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Sodium Arsenite Suppresses Human Papillomavirus-16 E6 Gene and Enhances Apoptosis in E6-Transfected Human Lymphoblastoid Cells

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Abstract The p53 tumor suppressor pathway is disrupted by human papillomavirus (HPV) in most cervical cancer cells. The E6 proteins, which could mediate p53 degradation, are related to cellular immortalization, transformation, and tumor formation. In order to study the E6 abrogated p53 function in stress, we transfected HPV-16 E6 gene to TK6 cells in this study. Here we showed that HPV-16 E6 mRNA levels decreased in a dose dependent manner after sodium arsenite (SA) treatment, but not after X-irradiation. P53, p21, and MDM2 were induced in E6-transfected TK6 cells, as well as in parental TK6 cells after arsenite treatment. But the above proteins were only induced in TK6 cells after X-irradiation. It indicated that arsenite, but not X-ray, could suppress the transcription of E6 gene and therefore activate the p53 tumor suppressor pathway in TK6-E6 cells. After arsenite treatment, TK6-E6 cells showed more sub-G1 apoptosis, activated caspase-3/CPP32 fragment, DNA ladder, and less viability than parental TK6 cells, indicating that arsenite enhanced apoptosis in E6-transfected TK6 cells. In contrast, after X-irradiation, TK6-E6 cells showed less sub-G1 apoptosis and higher viability than parental TK6 cells. Thus, it would be another possible strategy to promote arsenite as another potential candidate for the therapeutic purpose in HPV-positive cancer cells. J. Cell. Biochem. 84: 615–624, 2002.

Key words: sodium arsenite; human papillomavirus; E6; p53; apoptosis

Arsenic has been used as a therapeutic agent and poison for more than 2,400 years [Antman, 2001; Waxman and Anderson, 2001]. The mechanisms for its cytotoxic effects are not completely understood until recently. We had previously reported that sodium arsenite (SA) induced apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species (ROS) [Wang et al., 1996]. The arsenic compound, As_2O_3, presently used to cure acute promyelocytic leukemia (APL) patients was also reported through apoptosis [Chen et al., 1997; Shen et al., 1997]. The arsenic-induced apoptosis was through the generation of ROS [Jing et al., 1999]. Also, it was modulated by the cellular glutathione redox system [Huang et al., 1993; Dai et al., 1999]. Recently, As_2O_3 was applied in the treatment of androgen-independent prostate cancer cells [Maeda et al., 2001], and gynecological cancer cell lines [Du and Ho, 2001]. Maeda et al. suggested that the generation of ROS as a therapeutic target potentiated As_2O_3-induced apoptosis in their cancer cells. Since most of these above cells did not have normal p53 expression, it is not known how normal p53 may have some roles in arsenite induced cytotoxicity or apoptosis in other p53 normal cells.

It was reported that SA could induce p53 expression in human papillomavirus-18 (HPV 18) containing HeLa cells [Salazar et al., 1997]. Recently, Denk et al. [2001] reported that p53 mutations are rare events in recurrent cervical cancer. Cervical cancer cells usually possess wild type p53 alleles. Normally, E6 protein (one of the oncoproteins from HPVs) binds to normal p53, promoting its degradation through the ubiquitin pathway and thus abrogates its function [Scheffner et al., 1990; Crook et al., 1991; Munger et al., 1992]. It is possible that the SA seems to modulate the original p53 levels in

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these E6 containing HeLa cells. Most cervical cancer cells have HPVs infection [zur Hausen, 1996]. Besides E6 oncogene, there are numerous oncogenes, such as E1, E2, E4, E5, and E7 within these cells [Seedorf et al., 1985]. It is difficult to single out the influence of E6 from that of several above oncogenes in HPV-infected cervical cancer cells after exposure to a specific agent. In order to study the interaction between SA and E6 protein more directly, we transfected HPV-16 E6 gene to TK6 cells (wild type p53), human lymphoblastoid cells without HPVs infection. The p53 expression in TK6 cells and E6-transfected TK6 cells after SA treatment was examined in this study.

In addition, several E6-transfected cells did not produce p53 expression after treatment with several kinds of DNA damage agents, such as cisplatin [Hawkins et al., 1996], UV-radiation [El-Mahdy et al., 2000], ionizing radiation (IR) [Yu et al., 1997; Yu and Little, 1998], mitomycin C (MMC) [Xu et al., 1995], and taxol [Wahl et al., 1996]. However, their parental cells did produce p53 after treatment. The p53 abrogation in these E6-transfected cells after treatment of various DNA damage agents resulted in different apoptosis responses. Some of these E6-transfected cells showed more apoptosis after cisplatin, MMC, or taxol treatment than their parental cells [for review Rapp and Chen, 1998]. But, others, such as E6-transfected TK6 lymphoblastoid cells showed less apoptosis than their parental cells after IR exposure [Yu and Little, 1998]. Without enough p53 after drug treatment in these E6-containing cells, these E6-transfected cells contained E6-abrogated p53, but their corresponding induced apoptosis seemed not completely dependent on p53 among these E6-transfected cells. In this study, we treated SA in E6-transfected and parental TK6 cells and examined whether arsenite would regulate E6 gene expression. We also examined whether these different cells showed different arsenite-induced apoptosis and cytotoxicity.

MATERIALS AND METHODS

Materials

SA and propidium iodide (PI) were purchased from Merk (Schuchardt, Germany). RPMI-1640 medium was obtained from Gibco (Grand Island, NY). Methylthiozole tetrazolium (MTT) was purchased from Sigma (USA). Dimethyl sulfoxide (DMSO) was from Fisher Scientific. Monoclonal anti-p53 Do-1 antibody and anti-MDM2 antibody were from Santa Cruz Biotechnology. Monoclonal anti-p21 antibody was from BioLab. Polyclonal anti-caspase-3/CPP32 antibody was from Transduction Laboratories. Anti-mouse and anti-rabbit IgG horseradish peroxidase linked antibodies were from Amersham Life Science.

Cells and Culture Conditions

The TK6 cells (ATCC number: CRL-8015) derived from WIL-2 cells and TK6-E6 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) horse serum, 0.2% sodium bicarbonate, 0.03% L-glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C and with 5% CO_2.

Transfection of HPV-16 E6 Gene

Retrovirus carrying (a) both HPV-16 E6 and neo gene (neo-E6) or (b) neo gene from Dr. J.B. Little (originally provided by Dr. D.A. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) were used to transfect TK6 cells [Huang et al., 1996]. Approximately 10^6 cells were first incubated in 1 ml PRMI-1640 plus 3 ml virus-containing DMEM medium for 1 day and changed to fresh RPMI-1640 medium for another day. Cells were re-seeded at various densities in RPMI medium containing G418 (0.4 mg/ml) to selection for 14–20 days. G418-resistant clones were isolated and screened by means of polymerase chain reaction (PCR) using E6 specific primers and by Western analysis of p53 expression after irradiation with 0 or 13 Gy plus 2 h incubation.

Western Blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed on ice in lysis buffer containing 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 50 mM Tris-base at pH 8.0, 150 mM NaCl, 0.02 % NaN_3, 1% NP-40, and 1 μg/ml aprotinine. After centrifugation at 10,000*g* for 30 min, the supernatant containing proteins was collected, and protein concentration was determined by Bio-Rad DC Protein Assay kit (Bio-Rad laboratories, Richmond, CA). Equal amounts of proteins were electrophoresed on 10% SDS–polyacrylamide gel and electrotransferred onto PVDF membranes. Membranes were probed with appropriate

antibody and the signals were detected by an enhanced chemiluminescent (ECL) system.

Genomic DNA Extraction and PCR

In order to determine whether the HPV-16 E6 gene was integrated into the TK6 cells, genomic DNA was extracted from various G418 resistant cell clones. Genomic DNA from G418 resistant clones derived from E6-transfected TK6 cells showing abnormal p53 function by Western blotting were also prepared to confirm the integration of the HPV-16 E6 gene into the cells. Approximately $1-5 \times 10^5$ cells from single clone were lysed with 0.3 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 400 mM NaCl, and 1% SDS) and treated with proteinase K (100 μg/ml) at 50°C for 2 h or more. Saturated NaCl (0.1 ml) was then added to the lysis buffer and vortex. The supernatant was removed by 57,000*g* centrifugation for 20 min and precipitated with ethanol. The DNA was recovered after RNase (20 μg/ml) treatment and another ethanol precipitation. Amplification of E6 sequences integrated into the genome was carried out in buffer containing 100 ng genomic DNA, 200 ng primers, 1 U of Taq DNA polymerase (Pharmacia Biotech) in 30 μl solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, and 200 μM each of four different dNTP. PCR amplification was carried out for 35 cycles each of 1 min at 95°C and 48°C, and 30 s at 72°C using a programmable thermal controller (MJR PTC-100). After the last cycle of amplification, the samples were incubated at 72°C for 10 min. The oligonucleotide primers used in the experiments are listed below: HPV-16 E6 sense 5′-ATGCACCAAAAGAGAACTGCAATG-3′ and antisense 5′-TTACAGCTCCCTTTCTCTACGTGT-3′. The size of the PCR product of E6 is 476 bp [Huang et al., 1996].

RNA Extraction and Reverse Transcription–PCR (RT–PCR)

This method was performed and modified as previously described [Germolec et al., 1998]. Total cellular RNA was extracted by RNAzol B solution (Biotech Laboratories, Inc., Huston, TX), according to the manufacturer's instructions. For the synthesis of cDNA, 10 μg of total RNA was resuspended in a 20-μl final volume of the reaction buffer (50 mM Tris-HCl, pH 8.6, 40 mM KCl, 1 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 500 μM dNTP, and 40U RNasin) and 0.5 μg oligo(dT) 12-18 primer (Pharmacia). After the reaction reached 42°C, 400 U MML-V reverse transcriptase (200 U/μl) (USB) was added to each tube and incubated at 37°C for 30 min. The reaction mixture was stopped by denaturing the enzyme at 75°C for 15 min. Ten-microliter aliquots of the synthesized cDNA were added to a 30-μl final volume of the reaction buffer containing 200 ng primers, 1 U of Taq DNA polymerase (Pharmacia Biotech), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, and 200 μM each of four different dNTPs. PCR amplification was carried out for 35 cycles each of 1 min at 95 and 48°C, and 30 s at 72°C using a programmable thermal controller (MJR PTC-100). After the last cycle of amplification, the samples were incubated at 72°C for 10 min. RNA concentrations and PCR cycle were titrated to establish standard curves, to document linearity, and to permit semiquantitative analysis of single strength as previously described [Germolec et al., 1996]. The PCR products were visualized by UV illumination after electrophoresis through 2.0% agarose gel containing 0.5 μg/ml ethidium bromide at 100 V for 30 min in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.0). The PCR products were then quantified by a densitometer (Amersham Pharmacia Biotech). The primers of HPV-16 E6 gene were described previously, and the primers of human β-actin were as following: sense 5′-TGACTGACTACCTCATGAAG-3′ and antisense 5′-AAGGCTGGAAGAGTGCCTCA-3′. The size of the PCR product of β-actin is 239 bp, as an internal control.

DNA Analysis by Flow Cytometry

Cells were washed twice with cold PBS and fixed with 70% ethanol at 4°C over night. After removing ethanol, the cell pellets were stained with mixture containing 8 μg/ml PI and 50 μg /ml RNase A at room temperature in the dark for 30 min and applied to FACScan flow cytometry (Becton Dickinson) analysis. The percentages of cells with DNA content less than 2N (sub-G1) were analyzed by ModFit program (Becton Dickinson).

DNA Fragmentation Assay

This assay was performed and modified as previously described [Kondo et al., 1995]. Briefly, harvested cells (2×10^7) were centrifuged and washed twice with cold PBS. The cell pellet was lysed in 1.0 ml of a buffer containing 10 mM Tris-HCl, 10 mM EDTA, and 0.2% Triton

X-100 (pH 7.5). After 10 min on ice, the lysate was centrifuged (13,000*g*) for 10 min at 4°C in an Eppendorf microtube. Then, the supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted first with phenol and then with phenol-chloroform: isoamyl alchocol (24:1). The aqueous phase was brought to 300 mM NaCl, and nucleic acids were precipitated with 2 vol of ethanol. The pellet was washed with 70% ethanol, air-dried, and then dissolved in 20 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Following digestion of RNA with RNase A (0.6 mg/ml, at 37°C for 30 min), the sample was electrophoresed in a 2% agarose gel with TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.0). DNA was then visualized with ethidium bromide staining under UV light.

MTT Assay

Cellular viability was assessed by the ability of cells to convert the soluble salt of MTT to insoluble formazan precipitate in MTT assay. Cells were first seeded in 6-well dishes at an initial density of 1×10^5 cells/ml. For SA treatment, cells were treated in drug-containing medium for 4 h and then changed to fresh medium for indicated time. For X-ray irradiation, cells were exposed to 4 Gy X-ray and reincubated in 37°C for indicated time. MTT was added to medium to the final concentration of 0.5 mg/ml 4 h before harvesting cells. One milliliter of cultured cells was collected by centrifugation at 2,000*g* for 5 min and the supernatants were removed. The cell pellets containing intracellular formazan complex form were dissolved in 1 ml DMSO. The color formed was quantified by measuring the absorption value at 540 nm. The cellular viability was expressed relative to the set of control cells.

RESULTS

Selection of Neo and E6 Transfected Clones

TK6 cells were transfected with either the retrovirus-derived vector containing the (a) neo gene alone or (b) both the neo and HPV-16 E6 gene (neo/E6). Multiple clones resistant to G418 were isolated and screened by means of Western analysis of p53 after X-irradiation with 0 or 13 Gy plus 2 h incubation. At least nine individual clones (T10, T12, T14, T15, T25, T26, D6, D17, and D18) showing suppressed p53 expression were selected from 45 G418-resistant transfected clones (Fig. 1a). These candidates were then examined with PCR using E6 specific primers (Fig. 1b), indicating that all of the above mentioned E6-transfected clones showed E6 inserted gene. Therefore, TK6-E6-X cells were used to describe the cells that originated from the above X clones in the following studies.

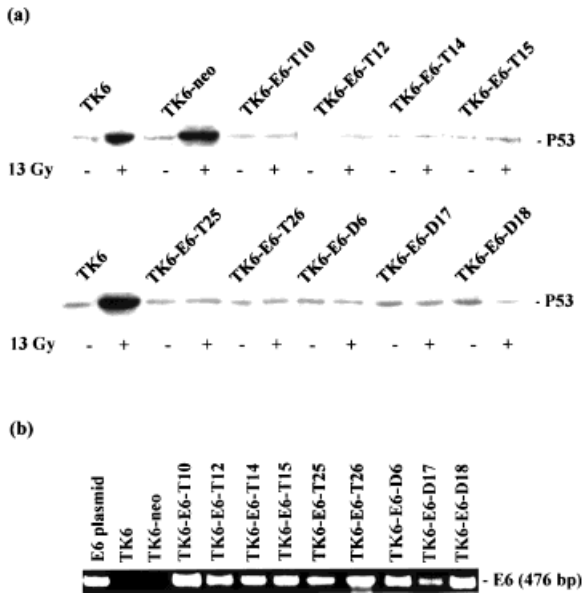


Fig. 1. Examination of p53 expression and E6 gene integration in subclones derived from TK6 cells. Cells were exposed to 0 (−) or 13 (+) Gy X-ray plus 2 h incubation. Whole cellular proteins were isolated, and Western analysis of p53 was performed as described in Materials and Methods. Suppressed p53 expression in TK6-E6 clones (T10, T12, T14, T15, T25, T26, D6, D17, and D18) was shown in figure (**a**). These TK6-E6 candidates were also confirmed with HPV-16 E6 integration by PCR examinations using E6 specific primers (**b**).

HPV-16 E6 mRNA Levels Were Downregulated by SA

Although the HPV-16 E6 mRNA levels were determined by RT–PCR (described in Materials and Methods) in several E6-transfected cells, the results from one of these E6 stable transfected clones, TK6-E6-T15 cells, were shown here in Figure 2 and the following studies. HPV-16 E6 mRNA was normalized by β-actin mRNA from each collected sample. After 20, 40, and 60 μM SA treatment in TK6-E6-T15 cells for 4 h, a downregulation of HPV-16 E6 mRNA, 47.18, 38.22, and 20.02% of those same mRNA compared with untreated control cells were found, respectively (Fig. 2a). These indicated that SA interfered with the transcription of HPV-16 E6 gene. However, HPV-16 E6 mRNA levels had no

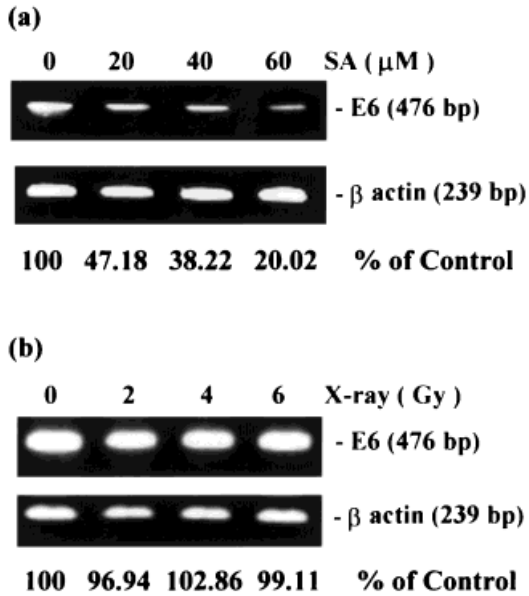


Fig. 2. The HPV-16 E6 and β-actin mRNA levels after SA treatment or X-ray exposure in TK6-E6-T15 cells. TK6-E6-T15 cells were treated with 0, 20, 40, and 60 μM SA for 4 h (**a**) or exposed to 0, 2, 4, and 6 Gy X-ray plus 4 h post-incubation (**b**). Total cellular RNA was isolated and RT–PCR was performed as described in Materials and Methods to determine relative difference of E6 mRNA levels normalized by β-actin. The percentages of E6 mRNA levels compared with untreated cells were shown at the bottom of the plot.

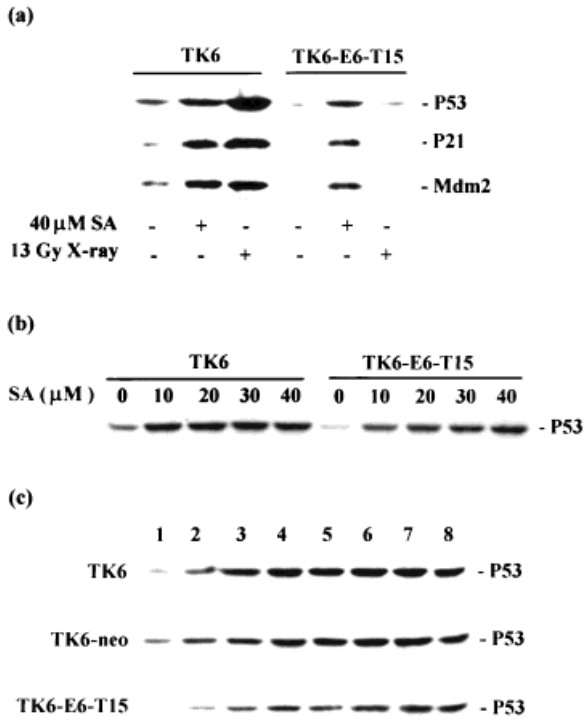


Fig. 3. Expressions of p53, $p21^{WAF1/CIP1}$, and MDM2 after SA or IR exposure. **a**: Cells were treated with 40 μM SA for 4 h or exposed to 13 Gy X-ray plus 2 h incubation. Whole cell lysates were collected and then examined by Western blotting of p53, $p21^{WAF1/CIP1}$, and MDM2. **b**: Cells were treated with different concentrations (0–40 μM) of SA for 4 h. **c**: Cells were treated with 40 μM SA for 1, 2, 3, and 4 h (**lane 1–lane 4**) and changed to fresh medium for another 1, 2, 3, and 4 h (**lane 5–lane 8**). Whole cell lysates were collected and then examined by Western blotting of p53.

difference among 0, 2, 4, and 6 Gy X-irradiated TK6-E6-T15 cells (Fig. 2b).

SA Altered p53, $p21^{WAF1/CIP1}$ and MDM2 Protein Levels

In comparison with untreated TK6 cells, about a 2-fold increase of p53, p21, and MDM2 in TK6 cells after 40 μM SA treatment for 4 h and about a 2–3-fold increase of the above proteins in the same cells after 13 Gy X-irradiation plus 2 h post-incubation were found. A more than 9-fold increase of p53 and its down stream proteins, $p21^{WAF1/CIP1}$ and MDM2, were detected in TK6-E6-T15 cells after 40 μM SA treatment, but not after 13 Gy X-irradiation (Fig. 3a). It indicated that arsenite could suppress the HPV-16 E6 gene and therefore reduced the E6-abrogated p53 expression in E6-transfected TK6 cells. On the other hand, TK6-E6-T15 cells also showed dose and time dependent p53 increase after arsenite treatment, as shown in their parental TK6 cells (Fig. 3b,c). About a 2–3-fold and a 7–15-fold increase of p53 were detected in TK6 cells and TK6-E6-T15 cells, respectively, after 10–40 μM SA treatment for 4 h. The E6 function in TK6-E6-T15 cells seems less effective when a larger arsenite dose or a longer time was applied in these cells.

SA Enhanced Apoptotic Responses in E6-Transfected TK6 Cells

Table I, column a showed that all six TK6-E6 transfected clones (T10, T12, T14, T15, T25, and T26) were more sensitive to produce sub-G1 apoptosis than their parental TK6 cells after 20 μM SA treatment for 4 h plus post-incubation for another 24 h. TK6-E6-T15 cells appeared most sensitive to arsenite among the above tested cells. In contrast, all of the above mentioned E6-transfected clones were more resistant to IR-induced sub-G1 apoptosis than their parental TK6 cells after 4 Gy X-ray exposure plus 48 h post-incubation (Table I, column b). TK6-E6-T14 cells appeared most resistant to IR among above tested cells. TK6-E6-T14, TK6-E6-T15 cells, and parental TK6 cells induced dose-dependent sub-G1 apoptosis after treatment

TABLE I. Sub-G1 Percentages of TK6 Cells and TK6-E6 Cells

Cell lines	20 μM SA[a]	4 Gy X-ray[b]	E6 gene insertion
TK6	10.42 ± 2.23	34.11	−
TK6-E6-T10	12.52	19.28	+
TK6-E6-T12	15.76 ± 3.40	12.19	+
TK6-E6-T14	23.55 ± 7.71	9.04	+
TK6-E6-T15	28.93 ± 5.10	10.86	+
TK6-E6-T25	24.90 ± 8.22	15.31	+
TK6-E6-T26	24.85 ± 4.09	10.6	+

[a]After 20 μM SA treatment for 4 h and recovering in fresh medium for another 24 h.
[b]After 4 Gy X-irradiation plus 48 h incubation.

with 0–40 μM SA for 4 h plus a 24 h post-incubation; however, the E6-transfected cells appeared more sensitive to arsenite-induced sub-G1 apoptosis than TK6 cells (Fig. 4a).

Because caspase-3/CPP32 has been implicated as playing a critical role in mediating apoptosis, we also asked whether the differential apoptotic responses in these cells are related to its activation. The activation of caspase-3/CPP32 was monitored by Western analysis. TK6-E6-T15 cells showed more activated caspase-3/CPP32 fragment (17 kDa) than TK6 cells immediately after 0–40 μM SA treatment for 4 h (Fig. 4b). When cells were treated with 20 μM SA plus a post-incubation for 0, 4, 8, or 12 h, significant amounts of 17 kDa caspase-3/CPP32 fragment appeared in each sample collected from TK6-E6 cells in comparison with that from parental TK6 cells (Fig. 4c). TK6-E6-T15 cells also produced more fragmented DNA than parental TK6 cells after the same treatment protocols (Fig. 4d). To sum up, SA enhanced apoptotic responses in E6-transfected TK6 cells.

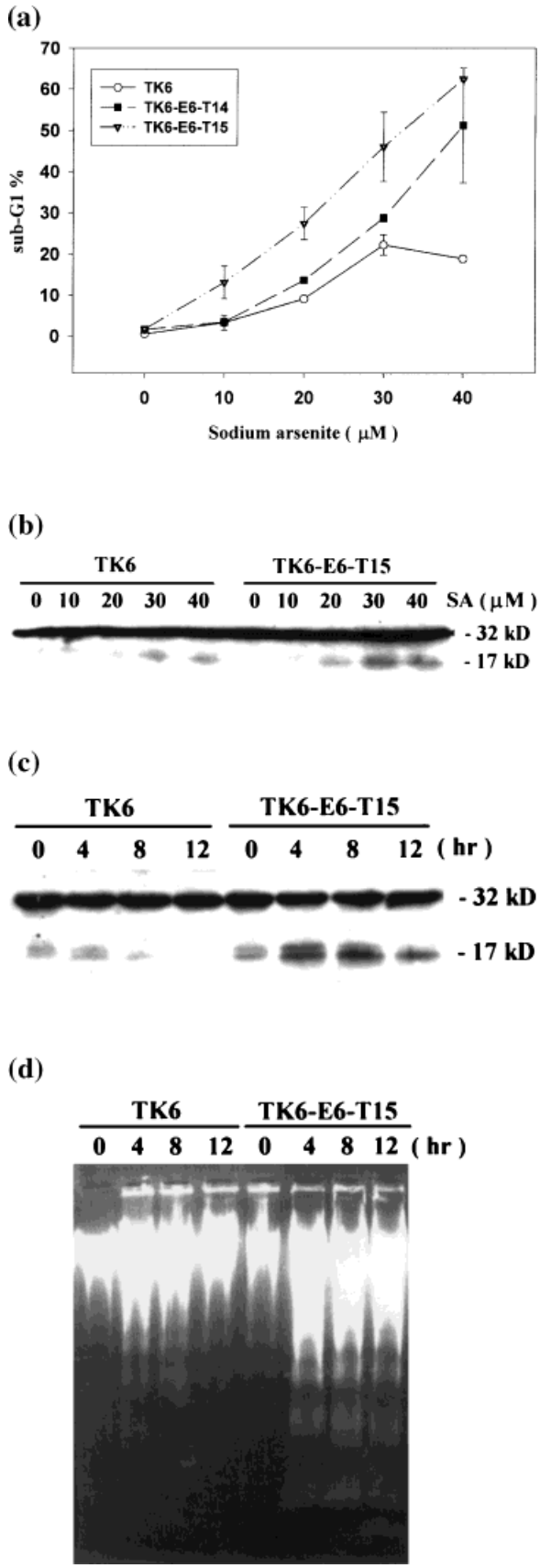
E6-Transfected TK6 Cells Were More Sensitive to SA, but More Resistant to IR Than Parental TK6 Cells

TK6-E6-transfected clones (T12, T14, and T15) had significantly lower cellular viability than parental TK6 cells after 20 μM SA treatment for 4 h and followed with post-incubation for 12, 24, 48, and 72 h by MTT assay as described in Materials and Methods (Fig. 5a). However, they showed significantly higher cellular viability than their parental TK6 cells after 4 Gy X-irradiation and recovering for 24, 48, and 72 h (Fig. 5b). The sensitivity of TK6-neo-transfected clone to SA or IR had no difference from that of the parental TK6 cells (Fig. 5). Another type of E6-transfected TK6 cells, TK6-E6-5E cells, kindly provided by Dr. J.B. Little, were also examined in this study. The results of E6 mRNA levels, p53 expression, apoptotic responses, and cellular viability from TK6-E6-5E cells were similar to those from our TK6-E6-T15 cells (data not shown).

DISCUSSION

In this study, we have found that the E6-transfected TK6 cells were more sensitive to SA than parental cells on both cellular viability and apoptotic responses (Figs. 4 and 5a). Arsenite significantly increased p53, $p21^{waf1/cip1}$, and MDM2 expression in TK6-E6-T15 cells, and the increased folds of above proteins were more than that in TK6 cells (Fig. 3a). In addition, more activated caspase-3/CPP32 fragment (17kDa) was observed in TK6-E6-T15 cells than in parental TK6 cells (Fig. 4b,c). In previous studies, caspase-3 has been reported to be involved in arsenite-induced apoptosis in NIH3T3 cells [Chen et al., 1998], U937 cells [McCabe et al., 2000], and human gastric cancer cells, AGS [Jiang et al., 2001]. It is possible that arsenite sensitizes TK6-E6 cells via suppression of E6 abrogated p53 and activation of caspase-3.

The increase of p53 expression might be partially due to the decrease of E6 mRNA expression in arsenite-treated TK6-E6 cells (Fig. 2a). Therefore, it would affect intracellular E6 levels to abrogate p53 protein. On the other hand, arsenite might directly or indirectly bind the thiol groups to E6 protein to affect its function or activity. Since E6 protein contains two zing finger sequences, cysteine residues at the base of each of the Cys-X-X-Cys motifs [Thomas et al., 1999], it would be possible for arsenite to change its abrogation ability to p53 protein through a binding to a cysteine or thiol groups of E6 protein. In fact, arsenite has been shown to bind compounds containing vicinal thiol [Delnomdedieu et al., 1993], or bind NF-kB on its critical cysteine. It did affect the function or activity of these proteins [Kapahi et al., 2000]. It should not rule out another possibility



to find out whether arsenite also affects another components besides E6 protein itself in E6/p53 degradation pathway. It is reasonable to believe that arsenite might also affect the integrity of another E6 associated protein or some other factors in E6 induced p53 degradation pathway. Therefore, E6 loses its abrogation ability to degrade p53, and apoptosis increases. Further evidence is required to support this arsenic-E6 interaction.

Several approaches have been proposed to control the growth of E6-expressing cancer cells recently. Hietanen et al. [2000] used some small molecules, leptomycin B and actinomycin D, to restore p53 activity in HPV-positive cervical carcinoma cells. Others apply (i) the papillomavirus E2 gene [Goodwin and DiMaio, 2000], (ii) antisense strategies [Steele et al., 1993], and (iii) the variant forms of E6 that interact both with full length E6 protein and with p53 [Pim et al., 1997], resulting in suppression of E6-mediated degradation of p53. In this study, we have confirmed that the restoration of p53 function was due to arsenite mediated reduction of E6 mRNA expression in E6-transfected cells (Fig. 2a). It would be another strategy here to promote arsenite as another potential candidate for the therapeutic purpose in HPV-positive cancer cells. In our preliminary data, we have found that arsenite again could reduce the E6 mRNA level and restore p53 expression in SiHa cervical cancer cells, which contain HPV16 (data not shown here).

In previous investigation, Yu and Little [1998] found that E6-transfected TK6 cells, TK6-E6-5E, did not induce p53 expression and did perform less apoptosis after 4 Gy γ-irradiation and 48 h post-incubation than those from parental TK6 cells. In addition, TK6-E6-5E cells did not alter radiosensitivity as measured by

Fig. 4. Apoptotic responses of TK6 cells and TK6-E6 cells after SA treatment. **a**: Cells were treated with various concentrations of SA for 4 h and changed to fresh medium for another 24 h. Cells were then applied to flow cytometric analysis for quantifying apoptotic cells with sub-G1 DNA contents by ModFit program. **b**: Cells were treated with various concentrations of SA for 4 h. Whole cell lysates were collected and then examined by Western blotting of Caspase-3/CPP32. Both pro-caspase-3 (32 kDa) and activated-caspase-3 (17 kDa) fragments were detected. Cells were treated with 20 μM SA for 4 h and changed to fresh medium for 0, 4, 8, and 12 h. Whole cell lysates were collected and then examined by Western blotting of Caspase-3/CPP32 (**c**). Fragmented DNA obtained from 2×10^7 cells in indicated time was resolved by agarose gel electrophoresis, ethidium bromide stained, and photographed (**d**).

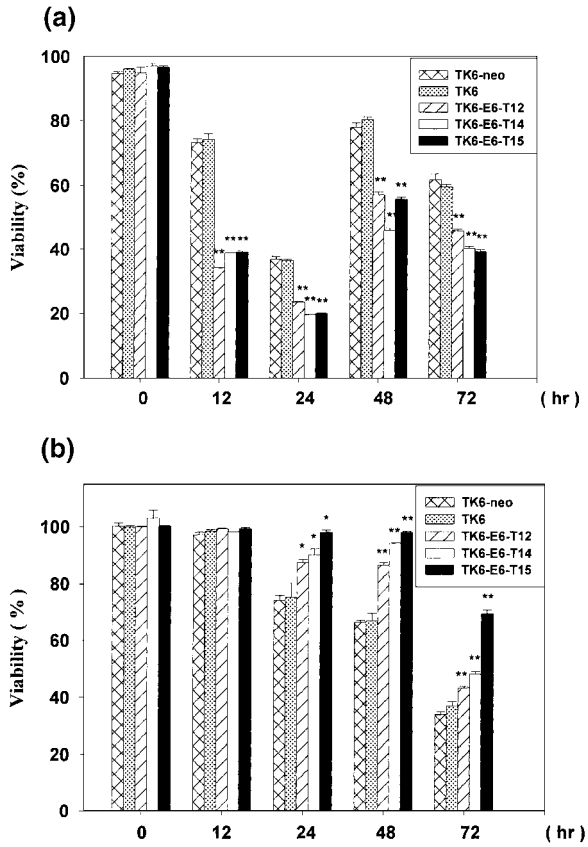


Fig. 5. SA and radiation sensitivity of TK6 cells and TK6-E6 cells. Cells were treated with 20 μM SA for 4 h and changed to fresh medium for the indicated time (**a**) or exposed to 4 Gy X-ray plus the indicated time of incubation (**b**). Viability was examined by MTT assay as described in Materials and Methods. TK6-E6 cells (T12, T14, and T15) were more sensitive to SA (a), but more resistant to X-irradiation (b) than TK6 cells. Data shown here were from three independent experiments and statistical analysis was performed with *t*-test. The symbols, ** and *, indicate $P < 0.01$and $P < 0.05$, respectively, in comparison with TK6 cells.

colony forming ability [Yu et al., 1997]. In this study, we also observed the similar apoptotic responses in various TK6-E6 clones (abrogated p53) (Table 1, column b), but the radiosensitivity was different, based on the data of cellular viability as measured by MTT assay (Fig. 5b). The E6-transfected TK6 cells were more resistant to IR than their parental TK6 cells at least in the beginning of 72 h after X-irradiation. IR has been used to cure several kinds of cancer including cervical cancer [Raimondi, 1972; Gerstner et al., 1986; Trinci et al., 1993; Greskovich and Macklis, 2000; Duan et al., 2001]. According to our results, TK6-E6 cells were more resistant to IR, but more sensitive to SA than their parental TK6 cells. Instead of IR, arsenite is worth to be further investigated for therapeutic purpose in other E6-containing tumor cells, such as SiHa or HeLa cells.

In conclusion, we have provided evidence to show that the arsenite treatment activated the p53 tumor suppressor pathway (p53, $p21^{waf1/cip1}$, and MDM2), increased cytotoxic effects and induced apoptosis in E6-positive TK6 cells. And the restoration of p53 function has been correlated with the decrease of E6 mRNA levels in E6-transfected cells after arsenite treatment. We speculate arsenite may interact with E6 protein. Additional studies are required to know more about the interaction among arsenite, E6, p53, and some associated proteins in E6/p53 degradation pathway.

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