Arsenic Trioxide-mediated Growth Inhibition in MC/CAR Myeloma Cells via Cell Cycle Arrest in Association with Induction of Cyclin-dependent Kinase Inhibitor, p21, and Apoptosis1

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ABSTRACT

We investigated the in vitro effect of As2O3 on proliferation, cell cycle regulation, and apoptosis in human myeloma cell lines. As2O3 significantly inhibited the proliferation of all of eight myeloma cell lines examined in a dose-dependent manner with IC50 of ~1–2 μM. DNA flow cytometric analysis indicated that As2O3 (2 μM) induced a G1 and/or a G2-M phase arrest in these cell lines. To address the mechanism of the antiproliferative effect of As2O3, we examined the effect of As2O3 on cell cycle-related proteins in MC/CAR cells in which both G1 and G2-M phases were arrested. Western blot analysis demonstrated that treatment with As2O3 (2 μM) for 72 h did not change the steady-state levels of CDK2, CDK4, cyclin D1, cyclin E, and cyclin B1 but decreased the levels of CDK6, cdc2 and cyclin A. The mRNA and protein levels of CDK1, p21 were increased by treatment with As2O3, but those of p27 were not. In addition, As2O3 markedly enhanced the binding of p21 with CDK6, cdc2, cyclin E, and cyclin A compared with untreated control cells. Furthermore, the activity of CDK6-associated kinase was reduced in association with hypophosphorylation of Rb protein. The activity of cdc2-associated kinase was decreased, which was accompanied by the up-regulation of cdc2 phosphorylation (cdc2-Tyr15 phosphorylation) resulting from reduction of cdc2B and cdc25C phosphatases. As2O3 also induced apoptosis in MC/CAR cells as evidenced by flow cytometric detection of sub-G1 DNA content and annexin V binding assay. This apoptotic process was associated with down-regulation of Bcl-2, loss of mitochondrial transmembrane potential (∆Ψm), and an increase of caspase-3 activity. These results suggest that As2O3 inhibits the proliferation of myeloma cells, especially MC/CAR cells, via cell cycle arrest in association with induction of p21 and apoptosis.

INTRODUCTION

Arsenic trioxide (As2O3) has recently been reported to induce complete remission in the patients with relapsed or refractory APL without severe marrow suppression (1, 2). Although the mechanism of the antileukemic effect of As2O3 is not well understood, it is known that As2O3 is able to degrade a PML protein and a PML/RARα fusion protein in APL with a t(15;17) (3–6). More recently, it has been shown that the antiproliferative effect of As2O3 is not limited to APL but can be observed in a variety of hematological malignancies without having the PML-RARα fusion protein (7–12), suggesting that the antiproliferative effect of As2O3 might be independent on a PML

1 The abbreviations used are: APL, acute promyelocytic leukemia; PML/RARα, promyelocytic leukemia gene-retinoic acid receptor; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; MM, multiple myeloma; PARP, poly(ADP-ribose) polymerase protein; IL-6, interleukin 6; PI, propidium iodide; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ICE, interleukin 1-convertase enzyme.

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of MTT (Sigma) solution (2 mg/ml in PBS) were added to each well, and the plates were incubated for additional 4 h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 200 µl of DMSO were added to each well before absorbance at 570 nm was measured.

**Cell Cycle Analysis.** Cell cycle distribution was determined by staining DNA with PI (Sigma) as previously described (25). Briefly, 1 x 10^6 cells were incubated with 10 µM bromodeoxyuridine (Sigma) at 37°C for 1 h. Cells then were washed in PBS and fixed in 70% ethanol. After incubation of cells with 1 ml of 2 N HCl containing Triton X-100 (Fisher Scientific, Fair Lawn, NJ) for 30 min at room temperature followed by two washes with PBS, cells were incubated with 20 µl of anti-bromodeoxyuridine for 30 min at room temperature. After two washes with PBS, cells were incubated with 1 µg of FITC-conjugated goat antimouse IgG (Caltag Laboratories, San Francisco, CA) for 30 min at room temperature. Cells were again washed with PBS and then incubated with 1 µg of PI. The percentage of cells in the different phases of the cell cycle was measured with FACSStar flow cytometer (Becton Dickinson, San Jose, CA), analyzed by using Becton Dickinson software (Lysis II, Cellfit).

**Northern Blot Analysis.** Total RNA was extracted by the TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA (15 µg/sample) was size fractionated through 1% agarose-formaldehyde gel and transferred to nylon membrane (Schleicher and Schuell, Dassel, Germany). The cDNA probes for p21, p27, p53, Bax, IL-6, and IL-6 receptor were labeled with [α-^32^P]dCTP (ICN, Costa Mesa, CA) to high specific activity by random primer labeling. The filter was hybridized for 20–24 h at 42°C washed, and exposed to Kodak XAR 5 film (Eastman Kodak, Rochester, NY). The filter was hybridized with the β-actin cDNA to normalize for RNA loading.

**Western Blot Analysis.** Samples containing 30 µg of total protein were resolved by a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) by electroblotting, and probed with anti-p21, anti-CDK2, anti-CDK4, anti-CDK6, anti-cyclin D1, anti-cyclin E, anti-cyclin A, anti-cyclin B1, anti-Rb, anti-cdk25B, anti-cdk25C, anti-Wee1, anti-Parp, anti-Bcl2, anti-Bax polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 polyclonal antibody, anti-caspase 3 monoclonal antibody (Oncogene Research Products, Cambridge, MA), and anti-cdc2 polyclonal antibody (Oncogene Research Products, Cambridge, MA), and anti-cdc2 phosphate-specific monoclonal antibody (New England Biolabs, Inc., Beverly, MA). The blots were developed by using the ECL kit (Amer sham, Arlington Heights, IL).

**Immunoprecipitation.** Samples of total protein (100 µg) were incubated with anti-cdk2, anti-CDK4, anti-CDK6, anti-cdk2, anti-cyclin A, anti-cyclin B1, anti-cyclin D1, and anti-cyclin E polyclonal antibodies for 2 h at 4°C followed by incubation with protein A-agarose conjugates (Santa Cruz Biotechnology) for 1 h. The protein complexes were washed three times with immunoprecipitation buffer [50 mM Tris (pH 7.5), 0.5% NP40, 150 mM NaCl, 50 mM NaF, 0.2 mM NaVO_4_, 1 mM DTT, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride] and released from the agarose bead by boiling in 2× SDS sample buffer [125 mM Tris (pH 6.8), 4% SDS, 10% β_2_-mercaptoethanol, 2% glycerol, 0.004% bromophenol blue] for 5 min, and the reaction mixture was resolved by a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane by electroblotting, and probed with anti-p21 and anti-p27 antibodies. The blot was developed by using the ECL kit.

**Kinase Reaction Assay.** Total lysates were prepared and immunoprecipitated with anti-cdk2, anti-CDK2, anti-CDK4, and anti-CDK6 polyclonal antibodies as described above. The beads were washed three times in immunoprecipitation buffer and then three times in kinase buffer [10 mM Tris (pH 7.5), 2 mM MgCl_2_]. The kinase reactions was carried out at 37°C for 30 min in 25 µl of kinase reaction buffer containing 2.5 mM EGTA, 0.1 mM NaVO_4_, 1 mM NaF, 20 µl ATP, 5 µCi [γ-^32^P]ATP, and 2 µg of histone H1 substrate. The reaction was stopped by adding 2× SDS sample buffer. After boiling for 5 min, the reaction products were electrophoretically separated on a 12% SDS-PAGE gel, and phosphorylated proteins were detected by autoradiography.

**Evaluation of Apoptosis.** Apoptosis was determined by staining cells with annexin V-FITC and PI labeling, because annexin V can identify the externalization of phosphatidylserine during the apoptotic progression and therefore detect early apoptotic cells (26). To quantitate the apoptosis of cells, prepared cells were washed twice with cold PBS and then resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2_] at a concentration of 1 x 10^6 cells/ml. Then, 5 µl of annexin V-FITC (PharMingen, San Diego, CA) and 10 µl of 20 µg/ml PI (Sigma) were added to these cells, which were analyzed with FACStar flow cytometer (Becton Dickinson). Also, during the cell cycle analysis described above, cells were considered to be in apoptosis if they exhibited sub-G1 DNA fluorescence and a forward angle light scatter the same as or slightly lower than that of cells in G1. Mitochondrial transmembrane potential (ψ_M) was determined by flow cytometry. Briefly, cells were washed twice with PBS and incubated with 0.1 µg/ml Rhodamine 123 (Sigma) at 37°C for 30 min. Subsequently, PI (1 µg/ml) was added, and Rhodamine 123 and PI staining intensity was determined by flow cytometry.

**RESULTS**

**Effect of As_2 O_3 on Growth Inhibition of Myeloma Cell Lines.** We examined the effect of As_2 O_3 on the cell proliferation of the MM cell lines using MTT assay. Significant dose-dependent inhibition of cell growth was observed in all of eight cell lines used in this study following the treatment of As_2 O_3 for 72 h (Fig. 1). As_2 O_3 at 2 µM exhibited >50% inhibition of growth in five MM cell lines (MC/CAR, ARH-77, MOPC315, NCI-H929, and U266). Whereas RPMI8266 cells were the most resistant to As_2 O_3 among the MM cell lines tested, NCI-H929 cells treated with As_2 O_3 were inhibited more effectively (IC_50 0.7 µM) in cell growth than any other MM cell lines tested in our study. With regard to MC/CAR cells, the concentration with a 50% growth inhibition to As_2 O_3 was <2 µM. As_2 O_3 also inhibited the proliferation in primary MM cells in a dose-dependent manner (data not shown). These results showed that As_2 O_3 was very potent in inhibiting the proliferation of MM cells in vitro.

**Cell Cycle Analysis in Myeloma Cells.** The effect of As_2 O_3 on the cell cycle was determined in MM cell lines by FACS analysis. As shown in Table 1, DNA flow cytometric analysis with three independent experiments indicated that As_2 O_3 induced a G1 and/or a G_2-M phase arrest in these cell lines following 72 h of exposure. Whereas ARH-77, NCI-H929, RPMI8266, and U266 cells showed a significant increase in the number of cells in the G1 phase of the cell cycle, HS-SULTAN and IM-9 cells significantly induced a G_2-M phase arrest of the cell cycle following the treatment of As_2 O_3, MOPC315 and MC/CAR cells had a significant increase in cells of G1 and G_2-M phases of the cell cycle. Compared with untreated control MC/CAR cells, the percentages of cells in G1 phase and G_2-M phases were increased by 13.1 and 7.8%, respectively, accompanied with a concomitant reduction of cells at the S phase of cell cycle after treatment of MC/CAR cells with As_2 O_3 for 72 h. Taken together,
these results indicated that As$_2$O$_3$ inhibited the cellular proliferation of MM cells via a G$_1$ and/or a G$_2$-M phase arrest of the cell cycle.

Expression of Cell Cycle-regulatory mRNAs and Proteins in MC/CAR Cells. Because As$_2$O$_3$ induced both G$_1$ phase and G$_2$-M phase arrests in MC/CAR cells, we wanted to determine the mRNA and protein levels of CDK1, p21, and p27, in MC/CAR cells exposed to 2 μM As$_2$O$_3$. The level of p21 protein increased with its mRNA were observed, although mild reduction was seen at 24 h after As$_2$O$_3$ treatment (Fig. 2). In particular, induction of p53 coincided with an increase in both protein and mRNA of p21 (Fig. 2A). In vertebrate cells, the G$_1$ progression and the G$_1$-S transition are also regulated by p-type cyclins that bind to and activate CDK4, CDK6, and cyclin E and A which activate CDK2, respectively (14–17). Treatment of MC/CAR cells with 2 μM As$_2$O$_3$ resulted in a down-regulation of the level of CDK4 in a time-dependent manner under the same condition (Fig. 3A). In contrast, CDK4 and cyclin E were expressed constantly for 72 h without the reduction of their proteins (Fig. 3A). In the case of cyclin A which is essential for CDK2 activation, its observed decrease was with time of treatment of As$_2$O$_3$ (Fig. 3A).

Also in MC/CAR cells, we assessed the levels of G$_2$-M phase-related proteins to know how these proteins were regulated by As$_2$O$_3$. Treatment of As$_2$O$_3$ resulted in a down-regulation of the level of cdc2 protein (Fig. 3B), which is an important role in the S phase and the G$_2$-M progression of the cell cycle (14, 15, 17). The expression of cyclin B1 which binds to and activates cdc2 was not changed at any times of incubation (Fig. 3B). Furthermore, because the progression from the G$_2$ phase into mitosis is negatively regulated by cdc2 phosphorylation on threonine 14 (T14) and tyrosine 15 (Y15) residues (15, 27, 28), we investigated the status of cdc2 phosphorylation on tyrosine 15 residues (cdc2-Y15) using a phosphorylation state-specific antibody capable of binding only to tyrosine 15-phosphorylated cdc2 protein. In MC/CAR cells treated with 2 μM As$_2$O$_3$ for 72 h, there was an increase of detectable cdc2-Y15 phosphorylation although the levels of cdc2-Y15 phosphorylation at the start time of As$_2$O$_3$ treatment were higher than expected as a basal level (Fig. 3B). In addition, we examined the changes of Wee1 (protein kinase), cdc25B, and cdc25C (protein phosphatase), which regulate the cdc2-

### Table 1: Effect of As$_2$O$_3$ (2 μM) treatment on cell cycle in myeloma cell lines using FACS analysis

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>G$_1$ (%)</th>
<th>S (%)</th>
<th>G$_2$-M (%)</th>
</tr>
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<tr>
<td>MOPC315</td>
<td>22.9</td>
<td>63.3</td>
<td>14.3</td>
</tr>
<tr>
<td>RPM18226</td>
<td>51.4</td>
<td>28.2</td>
<td>20.3</td>
</tr>
<tr>
<td>IM-9</td>
<td>50.6</td>
<td>32.6</td>
<td>16.8</td>
</tr>
<tr>
<td>ARH-77</td>
<td>51.3</td>
<td>28.3</td>
<td>20.3</td>
</tr>
<tr>
<td>Control</td>
<td>51.2</td>
<td>28.3</td>
<td>20.3</td>
</tr>
<tr>
<td>As$_2$O$_3$</td>
<td>50.6</td>
<td>32.6</td>
<td>16.8</td>
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<td>50.6</td>
<td>32.6</td>
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Fig. 2. Effect of As$_2$O$_3$ on p53, p21, and p27 expression in MC/CAR cells. Cells were harvested at the indicated times after incubation with 2 μM As$_2$O$_3$. A, total RNA (15 μg) was isolated, and RNA blot was subsequently hybridized with the human p53, p21, and p27 cDNA probe. Hybridization of β-actin cDNA probe was used to normalize for equal loading and blotting efficiency. B, aliquots of 30 μg of protein extracts were analyzed by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibodies, p21 and p27.
Regulating Effect of As₂O₃ on CDK-associated Kinase Activity. To determine whether the increased p21 and the decreased cdc2, CDK6, and cyclin A proteins result in the inhibition of CDK activity in As₂O₃-treated MC/CAR cells, we performed in vitro CDK activity assay on histone H1 substrate in immunoprecipitates with anti-cdc2, -CDK2, -CDK4, and -CDK6 antibodies. Treatment with As₂O₃ (2 μM for 72 h) demonstrated a dramatic decrease of cdc2- and CDK6-associated kinase activity on histone H1 substrate compared with As₂O₃-untreated control cells (Fig. 5A). However, there is no significant difference of CDK2- and CDK4-associated histone H1 kinase activity between As₂O₃-treated cells and -untreated cells. In addition, the decrease of CDK6-associated kinase activity was associated with the underphosphorylation of Rb protein (Fig. 5B). Taken together, these results suggested that As₂O₃ induced a G₁ and G₂-M phase arrest via the reducing activity of CDK6-associated kinase and cdc2-associated kinase, respectively, in association with increased p21 protein.

Induction of Apoptosis by As₂O₃ in MC/CAR Cells. We performed in vitro apoptosis detection assay to know whether As₂O₃ treatment could induce the apoptosis in MC/CAR cells by FACS analysis. Apoptosis was induced in a dose-dependent manner except at a dose of 1 μM As₂O₃ for 72 h (Fig. 6A). To confirm and evaluate the induction of apoptosis, we performed the staining of cells with annexin V and PI (Fig. 6B). As with the percentages of sub-G₁ group by FACS analysis, the proportion of apoptotic cells in the lower right quadrant was increased in a dose-dependent manner. Especially, at 2 μM As₂O₃, the percentages of apoptotic and dead cells were ~72.9%. Taken together, these results indicate that induction of apoptosis could be another mechanism of the antiproliferative effect of As₂O₃ besides G₁ and G₂-M phase arrests of the cell cycle in MC/CAR myeloma cells.

Apoptotic-related Proteins and Mitochondrial Transmembrane Potential (Δψ₅₃) in Treatment of As₂O₃ in MC/CAR Cells. Concerning the relationship between Bcl-2 and Bax regulation during apoptosis, the Bcl-2 protein level was down-regulated in a dose-dependent manner (Fig. 6C) and in a time-dependent manner (Fig. 7A). However, Bax protein was constitutively expressed in the absence of change of its protein level (Figs. 6C and 7A), suggesting that apoptosis by As₂O₃ may be mediated through the down-regulation of Bcl-2 apoptotic protein in MC/CAR cells. As shown in Fig. 7B, As₂O₃ also induced the subsequent loss of mitochondrial transmembrane potential (Δψ₅₃). Next, we wanted to investigate whether caspase-3 might be activated during the induction of apoptosis by As₂O₃, because the ICE/caspase family plays an essential role in apoptosis (29–32). The 32-kDa precursor (procaspase-3) was degraded in a time-dependent manner (Fig. 7A), implying that induction of apoptosis by As₂O₃ was related to activation of caspase-3 enzymatic protein. Concerning poly(ADP-ribose) polymerase (PARP) protein, which is a major substrate for active caspase-3 and a hallmark of apoptosis, Western blotting showed that the intact 113-kDa moiety of PARP was
degraded, as evidenced by increased 89-kDa cleavage products (Fig. 7A). Conclusively, these results indicated that As$_2$O$_3$-induced apoptosis of MC/CAR cells was accompanied by the changes of Bcl-2 protein levels, $\Delta\psi_m$ and caspase-3 activity.

DISCUSSION

In the present study, we have shown that As$_2$O$_3$ inhibited the cell proliferation of MM cells by inducing the cell cycle arrest as well as triggering the apoptosis. As$_2$O$_3$ exhibited a dose-dependent inhibition of cellular proliferation in primary MM cells as well as MM cell lines, although there is a little different susceptibility to As$_2$O$_3$ among the MM cells. We found that IC$_{50}$ of As$_2$O$_3$ was $\sim 2 \mu$M in five cell lines among eight MM cells, which was somewhat similar to that of As$_2$O$_3$ in growth inhibition of APL cells (3–6). Very recently, Rousselot et al. (7) reported that pharmacological concentration of As$_2$O$_3$ caused the inhibition of growth in MM cell lines as well as primary MM cells isolated from patients. They also showed almost same IC$_{50}$ of As$_2$O$_3$ as our results in NCI-H929 and U266 cells. These results suggest that As$_2$O$_3$ may be clinically useful in patients with MM at the therapeutic and pharmacological concentration, as demonstrated by studies on the treatment of APL patients (1, 2).

Our cell cycle analysis has revealed that As$_2$O$_3$ was able to prominently induce a G$_1$ phase and/or a G$_2$-M phase arrest of MM cells after their exposure to As$_2$O$_3$. These results were consistent with those of other investigators who showed that antiproliferative action of arsenical compound was linked to a G$_1$ phase arrest in lymphoid neoplasms (9) and a G$_2$-M phase arrest in NB4 cells (22) at lower doses. Therefore, it is likely that As$_2$O$_3$ may induce the cell cycle arrest of a G$_1$ phase and/or a G$_2$-M phase depending on the cell type, suggesting that the molecular mechanisms of cell cycle arrest by As$_2$O$_3$ have great variety. In this study, the G$_1$ and G$_2$-M phase arrests in MC/CAR cells were associated with a marked up-regulation of p21 protein and mRNA, suggesting transcriptional and translational regulation of p21 gene by As$_2$O$_3$. The p21 can be up-regulated by both p53-dependent and p53-independent pathways (33, 34). The expression of high level of the p53 can give rise to a G$_1$ arrest alone, a G$_2$-M arrest, or both G$_1$ and G$_2$-M arrests depending on the cell type (35–37). The expression of p53 mRNA was elevated in association with up-regulation of p21 mRNA. Therefore, it is probable that p21 induction and the cell cycle arrest of both G$_1$ and G$_2$-M phases in As$_2$O$_3$-treated MC/CAR cells may be mediated by p53. Among CDKs that regulate the cell cycle, CDK2, CDK4, and CDK6 are activated in association with d-type cyclins or cyclin E during the G$_1$ progression and the G$_1$-S transition. We found that the expression of CDK6 protein was decreased in a time-dependent manner in As$_2$O$_3$-treated MC/CAR cells, but those of CDK2, CDK4, cyclin D1, and cyclin E...
were not. In addition, the accumulated p21 protein in association with G1 arrest was detected largely in complexes with CDK6 and cyclin E without its increased binding to complexes with CDK2, CDK4, and cyclin D1, supporting the idea that only CDK6-associated kinase activity was markedly decreased in our kinase assay. Furthermore, reduced kinase activity of CDK6 was accompanied with the under-phosphorylation of Rb protein, which is known to sequester the transcription factor, E2F, thereby preventing cells from further entering the cell cycle progression. These results suggest that As2O3-induced p21 binds specifically to CDK6 and cyclin E proteins and inhibits the kinase activity of CDK6, ultimately resulting in hypophosphorylation of Rb protein. Taken together, G1 blocking MC/CAR cells from entry into S phase is mediated by down-regulation of CDK6-associated kinase activity in association with induction of CDK1, principally p21.

Cdc2 kinase is activated primarily in association with cyclin A and B in G2-M phase progressions. In this study, we have demonstrated that cdc2 and cyclin A proteins were decreased following treatment with As2O3. Rb-bound E2F suppresses a number of key genes needed for S phase progression including cyclin A (38–40) which is required in both S phase progression as well as the G2-M transition (14–17). Therefore, the decrease of cyclin A by As2O3 might be mediated via E2F sequestered by hypophosphorylation of Rb. The increased p21 was seen much higher in complexes with cdc2 and cyclin A in As2O3-treated MC/CAR cells. These effects could account for the decreased activity of cdc2-associated kinase in our kinase assay. Alternatively, cdc2 activity can be negatively regulated by cdc2 phosphorylation on threonine 14 and tyrosine 15 (15, 27, 28). These phosphorylations are enforced by protein kinases including Wee1 (41) and are also retarded by cdc25 phosphatases, especially cdc25C (42–44). Therefore, tyrosine phosphorylation of cdc2 at tyrosine 15 (cdc2-Y15 phosphorylation) inhibits kinase activity and is one of the mechanisms by which human cells inhibit mitosis after exposure to DNA-damaging agent (45). In MC/CAR cells treated with As2O3, there was an increase of detectable cdc2-Y15 phosphorylation. This result might be due to the down-regulation of cdc25B and cdc25C phosphatases, because no detectable change in Wee1 protein was observed. Thus, it is likely that the increased phosphorylation of cdc2 via down-regulation of cdc25B and cdc25C enhances the decreased activity of cdc2 kinase by p21 protein during a G2-M arrest. Conclusively, As2O3-induced cell cycle arrest in MC/CAR cells resulted from the inactivation of CDK6 in a G1 phase and cdc2 in a G2-M phase through the induction of p21.

In addition, our data showed that As2O3 markedly induced the apoptosis in a dose-dependent manner in all of the cell lines [ARH-77, HS-SULTAN, NCI-H929, and U266 (data not shown) including MC/CAR] tested. These results strongly support the notion that apoptosis induced by As2O3 can occur independently of PML-RARα fusion protein. To gain insight into understanding the molecular mechanism involved in apoptosis by As2O3, expression of the antiapoptotic protein, Bcl-2, was assessed in MC/CAR cells. It has been reported that nonorganic (As2O3) or organic (melarsoprol) arsenical compounds efficiently induced the apoptosis in myeloid and lymphoid cell lines through the down-regulation of Bcl-2 gene expression (9–12). Similarly, we showed that the induction of apoptosis was accompanied with the down-regulation of Bcl-2 protein, supporting the idea that alteration of Bcl-2 is directly or indirectly involved in the apoptotic effect of As2O3 in MC/CAR cells. By contrast, no detectable changes in Bcl-2 were reported during apoptosis in the T cell line and MM cell lines (NCI-H929 and U266) (7, 46). In addition to MC/CAR cells, we examined the modulation of Bcl-2 in ARH-77, NCI-H929, and U266 cells, resulting in the absence of any modification in Bcl-2 protein (data not shown). This discrepancy may be due to the existence of more than one distal pathway of apoptosis or different cell lineage specificity. In regard to regulation of the proapoptotic Bax gene, any induction of Bax protein was not observed during treatment with As2O3. However, it should be emphasized that the ratio of Bcl-2 to Bax determines the amount of Bcl-2/Bax heterodimers versus Bax/Bax homodimers and is important in determining susceptibility to apoptosis (47).

The ICE/caspase family plays a crucial role in apoptosis (29–32). In particular, caspase-3 (CPP32/Yama/apopain) has been shown to be a key component of the apoptotic machinery. Recently, it was reported that As2O3 appeared to induce apoptosis, coincident with conversion from inactive precursors to activated enzymes, especially caspase-3, in NB4 cells (9), primary APL cells (2), and mouse B cell leukemia cells (11). Similarly, our data demonstrated that caspase-3 was activated and PARP protein was degraded by As2O3. Although the collapse of mitochondrial transmembrane potential (ΔΨm) resulting from the low ratio of Bcl-2 to Bax was believed to cause the activation of caspase-3 in our study, the mechanism by which As2O3 activated caspase-3 protease remains to be elucidated. Furthermore, MC/CAR cells were protected from the apoptotic effect of As2O3 when treated with Z-VAD-FMK (50 μM) of the caspase-3 inhibitor (data not shown). Therefore, these results strongly provide the evidence that caspase-3 might be one of the critical steps in As2O3-induced apoptosis.

MM cells are tightly related to various cytokines including IL-6, recognized as a myeloma growth factor (48–50). It has been shown that IL-6 protects the myeloma cells against dexamethasone- and Fas-induced apoptosis (49, 50). However, mild cellular proliferation was noted in HS-SULTAN, NCI-H929, and U266 cells treated with IL-6 (10 ng/ml), and IL-6 could not inhibit As2O3-induced apoptosis of these MM cells (data not shown), which was similar to the result of Rousselot et al. (7). Thus, the mechanism by which As2O3 induces apoptosis in MM cells may be different from that of dexamethasone- or Fas-induced apoptosis. Furthermore, we could not identify the detectable mRNA changes of IL-6 and IL-6 receptor genes in all of the MM cell lines during the As2O3-induced cell cycle arrest and apoptosis (data not shown), suggesting that the antiproliferative effect of As2O3 in MM cells may not be mediated via the IL-6 signaling pathway. However, whether As2O3 disturbs the downstream signal transduction of IL-6/IL-6 receptor complex remains to be elucidated.

In summary, As2O3 inhibits the cell proliferation of MM cell lines by not only inducing the cell cycle arrest through p21 but also triggering the apoptosis through caspase-3, especially in MC/CAR cells. Finally, these results suggest that As2O3 may be useful as one of the investigational drugs in the treatment of MM patients. We are currently investigating the in vivo effect of As2O3 on MM using SCID mice.

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Arsenic Trioxide-mediated Growth Inhibition in MC/CAR Myeloma Cells via Cell Cycle Arrest in Association with Induction of Cyclin-dependent Kinase Inhibitor, p21, and Apoptosis

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