Neoplastic Transformation of Human Small Airway Epithelial Cells Induced by Arsenic

Gengyun Wen,[1] Gloria M Calaf,[1,2] Michael A Partridge,[1] Carlos Echiburú-Chau,[2] Yongliang Zhao,[1] Sarah Huang,[1] Yunfei Chai,[1] Bingyan Li,[1,3] Burong Hu,[1] and Tom K Hei[1,4]

[1]Center for Radiological Research, and [4]Department of Environmental Health Sciences, Columbia University Medical Center, New York, NY; [2]Instituto de Alta Investigación, Universidad de Tarapacá, Arica, Chile; [3]School of Radiation Medicine and Public Health, Soochow University, Sozhou, China

Human small airway epithelial cells (SAECs) previously immortalized with human telomerase reverse transcriptase (h-TERT) were continuously treated with sodium arsenite at a dose of 0.5 μg/mL in culture for up to 6 months. Arsenic-treated cells progressively displayed an increase in transformed phenotype including enhanced growth saturation density, plating efficiency, and anchorage-independent growth and invasion capability compared with their nontreated control cells. To determine whether arsenic-induced cell transformation was associated with genomic instability, treated and control cells were also analyzed for micronuclei formation. A 4.8-fold increase in micronuclei incidence in arsenic-treated cells was detected in conjunction with increased N-phosphonacetyl-L-aspartate (PALA)-resistant characteristics. In addition, arsenic-treated cells showed an increase in c-H-ras, c-myc, and c-fos protein expression relative to controls. The change in oncoprotein expression correlated with a decrease in wild-type p53 expression and hyperphosphorylated retinoblastoma. Taken together, these results strongly suggest that h-TERT immortalized human small airway epithelial cells underwent step-wise transformation after inorganic arsenic treatment.

INTRODUCTION

Arsenic is a trace element found naturally in the environment. Trace elements are of particular interest given that the levels of exposure to them are potentially modifiable (1). Although inorganic arsenic has been recognized as a human carcinogen for more than a hundred years (2), scientists have been unable to elucidate its carcinogenetic mechanisms. Arsenic is a naturally occurring metalloid, and humans are exposed through contaminated soil, food, and water (3). In addition, occupational exposure occurs mainly through inhalation of airborne particles derived from semiconductor and glass manufacturing or power generation by the burning of arsenic-contaminated coal (2,4,5). Arsenic can be classified into two types, inorganic and organic (6). The former arsenical species are metabolized by methylation to form monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in vivo, which are also carcinogenic.

Epidemiological studies have shown that chronic exposure to arsenic results in liver injury, peripheral neuropathy, keratosis, and increased incidence of cancer of the lung, skin, bladder, and liver (7). In addition, occupational exposure to arsenic increases lung cancer incidence among smokers and underground miners, suggesting a synergistic interaction of arsenic with tobacco smoke or radon found in mines (8,9). Currently, millions of people living in India and Bangladesh are known to be exposed to arsenic through contaminated groundwater and have developed various arsenic-induced diseases. In the U.S., there have been reports of arsenic contamination of groundwater in the Southwest, with a level 20 times higher than the current U.S. maximum contaminant level of 10 μg/L. An analytic case-control study conducted in northern Chile reported increased risk for lung cancer with increasing drinking water arsenic, providing confirmatory evidence of the ecologic results for this type of cancer (10-12).

Models of inorganic arsenic-induced carcinogenesis are crucial for a better understanding of the underlying carcinogenic process as well as for prevention and treatment of the disease. In fact, inorganic arsenic is one of the few human carcinogens that do not induce tumors in laboratory animals readily, except at extremely high doses that are irrelevant to human exposure conditions (13,14). In the absence of adequate animal models, in vitro studies become particularly important in assessing the carcinogenic mechanisms of arsenic. Arsenic and arsenical compounds can induce morphological transformants in Syrian hamster embryo and murine C3H 10T1/2 cells (15,16). These agents are also potent clastogens both in vivo and in vitro, as they can induce sister chromatid exchanges

---

Address correspondence and reprint requests to *Gengyun Wen, Columbia University Medical Center, Center for Radiological Research, VC11-218, 630 West 168th Street, New York, NY 10032. Phone: 212-305-9981; Fax: 212-305-3229; E-mail: gw2154@columbia.edu*

and chromosome aberrations in both human and rodent cells (13,15,17).

Arsenical compounds have also been shown to induce gene amplification, arrest mitosis, inhibit DNA repair, and increase c-fos and heme oxygenase expression in mammalian cells (18-20). In addition, arsenic and its derivatives have been implicated as promoters and comutagens for a variety of other agents (21). There is evidence that arsenic can promote cell transformation in vitro. For example, exposure to arsenite of mouse skin epidermal cell line JB6 Cl41 induced anchorage-independent growth in soft agar (22). Exposure of human lymphocytes to low concentrations of arsenite (0.8-10 μM) or to the pentavalent arsenate (2-10 μM) over a range of treatment periods from 1 h to six days resulted in enhanced blast transformation and DNA synthesis (23). Moreover, arsenite (0.5 μM) induced malignant transformation in rat liver epithelial TRL 1215 cell line, and these cells produced metastatic tumors upon injection into nude mice (24).

Previous human epithelial cell lines used for arsenic transformation and carcinogenesis study were established by incorporating a virus and other genes to immortalize the cells (25-27). It is possible that these factors affected the genomic stability and intracellular signaling in the cells, which may facilitate increased transformation. Human telomerase reverse transcriptase (h-TERT)-immortalized human small airway epithelial cells, which were established in our lab, have the characteristics of normal cells (28). In this study, we used this cell line to study the ability of inorganic arsenic to induce transformation in culture as an in vitro model of carcinogenesis.

MATERIALS AND METHODS

Cell Culture

The present study used h-TERT immortalized human small airway epithelial cells established in our lab as described (2). Control cells were cultured in SAGM medium supplemented with various growth factors supplied by the manufacturer (SAGM SingleQuots, cat. no. CC-4124; Cambrex Bio Science, Walkersville, MD, USA) and maintained at 37°C in a humidified 5% CO_2 incubator. The experimental group consisted of the control cells cultured in the presence of sodium arsenite (0.5 μg/mL) for up to 28 weeks (designated as SAEC-A0.5).

Growth Kinetics Assay and Plating Efficiency

To determine the effect of arsenic on growth rate and saturation density, SAEC and SAEC-A0.5 cells plated at a density of 2×10^5 cells from exponentially growing cultures were replated in 60-mm diameter dishes. At each time point studied, triplicate dishes from each group were trypsinized, and total number of cells per dish was determined. Plating efficiency (PE), a measure of the number of colonies originating from single cells, was used for determining the effects of arsenic treatment. PE is determined by the following formula: PE (%) = (number of colonies counted / number of cells inoculated) × 100.

Anchorage-Independent Assay

To test for soft-agar colony growth capacity, SAEC and SAEC-A0.5 cells were plated at a density of 1×10^3 cells in 1 mL 0.35% agarose over a 0.7% agar base in a 24-well culture plate. Cultures were fed every three days, and colonies with >50 cells were scored after 4 weeks under a dissecting microscope.

Determination of Frequency of PALA-Resistant Colonies

Exponentially growing cells were plated into 100-mm diameter dishes at a density of 2×10^3 each in medium containing *N*-(phosphonoacetyl)-L-aspartate (PALA) (Drug and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute). PALA was used at 180 μM, which corresponded to nine times the LD_{50} of the cells (LD_{50} for SAEC-A0.5 is 20 μM; data not shown). Cultures were maintained in the selective medium for 12-14 days with fresh medium replenished every three days. They were then fixed and stained, and the number of PALA-resistant colonies was counted. The frequency of resistant colonies was calculated as the ratio of plating efficiency in the presence or absence of PALA.

Micronucleus Assay

Briefly, for the micronucleus assay, cells were incubated with cytochalasin-B (3 μg mL/L) for 24 h (24). After a hypotonic shock in 0.075 M KCl, cells were stained with acridine orange and examined with a fluorescence microscope under UV light. Cells were counted in three independent experiments involving 1000 cells. The results are plotted as number of micronuclei per 1000 binucleated cells.

Cell Invasion Assay

Invasiveness was measured as described by the vendor (BD BioCoat Matrigel invasion chamber, cat. no. 354480; BD Biosciences, Bedford, MA, USA). Briefly, 8-mm-diameter filters (8 μm pore) of cell culture inserts coated with matrigel were placed in 24-well culture plates. Exponentially growing cells were trypsinized, harvested, resuspended in SAGM medium without supplemental growth factors, and passed repeatedly through a 25-gauge needle to produce a single-cell suspension. After determination of cell count and viability in a hemocytometer by the trypan blue exclusion test, the cells were added to the upper compartment of the chamber (1×10^5 cells/chamber). Growth factors added in the medium (complete medium) were used as chemoattractant and placed in the lower chamber. After incubation for 23 h at 37°C in a 5% CO_2 incubator, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab, and the cells that had traversed the matrigel and attached to the lower surface of the filter were studied. The filters were fixed, stained with Diff Quick (Sigma, St. Louis, MO, USA), cut out, and mounted on glass slides. The three fields of cell numbers that crossed the membrane were counted under a light microscope and the average value was calculated. Experiments

were performed three times with four chambers/cell line.

In Vitro Healing Wound Assay

For wound assays, the h-TERT cells were cultured in SAGM medium in the presence or absence of supplemented media with various growth factors supplied by the manufacturer (SAGM Single-Quots, cat. no. CC-4124; Cambrex Bio Science) and maintained at 37°C in a humidified 5% CO_2 incubator. Cells were seeded at a high density, 12×10^4 cells on fibronectin coated coverslips, and wounded one day later when the cells formed a confluent monolayer. The wound was made by scraping with a yellow tip through/across the cell monolayer. The wound was examined after 18 h.

Flow-Cytometry Studies

Cells were trypsinized, washed with PBS, and stained for 30 min on ice with primary antibodies, α2 or β4 integrins (BD Biosciences, San Jose, CA, USA), and then with secondary antibodies (Molecular Probes, Carlsbad, CA, USA). Surface expression of integrin receptors on arsenic-treated SAEC cells as well as controls was analyzed by flow-cytometric analysis of surface expression of these two endogenous integrins, the laminin receptor and the collagen receptor in SAEC + A0.05 cells and control SAEC cells determined by staining with anti-α2 and anti-β4 antibodies, respectively.

Lammelipodia Detection

Briefly, for lammelipodia detection, arsenic-treated and nontreated cells were washed, trypsinized, washed again, and added to fibronectin-coated coverslips (1 μg/mL) (Sigma) at 37°C for 1 h in DMEM, 2% bovine serum albumin, and 0.5% serum and incubated at 37°C for 90 min before fixation (PBS, 4% paraformaldehyde for 10 min) and permeabilization (PBS, 0.5% Triton X-100 for 5 min). Cells were stained for F-actin with rhodamine-conjugated phalloidin (Invitrogen, San Jose, CA, USA) for 30 min at 37°C, and images were captured using a laser confocal microscope (Nikon, Tokyo, Japan).

Determination of Protein Expression by Immunocytochemistry Coupled with Confocal Microscopy

Exponentially growing control and treated cells were plated on a glass chamber slide (Nunc, Naperville, IL, USA) as described (29). Cells were plated at a density of 1×10^4 in 1 mL medium and allowed to grow for two to three days until they reached 70% confluence. The cells were incubated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase, washed twice with buffer solution, and fixed with buffered paraformaldehyde in PBS (pH 7.4) at room temperature. Cells were covered with normal horse serum for 30 min at room temperature and incubated with the corresponding primary antibodies at a 1:500 dilution overnight at 4°C. The c-myc, c-fos, and c-Ha-ras (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were all mouse monoclonal antibodies. After washing in buffer solution, cultures were incubated for 60 min at room temperature with antimouse Rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at a 1:1000 dilution. After several washes of 5 min each with buffer solution, slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Controls included cultures stained with either the primary or secondary antibodies alone to monitor background staining. Cells were quantified as described (30) and viewed on Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY, USA) using a 40× (11.3 NA) objective lens equipped with a laser scanning confocal attachment (LSM 410; Carl Zeiss). To excite the Rhodamine secondary antibody, the fluorescent images were collected by an argon/krypton mixed-gas laser (488 nm). Fluorescent images were collected in black and white and changed to red color using Photoshop software, version 5.0. Composite images were quantified by using Photoshop. Protein expression of the control and transformed cells was determined by a semiquantitative estimation based on the

relative staining intensity. This computer program gives the area and the intensity of the staining of the cells present in the culture dishes. The experiments were repeated with three similar passages. Standard errors of mean are shown in the different figures. The number of immunoreactive cells was counted in five randomly selected microscopy fields per sample. Statistical analysis was done with the ANOVA F test (randomized block) and comparisons between groups with the orthogonal t test with significance value of <0.05.

Western Blot Analysis

Protein was extracted by lysing cells in extraction buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 1 mM phenylmethylsulfonyl fluoride). The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equivalent amounts of protein (30 μg) were fractionated by electrophoresis in SDS-polyacrylamide gel. The protein was subsequently transferred to PVDF membranes under semidry conditions. Antibodies against p53, phospho-Rb (Santa Cruz Biotechnology) were applied to probe the membranes. The secondary antibodies (antirabbit or antimouse) (Amersham Biosciences, Piscataway, NJ, USA) were conjugated to horseradish peroxidase (dilution 1:5000 to 1:10,000). Signals were detected using the ECL system (Amersham Biosciences).

RESULTS

The h-TERT immortalized SAEC cells grew as a contact-inhibited monolayer with a population doubling time of ~24 h. At confluence, these cells had a saturation density of ~1.19×10^5 cells/cm^2 dish (Figure 1A). The cells were treated continuously with sodium arsenite at 0.5 μg/mL for approximately 28 weeks, fresh medium was replenished weekly, and changes in growth kinetics and other transformed phenotypes were monitored periodically over a period of months. The population doubling time of the SAEC-A0.5 cells was similar to that of control
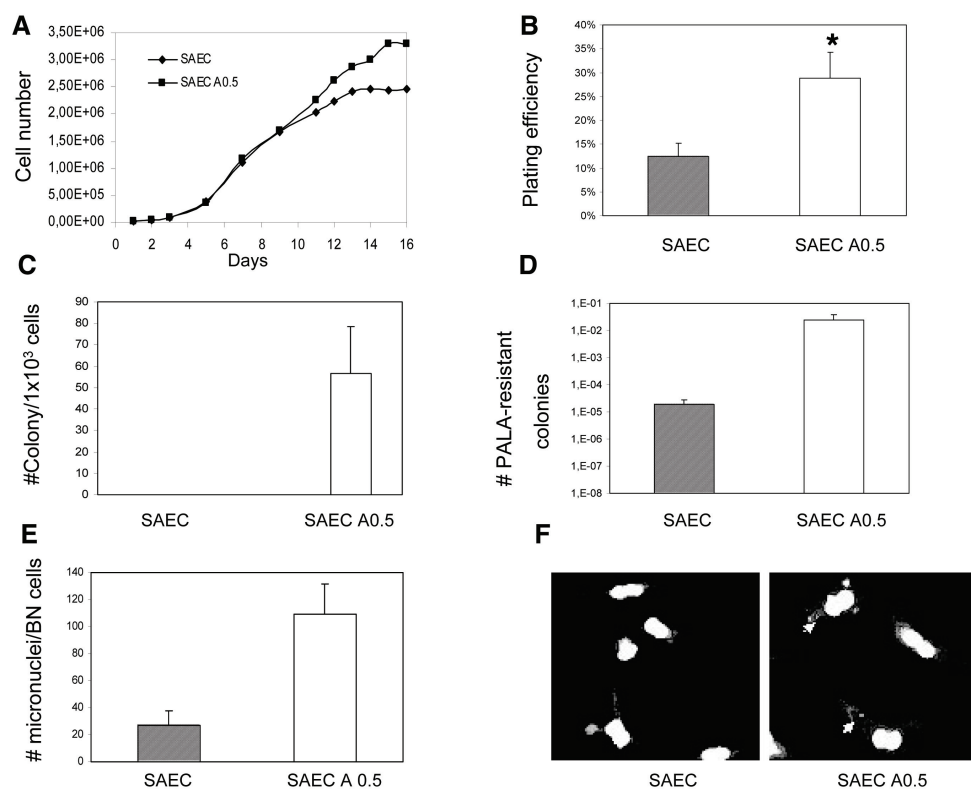
Figure 1. Growth kinetics (A) and plating efficiency (B) of SAEC cells and SAEC-A0.5 cells in SAGM medium. Data are the mean of triplicates. Bars represent ± SD, $P < 0.01$. C. Colony numbers in soft-agar: 1×10^3 cells in 1 mL 0.35% agarose were overlaid on a 0.7% agar base in a 24-well culture plate. Cultures were fed every three days, and colonies with >50 cells were scored after four weeks in cultures under a dissecting microscope. The experiment was carried out in triplicate. Bars represent ± SD. PALA-resistant colonies: Frequency determined at a concentration of 180 μM PALA that corresponds to nine times the LD_{50} of SAEC-A0.5 (data not shown): data are pooled from three independent experiments (D). Bars represent ± SD. Micronuclei (MN) formation: cells were incubated with cytochalasin-B (3 μg mL/L) for 24 h. After a hypotonic shock in 0.075 M KCl, cells were stained with acridine orange and examined with a fluorescence microscope under UV light. Cells with MN were counted in three independent experiments involving 1000 cells. Bars represent ± SD, $P < 0.01$ (E). Micronuclei can be observed in representative images of SAEC and SAEC-A0.5 cells (F).

SAEC cells, yet their saturation density increased to 1.57×10^5 cells/cm^2, suggesting that arsenic-exposed cells were able to partially overcome contact inhibition, a characteristic of transformed cells. Furthermore, SAEC-A0.5 cells had a much higher plating efficiency than SAEC cells (Figure 1B), 0.123 ± 0.028 in control versus 0.28 ± 0.053 in arsenic-treated cells, $P < 0.01$. Anchorage independent growth usually correlates strongly with invasiveness in many cell types. Our data demonstrated that only SAEC-A0.5 cells formed agar-positive clones, with a rate of >5% (Figure 1C). In contrast, control SAEC cells showed no anchorage-independent growth. Soft agar positive clones from arsenic-treated cells were isolated and used

for additional experiments to assess transformation capabilities.

Gene transformation is a frequent manifestation of genomic instability in cancer cells. Using resistance to the chemotherapeutic drug PALA as an index of gene amplification, our results demonstrated that SAEC-A0.5 cells exhibited a much higher frequency of PALA resistance than control SAEC cells. The incidence of PALA resistance was 1.3×10^{-5} in SAEC cells. In arsenic-treated cells, the frequency was 1.2×10^{-2}, a 10^3-fold increase in activity of CAD gene. These data indicate that arsenic exposure induced genomic instability in the SAEC cells during the neoplastic transforming process (Figure 1D).

Number of micronuclei is a conventional measure of genotoxicity and therefore used in this study to analyze arsenic-induced genotoxicity. Our results showed a 4.8-fold increase in the incidence of micronuclei in SAEC-A0.5 cells compared with control cells (Figure 1E and F). The incidence was substantially increased to more than 109 micronuclei per 1000 binucleated cells in SAEC-A0.5 cells, in contrast to only 27 micronuclei per 1000 binucleated cells in SAEC control cells (10% versus 3%), suggesting that chronic arsenic treatment induced genomic instability in SAEC cells.

Figure 2A shows invasive characteristics of control and arsenic-treated SAEC
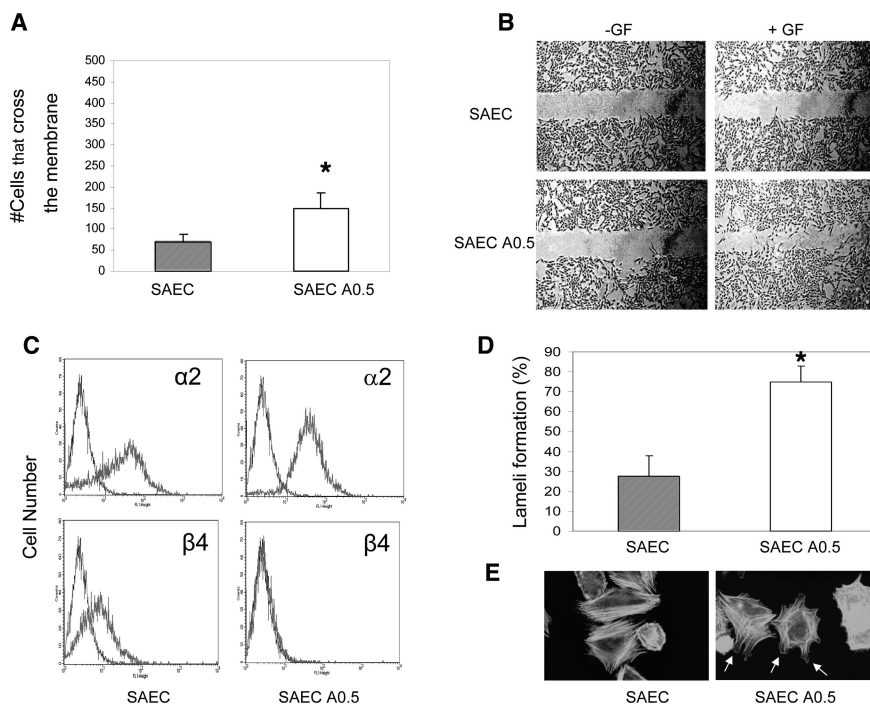
Figure 2. Invasion assay: 1×10^5 cells were added to the upper compartment of the chamber. Growth factors added in the medium (complete medium) were used as chemoattractant and placed in the lower chamber. After incubation for 23 h at 37°C in a 5% CO_2 incubator, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab, and the cells that had crossed the matrigel and attached to the lower surface of the filter were counted. The filters were fixed, stained with Diff Quick (Sigma), cut out, and mounted on glass slides. The three fields of cell number that crossed the membrane were counted under a light microscope and the average value was calculated. Experiments were performed three times with four chambers/cell line. Bars represent ± SD, $P < 0.05$ (A). In vitro wound-healing assay: the wound was created by scraping with loading tips. Images were captured 18 h after adding the growth factors in both SAEC and SAEC-A0.5 cells. The wound-closing trend was compared between the two types of cells upon addition of the growth factors (GF) (B). Surface expression of integrin receptors on arsenic-treated SAEC cells and controls. Fluorescence flow-cytometry analysis of surface expression of two endogenous integrins, the laminin receptor anti-α2 and the collagen receptor anti-β4, in arsenic-treated and untreated SAEC cells determined by staining with the correspondent antibodies, respectively (C). Rate of lamellipodia formation was compared between SAEC and SAEC-A0.5 cells by scoring the number of cells that actively form lamellipodia (D). Five hundred cells in three different coverslips were scored and recorded under a fluorescence microscope; percentage of cells with lamellipodia was calculated. Bars represent ± SD, $P < 0.01$ (D). Fluorescence microscopy of phalloidin stained SAEC and SAEC-A0.5 cells (E). Cells were grown on glass coverslips for 24 h, fixed, and labeled with Rhodamine-phalloidin to visualize actin filaments. Arrows point to lamellipodia areas. Magnification 600×.

cells when scored 23 h after plating onto matrigel basement membranes in Boyden chambers. Growth factors were used as chemoattractant. The number of cells that migrated through the membrane increased from 69.3 ± 18.4 in control to $149.3 \pm 35.6$ in arsenic-treated cells, $P < 0.05$, more than double, a clear indication that arsenic treatment resulted in an increase in invasive capabilities. Another indicator of transformation in arsenic-treated cells was in vitro wound-healing (Figure 2B), which is generally used for assessing cell motility. Arsenic-treated SAEC cells exhibited much greater cell migration ability in response to growth factors than control cells.

Because change in cell adhesion characteristics may imply change in integrin function, which plays an essential role in cell attachment, we examined the expression of various integrin receptor functions in control and arsenic-treated SAEC cells. Flow cytometry indicated that α2 integrin did not show any difference between control and arsenic-treated cells (Figure 2C). Similar results were also found in α3, α5, α1, and $\alpha_v\beta3$ integrins (data not shown). However, β4 integrin protein expression was lost in arsenic-treated cells while its expression was similar in control cells. Figure 2D shows lamellipodia formation in control and arsenic-treated cells. The arsenic-treated SAEC group had a higher percentage of lamellipodia formation than control cells (Figure 2D), 75 ± 8 versus 28 ± 10, $P < 0.01$; moreover, larger lamellipodia were formed in arsenic-treated cells than controls, indicating an alteration in epithelial morphology and attachment to the fibronectin substrate upon plating, both of which contribute to the process of transformation as seen in representative cells (Figure 2E).

Malignant transformation is frequently associated with changes in expression of specific oncogenes and tumor suppressors. Immunofluorescence studies indicated that there was a significant increase in c-myc, c-Ha-ras, and c-fos protein expression in arsenic-treated cells in comparison with control cells (Figure 3A). The expression of c-myc, c-fos, and c-Ha-ras was increased approximately up to 3-, 3.1, and 2.6-fold, respectively. Representative images of c-myc, c-Ha-ras, and c-fos cells are shown in Figure 3B.

Changes in c-Ha-ras, c-myc, and c-fos protein expression were confirmed by Western blot analysis in control and SAEC-A0.5 cells (Figure 4A and B). Phosphorylation of ERK and retinoblastoma in random growth conditions was examined by Western blot between SAEC and SAEC-A0.5 cells, and these changes were

A

B
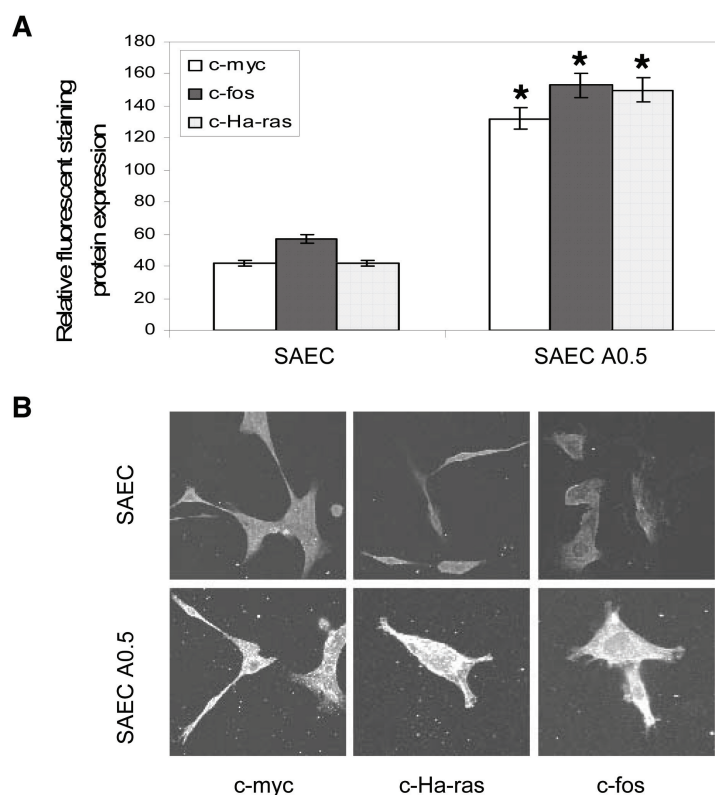
Figure 3. Bars represent the average and standard error of c-myc, c-Ha-ras, and c-fos protein expression of SAEC and SAEC-A0.5 (A). Representative immunofluorescence stained in confocal microscope images of c-myc, c-Ha-ras, and c-fos protein expression of SAEC and SAEC A0.5 cells. The primary antibodies used were mouse monoclonal antibody (Biotechnology) (B).

further confirmed by examining one of the soft-agar clones of SAEC-A0.5 cells, which showed even higher expression of c-fos and c-jun and more intensive phosphorylation of ERK (Figure 4A, B, and C). Under these culture conditions, the Rb and ERK proteins were hyperphosphorylated in SAEC-A0.5 cells, whereas the control cells maintained a basal level of phosphorylation. These results demonstrated that expression of molecules controlling cell proliferation and transformation, such as c-myc, c-fos, and c-Ha-ras, were altered by chronic arsenic treatment. Wild-type *p53* exhibited a significant decrease in SAEC-A0.5 cells compared with SAEC (Figure 4D). However, the coding region of *p53* in SAEC-A0.5 cells revealed no change in sequencing (data not shown).

DISCUSSION

The metalloid arsenic, a naturally occurring element, is a serious global environmental concern and a severe health problem affecting millions of people worldwide. To have an effective interventional approach in treatment and prevention of arsenic-induced carcinogenesis in vitro, a model of arsenic transformation assays based on human epithelial cells is needed. The objective of this study was to analyze whether arsenic was able to transform lung cells, based on the h-TERT immortalized human small airway epithelial cells previously described (2).

The initial indication that arsenic treatment altered SAEC cell phenotype was the change in growth characteristics. The cell population doubling time was similar in arsenic-treated and nontreated cells,

yet the saturation density substantially increased in SAEC-A0.5, suggesting that this phenotype may represent an early manifestation of transformation. The increased plating efficiency indicated a portion of highly proliferating cells that boosted the cell growth advantage and may be responsible for the loss of contact inhibition of growth and increased saturation density in the transformed cells.

Micronuclei are a reliable cytogenetic marker of chromosomal instability based on the presence of nuclear fragments in binucleated cells (29,31). In our study, the incidence of micronuclei in SAEC-A0.5 cells was >10%, in contrast to the 3% incidence in SAEC (Figure 1E), suggesting that chronic arsenic treatment induced genomic instability in the human small airway epithelial cells.

Transformation/carcinogenesis is a multistage process. The importance of maintaining genomic stability is evidenced by the fact that transformed cells often contain a variety of chromosomal abnormalities such as aneuploidy, translocations, and inversions. Genomic instability induced by chemicals or drugs may contribute to transformation by accumulating genetic changes that ultimately lead to malignant conversion. Our results based on micronuclei formation and the PALA assay indicated that arsenic resulted in genomic instability in the h-TERT immortalized human epithelial cells, consistent with previous studies in different cell types. The PALA assay is designed to measure directly the frequency with which gene amplification occurs in mammalian cells in culture (32,33). PALA prevents de novo pyrimidine biosynthesis through inhibition of the trifunctional enzyme CAD (carbamyl phosphate synthetase/aspartate transcarbamylase/dihydro-orotase), an essential enzyme in the synthesis of UMP (34). When cells are exposed to PALA, the synthesis of pyrimidines is inhibited, resulting in DNA mutations or chromosomal breaks (35). If PALA treatment is maintained, cells eventually undergo apoptosis (36). Cells become resistant to PALA through amplification of the *CAD* gene (37),
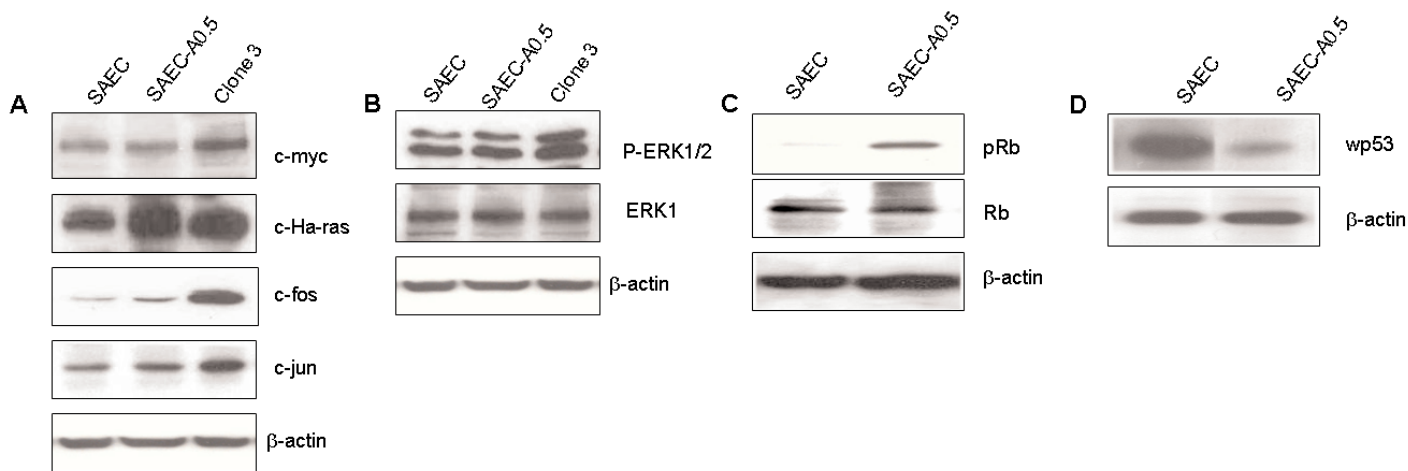
Figure 4. Western blot analysis: equal amount of whole cell lysates from SAEC and SAEC-A0.5 prepared in RIPA buffer, resolved on 12% SDS-PAGE and transferred to PVDF membrane, probed with correspondent antibodies shown in each panel (A-D).

which raises the cellular concentration of the CAD enzyme to overcome the inhibitory effects of the drug. Therefore, the frequency of the cells developing PALA resistance provides an objective measure of those cells undergoing gene amplification, which can be used as an indication of genomic instability.

Invasion capability of cells has been shown to correlate with their malignant characteristics in vivo (30). Transformed cells have enhanced invasive ability in vitro, which induces alterations in phenotype. In particular, arsenic-transformed cells displayed anchorage-independent growth as well as enhanced migration and invasive ability, suggesting that SAEC-A0.5 cells acquired some characteristics of oncogenic transformation through long-term arsenic treatment.

Accumulating evidence demonstrates that malignant transformation is associated with changes in the repertoire of expression of the integrin family of molecules, which mediate cell-matrix and cell-cell interactions. In the present study, we analyzed the presence of several major integrins on the cell surface of arsenic-treated cells versus the nontreated SAEC cells. Results indicated that α2 integrin, along with α3, α5, α1, and αβ3 integrins, did not show much difference between control and arsenic-treated cells. However, β4 integrin pro-

tein expression was lost in arsenic-treated cells, indicating a change in the extracellular matrix receptor affected by the chemical used. Lamellipodia formation was found in both control and experimental cells. The arsenic-treated SAEC cells had a higher percentage of lamellipodia formation than control cells, indicating a more potent protrusive force on the fibronectin substratum; this feature is possibly associated with the lost expression of β4 integrin.

Altered expression of genes such as oncogenes or tumor suppressors is likely affected by mutagens such as chemicals or drugs, and thus contributes to oncogenic transformation (38,39). Thus c-Ha-ras, c-myc, and c-fos have been used as prominent markers for malignant transformation in many cell types. Their upregulation leads to activation of several major signaling pathways in transformed or tumor cells, particularly in some types of arsenic-transformed cells (40-43). The change in expression of such proteins supported our hypothesis that, after a long period of treatment with arsenic, the cells exhibited characteristics of malignant transformation. Hence they was used in the present study to define cell transformation of arsenic-treated cells.

Consistent with these observations, we found upregulated phosphorylation of Rb and ERK in arsenic-treated and trans-

formed SAEC cells. The hyperphosphorylation of Rb in the transformed cell line is presumably due to the effects induced by upstream effectors, including the upregulation of c-Ha-ras oncogene as well as increased ERK activity. Because Rb hyperphosphorylation is responsible for the release of cell cycle block, c-myc, c-fos, and c-jun account for mediating the process, and thereby the SAEC-A0.5 gained cell growth advantage in comparison to control SAEC cells.

p53 plays a critical role in maintaining genome integrity and accuracy of chromosome segregation. The effects of arsenic on *p53* activation have been extensively studied and yet inconsistent. Some reports demonstrated that arsenic could induce *p53* accumulation in an ATM-dependent pathway (44); the overexpressed *p53* was a mutant form, and most frequent mutation sites were found in exons 5 and 8, so mutated *p53* subsequently led to its normal function loss. Other studies (45-47) documented that the promoter of *p53* in arsenic-exposed cells exhibited hypermethylation as some carcinoma appeared; consequently, *p53* expression decreased, resulting in its function loss or partial loss, so the cells were predisposed to uncontrolled proliferation, genomic instability, or apoptosis. In our study, *p53* did not show mutations in its coding region upon chronic arsenic

treatment, yet its protein expression was remarkably decreased relative to the control cells. Because *p53* is critical in maintaining genomic stability, this finding emphasizes many of the changes described in this study.

The benefits that this study provides include the technology needed to define signs of malignant transformation caused by arsenic that may lead to the design of specific tools for diagnosis, treatment, or prevention of lung cancer. The information presented here-related to protein expression of early oncogenes such as c-myc and c-fos, oncogenes such as c-Ha-ras, and a tumor suppressor gene such as p53-will help to define the damage related to transformation induced by arsenic exposure.

Studies of populations exposed to high levels of arsenic in drinking water have raised the possibility of a causal relationship for lung cancer. Marshall et al. (12) provided additional evidence linking arsenic to lung cancer. Our work used micronuclei as a biomarker in a h-TERT immortalized cell line that can be used for human lung cancer research. Arsenic-induced cell transformation was associated with genomic instability through analysis of micronuclei formation. A 4.8-fold increase in micronuclei incidence in arsenic-treated cells was detected. The arsenic-treated SAEC cells also presented a higher percentage of lamellipodia formation than control cells, enhanced by the lost expression of β4 integrin and in conjunction with increased PALA-resistant characteristics.

Human epithelial cell lines used for arsenic transformation and carcinogenesis studies have been previously established to immortalize the cells by incorporating a virus and other genes. It is possible that these factors affect the intracellular signaling in these cells, which may facilitate transformation. To a greater understanding of lung cancer, the arsenic-induced gene expression changes have been shown in prior work by many groups. Therefore, the novelty of this study was to use a more normal human lung cell line than previously reported. The h-TERT immortalized human SAEC is generally

genomically stable, taking much longer for such cells to be transformed, compared with other methods that use virus and T-antigen to immortalize the cells. Such cells had certain characteristics consistent with a cancer cell phenotype.

The novelty of these data is the use of an established immortalized h-TERT human small airway epithelial cell line that has the characteristics of normal cells. Thus, this work provides a combination of phenotypic and molecular tools to characterize the carcinogenic process induced by arsenic. Taken together, our results strongly suggest that malignant transformation occurred in the h-TERT immortalized human small airway epithelial cell after inorganic arsenic treatment in vitro.

REFERENCES

1. Navarro Silvera SA, Rohan TE. (2007) Trace elements and cancer risk: a review of the epidemiologic evidence. *Cancer Causes Control* 18:7-27.
2. Chan P, Huff JE. (1997) Arsenic carcinogenesis in animals and in humans: mechanistic, experimental, and epidemiological evidence. *Environ. Carcinog. Ecotox. Rev.* 15:83-122.
3. Huang C, Ke Q, Costa M, Shi X. (2004) Molecular mechanisms of arsenic carcinogenesis. *Mol. Cell Biochem.* 255:57-66.
4. Yager JW, Wiencke JK. (1993) Enhancement of chromosomal damage by arsenic: implications for mechanism. *Environ. Health. Perspect.* 101:79-82.
5. Zhang JJ, Smith KR. (2007) Household air pollution from coal and biomass fuels in China: measurements, health impacts, and interventions. *Environ. Health Perspect.* 115:848-55.
6. Shi HL, Shi XL, Liu KJ. (2004) Oxidative mechanism of arsenic toxicity and carcinogenesis. *Mol. Cell Biochem.* 255:67-78.
7. IARC (2004) Some drinking-water disinfectants and contaminants, including arsenic. Working Group on the Evaluation of Carcinogenic Risks to Humans. *IARC Monogr. Eval. Carcinog. Risks Hum.* 84:1-477.
8. Hertz-Picciotto I, Smith SH, Holtzman D, Lipsett M, Alexeeff G. (1992) Synergism between occupational arsenic exposure and smoking in the induction of lung cancer. *Epidemiology* 3:23-31.
9. Kusiak RA, Ritchie AC, Muller AC, Springer J. (1993) Mortality from lung cancer in Ontario uranium miners. *Br. J. Ind. Med.* 50:920-7.
10. Ferreccio C, Gonzalez C, Milosavjlevic V, Marshall G, Sancha AM, Smith AH. (2000) Lung cancer and arsenic concentrations in drinking water in Chile. *Epidemiology* 11:673-9.
11. Lubin JH, Beane Freeman LE, Cantor KP. (2007) Inorganic arsenic in drinking water: an evolving public health concern. *J. Natl. Cancer Inst.* 99:906-7.
12. Marshall G, Ferreccio C, Yuan Y, Bates MN, Steinmaus C, Selvin S, Liaw J, Smith AH. (2007) Fifty-year study of lung and bladder cancer mortality in Chile related to arsenic in drinking water. *J. Natl. Cancer Inst.* 99:920-8.
13. Barrett JC, Lamb PW, Wang TC, Lee TC (1989) Mechanisms of arsenic-induced cell transformation. *Biol. Trace Elem. Res.* 21:421-9.
14. Wang Z, Rossman R. (1993) Stable and inducible arsenite resistance in Chinese hamster cells. *Toxicol. Appl. Pharmacol.* 118:80-6.
15. Lee TC, Oshimura M, Barrett JC. (1985) Comparison of arsenic-induced cell transformation, cytotoxicity, mutation and cytogenetic effects in Syrian hamster embryo cells in culture. *Carcinogenesis* 6:1421-6.
16. Landolph J. (1989) Molecular and cellular mechanisms of transformation of C3H/10T1/2 Cl 8 and diploid human fibroblasts by unique carcinogenic, nonmutagenic metal compounds: a review *Biol. Trace Elem. Res.* 21:459-67.
17. Nakamuro K, Sayato YC. (1981) Comparative studies of chromosomal aberration induced by trivalent and pentavalent arsenic. *Mutat. Res.* 88: 73-80.
18. Natarajan AT, Boei JJ, Darroudi F, Van Diemen PC, Doulout F, Hande MP, Ramalho AT. (1996) Current cytogenetic methods for detecting exposure and effects of mutagens and carcinogens. *Environ. Health Perspect.* 104:445-58.
19. Lee TC, Tanaka N, Lamb PW, Gilmer TM, Barrett JC. (1988) Induction of gene amplification by arsenic. *Science* 241:79-81.
20. Vega L, Gonsebatt ME, Ostrosky-Wegman P. (1995) Aneugenic effect of sodium arsenite on human lymphocytes in vitro: an individual susceptibility effect detected. *Mutat. Res.* 334:365-73.
21. Keyse SM, Applegate LA, Tromvoukis Y, Tyrell RM. (1990) Oxidative stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblasts. *Mol. Cell Biol.* 10:4967-72.
22. Huang CS, Ma WY, Li JX, Goranson A, Dong ZG. (1999) Requirement of Erk, but not JNK, for arsenite-induced cell transformation. *J. Biol. Chem.* 274:14595-601.
23. Meng ZQ, Meng NY. (2000) Effects of arsenic on blast transformation and DNA synthesis of human blood lymphocytes. *Chemosphere* 41:115-9.
24. Zhao CQ, Young MR, Diwan BA, Coogan TP, Walkes MP. (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. *Proc. Natl. Acad. Sci. U. S. A.* 94:10907-12.
25. Bryan TM, Reddel RR. (1994) SV40-induced immortalization of human cells. *Crit. Rev. Oncog.* 5:331-57, 1994.
26. Willey JC, Broussoud A, Sleemi A, Bennett WO, Cerutti BP, Harris CC. (1991) Immortalization of normal human bronchial epithelial cells by human papillomaviruses 16 or 18. *Cancer Res.* 51:5370-7.

27. Achanzar WE, Brambila EM, Diwan BA, Webber MM, Waalkes MP. (2002) Inorganic arsenite-induced malignant transformation of human prostate epithelial cells. *J. Natl. Cancer Inst.* 94:1888-91.

28. Piao CQ, Liu L Zhao YL, Balajee AS, Suzuki M, Hei TK. (2005) Immortalization of human small airway epithelial cells by ectopic expression of telomerase. *Carcinogenesis* 26:725-31.

29. Fenech M. (2000) The *in vitro* micronucleus technique. *Mutat. Res.* 455:81-95.

30. Calaf GM, Hei TK. (2000) Establishment of a radiation and estrogen-induced breast cancer model. *Carcinogenesis* 21:769-76.

31. Hamada S, Nakajima K, Serikawa T, Hayashi M. (2003) The effect of aging on the results of the rat micronucleus assay. *Mutagenesis* 18:273-5.

32. Piao CQ, Hei TK. (2001) Gene amplification and microsatellite instability induced in tumorigenic human bronchial epithelial cells by alpha particles and heavy ions. *Radiat. Res.* 155:263-7.

33. Otto E, McCord S, Tlsty TD. (1989) Increased incidence of CAD gene amplification in tumorigenic rat lines as an indicator of genomic instability of neoplastic cells. *J. Biol. Chem.* 264:3390-6.

34. Wahl GM, Padgett RA, Stark GR. (1979) Gene amplification causes overproduction of the first three enzymes of UMP synthesis in *N*-(phosphonacetyl)-L-aspartate-resistant hamster cells. *J. Biol. Chem.* 254:8679-89.

35. Lemoine FJ, Marriott SJ. (2002) Genomic instability driven by the human T-cell leukemia virus type I (HTLV-I) oncoprotein. *Tax Oncogene* 21: 7230-4.

36. Chernova OB, Chernov MV, Ishizaka Y, Agarwal ML, Stark GR. (1998) MYC abrogates p53-mediated cell cycle arrest in *N*-(phosphonacetyl)-L-aspartate-treated cells, permitting CAD gene amplification. *Mol. Cell Biol.* 18:536-45.

37. Mondello C, Riboni R, Rady M, Giulotto E, Nuzzo F. (1995) Gene amplification in Chinese hamster DNA repair deficient mutants. *Mutat. Res.* 346:61-7.

38. Reichmann E. (1994) Oncogenes and epithelial cell transformation. *Semin. Cancer Biol.* 5:157-65.

39. Rosenwald IB. (2004) The role of translation in neoplastic transformation from a pathologist's point of view. *Oncogene* 23:3230-47.

40. Vogelstein B, Fearson ER, Bose JL. (1988) Genetic alteration during colorectal-tumor development. *N. Engl. J. Med.* 319:525-32.

41. Ling V Chambers AF, Harris JF, Hill RP. (1985) Quantitative genetic analysis of tumor progression. *Cancer Metastasis Rev.* 4:173-94.

42. Liu J, et al. (2006) Further studies on aberrant gene expression associated with arsenic-induced malignant transformation in rat liver TRL1215 cells. *Toxicol. Appl. Pharmacol.* 216:407-15.

43. Chen H, Liu J, Zhao CQ, Diwan BA, Merrick BA, Waalkes MP. (2001) Association of c-myc overexpression and hyperproliferation with arsenite-induced malignant transformation. *Toxicol. Appl. Pharmacol.* 175:260-8.

44. Menendez D, Mora G, Salazar AM, Ostrosky-Wegman P. (2001) ATM status confers sensitivity to arsenic cytotoxic effects. *Mutagenesis* 16:443-8.

45. Hsieh HJ, Chen JT, Hsieh SH, Jee GS, Chen CJ. (1994) Arsenic-related Bowen's disease and paraquat-related skin cancerous lesions showed no detectable ras and p53 gene alterations. *Cancer Lett.* 28:59-65.

46. Chanda S, et al. (2006) DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicol. Sci.* 89:431-7.

47. Boonchai W, Walsh M, Cummings M, Chenevix-Trench G. (2000) Expression of p53 in arsenic-related and sporadic basal cell carcinoma. *Arch. Dermatol.* 136:195-8.