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Biochemical and Biophysical Research Communications 300 (2003) 230–235
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Arsenic trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis

Woo Hyun Park, Yeon Hee Cho, Chul Won Jung,* Joon Oh Park, Kihyun Kim, Young Hyuck Im, Mark H. Lee, Won Ki Kang, and Keunchil Park

Division of Hematology/Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea

Received 31 October 2002

Abstract

Previously, we showed that arsenic trioxide potently inhibited the growth of myeloma cells and head and neck cancer cells. Here, we demonstrate that arsenic trioxide inhibited the proliferation of all the renal cell carcinoma cell lines (ACHN, A498, Caki-2, Cos-7, and Renca) except only one cell line (Caki-1) with IC_{50} of about 2.5–10 μM. Arsenic trioxide induced a G_1 or a G_2-M phase arrest in these cells. When we examined the effects of this drug on A498 cells, arsenic trioxide (2.5 μM) decreased the levels of CDK2, CDK6, cyclin D1, cyclin E, and cyclin A proteins. Although p21 protein was not increased by arsenic trioxide, this drug markedly enhanced the binding of p21 with CDK2. In addition, the activities of CDK2- and CDK6-associated kinase were reduced in association with hypophosphorylation of Rb protein. Arsenic trioxide (10 μM) also induced apoptosis in A498 cells. Apoptotic process of A498 cells was associated with the changes of Bcl-$_{XL}$, caspase-9, caspase-3, and caspase-7 proteins as well as mitochondria transmembrane potential ($\Delta\psi_m$) loss. Taken together, these results demonstrate that arsenic trioxide inhibits the growth of renal cell carcinoma cells via cell cycle arrest or apoptosis.

Keywords: Arsenic trioxide; Renal cell carcinoma; Cell cycle; CDK; Cyclin; CDKI; Apoptosis; Caspase

Arsenic trioxide (As_2O_3) has recently been reported to induce complete remission in the patients with relapsed or refractory acute promyelocytic leukemia (APL) without severe marrow suppression [1]. Although the mechanism of the antileukemic effect of arsenic trioxide is not well understood, it is known that arsenic trioxide is able to degrade a PML protein and a PML/RARα fusion protein in APL with a $t(15;17)$ [2,3]. More recently, it has been shown that antiproliferative effect of arsenic trioxide is not limited to APL cells but can be observed in a variety of hematological malignancies without having the PML/RARα fusion protein [4–6], suggesting that the antiproliferative effect of arsenic trioxide might be independent of a PML or PML/RARα fusion protein. The accumulating evidences indicated that arsenic trioxide could regulate many biological functions such as cell proliferation, apoptosis, differentiation, and angiogenesis in various cell lines [7]. For example, arsenic trioxide inhibits NB4 APL cells via the down-regulation of Bcl-2 and modulation of PML/RARα fusion proteins [3]. Also, we showed that arsenic trioxide inhibits the growth of myeloma cells and head and neck cancer cells [4,8,9]. These biological effects may be mediated by interactions between arsenic trioxide and proteins with a high cysteine content such as PML protein [10].

The cell cycle in eukaryotes is regulated by cyclin-dependent kinases (CDKs). The cyclins, members of the cell cycle regulators, bind to and activate CDKs. Sequential formation, activation, and subsequent inactivation of cyclins and CDKs are critical for control of the cell cycle [11–14]. Recently, proteins of a new functional class that inhibit CDK activity, called cyclin-dependent kinase inhibitors (CDKIs), have been identified. These CDKIs can play a key role in controlling cell cycle

* Corresponding author. Fax: +82-2-3410-0388.
E-mail address: leukemia@smc.samsung.co.kr (C. Won Jung).

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PII: S0006-291X(02)02831-0

progression by negatively regulating the CDK activities at appropriate time in the cell cycle [15–18].

Apoptosis is an important mode of cell death that occurs in response to a variety of agents including ionizing radiation or anticancer chemotherapeutic drugs [19]. In particular, antiapoptotic members of Bcl-2 family including Bcl-2 and Bcl-$_{XL}$ act to prevent or delay cell death, while proapoptotic Bax and Bcl-$_{XS}$ genes promote the apoptosis [20,21]. Several lines of evidence suggest that interleukin-1-converting enzyme (ICE)/caspase family plays a crucial role in apoptosis [22,23]. Especially, caspase-3 has been shown to be a key component of the apoptotic machinery. Caspase-3 has been shown to be activated in apoptotic cells and cleaves several cellular proteins including poly(ADP-ribose) polymerase (PARP) protein, the cleavage of which is a hallmark of apoptosis.

In the present study, we evaluated the effect of arsenic trioxide on cell growth inhibition in many renal cell carcinoma cell lines.

Materials and methods

Cell lines and culture. Renal cell carcinoma cell lines used in this study were ACHN, A498, Caki-1, Caki-2, Cos-7, and Renca. These cells were maintained according to the recommendation of the American Type Culture Collection. Cells were grown at 37 °C in an atmosphere of 5% CO_2 in air.

Reagent. Arsenic trioxide was purchased from Sigma Chemical (St. Louis, MO). Arsenic trioxide was dissolved in 1.65 M NaOH at 5×10^{-2} M as a stock solution. The maximum concentration of NaOH in culture had no influence on cell growth of these cell lines.

Growth inhibition assay. In vitro growth inhibition effect of arsenic trioxide on renal cell carcinoma cells was determined by measuring MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye absorbance of living cells [24]. Briefly, cells (2×10^5 cells per well) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark). After exposure to the drug for 72 h, 50 μl MTT (Sigma) solution (2 mg/ml in PBS) was 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 DMSO was added to each well before absorbance at 570 nM was measured.

Cell cycle analysis. Cell cycle distribution was determined by staining DNA with PI (propidium iodide) (Sigma). Briefly, 1×10^6 cells were incubated without arsenic trioxide or with arsenic trioxide for 72 h. Cells were then washed in PBS and fixed in 70% ethanol. Cells were again washed with PBS and then incubated with PI (10 μg) with simultaneous (RNase) treatment at 37 °C for 30 min. The percentage of cells in the different phases of the cell cycle was measured with FACStar flow cytometer (Becton–Dickinson, San Jose, CA) and analyzed by using Becton–Dickinson software (Lysis II, Cellfit).

Western blot analysis. Samples containing 30 μg 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-Bcl-2, anti-Bcl-$_{XL}$, and anti-Bax antibodies (Santa Cruz, CA), anti-p27 polyclonal antibody (Transduction Laboratories, Lexington, KY), anti-Rb polyclonal antibody (Pharmingen, San Diego, CA), and anti-caspase-9, anti-caspase-3, anti-caspase-7, anti-PARP, anti-p-ERK, anti-p-JNK, and anti-p-p38 antibodies (Cell Signaling Technology, Beverly, MA). The blots were developed by using the ECL kit (Amersham, Arlington Heights, IL).

Immunoprecipitation. Samples of total protein (100 μg) were incubated with anti-CDK2, anti-CDK4, and anti-CDK6 polyclonal antibodies for 2 h at 4 °C, followed by incubation with protein A–agarose conjugates (Santa Cruz) for 1 h. The protein complexes were washed three times with immunoprecipitation buffer [50 mM Tris (pH 7.5), 0.5% NP-40, 150 mM NaCl, 50 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 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, and 0.004% bromphenol 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. Using the ECL kit one developed the blot.

Kinase reaction assay. Total lysates were prepared and immunoprecipitated with 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 reaction was carried out at 37 °C for 30 min in 25 μl 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 histone H1 substrate. Adding 2× SDS sample buffer stopped the reaction. 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. 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, and 2.5 mM $CaCl_2$] at a concentration of 1×10^6 cells/ml. Then, 5 μl 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). Mitochondrial transmembrane potential ($\Delta\Psi_m$) was determined by flow cytometry. Briefly, cells were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml) (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 and discussion

Effect of arsenic trioxide on growth inhibition in renal cell carcinoma cell lines

We examined the effect of arsenic trioxide on the cell proliferation of renal cell carcinoma cell lines using MTT assay. Dose-dependent inhibition of cell growth was observed in all the cell lines but Caki-1 cells with IC_{50} of about 2.5–10 μM following the treatment of arsenic trioxide for 72 h (Fig. 1). The susceptibility to arsenic trioxide in renal cell carcinoma cells is lower than that in leukemia and myeloma cell lines [2–4] and is similar to that in solid tumors such as human-small-cell lung cancer, ovarian cancer, colon cancer, cervical cancer, and breast cancer cell lines [26, our unpublished data]. This different susceptibility of arsenic trioxide on cell lines might result from cell's different origins. It is very noticeable that renal cell carcinoma cells were as much sensitive to arsenic trioxide as the other solid

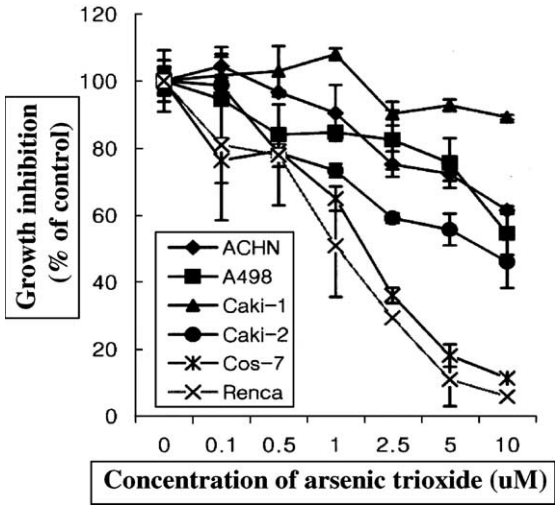

Fig. 1. Effect of arsenic trioxide on the growth inhibition of renal cell carcinoma cell lines in vitro. Exponentially growing cells were treated with the indicated concentration of arsenic trioxide for 72 h. Cell growth inhibition was assessed by MTT assay as described in "Materials and methods." The cellular growth of all the cell lines was significantly inhibited in a dose-dependent manner. Results represent means of at least four independent experiments; bars, SD.

tumors, because it has long been considered that renal cell carcinoma cells are more resistant to many chemotherapeutic drugs than other solid tumors. We have investigated the mechanism by which arsenic trioxide inhibited cell growth of renal cell carcinoma cells in terms of cell cycle arrest and apoptosis.

Table 1
Effect of arsenic trioxide treatment on cell cycle in renal cell carcinoma cell lines

Summary of cell cycle arrest by arsenic trioxide (2.5 μM)			
Cell lines	G_1 (%)	S (%)	G_2-M (%)
ACHN			
Control	45.9	40.9	13.1
Arsenic trioxide	41.8	38.8	19.3
A498			
Control	39.8	39.8	20.6
Arsenic trioxide	63.4	24.2	12.4
Caki-1			
Control	66.3	12.6	21.1
Arsenic trioxide	54.9	15.4	29.7
Caki-2			
Control	57.7	31.1	11.2
Arsenic trioxide	61.0	26.1	12.9
Cos-7			
Control	46.1	44.4	9.6
Arsenic trioxide	26.9	44.8	28.6
Renca			
Control	43.7	45.5	10.8
Arsenic trioxide	42.4	35.3	22.3

Effects of arsenic trioxide on the cell cycle in renal cell carcinoma cell lines

As shown in Table 1, DNA flow cytometric analysis indicated that arsenic trioxide induced a G_1 or a G_2-M phase arrest in these cell lines following 72 h of exposure. Whereas A498 cells showed increased cells in the G_1 phase, Caki-1, Cos-7, and Renca cells were arrested in a G_2-M phase of the cell cycle. ACHN cells showed a few increased cells in the G_2-M phase. There was little cell cycle change in arsenic trioxide-treated Caki-2 cells. Arsenic trioxide was likely to induce mostly a G_2-M phase arrest rather than a G_1 phase arrest in these cell lines. A G_2-M phase arrest by arsenic trioxide is also shown in the head and Neck cancer cell (PCl-1) and breast cancer cell (MCF-7) [9,26]. The changes of tubulin polymerization or microtubule network by arsenic trioxide may cause a G_2-M phase arrest in these cells [26]. In contrast, arsenic trioxide could induce a G_1 phase arrest in the A498 cells and myeloma cells [4] and lymphoid malignant cells [5]. Taken together, it is likely

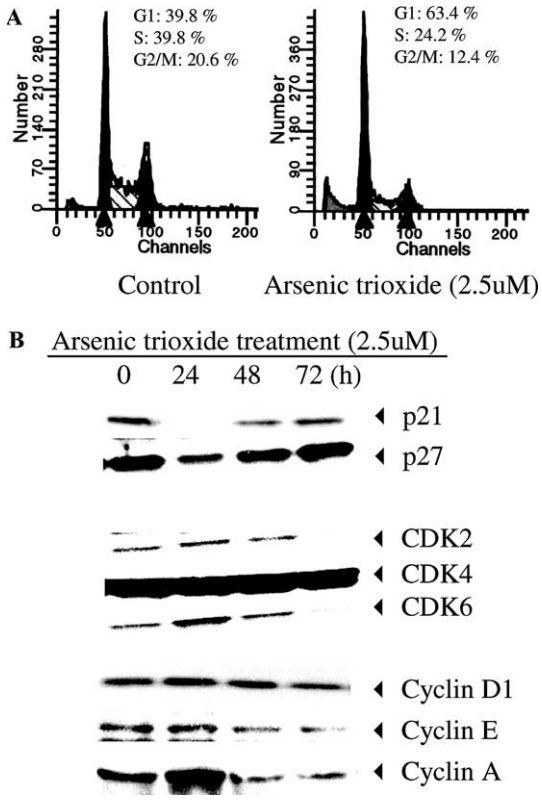

Fig. 2. Effect of arsenic trioxide on cell cycle-related proteins in A498 renal cell carcinoma cells. (A) The changes of cell cycle phase distribution in arsenic trioxide-treated A498 cells. (B) Cells were harvested at the indicated times after incubation with 2.5 μM arsenic trioxide. Cells were then lysed and the supernatants were subjected to Western blot analysis. Aliquots of 30 μg protein extracts were analyzed by 12% SDS–PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibodies; p21, p27, CDK2, CDK4, CDK6, cyclin D1, cyclin E, and cyclin A.

that arsenic trioxide induce a G_1 or a G_2-M phase arrest depending on the cell types.

Because arsenic trioxide induced definitely a G_1 phase arrest of cell cycle in A498 cells (Fig. 2A), we wanted to determine the levels of cell cycle-regulated proteins in this cell line exposed to 2.5 μM arsenic trioxide. A498 cells treated with arsenic trioxide resulted in a down-regulation of the protein of CDK2, CDK6, cyclin D1, cyclin E, and cyclin A (Fig. 2B). Next, we questioned whether p21 and p27 could be detected in complexes with CDKs in arsenic trioxide-treated A498 cells, even though these proteins were not increased by it. As shown in Fig. 3A, the complexes immunoprecipitated with anti-CDK2 antibody showed higher amounts of immunodetectable p21 protein from arsenic trioxide-treated cells than from control cells. However, p27 protein was not more bound to CDKs from arsenic trioxide-treated cells than from arsenic trioxide-untreated cells.

To determine whether the changed cell cycle regulatory proteins result in the inhibition of CDK activity in arsenic trioxide-treated cells, we performed in vitro CDK activity assay on histone H1 substrate in immunoprecipitates with anti-CDK2, -CDK4, and -CDK6 antibodies. A498 cells treated with arsenic trioxide demonstrated a dramatic decrease of CDK2- and CDK6-associated kinase activities on histone H1 substrate compared with arsenic trioxide-untreated control cells (Fig. 3B). In addition, this decrease of CDK-associated kinase activity was associated with the underphosphorylation of Rb protein (Fig. 3C), which is known to sequester the transcription factor, E2F. Rb-bound E2F suppresses a number of key genes needed for S phase progression including cyclin A that is required for both S phase progression. Therefore the decreased cyclin A by arsenic trioxide might be mediated via E2F sequestered by hypophosphorylation of Rb. Collectively, arsenic trioxide-induced G_1 phase arrest in A498 cells resulted from the changes of cell cycle-regulatory proteins.

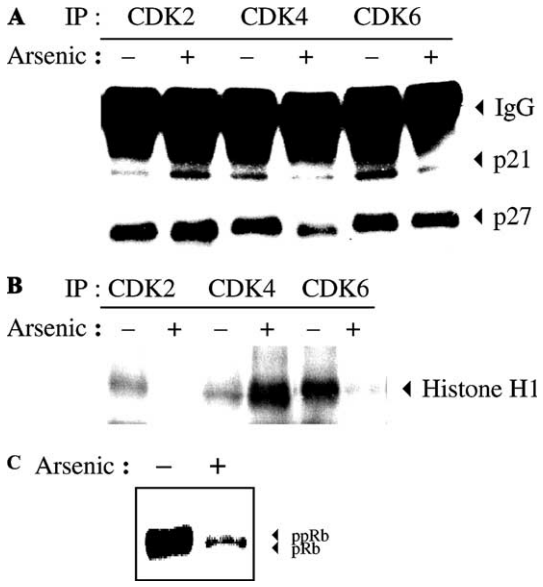

Fig. 3. (A) Association of p21 and p27 with CDKs in A498 cells. Cells were treated without (–) or with (+) arsenic trioxide at a dose of 2.5 μM for 72 h. Total lysates were immunoprecipitated with anti-CDK2, -CDK4, and -CDK6 antibodies. The bound p21 and p27 in each immunocomplex were determined by Western blot analysis. (B) CDK-associated histone H1 kinase activities. Cells were treated without (–) or with (+) arsenic trioxide at a dose of 2.5 μM for 72 h. Total protein lysates were prepared and CDK2, CDK4, and CDK6 kinase activities were determined by histone H1 kinase assay using the indicated antibodies. (C) Phosphorylation of Rb protein, Total protein lysates were resolved by 12% SDS–PAGE, transferred to a nitrocellulose membrane, and immunoblotted with an anti-Rb polyclonal antibody. The hypophosphorylated Rb (pRb) showed higher electrophoretic mobility than the hyperphosphorylated Rb (ppRb).

Induction of apoptosis by arsenic trioxide in renal cell carcinoma cell lines

We performed in vitro apoptosis detection assay in order to know whether arsenic trioxide treatment could induce the apoptosis in renal cell carcinoma cell lines by FACS analysis. Arsenic trioxide remarkably induced apoptosis or necrosis in the Cos-7, Renca, and ACHN cell lines tested in our experiment (Table 2; Sub-G_1 and Annexin V staining cell). Although arsenic trioxide (2.5 μM) could not induce cell death in A498, Caki-1, and Caki-2 cell lines, we could identify that arsenic trioxide (10 μM) could induce cell death in Caki-1 and Caki-2 cell

Table 2
Effect of arsenic trioxide treatment on apoptosis in renal cell carcinoma cell lines

Summary of apoptosis by arsenic trioxide (2.5 μM)		
Cell lines	Sub-G_1 (%)	Annexin V staining cell (%)
ACHN		
Control	6.4	14.3
Arsenic trioxide	22.5	13.7
A498		
Control	1.6	5.2
Arsenic trioxide	2.5	6.5
Caki-1		
Control	4.9	6.5
Arsenic trioxide	6.9	7.1
Caki-2		
Control	1.4	6.1
Arsenic trioxide	1.6	8.0
Cos-7		
Control	14.4	9.2
Arsenic trioxide	41.2	15.8
Renca		
Control	4.9	16.0
Arsenic trioxide	40.2	62.5

lines (data not shown) and A498 cells (Fig. 4A). These results indicate that induction of apoptosis or necrosis can be another antiproliferative mechanism of arsenic trioxide in renal cell carcinoma cells.

Concerning the relationship between Bcl-2 and Bax regulation during apoptosis of arsenic trioxide-treated A498 cells (Fig. 4A), Bax and Bcl-2 proteins were not changed but Bcl-$_{XL}$ proteins were decreased (Fig. 4B). This result supports the idea that alteration of Bcl-2 family proteins is directly or indirectly involved in the apoptotic effect of arsenic trioxide on A498 cells. It has been suggested that a high ratio of proapoptotic proteins such as Bax to antiapoptotic proteins such as Bcl-2 and Bcl-$_{XL}$ can cause the collapse of mitochondrial transmembrane potential ($\Delta\Psi_m$), resulting in releasing cytochrome *c* and apoptosis [25]. In our data, arsenic trioxide clearly induced the loss of mitochondrial transmembrane potential ($\Delta\psi_m$) in the A498 cells (Fig. 4A), suggesting that apoptosis by arsenic trioxide is very tightly related to or dependent on the loss of mitochondrial transmembrane potential ($\Delta\psi_m$). Cytochrome *c* in cytosol forms an apoptosome that is composed of Apaf-1 and procaspase-9, resulting in activation of caspase-9. Caspase-9 activates the effector procaspases, including procaspase-3 and -7, to execute the process of apoptosis [22]. In particular, many agents transform caspase-3 from inactive precursors to activated enzymes during apoptosis. Similarly, our data showed that procaspase-9, -3, and -7 were cleaved and activated by arsenic trioxide. This activation finally results in degradation of PARP protein, the cleavage of which is a hallmark of apoptosis. Therefore, these results strongly provide the fact that the loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and activation of caspase-3 or -7 might be a critical step in arsenic trioxide-induced apoptosis. And these results support the fact that mitochondrial transmembrane potential ($\Delta\psi_m$) or caspase-3 is very tightly related to the apoptosis by arsenic trioxide [1,4,8,26–28].

Because the role of MAPK (ERK, JNK, and p38 MAP kinase) activity in apoptosis has been extensively discussed, we want to check out the changes of these kinases' activity. As shown in Fig. 4C, arsenic trioxide does not alter the phosphorylated p42/44 ERK protein.

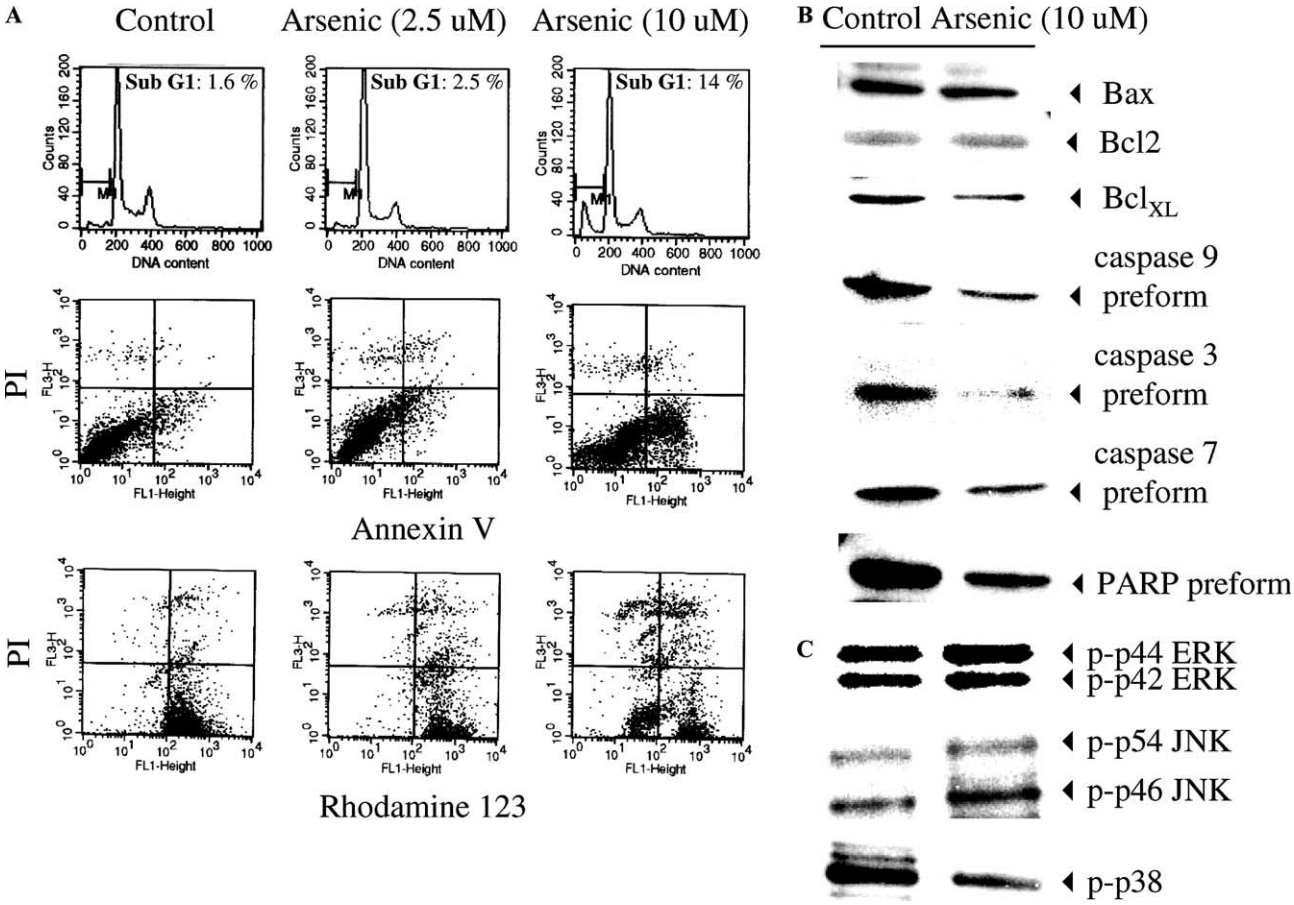

Fig. 4. Effect of arsenic trioxide on apoptotic-related proteins and on the mitochondrial transmembrane potential ($\Delta\Psi_m$) in A498 cells. (A) Arsenic trioxide (10 μM) increased Sub-G1 portion and annexin V staining, and triggered the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Cells were treated without or with arsenic trioxide at a dose of 10 μM for 72 h. Aliquots of 30 μg of protein extracts were analyzed by 12% SDS–PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibodies; (B) Bax, Bcl-2, Bcl-$_{XL}$, caspase-9, caspase-3, caspase-7, and PARP. (C) p-ERK, p-JNK, and p-p38.

While this drug increased phosphorylated JNK protein, this drug decreased phosphorylated p38 protein. The result that activity of JNK was increased in arsenic trioxide-treated A498 cells was similar to Maeda's result, showing that arsenic trioxide increases the activity of JNK in human androgen-independent prostate cancer cells [29]. However, p38 activity decreased by arsenic trioxide-treated A498 cells is opposite to the results that p38 activity is increased in arsenic trioxide-induced apoptosis of human androgen-independent prostate cancer cells [29], human leukemia U937 cells [30], and vascular cells [31]. Therefore, the roles of MAPKs in arsenic trioxide-triggered apoptosis need to be studied more.

In summary, arsenic trioxide inhibited the cell proliferation of renal cell carcinoma cell lines by inducing cell cycle arrest or by triggering apoptosis, especially in A498 cells. Finally, these results suggest that arsenic trioxide may be a useful drug in the treatment of renal cell carcinoma patients.

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