Induction of Centrosome Amplification during Arsenite-Induced Mitotic Arrest in CGL-2 Cells

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Abstract
Arsenite-induced mitotic abnormalities result in mitotic death in several cancer cell lines. However, how arsenite induces these effects is not known. We have previously shown that arsenite induces mitotic arrest, mitotic abnormalities, and mitotic death in CGL-2 cells. To further delineate the mechanism of action of arsenite, we examined its effect on centrosome duplication and the possible link between centrosome dysregulation and arsenite-induced mitotic death. Immunofluorescence staining of γ-tubulin revealed that centrosome amplification was induced in arsenite-arrested mitotic cells but not in nocodazole-arrested cells. When S phase–enriched cells were treated with arsenite, they progressed into and arrested at mitosis and then formed supernumerary centrosomes. A further increase in arsenite-induced centrosome amplification was seen during the prolonged mitotic arrest. The arsenite-induced supernumerary centrosomes might result from uneven fragmentation of centrosome, overexpression of pericentriolar materials, and inhibition of centrosomal coalescence during mitosis. Furthermore, termination of mitotic arrest by treatment of arsenite-arrested mitotic cells with cyclin-dependent kinase 1 inhibitors or by suppression of spindle checkpoint function by small interfering RNA–mediated silencing of BubR1 or Mad2 markedly reduced the induction of centrosome amplification and mitotic death in arsenite-treated cells. These results indicate that centrosome amplification is induced in arsenite-arrested mitotic CGL-2 cells in a spindle checkpoint-dependent manner and is involved in the induction of arsenite-induced mitotic death.

Introduction
Recently, arsenic trioxide (As$_2$O$_3$), a trivalent arsenite, has been added to therapeutic strategies for acute promyelocytic leukemia (APL), especially for APL patients resistant to all trans-retinoic acid (1). As$_2$O$_3$ has a dual dose-dependent effect on APL cells, inducing partial differentiation at low concentrations (0.1-0.5 μmol/L) and triggering apoptosis at high concentrations (1.0-2.0 μmol/L; ref. 2). However, arsenite-induced apoptosis is not restricted to APL cells, because several reports have shown that it can also occur in some solid tumor cells and in leukemia cells other than APL (3, 4). Although the mechanism of arsenite-induced apoptosis is very complicated, many reports had shown a tight link between arsenite-induced apoptosis and mitotic arrest, and that mitotic arrest is one of the major mechanisms for arsenite-induced apoptosis in cancer cells (5-8).

Arsenite-induced mitotic arrest is accompanied by the generation of several mitotic abnormalities, such as spindle multipolarity, chromosome missegregation, and aberrant mitotic division (9, 10). Accurate cell division requires error-free DNA replication and the equal segregation of the replicated chromosomes into the two newly formed daughter cells. To achieve this, microtubules emanating from the opposite poles of a bipolar mitotic spindle attach to the replicated chromosomes and coordinate chromosome congression to the metaphase plate. Then the sister chromatids segregate and move to the spindle poles. In mammalian cells, each spindle pole is centered around a centrosome. The centrosome, which consists of a pair of centrioles surrounded by pericentriolar material, is a major microtubule-organizing center in eukaryotic cells and plays an integral role in directing the organization of the cytoplasmic microtubules and the assembly of the mitotic spindles (11). The centrosome is duplicated in a remarkably semiconservative process that is completed in the S phase, whereas more than an entire cell cycle is required for the maturation of the daughter centriole (12). For accurate chromosomal division to occur at the correct time, centrosome duplication must be precisely coupled to DNA replication and mitotic division (13).

Aberrations in the number of centrosomes inevitably lead to mitotic defects and cause chromosome missegregation and centrosome abnormalities and are therefore frequent in many common cancers (14). In addition, recent molecular evidence suggests that the centrosome also plays an active role in the stress response and in cell cycle checkpoint control. Centrosome overduplication has been reported to be one of the underlying causes of radiation-induced cell death (15). Genotoxic stress induced by hydroxyurea or daunorubicin also leads to centrosome amplification in cells that have an inactive G1-S cell cycle checkpoint (16). Several viral proteins, such as E6 and E7 from human papilloma virus (17), E1A from adenovirus (18), and x protein from hepatitis B virus (19), have been reported to induce supernumerary centrosomes and therefore induce carcinogenesis. The effects of these stresses on the centrosome show that it is capable of responding to a wide variety of different stimuli. The centrosomal stress response is a part of the cell cycle checkpoint control system and can completely halt cell division or cell cycle progression under unfavorable conditions (20).

Several reports have shown that arsenite and its metabolites induce supernumerary centrosomes and multipolar spindles in many cell systems (21, 22). The induction of centrosome abnormalities can lead to abnormal chromosome segregation and result in aneuploidy and is associated with arsenite-induced morphologic cell transformation (22). However, how arsenite induces centrosome abnormality and whether induction of centrosome abnormality is involved in arsenite-induced mitotic
arrest and mitotic death remain largely unknown. Our previous studies indicated that treatment of HeLa S3 cells with 5 to 10 μmol/L arsenite can inhibit mitotic division, result in 30% to 35% cell population arrested at the mitotic stage, and then lead to mitosis-mediated cell death (5, 23). Recently, we have shown that arsenite at 2 μmol/L, a clinically achievable concentration (24), can induce significant mitotic arrest (the mitotic index is up to 50%) and mitotic death in CGL-2 cells (8). In the present study, we took advantage of this effect on CGL-2 cells to further delineate the mechanism involved in arsenite induction of mitotic abnormalities and the possible role of centrosome aberrations in arsenite-induced mitotic death.

Materials and Methods

Cell culture. The CGL-2 cell line (25), derived from a hybrid (ESH5) of the HeLa variant, D98/AIF2, and a normal human fibroblast strain, GM77, was kindly provided by Dr. E.J. Stanbridge (University of California-Irvine). The cells were routinely maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 0.37% sodium bicarbonate, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in an humidified incubator in air and 10% CO2. The cells were routinely passaged twice per week. The logarithmically growing CGL-2 cells were treated with 2 μmol/L sodium arsenite (Merck Biosciences, San Diego, CA) or 40 to 50 mmol/L nocardazole (Calbiochem, Merck Biosciences) for different times. A sodium arsenite stock solution (10 mmol/L) was freshly prepared in double distilled water before use.

Analysis of cell cycle progression and mitotic index. Cell cycle progression was monitored using DNA flow cytometry. DNA was stained with propidium iodide, and mitotic cells were quantified by measuring the expression of a mitosis-specific marker, phospho-histone H3 (26). In brief, the cells were trypsinized, washed once with PBS (pH 7.4), fixed with ice-cold 70% ethanol for 16 hours, and immunostained with a mouse anti-phospho-histone H3 antibody (Cell Signaling Technology, Beverly, MA) followed by a FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were then stained with 4 μg/ml propidium iodide in PBS containing 1% Triton X-100 and 0.1 mg/ml RNase A. Phospho-histone H3 levels and the DNA content of individual cells were analyzed using a fluorescence-activated cell sorter (FACSTAR, Becton, Dickinson and Company, Franklin Lakes, NJ), and the cell cycle distribution was analyzed using a fluorescence activated cell sorter (FACSTAR, Becton, Dickinson and Company). The percentage of cells with a given number of centrosomes, two or more, was calculated from 2000-3000 cells per sample.

Synchronization of CGL-2 cells. Cells were synchronized in G1 by the protocol of double-thymidine block (27). Logarithmically growing CGL-2 cells at 50% confluence were treated with 2 mmol/L thymidine for 12 hours, switched to thymidine-free medium for 12 hours, then again treated with 2 mmol/L thymidine for 12 hours. At this point, the majority of the cells were at the G1-S boundary (8). They were then allowed to progress forward by switching them to thymidine-free medium, and the cell cycle progression was monitored at 2- to 4-hour intervals using a DNA flow cytometer as described above.

Immunofluorescence staining. Cells seeded on glass coverslips were incubated for 24 hours at 37°C with or without 2 μmol/L arsenite, then were washed twice with PBS and fixed in situ with 90% methanol at −20°C for 10 minutes. Alternatively, arsenite-arrested or nocardazole-arrested mitotic cells were shaken off the plates and cytosplasm on glass slides using a cytocentrifuge (Shandon Cytospin 4, Thermo Electron Corporation, Pittsburgh, PA) before a PBS wash and methanol fixation. The cells were again washed twice with PBS and coimmunostained for 1 hour at 37°C with a rabbit anti-α-tubulin antibody (AKL5, Sigma, Saint Louis, MO) and either a mouse anti-α-tubulin antibody (clone B512, Sigma) or a mouse antidynein antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Nonbound antibodies were removed by extensive washing with PBS containing 0.2% Tween 20 (PBST), then the cells were incubated for 30 minutes at 37°C in the dark with FITC-coupled anti-mouse immunoglobulin and Texas red-coupled anti-rabbit immunoglobulin antibodies (Jackson Immunoresearch Laboratories), the nuclei being simultaneously counterstained with 0.1 μg/ml 4,6-diamino-2-phenylindole (DAPI, Sigma). After thorough rinsing with PBST, the cells were mounted with 90% glycerol solution containing 1 mg/ml phenylenediamine, pH 8.0 (Merck Biosciences) and examined under a fluorescence microscope (Axioskop 2, Zeiss, Oberkochen, Germany). To calculate the percentage of cells with a given number of centrosomes, two to three independent experiments were done, and at least 200 cells were analyzed in each experiment. To examine the presence of centrosomes within the supernumerary centrosomes, the cells were subjected to cold treatment and brief extraction (40 seconds) with extraction buffer [0.75% Triton X-100, 5 mmol/L PIPES, 2 mmol/L EDTA (pH 6.8)] before fixation, then coimmunostained with anti-γ-tubulin antibody (which detects the location of pericentriolar material) and anti-α-tubulin antibody (which detects centrioles; ref. 28). The cells were examined under a confocal microscope (Leica TCS NT, Leica Lasertechnik GmbH, Heidelberg, Germany). The number of centrosomes in a centrosome was determined from 100 mitotic cells.

Immunoblotting. Levels of nuclear mitotic apparatus protein (NuMA), dynein, BubR1, and mitotic arrest–deficient 2 (Mad2) in total cell extracts were examined by immunoblot analysis as described previously (8). Briefly, the treated CGL-2 cells were washed twice with ice-cold PBS, scraped off, collected in a 1.5-ml vial, and boiled in SDS-PAGE sample buffer (29). Samples containing equal amount of cellular proteins (20-50 μg) were resolved by 8% SDS-PAGE, transferred onto polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ), and incubated overnight at 4°C with the primary antibodies diluted in PBST; then for 1 hour at room temperature with the appropriate horseradish peroxidase–conjugated secondary antibody (Jackson ImmunoResearch Laboratories) diluted in PBST. Following extensive washes with PBST, bound antibody was visualized by chemiluminescence using SuperSignal West Pico chemiluminescence reagent (Pierce, Rockford, IL). β-Actin or HSC-70 was used as a loading control. The results shown are representative of at least two independent experiments. Mouse anti-NuMA antibody was purchased from BD Biosciences (Palo Alto, CA); mouse anti-dynein antibody, mouse anti-HSC-70 antibody, and goat anti-Mad2 antibody were from Santa Cruz Biotechnology; and mouse anti-BubR1 antibody and mouse anti-β-actin antibody were from Chemicon International (Temecula, CA). Protein concentrations were determined by the Bradford method (30).

Apopotosis assay. The number of apoptotic cells was determined using an Annexin V-FITC apoptosis detection kit (Oncogene, Boston, MA) as described previously (8). The cells were washed once with PBS and resuspended in 100 μl of binding buffer containing 5 μl of a 200 μg/ml solution of FITC-conjugated Annexin V and 5 μl of a 30 μg/ml solution of propidium iodide. After a 10-minute incubation at room temperature, FITC binding to individual cells was analyzed using a fluorescence activated cell sorter (FACSTAR, Becton, Dickinson and Company). The percentage of apoptotic cells (FITC positive) in 10,000 cells was calculated in each experiment.

RNA interference. RNA interference was carried out as described previously (31). The small interfering RNA (siRNA) was synthesized by Proligo Singapore Pte Ltd. (Helios, Singapore). Cells were plated at a density of 0.5 to 1 × 10^5 per 35-mm dish 1 day before transfection. siRNA was transfected into the cells using Oligofectamine (Invitrogen) at a final concentration of 0.1 μmol/L. Twenty-four hours after transfection, the medium were replaced with fresh medium, and the cells were treated with nocardazole or arsenite. The sequences targeted by BubR1 or Mad2 siRNA were 5'-CACTATTAGTCCGTTATATAT-3' and 5'-CTACTACATCCCAAAGATTT-3', respectively. A double-stranded RNA targeting luciferase (5'-CGUAGCCGG-GAAUACUGG-UAGG) was used as a control. Cellular expression of BubR1 and Mad2 was examined by immunoblot analysis at 24, 48, and 72 hours after siRNA transfection.

Analysis of cell death. Nontransfected or siRNA-transfected cells were light untreated or were treated with 2 μmol/L arsenite for 60 hours, then the number of viable cells was determined by trypan blue (Invitrogen) exclusion using a hemocytometer. Cells were seeded at a density of 5 × 10^5 per well in triplicates in six-well plates 1 day before transfection. After treatment, cells that remained in each well was collected, suspended in 0.2% trypan blue
solution, and counted with a hemocytometer (32). The percentage of cell death in arsenite-treated cultures was calculated as 100% minus the percentage of viable cells.

Results
Arsenite induces the formation of supernumerary centrosomes and multipolar spindles in mitotic-arrested CGL-2 cells. We have previously shown that arsenite-treated CGL-2 cells show significant arrest at mitosis and damaged DNA and subsequently undergo apoptosis (8). To define more precisely the effect of arsenite on mitosis, arsenite-treated CGL-2 cells were stained with immunofluorescent antibody to phospho-histone H3 (a mitotic marker) and propidium iodide, and analyzed by flow cytometry, mitotic cells being differentiated from G2 cells by the level of phospho-histone H3 (Fig. 1A). Because our previous results indicated that the metaphase chromosomes in arsenite-arrested cells were overcondensed and shorter than those in normal metaphase cells (9), the lower phospho-histone H3 level in arsenite-arrested mitotic cells (Fig. 1A) might be due to decreased antibody accessibility to the overcondensed chromosomes in the assay. Treatment with 2 μmol/L arsenite induced a time-dependent increase in the percentage of mitotic cells (from 3.1% at 0 hour to 40% at 14 hours and 45% at 24 hours) and a gradual decrease in the percentage of the G1 and S phase cells (Fig. 1B). These results show that arsenite induced a significant accumulation of mitotic cells, and that these mitotic cells were arrested in a prolonged mitosis. Similar accumulation of mitotic cells (55%) was seen in CGL-2 cells treated with 50 nmol/L nocodazole for 24 hours (Fig. 1B). Immunofluorescence staining of γ-tubulin and α-tubulin revealed that supernumerary centrosomes and multipolar spindles were induced in arsenite-arrested mitotic cells (Fig. 1C) but not in interphase cells (data not shown). After 20 hours of arsenite treatment, half of the arsenite-arrested mitotic cells contained supernumerary centrosomes, and cells with up to six centrosomes could be seen (Fig. 1D). In contrast, supernumerary centrosomes and multipolar spindles were not seen in nocodazole-arrested mitotic cells (Fig. 1D). Because arsenite could also induce mitotic arrest and mitotic death in HeLa S3 cells (5, 23), the effect of arsenite on centrosome was conducted. Induction of centrosome amplification was also observed in 5 μmol/L arsenite-arrested mitotic HeLa S3 cells (data not shown). These results indicate that arsenite treatment induces the accumulation of mitotic cells with supernumerary centrosomes.

Induction of centrosome amplification during arsenite-induced mitotic arrest. Centrosome amplification often occurs as a result of prolonged S phase or a failure of cytokinesis (33). However, the results shown in Fig. 1 and those of our previous study (8) showed that the arsenite-treated CGL-2 cells were not...
blocked in the S phase or did not become polyploid because of cytokinesis failure but, instead, were arrested at mitosis. To determine the cell cycle stage at which the centrosomes were amplified, CGL-2 cells were synchronized at G1 stage by double thymidine block. At 3 hours after release from thymidine block, the cells were synchronized in the S phase and were treated with 2 μM arsenite. The cell cycle distribution and centrosome number in these cells were then followed. Flow cytometric analysis showed that untreated cells progressed into the G2 stage at 7 hours (Fig. 2A, E), mitosis at 10 hours (Fig. 2A, column), and had exited from mitosis by 13 hours after thymidine release. Most of the arsenite-treated cells also progressed into the G2 stage at 7 hours (Fig. 2B, E), whereas they entered into mitosis at 10 hours after thymidine release and then arrested (Fig. 2B, column), showing that prolonged mitotic arrest was induced by arsenite. This result is consistent with our previous report (8). Immunofluorescence staining of γ-tubulin showed that no significant centrosome aberrations were observed in the interphase cells (data not shown) or the mitotic cells (Fig. 2C) in untreated cultures at 10 to 14 hours after thymidine release. The interphase cells from arsenite-treated cultures, mostly at the G2 stage, also contained the normal number of two centrosomes (data not shown). However, in arsenite-treated cultures, the percentage of mitotic cells with a normal centrosome number fell markedly from 94% at 10 hours to 18% by 24 hours after thymidine release (Fig. 2D). Furthermore, the percentage of mitotic cells with more than four centrosomes was increased during the prolonged mitotic arrest from 35% at 16 hours after thymidine release to 80% at 24 hours (Fig. 2D). These results show that arsenite-induced centrosome amplification occurs during mitotic arrest. To confirm this, we used the same protocol to induce centrosome amplification as in Fig. 2, then alsterpaullone or purvalanol A [cyclin-dependent kinase 1 (cdk1) inhibitors] was added to the culture medium at 13 hours after thymidine release (i.e., at the time the arsenite-arrested mitotic cells began to accumulate; Fig. 2B). Because addition of alsterpaullone or purvalanol A resulted in mitotic exit of the arsenite-arrested mitotic cells within 4 to 6 hours (the mitotic index was decreased from 53% to 3% and 6%, respectively), centrosome amplification in the total cell population (mitotic and interphase cells) was examined at 20 hours after thymidine release. The results showed that centrosome amplification was markedly decreased in arsenite-treated cultures by addition of cdk1 inhibitors (Fig. 3). These results indicated that termination of mitotic arrest by cdk1 inhibitors could attenuate the arsenite induction of centrosome amplification and confirmed that arsenite-induced centrosome amplification occurred during the prolonged mitotic arrest.

**Supernumerary centrosomes in arsenite-arrested mitotic CGL-2 cells either contain abnormal number of centrioles or are acentriolar.** Centrosomes can fragment in response to microtubule poison–induced cell cycle arrest (34) and in response to damaged or incompletely replicated DNA during mitosis (35). To determine whether the γ-tubulin spots seen in arsenite-arrested mitotic CGL-2 cells reflected fragmentation or duplication of the centrosome structure, the centriole profiles in arsenite-induced supernumerary centrosomes were examined (33). The normal centrosome profile contained two centrosomes, each with a pair of...
centrioles (Fig. 4A, N). The uneven fragmented centrosomes contained only one or more than two centrioles (Fig. 4B, F). The acentriolar centrosomes contained overexpressed pericentriolar material components but without centrioles (Fig. 4C, A). The profiles in arsenite-induced supernumerary centrosomes were summarized in Fig. 4D. Ninety-seven percent of mitotic cells from untreated cultures had a normal centrosome profile (Fig. 4D, N), whereas in arsenite-treated cultures, only 15% of the mitotic cells showed a normal centrosome profile, whereas 26% contained uneven fragmented centrosomes (Fig. 4D, F), 15% contained acentriolar centrosomes (Fig. 4D, A), and 41% contained both (Fig. 4D, F + A). These results show that arsenite-induced abnormal centrosome amplification is mainly due to uneven centrosome fragmentation and/or pericentriolar material overexpression.

In addition to reduplication of centrosomes, spindle multipolarity can also occur by inhibition of centrosomal coalescence (14, 36). To further dissect the effects of arsenite on centrosome amplification in mitotic-arrested CGL-2 cells, the expression of NuMA and dynein, the two critical proteins involved in the regulation of centrosome coalescence (37), was examined. Immunoblot analysis showed that NuMA expression showed a time-dependent increase in arsenite-treated cells, whereas dynein expression was not significantly changed (Fig. 5A). By immunofluorescence staining, NuMA was located at the mitotic spindle, and its expression was significantly increased in arsenite-arrested mitotic cells (Fig. 5B, left). Dynein was located at the centrosome and along the mitotic spindles in normal mitotic cells, whereas the spindle dynein signal was reduced and diffuse in arsenite-arrested mitotic cells (Fig. 5B, right). In addition, DAPI staining revealed that chromosomes in the untreated cell were congressed at metaphase plate. However, the chromosomes in the arsenite-treated cells were scattered in the cytoplasm, indicating the chromosome segregation was altered. This is consistent with our previous reports that arsenite could alter chromosome segregation (9, 10). These results show that arsenite treatment increases NuMA expression in mitotic-arrested cells and interferes with the localization of dynein in the spindle. This result implies that arsenite-induced uneven centrosome fragmentation and/or pericentriolar material overexpression in mitotic arrested cells was associated with the inhibition of centrosomal coalescence during mitosis.

![Image of Figure 3](http://cancerres.aacrjournals.org/)

**Figure 3.** cdk1 inhibitors suppress centrosome amplification in arsenite-arrested mitotic CGL-2 cells. Cells were synchronized at G1 and treated with arsenite as described in Fig. 2, then at 13 hours after thymidine release, were left untreated (A) or treated with 10 μmol/L alsterpaullone (Als) or purvalanol A (Pur; B). The centrosome number in each cell was then examined at 16 and 20 hours after thymidine release. Columns, mean of the results for two independent experiments. At least 200 mitotic cells were examined in each experiment.

![Image of Figure 4](http://cancerres.aacrjournals.org/)

**Figure 4.** Arsenite-induced supernumerary centrosomes contain fragmented centrioles and/or are acentriolar. A-C, representative immunofluorescence images showing the types of centrosome abnormalities induced in arsenite-arrested mitotic CGL-2 cells. The cells were untreated (A) or treated with 2 μmol/L arsenite for 20 hours (B and C) then were subjected to analysis of centrosome profiles. The cells were treated with anti-γ-tubulin antibodies to detect pericentriolar materials (red) and with anti-α-tubulin antibodies to detect centrioles (green). The centrosome number in a centrosome was determined under a confocal microscope. N, a normal centrosome containing two centriole spots; F, a centrosome containing three centriole spots; A, an acentriolar centrosome containing no centriole spots. D, quantification of the arsenite-induced abnormal centrosome profile in mitotic cells. N, mitotic cells with two centrosomes, each containing a pair of centrioles; F, mitotic cells with fragmented centrosomes containing an abnormal number of centrioles; A, mitotic cells with acentriolar centrosomes; F + A, mitotic cells with both fragmented and acentriolar centrosomes. The number of centrosomes in a centrosome was determined on a confocal microscope from at least 100 mitotic cells. Columns, mean of two independent experiments.
Reduction of centrosome amplification by cdk1 inhibitors decreases induction of apoptosis in arsenite-arrested mitotic cells. Recent reports have shown that entry into mitosis in the presence of damaged DNA leads to inactivation of centrosomes, formation of aberrant spindles, and blockage of chromosome segregation, which consequently affect mitosis progression, induce mitotic abnormalities, and trigger mitotic death (35, 38). To address the role of arsenite-induced centrosome amplification, the induction of mitotic death in arsenite-arrested mitotic cells was examined. The arsenite-arrested mitotic cells were shaken off the plates, replated in arsenite-free medium, and incubated for various times. The results showed that these mitotic cells underwent apoptosis, as indicated by a gradual increase in the percentage of Annexin V–positive cells from 6% at time 0 to 40% at 14 hours (Fig. 6). However, induction of mitotic death was significantly reduced if the arsenite-arrested mitotic cells were shaken off and replated in medium containing alsterpaullone or purvalanol A. Because alsterpaullone or purvalanol A treatment could prevent centrosome amplification in arsenite-arrested mitotic cells (Fig. 3), the prevention of centrosome amplification by cdk1 inhibitors in arsenite-arrested mitotic cells decreases induction of mitotic death.

Attenuation of spindle checkpoint function suppresses arsenite-induced centrosome amplification and mitotic death. Because termination of mitosis by cdk1 inhibitors reduced arsenite-induced centrosome amplification and mitotic death, we further examined whether spindle checkpoint function was required for these effects using siRNA-mediated silencing of the expression of the spindle checkpoint proteins BubR1 or Mad2. Figure 7A shows that expression of BubR1 was decreased to <10% and Mad2 was decreased to 40% of the levels in nontransfected cells at 24 hours after transfection with each siRNA and remained low for 72 hours. Induction of mitotic arrest by nocodazole was also marked reduced in BubR1 or Mad2 siRNA-transfected cells (Fig. 7B), showing that spindle checkpoint function was partially inhibited after knockdown of these spindle checkpoint proteins. Furthermore, arsenite induction of mitotic arrest (Fig. 7B), centrosome amplification (Fig. 7C), and cell death (Fig. 7D) were significantly decreased in BubR1 or Mad2 siRNA-transfected cells compared with nontransfected cells or cells transfected with control siRNA. These results indicate that spindle checkpoint function is crucial for arsenite induction of centrosome amplification and mitotic death.
Discussion

The results of this study show that centrosome amplification was induced in arsenite-arrested mitotic CGL-2 cells in a spindle checkpoint-dependent manner. The amplified centrosomes contained mainly fragmented centrioles and/or were acentriolar and might result from the inhibition of centrosome coalescence during mitosis. In addition, suppression of centrosome amplification by cdk1 inhibitors or by attenuation of spindle checkpoint function in arsenite-arrested mitotic cells resulted in decreased induction of mitotic death. These results imply that centrosome amplification is one of the underlying mechanisms of arsenite-induced cell death.

It has been shown that arsenite-induced supernumerary centrosomes could lead to spindle multipolarity and chromosome missegregation (21, 22). Our previous reports also showed that arsenite can induce prolonged mitotic arrest, disrupted mitotic spindles, and mitosis-mediated apoptosis in HeLa S3 cells (5, 23) and in CGL-2 cells (8). These results indicate that the induction of centrosome abnormalities might be an early and common event that leads to arsenite-induced mitotic abnormalities and mitotic arrest. Several recent studies have implicated DNA damage in the generation of aberrations of centrosome number. Defects in a number of key genes involved in DNA repair have been shown to cause aberrations in centrosome number (39, 40). In Drosophila syncytia embryos, mitosis attempted in the presence of DNA damage causes disruption of centrosome function and the loss of the damaged nucleus by mitotic death (38). During somatic cell mitosis in the presence of incompletely replicated or damaged DNA, centrosomes split into fractions containing only one centriole, leading to the formation of multipolar spindles with extra centrosome-like structures (35). Furthermore, centrosome amplification was recently observed in Rad51-deficient chicken cells that had accumulated a high level of DNA damage due to inability to repair endogenously generated DNA lesions by Rad51 (41). Our present results showed that marked centrosome amplification was induced in arsenite-arrested mitotic CGL-2 cells but not in nocodazole-arrested cells. Arsenite can induce DNA damage and override G2 arrest in CGL-2 cells, resulting in a significant accumulation of mitotic cells with damaged DNA (8). Impairment induced by arsenite, such as DNA damage, in cells that have escaped from G2 arrest might thus provoke centrosome amplification. Several mitotic abnormalities might therefore be induced by the generation of centrosome amplification during mitosis.

Our results showed induction of centrosome amplification during arsenite-induced mitotic arrest, with no significant change in centrosome number in nonmitotic cells. The arsenite-induced supernumerary centrosomes therefore might not be due to uncontrolled reduplication of centrosomes during the S and G2 phase. The molecular mechanism by which centrosome duplication is regulated in mammalian cells is not well understood. The p53 tumor suppressor is known to regulate centrosome duplication through p21, which binds to and inhibits the kinase activity of cdk2/cyclin E, the major regulator of the early events of centrosome duplication (12). p53-deficient cells therefore consistently display centrosome amplification through deregulation of the centrosome...
duplication cycle (42). The CGL-2 cell used in this study originated from a fusion hybrid of HeLa and normal fibroblasts, has no functional p53 proteins, and might therefore be vulnerable to arsenite-induced centrosome amplification. On the other hand, our results showed that supernumerary centrosomes in arsenite-arrested mitotic CGL-2 cells either contained fragmented centrioles and/or were acentriolar, indicating that the duplicated centrosome was unevenly fragmented. These results are distinct from the observation in Chinese hamster ovary cells that, in the presence of incompletely replicated or damaged DNA during mitosis, centrosomes fragment into fractions containing only one centriole (35). DNA damage-induced centrosome inactivation in *Drosophila* embryos requires the presence of centrosomal Chk2 (38). In addition, Chk1 is also reported to be associated with the centrosome and functions to shield centrosomal cdk1 from unscheduled activation by cytoplasmic Cdc25, thereby contributing to the proper timing of the initial steps of cell division, including mitotic spindle formation (43). The centrosome amplification induced by Rad51 deficiency occurs during a prolonged G2 arrest and is ATM dependent (41). These data imply that centrosome structure is monitored by DNA damage checkpoint proteins, which normally function to inactivate the centrosome to halt mitosis progression and prevent chromosome segregation. Our previous results showed that arsenite can override X irradiation–induced G2 arrest, indicating that it might alter the activation of G2 DNA damage checkpoint (8). Because DNA damage checkpoint proteins are involved in the induction of centrosome amplification, arsenite therefore does not induce centrosome amplification before the cells enter mitosis. In addition to reduplication of centrosomes, supernumerary centrosomes can also arise by inhibition of centrosomal coalescence during mitosis (14, 36). NuMA, an abundant protein with a microtubule binding capacity, is involved in mitotic centrosome function (44), and NuMA overexpression is frequently observed in several cancer cells with spindle multipolarity (37). Overexpression of NuMA is reported to deplete dynein, the microtubule motor protein from spindles and thus inhibit centrosomal coalescence during mitosis (37). Our results showed that NuMA expression was increased in a time-dependent manner in CGL-2 cells following arsenite treatment, and that the spindle localization of dynein was reduced in arsenite-arrested mitotic CGL-2 cells, indicating that arsenite might induce centrosome amplification via uneven centrosome fragmentation and/or pericentriolar material overexpression and inhibition of centrosome coalescence during mitosis.

Our results showed that termination of arsenite-induced mitotic arrest by cdk1 inhibitors markedly reduced the percentage of cells with supernumerary centrosomes and the induction of mitotic death. Furthermore, repression of spindle checkpoint function reduced induction of mitotic arrest, centrosome amplification, and cell death by arsenite. These results showed that centrosome amplification in arsenite-arrested mitotic cells was spindle checkpoint dependent and might result in the induction of mitotic death. Spindle checkpoint function is required for mitotic cell death induced by DNA-damaging agents (45), which can induce centrosome amplification, resulting in cell death (35, 38), and suppression of centrosome amplification can reduce radiation-induced mitotic death (15, 46). These results suggest that DNA damage might induce centrosome amplification and mitotic death in a spindle checkpoint-dependent manner. On the other hand, inhibition of the G2 DNA damage checkpoint in the presence of DNA damage is reported to result in forced mitotic entry with massive mitotic abnormalities followed by mitotic death (45, 47). Because arsenite can induce DNA damage and attenuate the G2 DNA damage checkpoint and thus induce accumulation of mitotic cells with damaged DNA (8), the mitotic abnormalities in arsenite-arrested mitotic cells might result from abnormal amplification of centrosomes. The subsequent activation of the spindle checkpoint might further provoke centrosome amplification in arsenite-arrested mitotic cells, and these severely disrupted mitotic cells would therefore undergo apoptosis.

Centrosome abnormalities have been implicated in chromosome missegregation and generation of aneuploid cells, which are commonly found in a variety of human neoplasms. Arsenite-treated cells with supernumerary centrosomes, if survived from mitotic insults, might therefore develop chromosomal instability and become aneuploid. We have previously shown that arsenite could induce c-anaphase, chromosome endoreduplication, and tetraploidy in normal diploid human fibroblasts via induction of mitotic abnormalities and disruption of mitosis progression (9, 10). These results indicated that arsenite might induce chromosome instability via alteration of mitosis progression. Further investigation of the effect of arsenite-induced centrosome amplification on the growth behavior and tumorigenicity of CGL-2 cells is of great importance in elucidating the carcinogenicity of arsenite. Chromosome and/or chromosome segregation machinery are also targets of a variety of therapeutic agents. Our previous study showed that 96% of arsenite-arrested mitotic CGL-2 cells died as analyzed by colony formation assay (8). The present results provide evidence for a close association of centrosome amplification and arsenite induction of mitotic arrest and cell death. The genotoxic or therapeutic effects of arsenic on mitotic machinery may depend on the cell type and doses used. Our present results might be of a great help in terms of cancer therapy, especially in tumors with p53 mutations, and will be critical in realizing the potential of the synergy between arsenite and other chemotherapeutic agents.

**Acknowledgments**

Received 7/5/2005; revised 10/31/2005; accepted 12/5/2005.

**Grant support:** Academia Sinica and National Science Council, Republic of China grants NSC 92-2520-B-001-059 (L.J. Yih), NSC-93-2520-B-001-028 (L.J. Yih), NSC92-3112-B010-019 (T.C. Lee), and NSC93-3112-B010-005 (T.C. Lee).

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We thank Drs. K.Y. Jan and T.C. Wang (Institute of Cellular and Organismic Biology, Academia Sinica) and Dr. Jeng-Fan Lo (Institute of Oral Biology, National Yang-Ming University) for carefully reading the article and assisting with the operation of the flow cytometer and fluorescence microscope and the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica for assistance in confocal microscopy.

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