded promptly during mitotic transition from metaphase to anaphase [29, 34, 35]. It has been found that several different types of stress, such as DNA damage, growth factor withdrawal, and treatment with the spindle poison taxol, can induce unscheduled $p34^{cdc2}$/cyclin B kinase activity, which is prior to or simultaneous with the induction of apoptosis [36–39]. Prevention of $p34^{cdc2}$/cyclin B kinase activation by an antisense oligomer or a specific inhibitor abrogates taxol-induced apoptosis, suggesting that $p34^{cdc2}$/cyclin B kinase plays an important role in taxol-induced apoptosis [39]. However, stress-induced unscheduled $p34^{cdc2}$/cyclin B activation did not correlate with the entrance into mitosis. Whether $p34^{cdc2}$/cyclin B kinase is involved in arsenite-induced mitosis-mediated apoptosis is still unclear. In the present study, the delayed

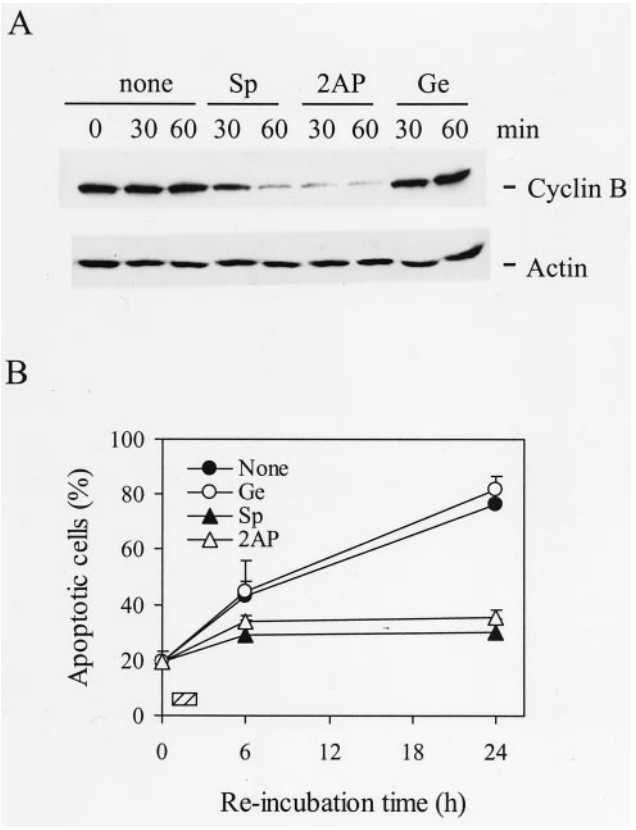

FIG. 6. Effects of kinase inhibitors on cyclin B degradation and apoptotic frequency. (A) Immunoblot analysis of cellular cyclin B and actin levels in arsenite-arrested mitotic cells, which were re-incubated with arsenite-free medium but supplemented with nothing (none), 44 nM staurosporine (Sp), 10 mM 2-aminopurine (2AP), or 100 μM genistein (Ge) for 0, 30, and 60 min. (B) Apoptotic frequencies. Arsenite-arrested mitotic cells were treated with various kinase inhibitors as described in panel A for 2 hr. Afterward, the cultures were washed with PBS and re-incubated with drug-free medium. At the time indicated, cells were stained with Annexin-V–FITC and examined under a fluorescent microscope as described in Materials and Methods. Bars represent SD of 3 independent experiments. The hatched area indicates the time period for the treatment with kinase inhibitors.

cyclin B degradation was coupled with anachronistic nuclear envelope reformation and mitotic exit without division, culminating in apoptosis induced by re-incubation of arsenite-arrested mitotic cells in arsenite-free medium. These results suggest the presence of a conflict between cyclin B degradation and mitotic exit in arsenite-arrested mitotic cells. As hypothesized by Kung *et al.* [40], the cytotoxic effects of cell cycle phase-specific agents do not derive directly from their biochemical actions *per se* but from processes that are evoked by dissociation of normally integrated cell cycle events. Since cyclin B degradation controls the transition from metaphase to anaphase, its delayed degradation would disturb the signal pathway of mitotic checkpoints, and, hence, trigger apoptosis. The usage of staurosporine or 2-aminopurine, which accelerates cyclin B degradation, overrides mitotic arrest, and prevents apoptosis (Figs. 6 and 7), supports the hypothesis that

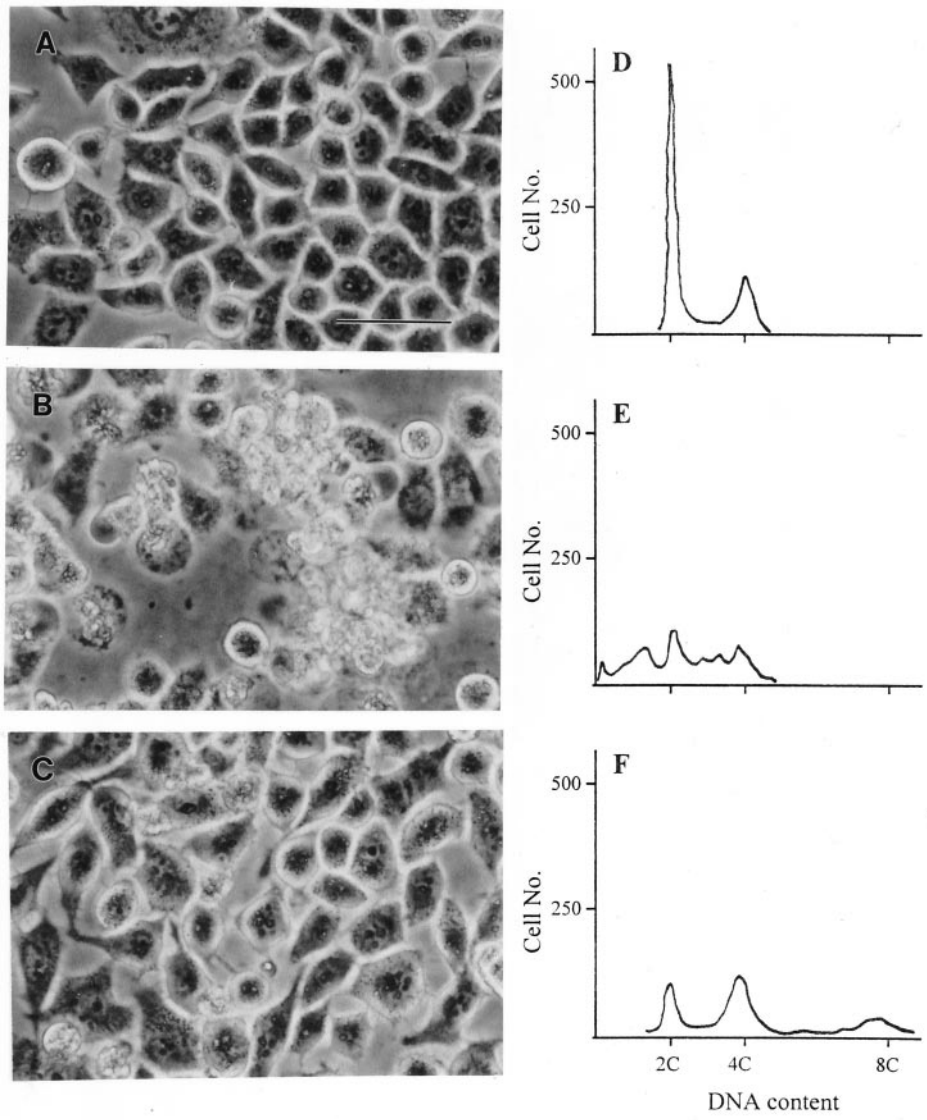

FIG. 7. Induction of mitotic exit by 2-aminopurine. Arsenite-arrested mitotic cells and the remaining attached cells were separated by a shake-off technique. The attached cells were re-incubated with fresh medium for 24 hr. The arsenite-arrested mitotic cells were treated with or without 10 mM 2-aminopurine for 2 hr and then re-incubated with drug-free medium for another 22 hr. At the end of treatment, the cellular morphology was examined under a phase-contrast microscope (A to C), and DNA content was analyzed by a flow cytometric technique (D to F). (A and D) Remaining attached cells; (B and E) arsenite-arrested mitotic cells without 2-aminopurine treatment; (C and F) arsenite-arrested mitotic cells treated with 2-aminopurine. Bar represents 50 μm.

prolonged $p34^{cdc2}$/cyclin B kinase activation dislocates integrated cell cycle events. The internal conflict between cellular injury and cell cycle progression has been observed frequently in xenobiotic- or cytokine-induced apoptosis [41]. Thus, mitosis-mediated apoptosis induced by arsenite may be initiated through the internal conflict of mitotic checkpoints and mitotic exit.

Degradation of cyclins generally occurs through ubiquitin-dependent proteolytic pathways [42, 43] or, occasionally, caspase cascades [44]. We have incubated *in vitro* translated human cyclin B with recombinant caspases 3, 6, 8, and 9. No cyclin B was cleaved by these caspases (data not shown). Alternatively, arsenite was shown to inhibit ubiquitin-dependent proteolytic pathways [45, 46]. The delayed cyclin B degradation in arsenite-arrested mitotic cells probably may be attributed to the inhibitory effect of arsenite on ubiquitin-dependent proteolysis. Since the efflux of arsenite is quite fast [47, 48], more than 80% of accumulated arsenite was extruded from the arsenite-arrested mitotic cells within 2 hr when they were re-incubated in arsenite-free medium. We suspect that the cyclin B degradation in mitosis-mediated apoptosis was gradually reversed by extruding arsenite from the cells. More experiments are required to address how cyclin B is degraded during mitosis-mediated apoptosis.

Accumulated evidence has shown that numerous phosphorylation-regulated proteins are involved in signaling systems and apoptosis [49]. Since delayed cyclin B degradation in arsenite-arrested mitotic cells is rescued by wide-spectrum kinase inhibitors such as staurosporine or 2-aminopurine, a cyclin-dependent kinase or other related protein kinases may also be involved in the regulation of cyclin B degradation. Since genistein (Fig. 6B) or H7 (data not shown) did not enhance cyclin B degradation or prevent the arsenite-arrested mitotic cells from undergoing apoptosis, protein kinase C or protein tyrosine kinase is unlikely to be involved in cyclin B degradation and mitosis-mediated apoptosis.

Our present results are similar to a recent study showing that taxol treatment induces cyclin B accumulation and

apoptosis in human epidermoid carcinoma KB cells [50]. However, apoptosis induced in arsenite-arrested mitotic cells has faster kinetics than that induced by taxol. In our cases, apoptosis occurred within a few hours after re-incubation of arsenite-arrested mitotic cells in arsenite-free medium (Fig. 4). Apoptosis induced by low doses of taxol appeared at least 24 hr after the removal of taxol [18, 50]. Furthermore, the protein and RNA synthesis inhibitors cycloheximide and actinomycin D were shown to abrogate the taxol-induced cellular effects, including mitotic arrest, cyclin B accumulation, and apoptosis [50]. However, the protein and RNA synthesis inhibitors cycloheximide, emetine, and α-amanitin did not prevent arsenite-arrested mitotic cells from apoptosis. Therefore, in addition to delayed cyclin B degradation, which has been inferred to participate in initiating apoptosis in arsenite-arrested mitotic cells, other unknown factors are certainly required for induction of apoptosis.

Arsenic trioxide (As_2O_3), another trivalent inorganic arsenic compound, has been used as a chemotherapeutic agent for APL [4, 5]. However, its pharmacological mechanism is not clear. The therapeutic effects of As_2O_3 on APL cells were suggested to promote the degradation of PML/RARα, a nuclear structural protein (PML) and retinoic acid receptor α fusion protein specifically expressed in APL cells, to down-regulate the expression of bcl-2, and finally to induce apoptosis [9]. Zhu *et al.* [51] demonstrated that As_2O_3 exerts its cytotoxic effects by damaging mitochondrial transmembrane potential, and hence depleting cellular ATP levels. Therefore, As_2O_3 has been suggested to be a novel mitochondriotoxic anticancer agent in malignant lymphocytes. In our previous study, arsenite at concentrations of 50–100 μM caused destruction of mitochondrial cisternae and ATP depletion in HeLa S3 cells [52]. Under the present experimental conditions (5 μM arsenite for 24 hr), mitochondrial membrane potential collapse was not observed by using the Mitotracker (Molecular Probes) technique [53] at the times when apoptosis examination was performed. In HeLa cells, arsenite-induced mitosis-mediated apoptosis is unlikely to be due to the collapse of mitochondrial membrane potential. Recently, Li and Broome [15] reported, similarly to our previous report for arsenite [13], that As_2O_3 interacts with tubulin to induce mitotic arrest and apoptosis in myeloid leukemia cells. Our present study demonstrated that arsenite induces a mitosis-mediated apoptosis, and it extends a potential role of arsenic compounds as chemotherapeutic drugs for other cancers. Furthermore, delayed cyclin B degradation and altered mitotic exit in arsenite-arrested mitotic cells may be used as a molecular clue to differentiate the biochemical pathways for the processes of mitosis and apoptosis.

We thank Drs. J. J. Yen and H. F. Yang-Yen for valuable suggestions. We also thank Mr. Douglas Platt for carefully reading the manuscript. This work was supported by grants from Academia Sinica and the National Science Council, Republic of China.

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