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Diarsenic and Tetraarsenic Oxide Inhibit Cell Cycle Progression and bFGF- and VEGF-Induced Proliferation of Human Endothelial Cells

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Abstract Arsenic trioxide (As_2O_3, diarsenic oxide) has recently been reported to induce apoptosis and inhibit the proliferation of various human cancer cells derived from solid tumors as well as hematopoietic malignancies. In this study, the in vitro effects of As_2O_3 and tetraasrsenic oxide (As_4O_6) on cell cycle regulation and basic fibroblast growth factor (bFGF)- or vascular endothelial growth factor (VEGF)-stimulated cell proliferation of human umbilical vein endothelial cells (HUVEC) were investigated. Significant dose-dependent inhibition of cell proliferation was observed when HUVEC were treated with either arsenical compound for 48 h, and flow cytometric analysis revealed that these two arsenical compounds induced cell cycle arrest at the G_1 and G_2/M phases—the increases in cell population at the G_1 and G_2/M phase were dominantly observed in As_2O_3- and As_4O_6-treated cells, respectively. In both arsenical compounds-treated cells, the protein levels of cyclin A and CDC25C were significantly reduced in a dose-dependent manner, concomitant to the reduced activities of CDK2- and CDC2-associated kinase. In G_1-synchronized HUVEC, the arsenical compounds prevented the cell cycle progression from G_1 to S phase, which was stimulated by bFGF or VEGF, through the inhibition of growth factor-dependent signaling. These results suggest that arsenical compounds inhibit the proliferation of HUVEC via G_1 and G_2/M phase arrest of the cell cycle. In addition, these inhibitory effects on bFGF- or VEGF-stimulated cell proliferation suggest antiangiogenic potential of these arsenical compounds. J. Cell. Biochem. 95: 120–130, 2005.
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Key words: arsenic trioxide; basic fibroblast growth factor; diarsenic oxide; human umbilical vein endothelial cells; tetraarsenic oxide; vascular endothelial growth factor

Angiogenesis denotes the formation of new blood vessels from pre-existing vessels. Physiological angiogenesis, which is required for embryonic development, wound healing, and the menstrual cycle, is characterized by tight spatial and temporal regulation. In adults, new blood vessels arise through the critical process of angiogenesis, which facilitates the growth of solid tumors [Folkman, 1995a,b]. Thus, the inhibition of angiogenesis is a promising new approach in cancer therapy. Currently, a wide variety of compounds are on clinical trial, together with the frequent addition of new angiogenesis inhibitors. Since endothelial cells are the primary structural units of blood vessels, the principal target cell of most angiogeneisis inhibitors is the endothelial cell. Inhibitors also

Abbreviations used: As_2O_3, diarsenic oxide; As_4O_6, tetraarsenic oxide; bFGF, basic fibroblast growth factor; ECGS, endothelial cell growth supplement; HUVEC, human umbilical vein endothelial cells; PI, propidium iodide; VEGF, vascular endothelial growth factor.

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selectively affect a number of endothelial cell functions acquired during angiogenesis, including activation, proliferation, migration, invasion, and survival [Kong and Crystal, 1998; Griffioen et al., 1999].

Many countries have applied arsenical compounds to traditional medicine, particularly in the treatment of several diseases, including cancer. These applications have received renewed attention in recent years due to the successful clinical application of arsenic trioxide (As_2O_3, diarsenic oxide) to the treatment of acute promyelocytic leukemia (APL) [Shen et al., 1997; Soignet et al., 1998]. Long-term clinical trials have indicated that As_2O_3 is effective for inducing complete remission of APL in patients who are resistant to both cytotoxic chemotherapy and all-trans retinoic acid. Although the mechanism of the anti-leukemic effect of As_2O_3 has not been confirmed, treatment with clinically allowable concentrations of As_2O_3 has been shown to induce apoptosis in the APL cell line, NB4, which may be mediated through down-regulation of bcl-2 and modulation of the PML–RARα fusion protein [Chen et al., 1996]. Furthermore, As_2O_3 has an antivascular effect on a murine fibrosarcoma model via vascular shutdown without adverse effects on normal skin, muscle, and kidney. Its precise mechanisms remain unclear, though [Lew et al., 1999]. Since arsenic affects numerous cellular and physiological pathways, a wide variety of malignancies, including both hematologic cancer and solid tumors derived from several tissue types, may be susceptible to therapy with As_2O_3 [Seol et al., 1999; Ora et al., 2000; Park et al., 2000; Maeda et al., 2001; Woo et al., 2002; Hyun Park et al., 2003].

Tetraarsenic oxide (As_4O_6; 2,4,6,8,9,10-Hexaoxa-1,3,5,7-tetraarsatricyclo[3.3.1.1^{3,7}]decane) is a trivalent arsenical compound with physical and chemical properties that are different from those of As_2O_3 [Park et al., 2003a,b]. The antiangiogenic properties of As_4O_6 in in vitro and in vivo studies were previously reported [Park et al., 2003b]. As_4O_6 inhibited the proliferation, invasion, and migration of basic fibroblast growth factor (bFGF)-stimulated bovine capillary endothelial cells (BCEC). It also inhibited bFGF-induced new vessel formation and lung metastasis of melanoma cells. Moreover, it potently induced the apoptotic cell death of As_2O_3-resistant U937 leukemic cells through generation of reactive oxygen species (ROS) [Park et al., 2003a]. On the basis of the anti-angiogenic activity of arsenical compounds reported by us as well as others, the molecular mechanism(s) of arsenical compound-induced cell cycle arrest of human umbilical vein endothelial cells (HUVEC) were investigated. We observed that HUVEC displayed cell cycle arrest by As_4O_6 or As_2O_3 at the G_1 and G_2/M phase. This occurred through the degradation of cyclin A, CDC2, and CDC25C, which led to the inactivation of CDK2 and CDC2 (also called CDK1) activities. In partly G_1-arrested HUVEC by removal of endothelial cell growth supplement (ECGS) and serum, As_4O_6 and As_2O_3 effectively inhibited the cell cycle progression that was stimulated by bFGF or vascular endothelial growth factor (VEGF). The results of this study provide an alternative therapeutic means for the treatment of tumor-associated angiogenesis by inducing endothelial cell cycle arrest.

MATERIALS AND METHODS

Cell Culture and Reagents

HUVEC (obtained from ATCC, Rockville, MA) were maintained in medium 199 containing 20% fetal bovine serum (Hyclone, South Logan, UT), 2 mM L-glutamine, 100 μg/ml of heparin (Sigma, St. Louis, MO), and 30 μg/ml of bovine ECGS (Sigma) under 5% CO_2 in a humidified incubator at 37°C. As_4O_6 (more than 99.9% purity; Chonjisan Institute, Seoul, Korea) was dissolved at 5×10^{-2} M concentration in 1N NaOH as a stock solution. The concentration of 1N NaOH in the culture medium had no influence on the growth of endothelial cells. Recombinant human bFGF and VEGF were purchased from R&D System (Minneapolis, MN).

Cell Growth and Cell Cycle Analysis

For cell growth analysis, cells were collected by trypsin treatment, mixed with an equal volume of PBS containing 0.4% trypan blue dye, and manually counted. For cell cycle analysis, cells were grown in 60 mm culture dish in the absence or presence of arsenical compound, trypsinized, and resuspended in PBS. Cell cycle distribution was determined by staining DNA with propidium iodide (PI), using CycleTest™ Plus (Becton Dickinson, San Jose, CA), as described in the manufacturer's protocol. The

DNA content was determined by using FACStar flow cytometer (Becton Dickinson).

Western Blot Analysis

Control and reagents-treated cells were collected, washed with cold PBS, and lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS] supplemented with protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). The lysates were clarified by centrifugation at $15{,}000 \times g$ for 15 min at 4°C. Protein content was measured through the Bradford method. An aliquot (30 or 50 μg of protein per lane) was resolved by SDS–PAGE and blotted to the nitrocellulose membrane. Immunoblotting was carried out, using the primary antibodies (antibodies against cyclin A, cyclin B1, cyclin D1, cyclin E, CDC2, CDK2, CDK4, CDK6, $p16^{Ink4a}$, $p21^{Cip1}$, $p27^{Kip1}$, CDC25A, CDC25B, CDC25C, and β-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-T [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20], and then with the secondary antibody of HRP-linked anti-rabbit or anti-mouse IgG by using Amersham (Piscataway, NJ) ECL systems, as described in the literature [Park et al., 2000].

Evaluation of Apoptosis

Apoptosis was evaluated by staining cells with annexin V-FITC and PI (PharMingen, San Diego, CA). To quantitate apoptosis, prepared cells were washed with cold PBS and resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] at a concentration of 1×10^6 cells/ml. Then, 5 μl of annexin V-FITC and 10 μl of PI were added to these cells, and were analyzed with FACScan flow cytometer (Becton Dickinson).

Kinase Reaction Assay

The cells were lysed in TNN buffer [40 mM Tris · Cl (pH 8.0), 120 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, 0.2 mM $NaVO_4$, 25 μg/ml aprotinin, 25 μg/ml leupeptin]. Equal amounts of protein were immunoprecipitated with anti-CDC2, anti-CDK2, anti-CDK4, and anti-CDK6 polyclonal antibodies (Santa Cruz Biotechnology) [Park et al., 2000]. The beads were washed three times in a TNN buffer and were again washed three times in a kinase buffer [10 mM Tris (pH 7.5), 2 mM $MgCl_2$]. The kinase reactions were carried out at 37°C for 30 min in 20 μl of a kinase reaction mixture containing 0.1 mM $NaVO_4$, 1 mM NaF, 20 μl ATP, 5 μCi [γ-^{32}P]ATP, and 2 μg of histone H1 as substrate. The addition of a 5× SDS sample buffer stopped the reaction. After boiling for 5 min, the reaction products were electrophoretically separated on a 12% SDS–PAGE gel. Phosphorylated proteins were detected by autoradiography.

RESULTS

Effect of As_2O_3 and As_4O_6 on HUVEC Growth

The effects of As_2O_3 and As_4O_6 on the proliferation of HUVEC were examined using trypan blue exclusion assay. Significant dose-dependent inhibition of cell growth was observed following the treatment with As_2O_3 or As_4O_6 for 3 to 4.5 days (Fig. 1A,B). As_2O_3 at 1–2 μM concentrations exhibited more than 70% inhibition of growth (Fig. 1A). On the other hand, the number of viable cells after treatment with 2.5 μM As_2O_3 decreased to less than the number of initial plated cells, possibly due to its cytotoxic effect as well as inhibition of growth. The growth inhibition was also observed in As_4O_6-treated cells. A higher cytotoxicity was shown in the cells treated with 2.5 μM As_4O_6 compared with As_2O_3-treated cells (Fig. 1B). Both arsenical compounds dose-dependently inhibited the proliferation of HUVEC, demonstrating that they are very potent inhibitors of HUVEC proliferation in vitro.

Effects of As_2O_3 and As_4O_6 on Apoptosis and Cell Cycle in HUVEC

It is possible that either arsenical compound-induced apoptosis or cell cycle arrest is responsible for the inhibition of cell growth of HUVEC. Thus, it was first examined whether apoptotic cell death was responsible for As_2O_3- or As_4O_6-mediated growth inhibition of HUVEC, by treating HUVEC with either 2 μM As_2O_3 or As_4O_6 for 48 h and assessing the induction of apoptosis after staining with PI and annexin V-FITC. As shown in Figure 2A, the fraction of apoptotic cells was not significant in HUVEC that was treated with arsenical compounds for 48 h. Moreover, the arsenical compounds had no detectable effect on apoptotic cell death even after 72 h following treatment with 0.5–2 μM (Fig. 2B). Since PI is generally used to detect necrotic cells in culture for its membrane-

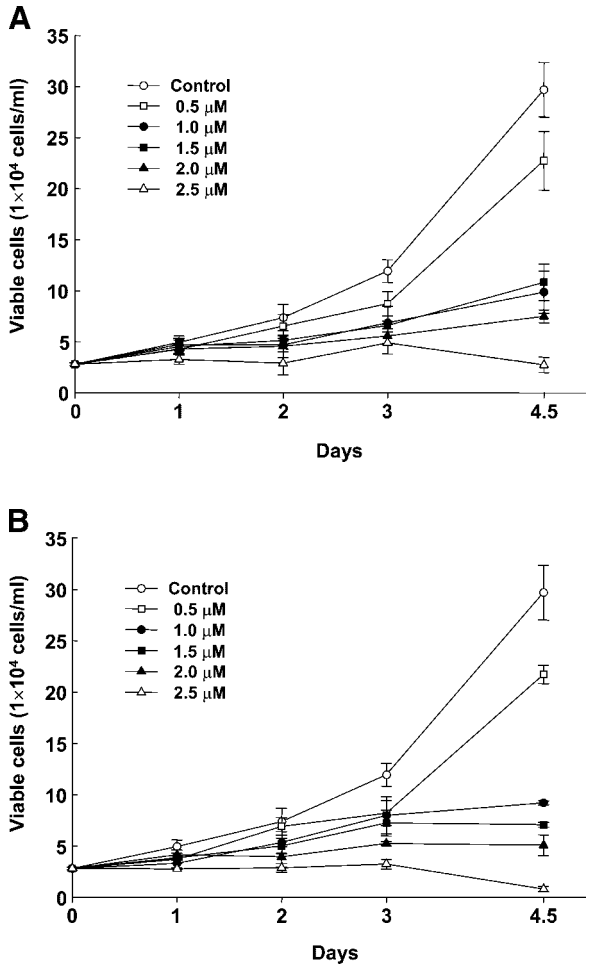

Fig. 1. Effects of arsenical compounds on the growth of HUVEC. HUVEC were exposed to various concentrations of As_2O_3 (**A**) or As_4O_6 (**B**) for different periods of time and cell numbers were determined. The results represent means of at least three independent experiments; the bar denotes standard deviation (SD).

impermeant property [Unal Cevik and Dalkara, 2003], the absence of an increase of PI positive cells suggests no necrotic cell death occurring in 2 μM As_2O_3 or As_4O_6-treated HUVEC. Next, it was examined whether the arsenical compounds would induce cell cycle arrest of HUVEC. Asynchronous HUVEC were treated with 0.5–2 μM As_2O_3 or As_4O_6 for 48 h, and cell cycle profiles were monitored by flow cytometric analysis of DNA content. Figure 3 shows that, compared with untreated control HUVEC, the percentages of cells in the G_1 and G_2/M phases increased after treatment with arsenical compounds, accompanied by the concomitant reduction of cells at the S phase of the cell cycle. Although the increase in the number of cells in the G_1 and G_2/M phases and the marked reduction of S-phase fraction by As_2O_3 were dose-dependent, the increase in cells in the G_1 phase reached a maximum after incubation with 1 μM As_4O_6 concentration. In addition, the accumulation of G_2/M phase cells was prominent in cells treated with a higher dose of As_4O_6 (1.5 and 2 μM). Taken together, these results indicate that the arsenical compounds inhibit the proliferation of HUVEC through the G_1 and G_2/M phase arrest of the cell cycle.

Effects of As_2O_3 and As_4O_6 on the Expression of Cell Cycle Regulatory Proteins in HUVEC

To further demonstrate the effects of the arsenical compounds on HUVEC, we investigated the expression of the genes involved in the regulation of cell cycle progression and transition through restriction checkpoints. First, the expression levels of cyclins and CDKs were examined through Western blot analysis. The antibody against cyclin A used in this study also possessed the ability to recognize cyclin A1, a related cyclin sharing 48% sequence identity with cyclin A, therefore, its band appeared right above the cyclin A band in Western blot analysis. As shown in Figure 4, the treatment of HUVEC with As_2O_3 for 48 h resulted in the down-regulation of the cyclin A protein level in a dose-dependent manner. Similarly, a reduction of cyclin A expression in As_4O_6-treated HUVEC was observed, although it showed no dose-dependency. The lower dose of As_4O_6, compared with that of As_2O_3, potently inhibited the expression of cyclin A. On the other hand, there was no significant change in the protein levels of cyclin B1, cyclin D1, cyclin E, CDK2, CDK4, and CDK6, except that CDC2 protein was prominently down-regulated in HUVEC after exposure to 2 μM As_4O_6—CDC2 protein plays an important role in the S phase and the G_2/M progression of the cell cycle [Lee and Nurse, 1988; Takizawa and Morgan, 2000]. With regard to cyclin-dependent kinase inhibitors (CKIs), $p27^{Kip1}$ and $p16^{Ink4a}$, no detectable increments were observed after the As_2O_3 and As_4O_6 treatments, whereas the expression of $p21^{Cip1}$, another CKI, was slightly up-regulated. Although $p16^{Ink4a}$ was down-regulated in HUVEC after treatment with 1.5 and 2 μM As_4O_6 in the present study, there has been no study to suggest that the reduction of $p16^{Ink4a}$ expression was directly related to cell cycle arrest.

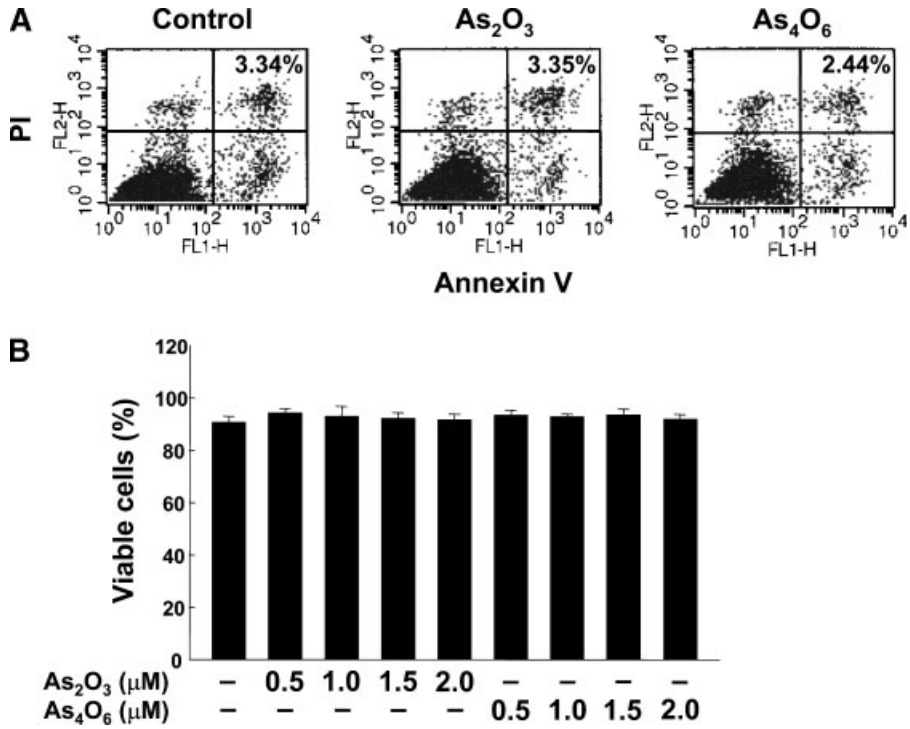

Fig. 2. Effects of arsenical compounds on apoptotic cell death in HUVEC. HUVEC were treated for 48 h with 2 μM arsenical compounds (**A**) or for 72 h with the indicated concentrations of arsenical compounds (**B**). For the detection of apoptotic cell death, prepared cells were stained with annexin V-FITC and PI. Apoptotic (positive for Annexin V-FITC) cells are shown in the second and forth quadrants of the plots (A), and the fractions of the cells that stain negative for both annexin V-FITC and PI are represented in the vertical bar chart (B). The results represent means of at least three independent experiments. No statistical differences were shown in the paired Student's *t*-test; the error bars represent SDs.

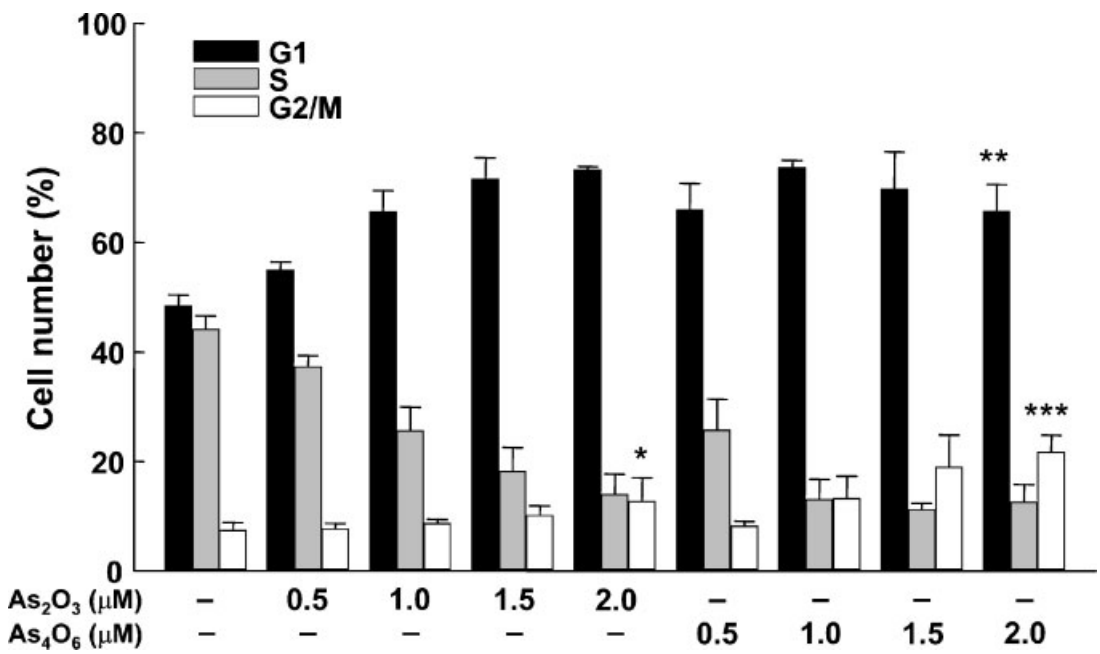

Fig. 3. Effects of arsenical compounds on cell cycle in HUVEC. Subconfluent (70–80%) and asynchronous HUVEC were incubated for 48 h with the indicated concentrations of arsenical compounds. Prepared cells were stained with PI and analyzed by flow cytometry. The results are expressed as mean ± SD of three independent experiments (single, double, and triple asterisks, $P < 0.05$, significantly different from untreated).

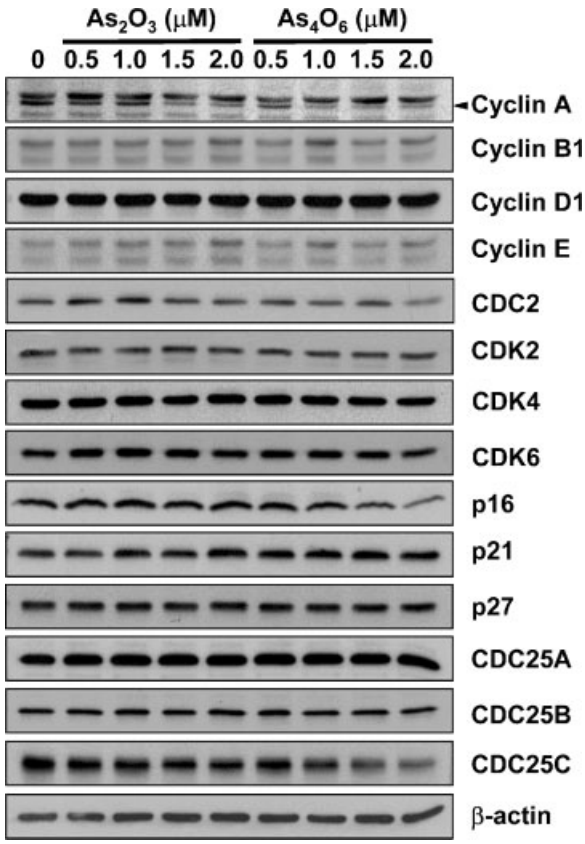

Fig. 4. Effects of arsenical compounds on cell cycle-related proteins in HUVEC. HUVEC were harvested at 48 h after incubation with the indicated concentrations of arsenical compounds. Cells were then lysed and the supernatants were subjected to Western blot analysis. β-actin was reprobed to indicate evenness of loading of protein extract from each treatment. Each blot is representative of three similar experiments.

The activation of CDKs requires dephosphorylation of Thr14 and Tyr15 by CDC25 phosphatases [Nilsson and Hoffmann, 2000]. In mammals, the CDC25 family includes three homologs—CDC25A, B, and C [Sadhu et al., 1990]. CDC25A induces S-phase entry through the activation of cyclin E(A)–CDK2 complexes. On the other hand, CDC25B and C are regarded as mitotic regulators, which induce the activation of mitosis-promoting CDKs, including cyclin B-CDC2. Interestingly, although no changes were observed in the expression of CDC25A and B proteins (Fig. 4), the expression of CDC25C in the As_4O_6-treated cells was markedly reduced in a dose-dependent manner. HUVEC exposed to As_2O_3 displayed only a slight decrease in its CDC25C level, as compared with As_4O_6-treated cells. Collectively, therefore, these results suggest that the down-regulation of CDC2 and CDC25C, as well as of cyclin A, which was also down-regulated by As_2O_3, may play a key role in G_1 and G_2/M phase arrests in As_4O_6-treated HUVEC.

Regulatory Effect of As_2O_3 and As_4O_6 on CDK-Associated Kinase Activity

To determine whether the reduced level of proteins, which are involved in cell cycle regulation, resulted in the inhibition of CDK activity in As_2O_3- and As_4O_6-treated HUVEC, in vitro CDK activity assay was performed with histone H1 as substrate in immunoprecipitates with anti-CDC2, -CDK2, -CDK4, and -CDK6 antibodies. As shown in Figure 5, the treatment of cells with 2 μM arsenical compounds for 48 h dramatically reduced CDC2- and CDK2-associated kinase activity assayed with the histone H1 substrate, compared with untreated control cells. There was no significant difference, however, in CDK4- and CDK6-associated histone H1 kinase activity between the arsenical compound-treated and untreated cells. The decrease in CDC2-associated kinase activity was more prominent in As_4O_6-treated cells than with As_2O_3-treated cells, being consistent with the down-regulation of the CDC25C protein, shown in the Western blot analysis (Fig. 4). Taken together, these results suggest that arsenical

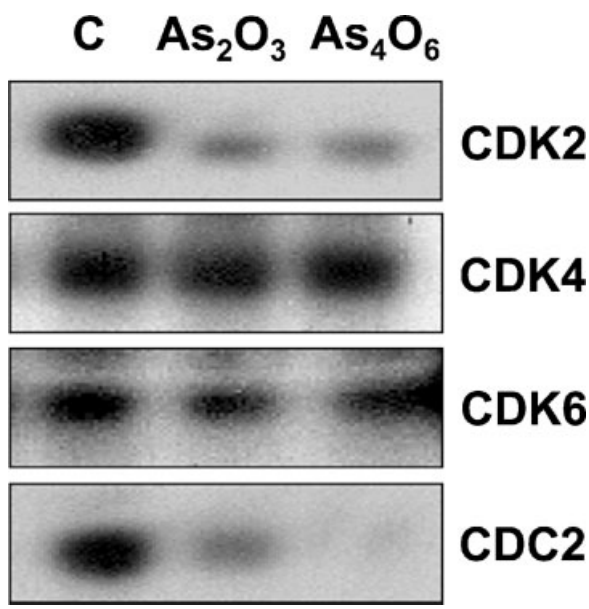

Fig. 5. CDK-associated histone H1 kinase activities. HUVEC were treated with or without 2 μM arsenical compounds for 48 h. Total protein lysates were prepared, and CDC2, CDK2, CDK4, and CDK6 kinase activities were determined using histone H1 kinase assay. C, control.

compounds may induce G_1 and G_2/M phase arrest by reducing CDK2- and CDC2-associated kinase activity.

Effects of As_2O_3 and As_4O_6 on Cell Cycle Progression Induced by VEGF and bFGF

In the recent in vitro and in vivo studies of the researchers, it was demonstrated that As_4O_6 exerted a potent inhibitory action on the angiogenesis of bFGF-stimulated BCE cells [Park et al., 2003b]. Since endothelial cell proliferation is critical for angiogenesis in response to mitogenic stimuli, such as VEGF and bFGF [Cross and Claesson-Welsh, 2001], the ability of arsenical compounds to inhibit VEGF- or bFGF-induced cell cycle progression of HUVEC was assessed. To verify the mitogenic response of VEGF and bFGF, ECGS was omitted from the complete growth medium and serum was reduced to 5%, the cells were cultured for 12 h to partially synchronize HUVEC. As seen in Figure 6, the flow cytometric analyses of cell cycle distribution showed that 62% of the cells were in the G_1, 16% in the S, and 16% in the G_2/M phase. Upon VEGF or bFGF stimulation, the cells entered the S phase, and the percentages of the S phase were 24% and 30%, respectively. Treatment with 2 μM As_4O_6 concurrent with VEGF or bFGF stimulation decreased the S phase population to the unstimulated level, although As_2O_3 exhibited the effect only in the VEGF-stimulated cells, demonstrating inhibitory potency of the arsenical compounds on VEGF- or bFGF-stimulated cell cycle progression from the G_1 to the S phase.

Inhibitory Effects of As_2O_3 and As_4O_6 on Growth Factor-Dependent Signaling

The MAPK and PI3K pathways have been shown to contribute to the mitogenic and survival responses of HUVEC to growth factors, such as VEGF and bFGF [Gerber et al., 1998; Wu et al., 2000]. To examine the effects of arsenical compounds on the VEGF- or bFGF-stimulated activation of MAPK (ERK1 and ERK2) and Akt, the downstream effector of PI3K, HUVEC were preincubated in 5% serum for 12 h in the absence of ECGS, and then treated with VEGF or bFGF. As expected, Akt phosphorylation was

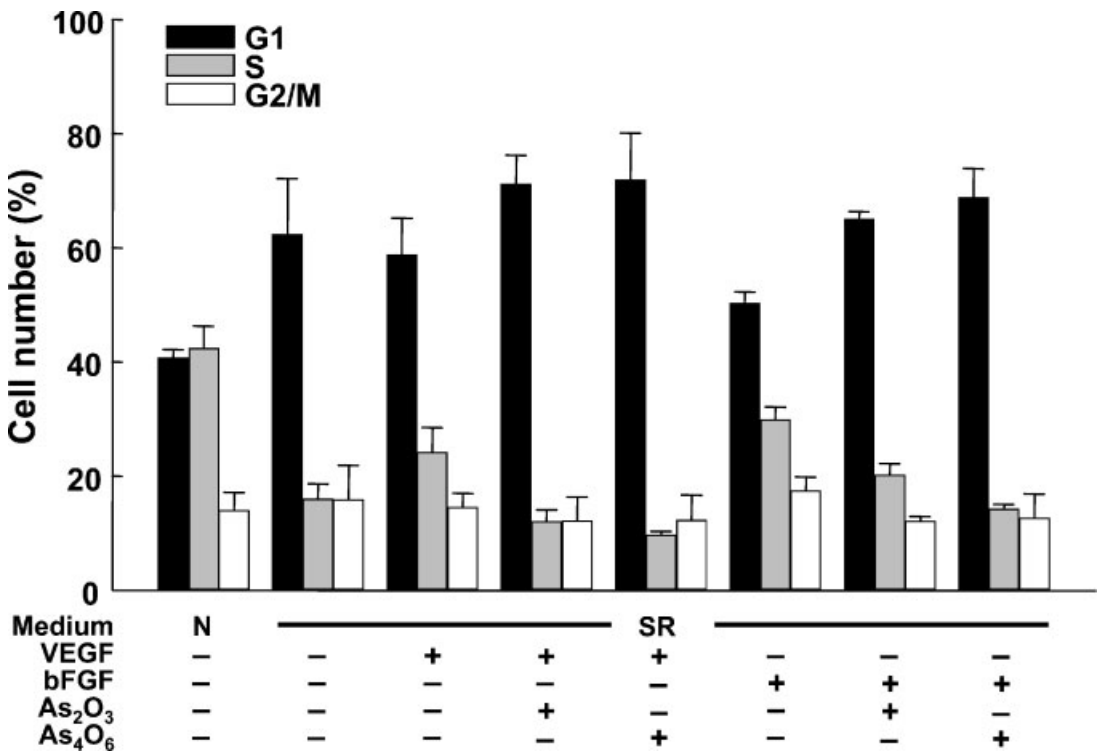

Fig. 6. Effects of arsenical compounds on VEGF- or bFGF-stimulated progression of the G_1 into the S phase in HUVEC. HUVEC were cultured in a complete culture medium (N) or a serum-reduced medium supplemented only with 5% serum (SR) for 12 h and then treated in a medium containing VEGF (10 ng/ml) or bFGF (10 ng/ml), with or without 2 μM arsenical compounds, for 24 h. Prepared cells were stained with PI and analyzed using flow cytometry.

observed after 24 h of exposure to 10 ng/ml VEGF or bFGF. Interestingly, bFGF induced a more profound phosphorylation of ERK1 and ERK2 than VEGF, indicating that bFGF is a much stronger mitogen for HUVEC than VEGF is in vitro (Fig. 7). Exposure of HUVEC to 2 μM As_4O_6 inhibited the VEGF- or bFGF-induced phosphorylation with both Akt and ERK1/2, whereas As_2O_3 was effective only in VEGF- or bFGF-stimulated ERK1/2 phosphorylation. These data indicate that As_4O_6 has a more potent antimitogenic activity than As_2O_3 has, probably through the inactivation of Akt and ERK activity induced by VEGF- or bFGF-stimulation.

DISCUSSION

During the last decade, As_2O_3 has been well established as an effective tool in treating patients with APL through the induction of apoptosis of malignant cells [Shen et al., 1997; Soignet et al., 1998]. It also inhibits proliferation and induces apoptosis of different types of solid tumors, including carcinoma, myeloma, and neuroblastoma. Various mechanisms have been known to influence numerous signal transduction pathways, resulting in a vast range of cellular effects, including apoptosis induction, growth inhibition, and promotion or inhibition of differentiation [Seol et al., 1999; Ora et al., 2000; Park et al., 2000; Maeda et al., 2001; Miller et al., 2002; Woo et al., 2002; Hyun Park et al., 2003]. Little attention has been paid, however, to the effects on normal cell proliferation, particularly in vascular endothelial cells, whose proliferation is an essential component of the angiogenic response.

In this study, it was shown that 1–2 μM of As_2O_3 (which is clinically achievable and does not cause severe side effects) inhibited the proliferation of HUVEC [Shen et al., 1997; Agis et al., 1999]. It was recently shown that As_4O_6 significantly decreased proliferation, migration, invasion, and tube formation of BCEC induced by the angiogenic factors bFGF [Park et al., 2003b]. It was also reported that As_4O_6 inhibited angiogenesis in the rat corneal micropocket model and lung metastasis in the mouse model in vivo. As shown in Figure 1B, 1–2 μM of As_4O_6 potently inhibited the growth of HUVEC in a dose-dependent manner. Although it was previously reported that As_2O_3 significantly decreased the cell proliferation of HUVEC through induction of apoptosis, no significant increment of apoptosis was observed in the As_2O_3- and As_4O_6-treated HUVEC [Roboz et al., 2000]. This suggests that arsenical compounds at clinically achievable concentration may not cause severe cytotoxicity of HUVEC. This

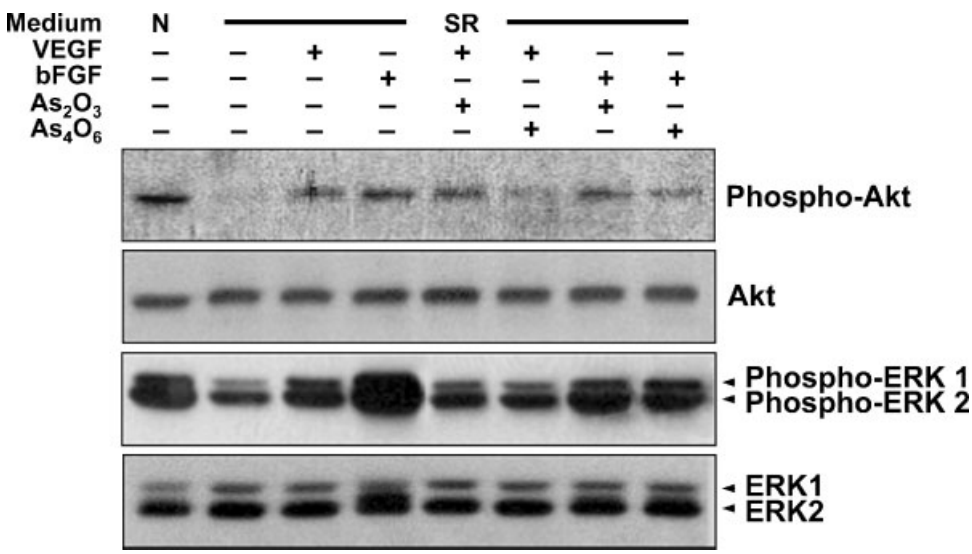

Fig. 7. Effects of arsenical compounds on activation of Akt and ERK in VEGF- or bFGF-stimulated HUVEC. HUVEC were cultured in a normal culture medium (N) described in ''Materials and Methods,'' or in a serum-reduced medium supplemented only with 5% serum (SR) for 12 h, and were then treated in a medium containing VEGF (10 ng/ml) or bFGF (10 ng/ml), with or without 2 μM arsenical compounds, for 24 h. The total cell lysate (30 μg) was analyzed by immunoblotting with specific antibodies for Akt, p-Akt, ERK, and p-ERK.

contradicting result may have been due to differences in cell culture and drug treatment conditions.

The cell cycle analysis that was performed in this study revealed that arsenical compounds were able to induce G_1 and G_2/M phase arrest of HUVEC. These results are consistent with those of other studies, which showed that the antiproliferative action of arsenical compound in several cancer cell lines was linked to G_1 and/or G_2/M phase arrest [Zhang et al., 1998; Seol et al., 1999; Park et al., 2000, 2001]. Furthermore, our recent study on BCEC showed that As_4O_6 specifically induced cell cycle arrest of bFGF-stimulated BCEC at the G_2/M phase [Park et al., 2003b]. Interestingly, the G_1 phase arrest was more prominent than the G_2/M phase arrest in As_2O_3-treated HUVEC. On the other hand, the G_1 phase arrest in As_4O_6-treated cells peaked at lower concentrations (0.5–1.0 μM) and the G_2/M phase arrest was dominant at higher concentrations (1.5–2.0 μM), implying that As_4O_6 strongly regulates the activity of cell-cycle components which are involved in the progression from the G_2 to the M phase.

The G_1/S transition is one of the most critical steps in determining cell cycle progression in mammalian cells and is, therefore, precisely regulated by the concerted actions of cyclins, CDKs, and CKIs. Cyclins and CDKs form active kinase complexes that promote cell cycle progression. For example, early in the G_1 phase, CDK4 and CDK6 assemble into holoenzymes with D-type cyclins of which expression is induced as a delayed response to mitogens. As the cell cycle progresses, the G_1 kinase CDK2 associates with another cyclin, cyclin E. Once the cell enters the S phase, cyclin E is degraded while CDK2 combines with cyclin A [Pardee, 1989; Pagano et al., 1992; Sherr, 1994]. Among the cyclins, cyclin A is of particular interest, because it is associated with both CDC2 and CDK2 and functions in both the S phase and mitosis [Pagano et al., 1992]. Recent studies suggest that the cyclin A/CDC2 complex may function before cyclin B does and may control the half-life of cyclin B [Roy et al., 1991; Pagano et al., 1992; Lukas et al., 1999]. In the present study, it was found that the cyclin A protein expression was reduced in a dose-dependent manner in both As_2O_3- and As_4O_6-treated HUVEC, although those of CDK2, CDK4, CDK6, cyclin B1, cyclin D1, and cyclin E were not. Thus, it is highly likely that the decrease in the cyclin A level may be related to the down-regulation of the CDK2 and CDC2 kinase activities, which leads to the underphosphorylation of the Rb protein in As_2O_3- or As_4O_6-treated HUVEC (data not shown).

When cells progress from the G_2 to the M phase, at least two events must occur in order to prompt the activation of mitotic CDC2 kinase. CDC2 has to bind with an appropriate B-type cyclin, and has to be dephosphorylated by CDC25 phosphatases. The latter is required, since CDC2 is kept inactive by phosphorylation on the Thr14 and Tyr15 residues by the protein kinases Wee1 and Myt1 [Lee and Nurse, 1988; Igarashi et al., 1991; Takizawa and Morgan, 2000]. CDC25B, as a starter phosphatase, triggers the initial activation of mitosis-promoting CDKs, including cyclin B-CDC2, which in turn activates CDC25C and creates a positive amplification loop required for irreversible commitment to mitosis [Nilsson and Hoffmann, 2000]. Treatment of HUVEC with different concentrations of As_4O_6 for 48 h led to a marked dose-dependent decrease in CDC25C. In addition, the expression of the CDC2 protein was also reduced after 2 μM As_4O_6 treatment. In contrast, however, the same concentrations of As_2O_3 had a mild down-regulating effect on CDC25C expression. These differences between As_2O_3 and As_4O_6 may explain the higher extent of the G_2/M phase arrest induced by As_4O_6 than that induced by As_2O_3.

The up-regulation of CKI proteins also inhibits cyclin–CDK complexes and causes cell cycle arrest [Sherr and Roberts, 1995]. As shown in Figure 4, however, neither As_2O_3 nor As_4O_6 were able to increase the expression of $p27^{Kip1}$ and $p16^{Ink4a}$. Although several studies have shown that the expression of $p21^{Cip1}$ prominently increased after the exposure of tumor cells to As_2O_3 [Seol et al., 1999; Park et al., 2000; Hyun Park et al., 2003], only a slight increase of $p21^{Cip1}$ expression was observed in As_2O_3- or As_4O_6-treated HUVEC. Furthermore, $p27^{Kip1}$ and $p21^{Cip1}$ in the complexes immunoprecipitated with CDK2 or CDC2 did not increase (data not shown). Thus, it is quite likely that CKIs may not play a significant role in the cell cycle arrest of HUVEC induced by arsenical compounds.

Abnormal angiogenesis is an important prerequisite for abnormal cell growth in a series of human diseases, especially in cancer [Folkman,

1995a,b]. Therefore, the inhibition of angiogenic factor-driven mitogenesis is critical in achieving selective antiangiogenic action, as vascular endothelial cells in normal adult tissues are essentially quiescent. In HUVEC that are partly synchronized by ECGS-depletion and serum-reduction, arsenical compounds were found to inhibit bFGF- or VEGF-stimulated HUVEC cell cycle progression into the S phase, measured by flow cytometry. The serine/threonine protein kinase Akt is a downstream effector of PI3K that is activated by various growth factors, including those known to induce angiogenesis such as VEGF and bFGF [Gerber et al., 1998]. Therefore, it is highly probable that a better inhibitory potency of As_4O_6 may be due to the reduction in the level of phospho-Akt as well as those of ERK1/2.

In summary, the data described herein strongly support the idea that a potent anti-mitogenic action of clinically achievable levels of arsenical compounds is possible by a mechanism, targeting to control G_1-S and G_2-M transitions of the HUVEC cell cycle. Recent report indicates that up to 20 μM of As_2O_3 does not significantly affect cell viability in cultured human fibroblasts [Burnichon et al., 2003], and we also confirmed no effects of 2 μM As_2O_3 or As_4O_6 treatment on cell viability of human dermal fibroblast (data not shown). Therefore, it is highly unlikely that arsenical compounds would have nonspecific antimitogenic activity on normal cells. Our findings suggest that arsenical compounds might be good therapeutic agents not only for tumor cell cytotoxicity, but also for the inhibition of angiogenesis of endothelial cells.

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