The Target Factor of Heavy Metal Toxicity and Its Molecular Mechanism

Current Topics

BubR1 Is Essential for Thio-Dimethylarsinic Acid-Induced Spindle Assembly Checkpoint and Mitotic Cell Death for Preventing the Accumulation of Abnormal Cells

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INTRODUCTION

Chronic exposure to arsenic from arsenic-contaminated drinking water, and high level of arsenic-containing ground-water were presumed to be the major cause for patients suffering from cancers of the skin, lungs, and urinary bladder.12-13) However, the mechanism underlying arsenic-induced carcinogenicity in the target organs and the type of arsenic species involved in and responsible for the arsenic-induced toxicity and carcinogenesis remain unclear.

In humans, inorganic arsenic such as arsenite (iAs III) or arsenate (iAs V) is transformed into methylated metabolites such as monomethylarsinic acid (MMA V) and dimethylarsinic acid (DMA V). Toxicological studies of various types of arsenic compounds in vitro showed that the cytotoxicity of dimethylarsinic acid (DMA V) was 100-times or 10-times weaker than that of iAs III or iAs V, respectively. Therefore, methyl metabolism of inorganic arsenicals was presumed to be an important detoxication process in humans. However, subsequent studies have shown that trivalent methylated arsenicals, such as methylarsinous acid (MAA III) and dimethylarsinous acid (DMA III), indicated stronger cytotoxic and genotoxic effects in vitro than inorganic arsenicals.3-9) These observations indicated that trivalent methyl arsenicals produced by the methylation process were considered a part of the active metabolic pathway for cytotoxicity and genotoxicity.

Thio-dimethylarsinic acid (thio-DMA), which is a pentavalent methyl arsenic compound in which the oxygen atom of dimethylarsinic acid (DMA V) is substituted with a sulfur atom, has been identified in the urine of Bangladeshi women exposed to inorganic arsenic in drinking water and in the urine of humans who had consumed marine algae.9-11) Based on toxicological studies, it is reported that thio-DMA exerted up to 100-fold higher cytotoxicity than DMA V and was shown to be equitoxic or slightly more toxic than iAs III.12-17) Naranmandura et al. have reported that the LC 50 value of thio-DMA and iAs III calculated by the cytotoxicity of human bladder carcinoma EJ-1 cells after 24h, was 16.7 and 112 µM, respectively.12) These results indicate that the cytotoxic effect of thio-DMA on EJ1 cell is much stronger than that of iAs III. Moreover, they showed that the LC 50 value of thio-DMA is only 10-fold higher than the concentration of iAs III found in the urine samples obtained from Bangladeshi women exposed to inorganic arsenic (0.3–1.6 µg As).12) In addition to the cytotoxic effects of thio-DMA, genotoxicity has been reported. Ochi et al. showed that thio-DMA induced chromosomal instability such as chromatid brakes, chromatid gaps, and chromatid exchanges. Additionally, these chromosomal abnormalities were associated with mitotic arrest.13) They also reported that the cytotoxic effects of thio-DMA may in part...
be associated with an apoptotic mode of cell death. It was observed that cell death would accompany the mitotic arrest. Moreover, they observed that mitotic associated apoptotic cell death was enhanced in the presence of glutathione (GSH), otherwise attenuated in GSH-depleted conditions. Thio-DMA-induced cell accumulation in G2/M phase was also observed in various type of cells such as UROtsa cells, A549 cells, and HepG2. However, the biological significance of thio-DMA-induced mitotic cell death was not determined.

In the mitotic phase, nuclear division and cytoplasmic division occurs precisely. It is established that spindle assembly checkpoint (SAC) is activated during prometaphase to accomplish sister-chromatid separation, and BubR1 plays a central role in this process. In instances, where the microtubule-unattached kinetochores are present or there is an absence of tension between the paired kinetochores, SAC is activated, and the cell cycle pauses in the prometaphase. During the active period of SAC, the inhibitory complex, which includes Mad2, BubR1, Bub3, and CDC20, is formed and prevents the activation of the anaphase-promoting complex/cyclosome (APC/C). BubR1 phosphorylation by various kinases such as Aurora B, polo-like kinase 1 (Plk1), Mps1, and Cdk1 during SAC is essential for monitoring the microtubule-kinetochore binding and sensing the kinetochore tension. When the mitotic checkpoint was completed, phosphorylated BubR1 and Mad2 were removed from CDC20, followed by the activation of APC/C and promotion of the proteolysis of securin and cyclin B to enter the anaphase.

Preparation of Cell Extract Cells incubated with various concentrations of thio-DMA for different periods were scraped off, centrifuged, and washed twice with ice-cold phosphate buffered saline (PBS). The cell pellet was suspended in cell lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1.0% sodium deoxycholate, 50 mM dithiothreitol, 0.1% sodium dodecyl sulfate (SDS), standard phosphatase inhibitor, and protease inhibitors (Sigma-Aldrich), incubated for 30 min on ice and sonicated. The cell suspension was centrifuged at 20000 × g for 15 min at 4°C and the collected supernatant was retained for further analysis. Protein content of the centrifuged supernatant was determined by Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Inc., CA, U.S.A.) using bovine serum albumin (BSA) as the standard. Cellular proteins were utilized for immunoprecipitation and immunoblotting.

Immunoblotting Protein samples were electrophoretically separated using 7.5, 10, or 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked overnight at 4°C with 0.5% skim milk or 0.5% BSA dissolved in TBS-T and protease inhibitors (Sigma-Aldrich) containing 0.1% Tween 20. Subsequently, the membrane was incubated with antibody for 6 to 16 h at 4°C with gentle rocking. The blot was washed twice with TBS-T for 10 min and once for 5 min. Thereafter, the membrane was incubated with 1:20000 diluted anti rabbit IgG or anti mouse IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) for 2 h at 4°C. The blot was washed with TBS-T and the membrane developed using the EzWestLumi plus (ATTO Corp, Tokyo, Japan) followed by analysis with the LAS-3000 mini luminoimage analyzer (FUJIFILM Corp, Tokyo, Japan).

Immunoprecipitation Mouse monoclonal anti-Cdc20 antibody was incubated with 300 µg of the cell lysate for 2 h in a revolving tube carousel at 4°C. After incubation, protein A-Sepharose beads were added to each sample, followed by overnight incubation in a revolving tube carousel at 4°C. Samples were centrifuged at 20000 × g for 30 s at 4°C, pelleted beads were washed three times, and the immune complex was released with 30 µL SDS-PAGE sample buffer and 5 min boiling. To confirm the formation of inhibitory complex, the presence of Mad2 or BubR1 was determined by immunoblotting.

Immunocytochemistry Immunochemistry was performed as described previously. Briefly, cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature, soaked in methanol for 5 min at −20°C, and washed with PBS. After blocking by PBS containing 2% skim milk (PBS-2% SM) for 30 min at room temperature, cells were incubated with anti-α-tubulin antibodies (Sigma-Aldrich) diluted (1 : 100) in PBS-2% SM for 6 h at room temperature. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibodies (Bethyl Laboratories, TX, U.S.A.) diluted (1 : 100) with PBS-2% SM in a moist chamber at 37°C for 90 min. Cells were washed five times with PBS and mounted on medium (glycerol : PBS = 9 : 1 solution).

MATERIALS AND METHODS

Materials Thio-DMA was synthesized from sodium dimethylarsinate trihydrate and aqueous H2S as described previously. The following monoclonal antibodies were obtained: mouse monoclonal anti-β-actin antibody (Abcam plc, Cambridge, U.K.); mouse monoclonal anti-β-tubulin antibody (Sigma-Aldrich, MO, U.S.A.); mouse monoclonal anti-BubR1 antibody, mouse monoclonal anti-Mad2 antibody, and mouse monoclonal anti-cyclin E antibody (BD Biosciences, San Diego, CA, U.S.A.); and mouse monoclonal anti-Cdc20 (p55 CDC H-7) antibody and mouse monoclonal anti-cyclin B1 antibody (Santa Cruz Biotechnology, Inc., CA, U.S.A.). Rabbit polyclonal anti-poly (ADP-ribose) polymerase (PARP) antibody was purchased from Cell Signaling Technology (MA, U.S.A.); and peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (PA, U.S.A.). Protein A sepharose beads were obtained from GE Healthcare Bio-Sciences Corp. (NJ, U.S.A.).

Cell Culture Cells of the human hepatocarcinoma cell line, HepG2, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biovest, Nuaillé, France) and the cells of human cervical carcinoma cell line, HeLa, were cultured in Eagle’s minimum essential medium (MEM) (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% (v/v) FBS under standard culture conditions at 37°C, 5% CO2 in humidified air.
**Cell Size Analysis** Cells incubated with thio-DMA for various time periods were detached by the addition of trypsin and suspended in culture medium. Trypan Blue Dye (Bio-Rad Laboratories, Inc., CA, U.S.A.) (0.4%) was added to the mixture, and the cell suspension was loaded onto the cell counting slides. Viable cell number and cell size distribution was measured by TC20™ Automated Cell Counter (Bio-Rad).

**Mitotic Index** Cells incubated with thio-DMA for various time periods were detached by the addition of trypsin and suspended in culture medium. The cell suspension was centrifuged at $270 \times g$ for 5 min at room temperature and the cell pellet was suspended in 3 mL of 37°C pre-warmed hypotonic solution (75 mM KCl) and incubated for 15 min at 37°C. Fixing solution (0.5 mL, methanol: acetic acid = 3 : 1, freshly prepared before use and chilled on ice) were added and mixed gently. After incubation for 5 min, the cell suspension was centrifuged at $270 \times g$ for 5 min at room temperature and the supernatant was discarded. Fixing solution (3 mL) was added and incubated for 5 min, and the cells were collected by centrifugation. Fixation was performed thrice and the cell pellet was re-suspended in 0.5 mL of the fixing solution. The fixed cell suspension was dropped on the glass slide and was allowed to dry for 1 h at room temperature. After drying, the glass slide was filled with 3% Giemsa solution and the cells were stained for 15 min at room temperature. After washing off the staining solution, mitotic cells were counted by microscope. Cleaved PARP: marker for apoptosis induction. Cyclin B1: marker for G2/M phase. Cyclin E: marker for G1 phase.

**Flow Cytometry** Cells were harvested via trypsinization, washed with PBS, and fixed in 70% ethanol overnight at 4°C. Fixed cells were incubated in permeabilizing solution (0.25% Triton X-100 containing PBS) for 5 min at room temperature and washed twice with rinsing solution (1% BSA resolved in PBS). Consequently, cells were incubated in 0.1 mL of the rinsing solution containing anti-cyclin B1 antibody or normal mouse IgG (1 µg of anti-cyclin B1 antibody or normal mouse
IgG per 10⁶ cells) for 60 min at room temperature with gentle agitation and washed with 5 mL of rinsing solution. Cells were suspended in 0.1 mL of the rinsing solution containing FITC-conjugated anti-mouse IgG antibody (1:40) and incubated for 60 min at room temperature, with gentle agitation in the dark. After incubation, 5 mL of rinsing solution was added, and cells were washed. Cells were re-suspended with 1 mL of PBS containing 5 µg/mL propidium iodide (PI) (to label DNA) in the presence of ribonuclease (RNase) A (100 µg/mL) and incubated for 60 min at room temperature. PI and FITC fluorescence was detected using BD FACSCanto™II (BD Biosciences, San Jose, CA, U.S.A.). A minimum of 20,000 cells per sample were analyzed.

RNA Interference (RNAi) Experiments Small interfering RNAs (siRNAs) targeting human BubR1 (BubR1-1; target sequence: GUA AUG AGG AUU ACU GCA UTU, BubR1-2; target sequence: GCA CAC UAG CUG AAC UAA ATT), or control siRNA (sequence: GGC UAU UAC GAC GUU AAU CTT) were prepared by TaKaRa Bio, Inc. (Shiga, Japan). Transfection of the siRNAs into HeLa cells was performed using the Lipofectamine™ RNAiMAX Reagent (Invitrogen Corp. CA, U.S.A.) according to the manufacturer’s instructions. Twenty-four hours after siRNA transfection, cells were cultured in fresh growth medium for 24 h, and incubated with thio-DMA. Following treatment with thio-DMA for the indicated time, cells were harvested and used for immunoblotting or immunoprecipitation analyses.

Statistical Analysis Data were shown as the mean ± standard deviation (S.D.). Statistical comparisons between the two groups were made by means of unpaired t-test. Values of p < 0.05 were considered significant.

RESULTS

Thio-DMA Promotes the Accumulation of Mitotic Cells and Mitotic Cell Death in HeLa Cells but Not in HepG2 Cells Our previous study has shown that thio-DMA promotes the accumulation of HepG2 in mitosis because an increase in 4N cells was detected by flow cytometry analysis. To confirm whether thio-DMA arrests other types of cells in mitosis, the human cervical carcinoma cell line, HeLa was examined. Phase-contrast microscopic observation of the cells after exposure to thio-DMA indicated an increase in the ratio of round cells that were confirmed to be mitotic by Giemsa staining. Figure 1A shows the time course of changes in the mitotic index in both cells after the addition of 10 μM thio-DMA. In HeLa cells, the mitotic index began to increase 6 h after the addition of thio-DMA and reached the maximum level at 24 h. Approximately 50% of cells were accumulated in mitotic phase after 24 h treatment with thio-DMA. However, we could not determine the mitotic index after 48 h treatment with thio-DMA, because almost all HeLa cells detached from culture dish and indicated apoptotic-like morphology (Fig. 1B, upper). The mitotic index of HepG2 cells began to increase 6 h after the addition of thio-DMA, reached the maximum level at 24 h, held the plateau for up to 48 h, and then declined at 72 h. Approximately 43% of cells were accumulated in the mitotic phase from 24 to 48 h after treatment with thio-DMA. Phase-contrast microscopic analysis indicated that detached cells were observed in HepG2 cells as well as HeLa cells at 48 h; however, attached cells were also observed. Moreover, after 48 h treatment with thio-DMA, attached HepG2 cells increased as the mitotic index was observed to decrease. (Fig. 1B, lower). Similar results were also observed when both cell groups were treated with nocodazole, which is a microtubule-depolymerizing reagent and known as an inducer of mitosis. These results indicate that equimolar thio-DMA or nocodazole promotes the accumulation of mitotic cells in both HeLa and HepG2 cells. However, the outcome of the cells after 24 h treatment with thio-DMA or nocodazole was different for each cell type.

In addition to the appearance of mitotic-like round-type cells, cells with membrane blebs and cell fragments were observed, especially in HeLa cells after treatment with thio-DMA. Previous studies have indicated that arsenic trioxide-induced mitotic arrest is associated with the initiation of apoptosis. To confirm whether thio-DMA-induced cell death was due to the mitotic associated apoptosis, the changes in cleaved PARP (used as apoptotic marker), cyclin B1 (G2/M cyclin used for mitotic arrest marker), and cyclin E (G/S cyclin used for interphase marker) in HeLa and HepG2 cells were determined by immunoblotting. As shown in Fig. IC left, cyclin B1 level in HeLa cells was elevated time-dependently but cyclin E levels decreased with increase of cyclin B1 after treatment with thio-DMA. At 48 h of treatment with thio-DMA, full length PARP was hardly detected in HeLa cells, and the cleaved PARP significantly increased following the accumulation of cyclin B1. However, cyclin B1 and cleaved PARP levels in HepG2 were lower than that of HeLa after 24 h treatment with thio-DMA. Although cyclin E levels in HepG2 cells were observed to marginally decrease time dependently, cyclin E continued to be detected at all points of time after treatment with thio-DMA. Nocodazole promoted cyclin B1 accumulation and cleaved PARP increase in HeLa cells but not in HepG2 cells. Concentration dependency of thio-DMA-induced mitotic arrest and apoptotic cell death were also investigated. As shown in Fig. 1C right, increased cleaved PARP and accumulation of cyclin B1 were observed in HeLa after treatment with 5–50 μM thio-DMA. Attached cells were hardly detected and all were observed to be floating (data not shown). Alternatively, cyclin B1 accumulation was observed in HepG2 cells after 24 h treatment of 5–50 μM thio-DMA. However, cyclin E1 was constitutively detected and cleaved PARP was marginally detected after treatment with 10 μM thio-DMA for 24 h. These results indicated that HeLa cells were more susceptible for thio-DMA induced mitotic accumulation and mitotic associated apoptotic cell death than HepG2 cells.

Thio-DMA Promotes Mitotic Checkpoint Complex (MCC) Formation in HeLa Cells but Not in HepG2 Cells McNee et al. indicated that BubR1, which is known as a constituent of the SAC, plays an important role in arsenic trioxide-induced mitotic arrest and apoptosis. In the metaphase cells, sister chromatids were arrayed in metaphase plate and precise kinetochore-spindle attachment was monitored by SAC. If the kinetochore-spindle attachment was incorrect, the APC/C was inhibited by formation of the MCC (Fig. 2A). During the SAC activation, BubR1 was phosphorylated and Mad2 was recruited to bind the APC/C activator CDC20, followed by formation of the MCC. The MCC prevents the onset of anaphase by sequestering the CDC20 from APC/C. When the SAC is completed, Mad2 is released from CDC20 and APC/C, which is known as ubiquitin ligase, activated, and ubiquitinated both securin and cyclin B for degradation by proteasome to allow...
for mitotic exit.\textsuperscript{22-27} To confirm whether MCC formation was related to the thio-DMA-induced mitotic arrest, the binding of Mad2 and CDC20 was determined by the immunoprecipitation assay using anti-CDC20 antibody. As shown in Fig. 2A, in HeLa cells, the amount of Mad2 co-immunoprecipitated with CDC20 increased after 6–24 h treatment with 10 \( \mu \)M thio-DMA. However, in HepG2 cells, Mad2 associated with CDC20 was slightly detected after 12–24 h treatment with 10 \( \mu \)M thio-DMA. Similar to Mad2, BubR1, which co-immunoprecipitated with CDC20 in HepG2 cells, was slightly detected at 24 h, but hardly detected during the indicated time points. Moreover, binding of Mad2 and CDC20 levels did not show significant difference in each cell, and time-dependent decrease of Mad2 was observed in both groups of cells after treatment with thio-DMA. Similar to Mad2, BubR1, which co-immunoprecipitated with CDC20 in HepG2 cells, was slightly detected at 24 h, but hardly detected during the indicated time points. Moreover, binding of Mad2 and CDC20 levels did not show significant difference in each cell, and time-dependent decrease of Mad2 was observed in both groups of cells after treatment with thio-DMA. Similar to Mad2, BubR1, which co-immunoprecipitated with CDC20 in HepG2 cells, was slightly detected at 24 h, but hardly detected during the indicated time points. Moreover, binding of Mad2 and CDC20 levels did not show significant difference in each cell, and time-dependent decrease of Mad2 was observed in both groups of cells after treatment with thio-DMA.

**BubR1 Is Essential for the Formation of MCC, Mitotic Arrest, and Mitotic Cell Death after Treatment with Thio-DMA** To confirm the necessity of BubR1 for MCC formation and mitotically associated apoptotic cell death induced by thio-DMA, BubR1 expression in HeLa cells were abated by siRNA. As shown in Fig. 3A, the binding of Mad2 and CDC20 was not observed in siRNA BubR1-1 transfected HeLa (BubR1 knockdown) cells after 24 h treatment with 10 \( \mu \)M thio-DMA. Otherwise, the total cellular Mad2 and CDC20 levels in both control siRNA (siRNA SNC) transfected HeLa (control HeLa) cells and BubR1 knockdown cells did not show significant changes. A decrease in total Mad2 proteins was also observed in both control and BubR1 knockdown cells as well as HeLa cells as seen in Fig. 2B after treatment with thio-DMA (Fig. 3A-left). To confirm thio-DMA-induced apoptosis, cleaved PARP protein levels were determined in BubR1 knockdown cells. The cleaved PARP levels significantly decreased in the BubR1 knockdown cells after treatment with thio-DMA. Moreover, accumulation of cyclin B1 was not observed, and cyclin E was constitutively detected in BubR1 knockdown cells after treatment with thio-DMA (Fig. 3A-right). These results indicate that BubR1 knockdown cells could not be arrested in the mitotic phase and were presumed to enter the G1 phase through mitotic phase after treatment with thio-DMA. The cytotoxicity of thio-DMA in BubR1 knockdown cells was evaluated in terms of changes in cell viability. In control HeLa cells, cell viability decreased concentration-dependently and only 15% of the cells cultured for 48 h could survive in the medium containing 10 \( \mu \)M thio-DMA. Although concentration-dependent decrease in cell viability was observed in BubR1 knockdown cells, approximately 44% of BubR1 knockdown cells were observed to survive after 48 h treatment with 10 \( \mu \)M thio-DMA. Moreover, the cell viability curve of BubR1 knockdown cells showed similarity to that of HepG2 (Fig. 3B). Changes in the mitotic index in both control and BubR1 knockdown HeLa cells after treatment with thio-DMA were determined. In control HeLa cells, the mitotic index increased time-dependently and reached a maximum level at 24 h. Since the amount of cells detached from the culture dish increased, the determination of the mitotic index after 24 h treatment of thio-DMA was not possible. However, BubR1 knockdown cells did not show an increase in mitotic index, and attached interphase cells were observed even after 72 h treatment with thio-DMA (Fig. 3C).
These results were also observed in BubR1 knockdown HeLa cells transfected with siRNA designed for another target of BubR1 sequence (siRNA BubR1-2).

**Cells with Lower BubR1 Protein Were Able to Survive with Abnormality Induced by Thio-DMA**

BubR1 knockdown HeLa cells could survive in the higher concentration of thio-DMA in which normal HeLa cells could not survive. These results indicated that thio-DMA associated cell death might be controlled by BubR1. To confirm whether the cells preventing mitotic cell death are normal or not, morphological changes in BubR1 knockdown cells after treatment with thio-DMA were examined. According to the observation by phase contrast microscopy, most of the control HeLa cells showed spherical mitotic or apoptotic-like morphology and detached from the culture dish after 24–48 h treatment with 10 µM thio-DMA. Alternatively, although detached cells were slightly observed, attached cells were observed after 48 h treatment with 10 µM thio-DMA in BubR1 knockdown HeLa cells (Fig. 4A). Moreover, it appeared that the size of attached BubR1 knockdown cells increased after 48 h treatment with thio-DMA. Accordingly, we confirmed the cell size distribution of viable cells in both control and BubR1 knockdown cells after treatment with thio-DMA. As shown in Fig. 4B, viable cells with large cell size (diameter larger than 16 µm) were detected. Also, the percentage of large cells increased in the BubR1 knockdown HeLa cells treated with 10 µM thio-DMA for 48 h. Moreover, knockdown of BubR1 promoted the appearance of large cells. Morphological changes of each cell were examined by immunofluorescence by using anti-β-tubulin antibodies and showed multinucleated cells in both control and BubR1 knockdown HeLa cells after 24 h treatment with thio-DMA. However, in control HeLa cells, most of the multinucleated cells detached from the dish after 48 h treatment with thio-DMA. However, BubR1 knockdown HeLa cells having multinuclei or huge nuclei were observed after 48 h treatment with 10 µM thio-DMA (Fig. 4B). These abnormal cells were observed at 72 h treatment with 10 µM thio-DMA (data not shown).

**BubR1 Knockdown Cells Permit Tetraploid Mitotic Cells to Enter the G1 Phase without Cell Division**

Multi-nucleated cells or cells having large nuclei were observed in...
thio-DMA treated BubR1 knockdown cells. Consequently, it was confirmed whether the cells having the extra DNA eluded from mitotic cell death by flow cytometry using PI for monitoring the DNA contents. As shown in Fig. 5A, the profile in control HeLa cells showed two discrete peaks, containing cells with 2N DNA (G₀ + G₁) and 4N ≤ DNA (G₂ + M), and no fractions of cells with DNA less than 2N and polyploidy (8N) were observed. When the control HeLa cells were incubated with 10 µM thio-DMA for more than 24 h, they were not divided and detached from dish and could not be collected for viable cell counting. (C) Morphological changes in attached control and BubR1 knockdown HeLa cells after treatment with thio-DMA. Attached control and BubR1 knockdown HeLa cells after treatment with thio-DMA were immunostained using an anti-β-tubulin antibody.

DNA as compared to control HeLa cells.

In both control and BubR1 knockdown HeLa cells, thio-DMA induced the accumulation of 4N ≤ DNA containing cells. However, these cells did not necessarily get arrested in the mitotic phase. To confirm this presumption, we used anti-cyclin B1 antibody for monitoring cells in G₂/M phase in combination with PI staining. As indicated in Fig. 5B, cyclin B1 positive cells were distinguished by the fluorescence intensity of control IgG antibody. In control HeLa cells, over 98% of 4N ≤ DNA containing cells tested positive for cyclin B1. Moreover, cyclin B1 positive cells were observed to increase after being treated with thio-DMA. Alternatively, in BubR1 knockdown cells, among the 4N ≤ DNA containing cells, cyclin B1 negative cells were marginally observed in basal conditions (4.6%). However, the increase of cyclin B1 negative cells was marginally observed in basal conditions (4.6%). However, the increase of cyclin B1 negative 4N ≤ DNA cells was observed with the decrease in cyclin B1 positive 4N ≤ DNA cells after treatment with thio-DMA. These results indicated that 25.2% of cyclin B1 negative 4N ≤ DNA cells entered the interphase though mitotic phase.
DISCUSSION

In the present study, we confirmed that thio-DMA promotes the mitotic cell accumulation in both HeLa and HepG2 cells, but the outcomes of both types of cells after the accumulation of mitotic phase were different from each other. HeLa cells were induced to undergo apoptotic cell death after the accumulation of cells in mitotic phase, but HepG2 cells showed resistance to apoptotic cell death despite accumulation of cells in mitotic phase (Figs. 1A, C). Moreover, HepG2 cells continue to survive after 72h treatment with thio-DMA through mitotic phase accumulation (Fig. 1B). In general, a healthy cell cycle progresses through the G1, S, G2, and M phases to complete a normal cell cycle. In normal M phase, synthesized daughter chromatids during S phase were precisely divided into two daughter cells and the SAC was accountable for this process.

We also examined the reasons for the different outcomes of both cells after treatment with thio-DMA with regard to SAC. Additionally, it was confirmed that in HeLa cells, the basal levels of BubR1, which is an SAC component, were 10-fold higher than that in HepG2. Moreover, a time-dependent increase of phospho-BubR1 levels was observed in HeLa cells by thio-DMA (Fig. 2B). Thus, we surmised that BubR1 expression levels might be related to the thio-DMA induced mitotic cell accumulation through the activation of SAC.

The main components of the SAC are mitotic arrest deficient l(Mad1) and Mad2; BubR1, Bub1, and Bub3. The SAC operates by inhibiting the E3 ubiquitin ligase activity of the APC/C, which normally targets mitotic regulators such as cyclin B1 and securine for degradation to allow for mitotic exits. If unattached kinetochores are present or if there is a lack in the tension, the SAC is active and recruits several proteins to form the MCC, a diffusible anaphase inhibitor that includes activated Mad2, BubR1, and Bub3 and functions by sequestering the APC/C activator CDC20 at the kinetochores that are unattached or lack tension. The MCC generates a signal that halts the progression of cells from metaphase to anaphase until all the chromosomes are perfectly oriented and aligned to the metaphase plate. To confirm the inhibitory complex formation after treatment with thio-DMA, binding of CDC20 and Mad2 proteins was determined by co-immunoprecipitation assay using anti CDC20 antibody. Moreover, we showed that the MCC was strongly formed in HeLa cells but not in HepG2 (Fig. 2B). Furthermore, we confirmed that the MCC was not formed in BubR1 knockdown HeLa cells, and these cells could not halt the mitotic phase (determined by cyclin B1, known as G2/M cyclin, and mitotic index as shown in Fig. 3C) and prevent the mitotic associated cell death (shown as cleaved PARP levels in Fig. 3A and cell viability as shown in Fig. 3B).

The relationship of SAC and mitotic cell death has been reported and three types of mitotic cell death were proposed. The first pattern is ‘mitotic death’ where the cell dies without exiting mitosis. In this pattern of cell death, elevated cyclin B1 levels were observed. The second pattern of cell death was observed in the G2 phase following mitotic arrest. In this case, cell death can occur quickly, within hours of mitotic exit. The third pattern consists of cell senescence, which does not induce cell death but senescence, an irreversible cell cycle arrest, with exiting mitosis. In our present results, cyclin B1 and phospho-BubR1 were observed during apoptotic cell death in HeLa cells. Consequently, we presumed that ‘mitotic death’ was induced by thio-DMA. However, it was observed that the mitotic index transiently increased but apoptotic cell death was not observed in thio-DMA-treated HepG2 cells. Thus, we thought that HepG2 cells might exit the mitotic phase and enter the G1 phase to survive or for senescence. Moreover, Vi-tale et al. proposed that mitosis incompetent cells can escape the mitotic arrest, either by ‘slipping’ into the next interphase or by undergoing one round of aberrant mitosis. Often, this division cannot be completed, and cytokinesis fails. Upon cytokinesis failure and mitotic slippage, tetraploid cells are generated, most of which die or become senescent. Our results indicated that in HepG2 cells, BubR1 protein levels were much lower than that of HeLa cells. If insufficient SAC activation occurred, mitotic slippage might happen and this caused the cells to enter the next interphase to survive. These results were also observed in HeLa cells transfected with siRNA target for endogenously expressed BubR1 (Figs. 3B, 4A).

Mitotic slippage is believed to enable tetraploid cell generation; morphological changes in BubR1 knockdown cells and cell size distributions were determined. As shown in Fig. 4A, large attached cells and the accumulation of viable cells, with diameter more than 16 μm, were observed after 48h treatment with thio-DMA (Figs. 4A, B). Moreover, it was observed that thio-DMA transiently promoted the appearance of multinucleated cells, and that most of these cells died after 48h in BubR1-expressing cells (control siRNA transfected HeLa cells). However, attached multinucleated cells and cells having large cell size were not excluded and were observed to live in BubR1 knockdown HeLa cells (Fig. 4B).

Furthermore, we confirmed that thio-DMA promotes the accumulation of tetraploid cells in both control and BubR1 knockdown HeLa cells. However, 25.2% of tetraploid cells in BubR1 knockdown cells were cyclin B1 negative (Fig. 5). These observations indicated that negative or weakened BubR1 expressed cells might incur ‘mitotic slippage,’ enter the next interphase and then survive.

We require information on the reasons and mechanism of activation of SAC by thio-DMA. Previous reports show that SAC is activated in certain conditions. The cells with extra centrosomes, which are prone to undergoing multipolar divisions, activate SAC. Mitotic spindle disruptors such as vinca alkaloids, known as microtubule depolymerizing agents; or taxanes, known for hyperpolymerizing the microtubules, activate SAC. Moreover, inhibition of microtubule passenger complex (CPC) coordinates chromosomal and cytokinetic events during mitosis or inhibition of the CPC components (such as Aurora kinase B and survivin) activate SAC. We did not confirm the detail mechanism of thio-DMA-induced SAC activation. However, it is reported that multipolar spindle formation and abnormal centrosome formation were induced by thio-DMA treatment. In our present study, nocodazole is a microtubule depolymerizer, used for accumulating cells in mitosis. Consequently, we used this as a positive control reagent in this study. As shown in Figs. 1 and 2, in nocodazole-treated HeLa cells, elevated levels of phospho-BubR1, cyclin B1, and cleaved PARP were also observed but not in BubR1 knockdown HeLa cells (Figs. 1, 2). We believe that thio-DMA might directly affect microtubule integrity. Therefore, detailed mechanisms of thio-DMA-induced SAC activation are now under investigation in our laboratory.

In conclusion, thio-DMA induced cell cycle arrest in mi-
tocic phase and mitotic associated apoptotic cell death by activating of SAC. In addition, thio-DMA-induced SAC activation and MCC formation were BubR1 dependently occurred. If the cell with lower BubR1 or without BubR1 expression, abnormal cells having multiple nuclei or abnormal DNA contents could not be excluded. Consequently, we proposed that BubR1-mediated SAC activation and MCC formation are one of the defense systems for preventing the accumulation and the survival of abnormal cells induced by thio-DMA.

**Conflict of Interest** The authors declare no conflict of interest.

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