Environmentally Relevant Concentration of Arsenic Trioxide and Humic Acid Promoted Tumor Progression of Human Cervical Cancer Cells: *In Vivo* and *In Vitro* Studies

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ABSTRACT: In a previous study, treatment at higher concentrations of arsenic trioxide or co-exposure to arsenic trioxide and humic acid was found to be inhibited cell growth of cervical cancer cells (SiHa cells) by reactive oxygen species generation. However, treatment at lower concentrations slightly increased cell viability. Here, we investigate the enhancement of progression effects of environmentally relevant concentration of humic acid and arsenic trioxide in SiHa cell lines *in vitro* and *in vivo* by measuring cell proliferation, migration, invasion, and the carcinogenesis-related protein (MMP-2, MMP-9, and VEGF-A) expressions. SiHa cells treated with low concentrations of humic acid and arsenic trioxide alone or in co-exposure significantly increased reactive oxygen species, glutathione levels, cell proliferation, scratch wound-healing activities, migration abilities, and MMP-2 expression as compared to the untreated control. *In vivo* the tumor volume of either single drug (humic acid or arsenic trioxide) or combined drug-treated group was significantly larger than that of the control for an additional 45 days after tumor cell injection on the back of NOD/SCID mice. Levels of MMP-2, MMP-9, and VEGF-A, also significantly increased compared to the control. Histopathologic effects of all tumor cells appeared round in cell shape with high mitosis, focal hyperkeratosis and epidermal hyperplasia in the skin, and some tumor growth in the muscle were observed. Our results may indicate that exposure to low concentrations of arsenic

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INTRODUCTION

Dietary exposures to total arsenic were highly variable, with a mean of 50.6 (females) and 58.5 (males) μg/day, and a range of 0.21 to 1276 μg/day (ATSDR, 2007). In addition, epidemiological and geochemical studies revealed the presence of a high concentration of arsenic (0.1–1.8 ppm; maximum of 200 to 3600 μg/day in 2 L drinking water) and humic acid (HA) (approximately 200 ppm; maximum of 400 mg/day in 2 L drinking water) in artesian well water within the Blackfoot disease (BFD) endemic area (southwest coast of Taiwan), and there are also high incidences of cancer (skin, lung, liver, and bladder), and cardiovascular anomalies, hypertension, and diabetes mellitus in these regions (Hseu et al., 2008; Lu, 1990)

Arsenic is a metallic element that is present in food, soil, water, and drug from both natural and man-made sources (Chakraborti et al. 2004). For example, arsenic is used in metallurgy for hardening alloys of copper and lead, as a dope in semiconductor production, in the manufacturing of pigments, glass, and in organic pesticides (Hall, 2002). Exposure of humans to arsenic occurs by both inhalation and consumption of contaminated water and food, which may be a hazard to human health (Florea and Busselberg, 2008). It has been shown that lower, environmentally relevant doses of arsenic trioxide (ATO) are in fact pro-angiogenic and tumorigenic (Bishop and Kipling, 1978). Chronic exposure to arsenic increases the risk of skin, liver, lung, and bladder cancer, and it is a human carcinogen (Bashir et al., 2006; Chiou et al., 2001; Smith et al., 1998; Steinmaus et al., 2000). However, the effects of arsenic at low doses are not well understood. Many studies have failed to show a dose–response relationship between incidence rates of various cancers and exposure to low levels (10 μg/L) of inorganic arsenic (Josyula et al., 2006; Lamm et al., 2004; Moore et al., 2002). On the other hand, Florea and Busselberg (2008) reported that ATO had cell type specific cytotoxic effects on neuroblastoma cells within the environmentally and clinically relevant concentrations (100 pM–1 μM).

Angiogenesis, the formation of new blood vessels from established vessels, occurs under a variety of normal and pathological conductions (Di Benedetto et al., 2003). The delivery of blood-borne nutrients to the tumor cells is essential for their survival and spread. The induction of angiogenesis was observed to precede the development of invasive tumor. One of the most specific and critical regulators of angiogenesis is vascular endothelial growth factor (VEGF), which regulates endothelial proliferation, permeability, and survival (Ramanujan et al., 2000; Weidner et al., 1991). On the other hand, tumor-secreted matrix metalloproteinases (MMPs) destroy extracellular matrix components in the surrounding tissues of the tumor, and subsequently, tumor cells invade through the basement membrane of blood vessels and facilitate the spread to distant organs, resulting in organ failure and patient mortality (Johnsen et al., 1998).

Exposure to humic acid damages tissues and cells and is involved in goiter and cancer and has been implicated as a causal factor of Blackfoot disease (Lu, 1990; Yang et al., 2002). HA is a group of high-molecular-weight polymers that come from the decomposition of organic matter, in particular dead plants. HA exists abundantly in peat, soil, well water, and other sources (Hartenstein 1981). A hypothetical structure for HA has been proposed as follows: a polymer that is composed of aromatic rings of the di- and trihydroxybenzene type that are bridged by ether, methylene, amine, imine, and other linkages (Cheng et al., 2003). HA has been shown to generate reactive oxygen species (ROS), such as superoxide anion (Ho et al., 2003), and causes a depletion of glutathione and several antioxidant enzymes (Cheng et al., 1999). ROS, known to damage DNA, proteins, and lipids, may cause human disorders. HA produces genotoxicity in intestinal cells after administration of a single oral dose (100 mg/Kg BW) to mice (Bernacchi et al., 1996), and human peripheral blood lymphocytes (Hseu et al., 2008). In addition, low-concentration HA was observed to promote neoplastic transformation of mouse epidermal JB6 cells (Lu et al., 2006) and to enhance the progression of lung cancer A549 cells *in vitro* (Lee et al., 2009).

Cervical cancer was the second leading cause of cancer death among 20 to 39 year old women in 2006, with about 11,270 new cases and more than 4070 deaths estimated in the United States in 2009 (Jemal et al., 2009). In addition, cervical cancer was the seventh leading cause of death for females in Taiwan for 2012 (Ministry of Health and Welfare, Executive Yuan, R.O.C.). In our previous study, treatment at higher concentrations (1.0–100 μM) of ATO or co-exposure to ATO (1.0–100 μM) and HA (300 and 500 μg/mL) inhibited cell growth of cervical cancer cells by way of ROS generation, glutathione (GSH) depletion, and caspase 3 activation, and lower concentrations of ATO (< 1.0 μM) or HA (< 100 μg/mL) slightly increased cell viability (Ting et al., 2010). However, lower concentrations (≤ 500 μg/mL) of HA alone did not result in a decrease in cervical cancer cells viability (Ting et al., 2010). It is interesting that the toxicokinetic processes of different concentrations of HA and ATO co-exposure are more complicated than single exposure about which further understanding is required of the toxicity mechanism from future studies.

In the present study, we investigated the enhancement of progression effects of environmentally relevant concentrations of HA and ATO in human cervical cancer cell lines *in vitro* and *in vivo* by measuring cell proliferation, migration, invasion, and protein expression of MMP-2, MMP-9, and VEGF-A.

MATERIALS AND METHODS

Ethics Statement

The experimental animals of this study were handled according to the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMU), and the protocol was approved by IACUC, CSMU (Permit Number: 967). All surgery was performed under isoflurane-inhalant anesthesia, and all efforts were made to minimize suffering.

Chemicals

Fetal bovine serum, PBS (phosphate buffered saline), RPMI-1640 medium, and penicillin/streptomycin (PS) were obtained from Hyclone Co. (Logan VT, USA). Humic acid, Tris–HCl, gelatin, polyacrylamide, SDS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), QuantiPro^{TM} BCA kit, and 2′-7′-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Monochlorobimane (mBCL) was obtained from Molecular Probes, Inc. (Fingene, OR, USA). Primary antibodies of VEGF A (A-20), MMP-2 (H-76), MMP-9 (C-20), and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Delaware Avenue, CA, USA)

Purification of HA

Chromatographic and fluorescence data indicate that Sigma-Aldrich HA is similar to HA obtained from coral skeletons, sea water, river water, soil, and plant leaves (Susic and Boto, 1989). Therefore, all experiments were performed with the same batch of HA, which was obtained from Sigma-Aldrich Co.

HA was first dissolved in 1N NaOH solution (pH $>$ 10), and any undissolved material was removed by filtration. The solution was then acidified with 1N HCl to a pH $<$ 2.0 to precipitate the HA. Any precipitate formed was collected by centrifugation at 3000xg for 30 min and redissolved in 1N NaOH. The alkaline–acid treatment was repeated thrice to obtain the purest HA, as described by Schnitzer (Schulze et al., 1994). After the third round of acid precipitation, the precipitate was dissolved in 0.1N NaOH, and the pH of the resultant solution was adjusted to 7.2–7.4. The purified HA was then freeze-dried to a powder. The HA was stored as a dried powder and was dissolved in PBS (pH 7.4) or double-deionized (DI) water before the experiments.

Preparation of ATO

ATO was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). ATO was dissolved in double-distilled water, and a stock solution of 1.0 mM ATO was obtained. The cells treated with various concentrations of ATO in this study were daily prepared by diluting 1.0 mM stock with double-deionized water.

Cell Culture

Two different types of human cervical cancer cells, HeLa (CCL-2, human cervical epithelioid cell carcinoma) and SiHa cells (HTB-35, human cervical squamous cell carcinoma), were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 100 unit/mL of penicillin, and 100 unit/mL of streptomycin at 37°C in a humidified atmosphere of 5% CO2. The medium was exchanged every 2–3 days.

Assessment of Cell Proliferation

Cells (2×10^5 mL^{-1}) were seeded onto 24-well culture plates and then treated for 48 h with various concentrations of HA, ATO, or both. The cells were washed twice with PBS, and then, a new medium containing MTT (2 mg/mL) was added and incubated for another 4 h at 37°C. Cell growth was determined using the MTT assay (Denizot and Lang, 1986). The values of cell proliferation are expressed in relation to the absorption of untreated control cells that were considered to be 100%.

Intracellular ROS

The amount of intracellular ROS, especially H_2O_2 produced by drug treatment, was determined using DCFDA (Guo et al., 1998). SiHa cells were treated for 2 h with various concentrations of HA and ATO or both of them. After removing the supernatant, cells were washed twice with PBS, and then, a new medium containing 10 μM DCFDA was added and incubated for a further 20 min. Cells were lysed in 110 mM Tris and 0.25 M sucrose in 0.05% Triton X-100 solution (pH 7.5). The cytoplasmic fluorescence intensity was measured using a fluorescence spectrometer (Perkin Elmer LS-30, Perkin Elmer Inc., USA), with excitation and emission wavelengths of 405 nm and 520 nm, respectively.

Intracellular GSH Levels

After treatment for 4 h, the SiHa cells were washed with PBS, and then, a new medium containing 60 μM mBCL, specified to GSH, was added and incubated for a further

30 min. Cells were lysed in lysing buffer (110 mM Tris and 0.25 M sucrose in 0.05% Triton X-100, pH 7.5). The mBCL-GSH-related fluorescence in the cells was monitored using a fluorescence spectrometer (Perkin Elmer LS-30) with excitation and emission wavelengths of 385 nm and 485 nm, respectively (Chatterjee et al., 1999).

Wound-Healing Assay

SiHa cells were plated onto 6-well culture plates at about 5×10^5 cells per well and grown overnight in serum-containing medium. The medium was changed to serum-free medium and starved overnight, and then, cells were treated with various concentrations of HA and ATO or both of them for 24 h. After exposure, the culture was wounded by scratching the monolayer of cells with a p200 pipette tip, and then, the cells were washed twice with PBS to removed floating cells (Lu et al., 2006). A new serum-free medium was added into plates and incubated for another 48 h at 37°C. Cells migrating into the scratched areas were observed and counted at five random fields with phase contrast microscopy (Axiovert 200, Carl Zeiss Inc., Germany).

Preparation of Conditioned Medium and MMP-2 Activity Assay

After the SiHa cells were treated with various concentrations of HA and ATO separately or together in serum-free medium for 24 h, the supernatant of conditioned medium was collected and centrifuged at 3000 rpm, 4°C for 10 min and then kept frozen at −85°C until the MMP analysis. MMP-2 activity was quantified by using the fluorimetric SensoLyte MMP-2 assay kit (AnaSpec,Inc., San Jose, CA, USA) according to the manufacturer's instructions. The supernatant of conditioned mediums was incubated with 4-aminophenlmrcuric acetate for activating pro-MMP-2 followed initiating the enzyme reaction, and the activity of MMP-2 was assayed with a fluorescence microplate reader (FLx 800, Bio-Tek Instruments, Burlington, VT, USA) at excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Xenorafts in NOD/SCID Mice

Female NOD/SCID mice, 4–5 weeks of age, were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Animals were kept in a full ventilation environmental rodent housing systems in a temperature controlled room on a 12:12 light/dark schedule with food and water *ad libitum*. The animal study was conducted in accordance with the guidelines for the care and use of laboratory animals of the animal research committee of the Chung Shan Medical University. NOD/SCID mice were divided into the following groups (5 mice in each group, drugs dissolved in 0.5 mL saline were administrated by i.p. 3 times per week): (1) control group: saline; (2) HA group: 7.8 mg HA/Kg BW; (3) As H group: 0.3 mg ATO/kg BW; (4) As L group: 0.1 mg ATO/kg BW; (5) HA+As H group: 7.8 mg HA/kg BW and 0.3 mg ATO/kg BW; and (6) HA+As L group: 7.8 mg HA/kg BW and 0.1 mg ATO/kg BW. After 5 weeks of pretreatment, xenografts were produced by single cell inoculation of 1×10^6 cells/mL of SiHa cells in single cell suspension in 0.1 mL PBS into the backs of the mice. The drugs were persistently administrated 3 times a week for an additional 45 days after tumor cell injection. The volumes (cm^3) of tumors were estimated in accordance with the formula (tumor width^2 × tumor length)/2 after tumor development (Choi et al., 2006). In addition, tumors and surrounding skin and muscle were rapidly removed and used for histopathological examination. A portion of the tissue was fixed overnight in a 10% neural formaldehyde solution and embedded in paraffin. Tissue slices were subjected to hematoxylin and eosin staining and histological study using light microscopy. Slides were coded and examined blind by the pathologist for the histological alterations.

Western Blot Analysis

Fifty micrograms of protein of each tumor sample was subjected to electrophoresis on 10% SDS-polyacrylamide gels. The samples were then electroblotted on polyvinylidene difluoride membranes. After blocking, blots were incubated with primary antibodies of MMP-2, MMP-9, VEGF or α-tubulin (Santa Cruz Biotechnology) in PBS and 0.1% Tween 20 for 1 h followed by two washes (15 min each) in PBS and 0.1% Tween 20. Then, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amersham) were used to depict the protein bands on membranes.

Statistical Analysis

Student's t-test was used to determine the statistical significance between treatment groups. Differences were considered statistically significant at a value of $p < 0.05$.

RESULTS

The Promoted Cell Proliferation Effects of HA and ATO on Cancer Cells

Cell growth was determined by the MTT assay, and the values were expressed in relation to the absorption of control cells that were considered to be 100%. HA in concentrations ranging from 0–100 μg/mL with stepped increases was used to treat the HeLa and SiHa cells for 48 h. As shown in Figure 1(A), the HeLa or SiHa cells treated with lower concentrations of HA for 48 h slightly increased cell growth.

Figure 1(B) illustrates the cell proliferation effects of HA and ATO in the SiHa cells for 48 h. Treatments at lower concentrations of ATO alone (≤1 μM for 48 h) slightly increased

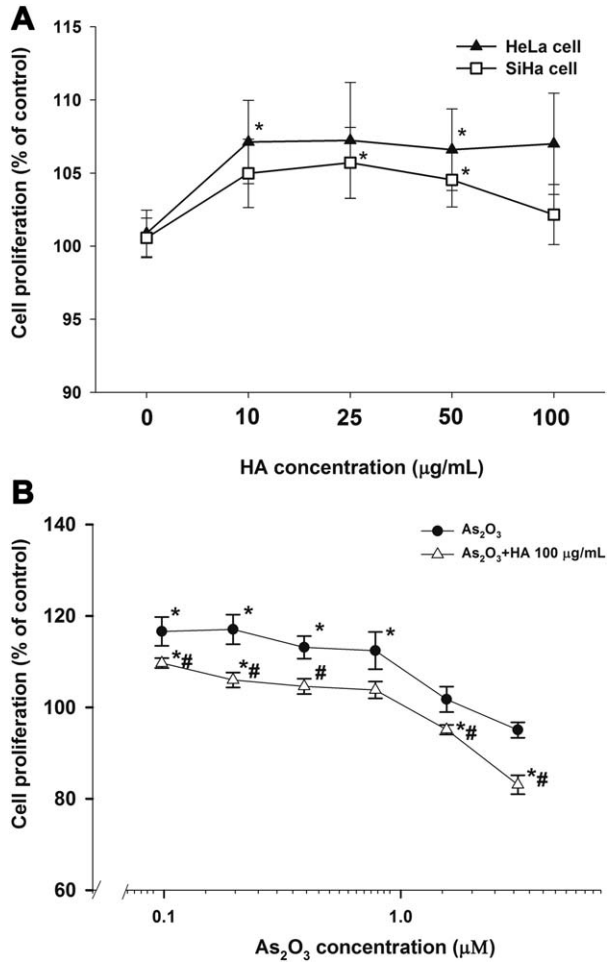

Fig. 1. Effects of HA or ATO on the cell proliferation of cervical cancer cells. (A) The cells were exposed to different concentrations of HA for 48 h. Cell growth was determined by the MTT assay, as described in the Materials and Methods. The values (%) are expressed in relation to the untreated control cells. (B) SiHa cells were exposed to the different concentrations of ATO in the presence or absence of HA (100 ug/mL) for 48 h. The values (%) are expressed in relation to untreated control cells. Data are presented as the mean ± SEM ($n = 6$–10). *$P < 0.05$ as compared with control group; #$P < 0.05$ as compared with ATO group.

cell growth by about 12–17% ($p < 0.05$), but the higher concentrations did not induce the cell proliferation. After 48-h treatment, lower concentrations of ATO (≤1 μM) and HA (100 μg/mL) co-exposure conferred no cytoxicity to SiHa cells and show the inhibitory effects on ATO alone-induced cell growth. Based on our observations, lower concentrations of single exposure of HA or ATO alone could promote cell proliferation more effectively than their co-exposure.

Intracellular ROS and GSH Levels

Because the change of ROS or GSH levels are early effects, we observed the cells exposure to lower concentrations of HA, ATO or both compounds after 2 or 4 h. Exposure to lower concentrations of drugs for 2 h could slightly induce oxidative stress in the cells, and there was dose dependence in the treatment of single drug. In addition, HA can enhance the ROS production effects of ATO [$p < 0.05$; Fig. 2(A)]. GSH concentrations also increased in the SiHa cells after exposure to HA, ATO, or both of them for 4h, and there was dose dependence in the treatment of single drug [$p < 0.05$; Fig. 2(B)]. Based on these observations, HA and ATO or both of them elevated the oxidative stress and induced GSH synthesis in the SiHa cells. After exposure to different concentrations of HA and ATO in the medium for 4 h, the cellular GSH content rose to 1.05–1.14-fold of control levels, especially when lower concentrations of ATO were

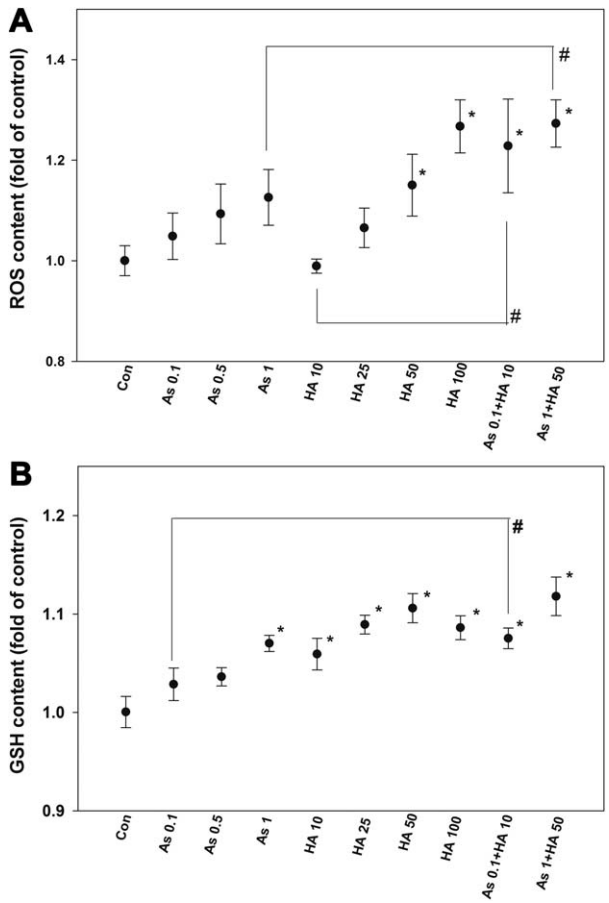

Fig. 2. Effect of HA and ATO on the intracellular oxidative property and GSH content of SiHa cells. (A) The cells were exposed to different concentrations of HA (5–100 μg/mL), ATO (0.1–1 μM), or both, for 2 h as described in the Materials and Methods. The values are expressed in relation to the untreated control cells. (B) The cells were exposed to different concentrations of HA (5–100 μg/mL), ATO (0.1–1 μM), or both, for 4 h as described in the Materials and Methods. The values are expressed in relation to the untreated control cells. Data are presented as the mean ± SEM ($n = 10$–15). *$p < 0.05$ as compared with the control; #$p < 0.05$ as compared between two group.

combined with HA, which may be caused by up-regulation of GSH synthesis in the cells (Li and Chou, 1992).

Pretreatment of HA- and ATO-Promoted Cell Migration of SiHa Cells

Wound-healing assays were used to investigate the migration effects of HA and ATO on SiHa cells after 24-h pretreatment and then 48-h observation. Figure 3(A) shows that pretreated lower concentrations of HA, ATO, or their combination, could significantly enhance the ability of cell migration for the SiHa cells. When we counted the number of cells migrating into the wounded area, it was found that pretreatment of SiHa cells with HA and ATO for 24 h significantly increased the migrating cells compared to the control [Fig. 3(B)]. Co-exposure of difference concentrations HA and As significantly increased migration activity than control, but which was less potency than HA and As along. The mechanism may be mediated via chelating of As by HA leading to less available As and decrease HA functional groups (Cheng et al., 1999) which may lead to decrease the promoted effects to cells.

Effects of HA and ATO on the Enzyme Activity of MMP-2 in Conditioned Medium

Matrix-degrading proteinases are required for extra-cellular matrix degradation, which is one of the critical steps for cancer cells invasion (Lee et al., 2009). The enzyme activity of MMP-2 was quantified by SensoLyte® 490 MMP-2 Assay Kit (Ana Spec. Inc., San Jose, CA, USA). Enzyme activity of MMP-2 was significantly enhanced in conditioned medium of SiHa cells after HA or ATO exposure for 24 h ($p < 0.05$; Fig. 4). Based on the observation described previously, HA or ATO exposure can be seen as enhancing MMP-2 expression and may facilitate SiHa cell invasion.

Pretreatment of HA- and ATO-Enhanced Tumor Growth in SiHa Xenograft Model of NOD/SCID Mice

After 5 weeks of pretreatment of environmentally relevant doses of HA, ATO or both of them together, xenografts were produced using single-cell inoculation of the SiHa cells, and the drugs were persistently administrated for additional 45 days after tumor cell injection. The tumor volume in both single drug (HA or ATO) and combined drug-treated group was significantly larger than that of the control ($p < 0.05$; Fig. 5). However, the tumor volume enhanced by treatment of HA and ATO alone did not differ significantly from that of co-treatment of HA and ATO.

Histopathological Examination of SiHa Cells Pretreated With HA and ATO

In addition, tumors and surrounding tissues were rapid removed and used for histopathological examination. The SiHa cells appeared round in cell shape with high mitosis in the tumor of all animals and induced focal hyperkeratosis and epidermal hyperplasis in most animals [Fig. 6(A)]. Infiltration of tumor cells was also observed in the muscle of some of the mice controls, as well as the AsL, HA+AsL, and HA+AsH groups [Fig. 6(B)]. Furthermore, there was no difference in body weight of the SCID mice between each group, suggesting that the treatment of drugs was well tolerated (data not shown).

Effects of HA and ATO on the Protein Expression of MMPs and VEGF A in Tumor Tissue of NOD/SCID Mice

VEGF-A, MMP-2, and MMP-9 have been associated with the malignant phenotype of tumor cells because of their unique ability to induce angiogenesis and degrade collagen (Masood et al., 2001; Nelson et al., 2000). To investigate how HA and ATO affect VEGF-A, MMP-2, and MMP-9 levels in NOD/SCID mice, the tumor homogenates were subjected to western blot assay and probed with anti-VEGF-A, MMP-2, MMP-9, and α-tubulin (αT) used for equal loading as control. Figure 7(A) indicates that pretreated lower concentrations of HA or ATO could significantly increase the protein expressions of VEGF-A, MMP-2, and MMP-9 in the tumor tissue. Otherwise, the protein expressive effects of HA and ATO pretreatment were slightly less than those from HA and ATO alone, which were similar to the tumor volumes. The expressions of VEGF-A, MMP-2, and MMP-9 in the tumor of NOD/SCID mice that were pretreated with ATO or HA were increased by 1.10 to 1.25-fold, 1.03 to 1.29-fold, and 1.11 to 1.27-fold of control, respectively [Fig. 7(B)].

DISCUSSION

With respect to its vascular effects, ATO has been shown to be anti-angiogenic at higher doses, such as those used in the treatment of cancer. ATO has been implicated as a promising anticancer agent for leukemia and some solid tumors (Wei et al., 2005; Yu et al., 2007). On the other hand, arsenic is a known human carcinogen, and it has also been shown that lower environmentally relevant doses of ATO are in fact pro-angiogenic and tumorigenic (Bishop and Kipling, 1978). The paradox of arsenic has been theorized that arsenic at different doses may act according to different mechanisms, perhaps producing different patterns of biological effects (Cohen et al., 2006). In addition, ATO is well known for its ability to induce the production of superoxide and H_2O_2 (Barchowsky et al., 1999; Woo et al., 2002), which is one of several proposed mechanisms of action for arsenic-induced carcinogenesis (Schoen et al., 2004).

Due to the chemical properties of HA, it strongly interacts with both inorganic and organic agents, and thus might participate in redox regulation. HA has been shown to generate

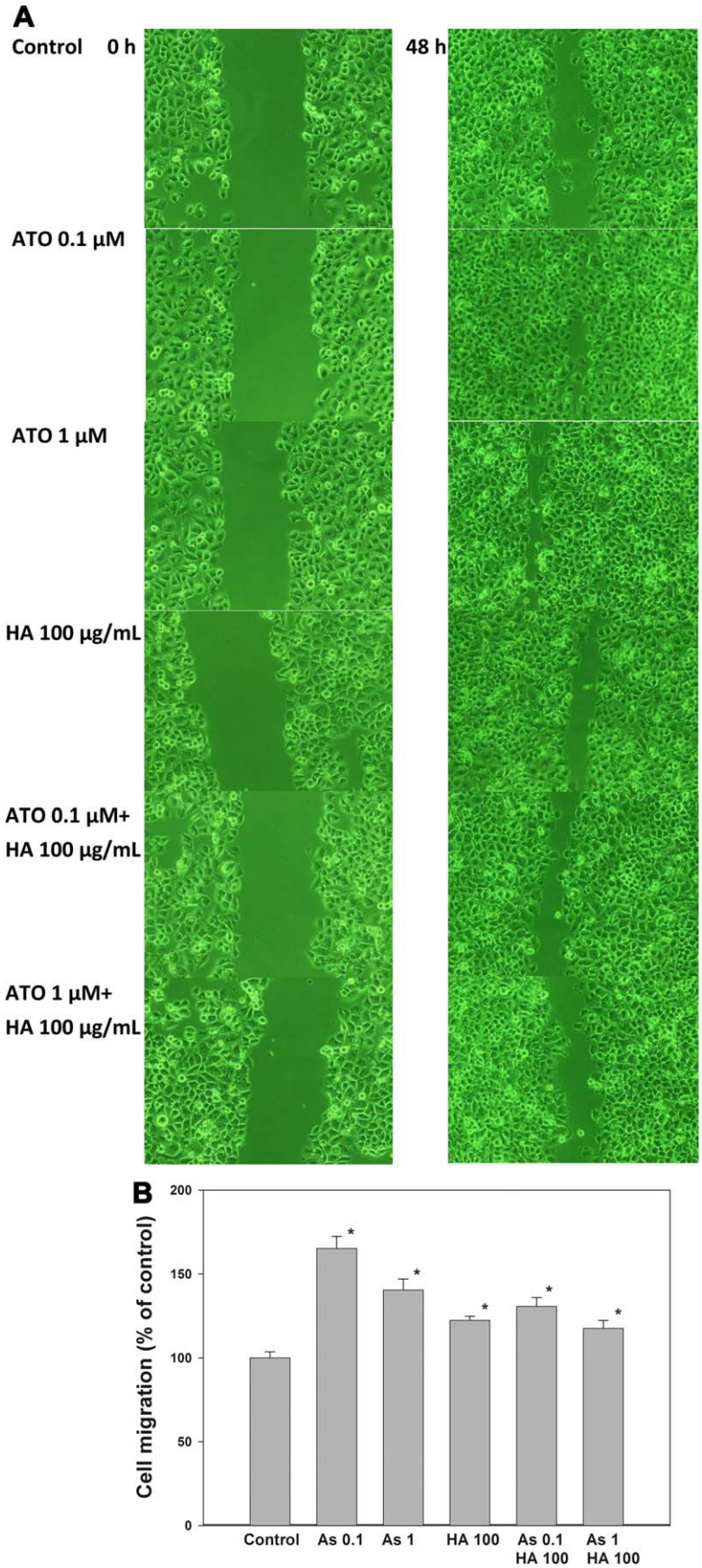

Fig. 3. Effects of HA and ATO on the cell migration of SiHa cells. (A) The cells were pretreated with different concentrations of HA, ATO, or both, for 24 h, and then, the serum-starved monolayer cells were scratched with a p200 pipette tip. The results were observed under phase contrast microscope (400x) and photographed as described in the Materials and Methods. (B) Quantitative assessment of the average number of cells in the denuded zone was as described at Materials and Methods. The values (%) are expressed in relation to the untreated control cells. Data are presented as the mean ± SEM ($n = 5$). *$p < 0.05$ as compared with the control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

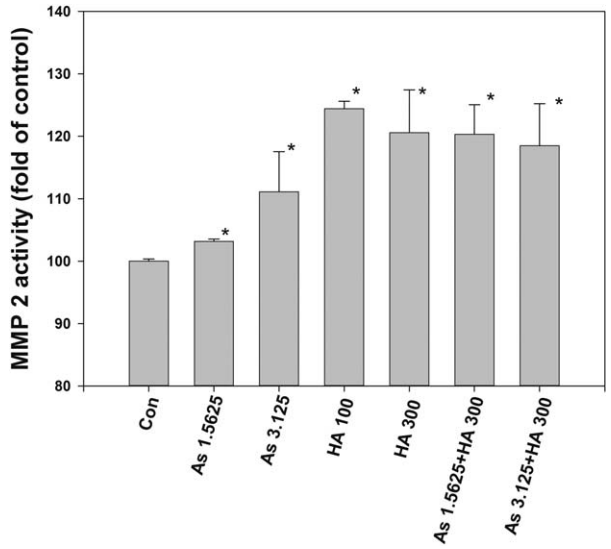

Fig. 4. Effects of HA and ATO on the activity of MMP-2 in the culture medium of SiHa cells. SiHa cells were treated with HA, As_2O_3, or both, for 24 h, and then, the supernatant of the medium was collected to analyze the activity of MMP-2 as described in Materials and Methods. Data are presented as the mean ± SEM ($n = 6$–8). *$p < 0.05$ as compared with the control.

ROS, such as superoxide anion (Cheng et al., 2003), and to cause a depletion of glutathione and several antioxidant enzymes (Cheng et al., 1999). Latch and McNeill observed that irradiated HA solutions can produce singlet oxygen in aquatic systems that can react with proteins, DNA, and other biomolecules, which have also been reported in previous studies (Cheng et al., 1999; Cheng et al., 2003; Latch and McNeill, 2006; Ting et al., 2010; Yen et al., 2007). HA has been proposed as a risk factor of cancer in the Blackfoot disease endemic region (Hseu et al., 2008), and low-concentration HA promoted neoplastic transformation of mouse epidermal JB6 cells (Lu et al., 2006) and enhanced the progression of lung cancer A549 cells in vitro (Lee et al., 2009). In our previous studies, high concentration of HA and ATO could inhibit cell proliferation of human cervical cancer cells by way of ROS and apoptosis induction. However, we also observed that noncytotoxic concentration of HA and ATO could enhance tumor cell proliferation (Ting et al., 2010).

We used DCFDA as a free-radical probe to study the intracellular oxidation in SiHa cells that were treated for 2 h with HA or ATO. As shown in Figure 2(A), ROS (as H_2O_2) significantly increased in SiHa cells that were exposed to HA combined with ATO, as well as higher concentrations of HA ($p < 0.05$). On the other hand, ATO (≤ 1 μM) only slightly enhanced the oxidative stress at this stage. It seems that HA could be a more stable free radical donor when compared with As (Cheng et al., 1999; Cheng et al., 2003; Lu et al., 1988).

After exposure to different concentrations of HA and ATO in the medium for 4 h, the cellular GSH content rose to 1.03–1.12-fold that of the control levels [Fig. 2(B)]. Intracellular GSH significantly increased after exposure to ATO combined with HA or higher concentrations HA alone for 4 h ($p < 0.05$), which was similar to the production of ROS. In addition, this increase may have been caused by the activation of antioxidant mechanisms and up-regulation of GSH synthesis in the cells. Based on our data, the increased oxidative stress induced by HA and ATO, or the combination of HA and ATO, improved this effect, which agrees with previous studies (Cheng et al., 1999; Cheng et al., 2003; Yen et al., 2007). Elevation of GSH in response to HA and ATO exposure may thus reflect a self-protective mechanism against cellular injury (Yen et al., 2007).

VEGF-A, MMP-2, and MMP-9 play critical roles in tumor angiogenesis, invasion, and metastasis of cervical cancer (Frumovitz and Sood, 2007; Roomi et al., 2010). Consistent with stimulation of growth, arsenic-induced expression of VEGF-A in endothelial cells and ovarian cancer cells. However, this response was not uniform, since arsenic inhibits VEGF expression in leukemic cells. These conflicting results suggest that the effects of arsenic on vascular growth factor expression are dose, cell, and context-specific. Arsenic exposure has been associated with the activation of MMPs in prostate cells (Achanzar et al., 2002), keratinocytes (Cooper et al., 2004), and sputum of humans (Josyula et al., 2006) exposed to arsenic in drinking water. Otherwise, higher concentration of ATO (2–5 mg/Kg BW)

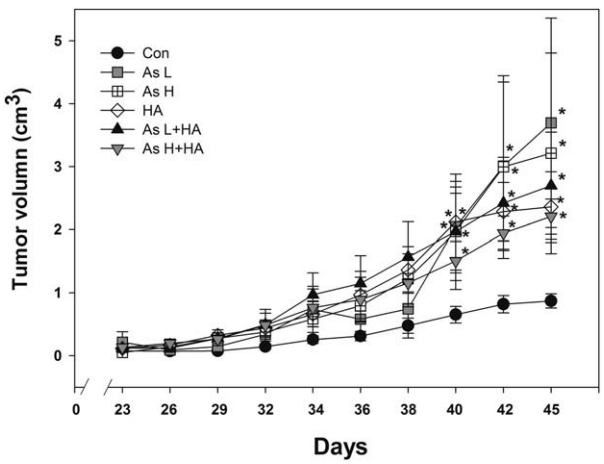

Fig. 5. The tumor volume of NOD/SCID mice inoculated with SiHa cells. The tumor volume of the NOD/SCID mice that underwent single-cell inoculation of SiHa cells into their back after 5 weeks pretreatment of HA and ATO and then were observed for an additional 45 days, as described in Materials and Methods. The volumes (cm^3) of tumors were estimated in accordance with the formula (tumor width^2 × tumor length/2) after tumor development. Data are presented as the mean ± SEM ($n = 5$). *$p < 0.05$ as compared with the control.

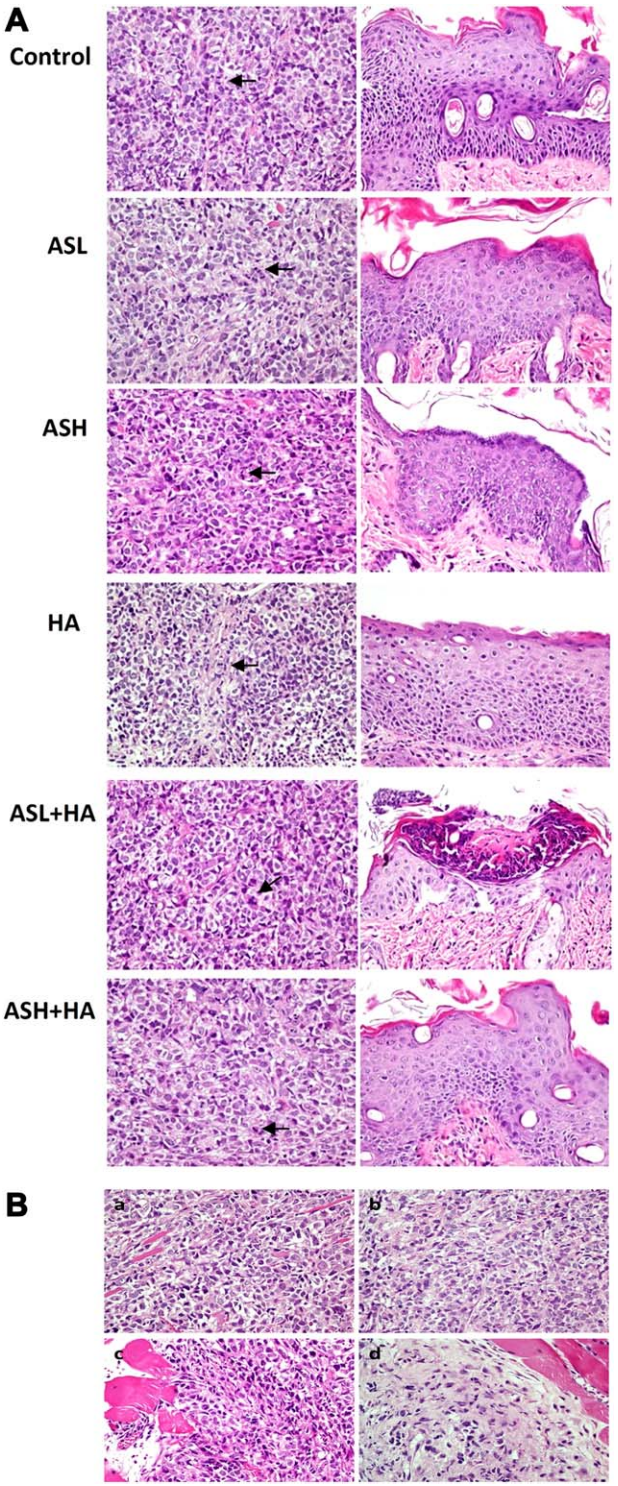

Fig. 6. Histopathological changes of NOD/SCID mice inoculated with SiHa cells. (A) Tumor cells appeared round in cell shape with high mitosis (Left, arrow), focal hyperkeratosis and epidermal hyperplasia in the skin (Right). (B) Some of tumor grown in the muscle was observed; a, b, c, and d, respectively, represent the Control-, ASL-, ASL + HA-, and ASH + HA-treated mice inoculated with SiHa tumor cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

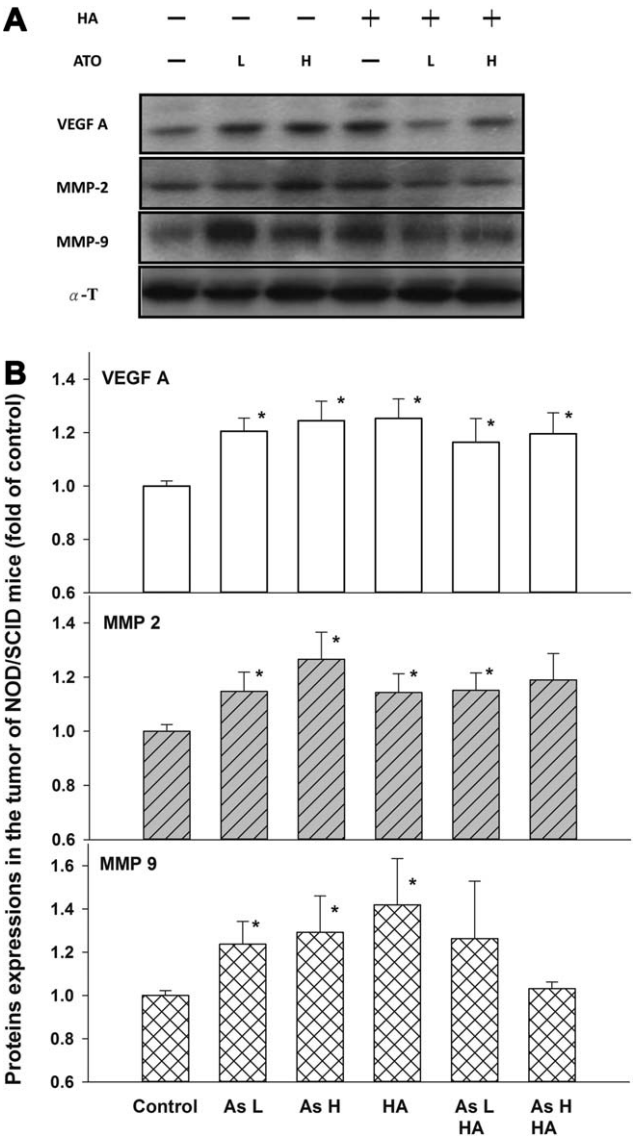

Fig. 7. Protein expressions of VEGF-A, MMP-2, and MMP-9 in the tumor of NOD/SCID mice. (A) The tumor homogenates were subjected to western blot assay as described in the Materials and Methods and probed with anti-VEGF-A, MMP-2, MMP-9, and α-tubulin (α*T*) used for equal loading as control. The results are shown as one data set representing five independent experiments. (B) Determined expression of these proteins was subsequently quantified by densitometry analysis with the fold of control as shown in the graph. The quantitative data are presented as mean ± SEM ($n = 5$).*$p < 0.05$ as compared with the control.

reduced protein level of MMP-2 and as an inhibitor of cervical cancer invasion (Yu et al., 2007). In addition, Lee et al. have reported that humic acid enhanced the activity of MMP-9 (Lee et al., 2009). The findings of the aforementioned study were consistent with our results. In our present

study, the protein expressions of VEGF-A, MMP-2, and MMP-9 of NOD/SCID mice that were co-exposured to both HA and ATO were significantly higher than those of the control and slightly lower than those of HA- or ATO-treated alone. On the other hand, the tumor volumes of the single drug- (HA or ATO) or combined drug-treated groups were also significantly larger than those of the control. In addition, the expressions of VEGF-A, MMP-2, and MMP-9 in the tumor tissue of single drug (HA or ATO) are higher than combined drug, which are similar with tumor volumes. These findings implied that biological effects of ATO and HA slightly decreased by co-exposure of them are similar to the study of Hseu and Yang (2002) which the HA could bind As and inhibit the enhancement effects of HA on the hemolysis. Furthermore, histopathological examination of tumors and surrounding tissues removed from the NOD/SCID mice displayed infiltration of tumor cells in the skin and muscle. These findings may be compatible and may demonstrate that the invasion and progression of cancer is associated with the increasing expressions of VEGF-A, MMP-2, and MMP-9 which may be enhanced by either HA or ATO, or a combination of them both. Overall, single drug (HA or ATO) or combined drug can promoted tumor growth and the tumor progression potential of ATO is great than HA or both of them.

CONCLUSIONS

In our study, human cervical cancer cells exposed to environmental relevant concentration of arsenic trioxide and humic acid significantly increased the abilities of cell proliferation, migration, invasion, and protein expressions of MMP-2, MMP-9, and VEGF-A. These findings suggest that exposure to environmentally relevant doses of arsenic trioxide and humic acid by human cervical cancer cells is associated with the progression of cancer. The toxicokinetic processes of HA and ATO complex are more complicated than single exposure, which are needed more and further studies of the intervention of them in the future. Overall, single or combined drug can promoted tumor growth and the tumor progression potential of ATO is great than HA or both of them. This study has important implications in the carcinogenicity effect of humic acid and arsenic trioxide which are co-present in artesian well water within the Blackfoot disease (BFD) endemic area (southwest coast of Taiwan).

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