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Effects of arsenite on cell cycle progression in a human bladder cancer cell line

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Abstract

Bladder cancer is one of the most important diseases associated with arsenic (As) exposure in view of its high prevalence and mortality rate. Experimental studies have shown that As exposure induces cell proliferation in the bladder of sodium arsenite (iAsIII) subchronically treated mice. However, there is little available information on its effects on the cell cycle of bladder cells. Thus, our purpose was to evaluate the effects of iAsIII on cell cycle progression and the response of p53 and p21 on the human-derived epithelial bladder cell line HT1197. iAsIII treatment (1–10 μM) for 24 h induced a dose-dependent increase in the proportion of cells in S-phase, which reached 65% at the highest dose. A progressive reduction in cell proliferation was also observed. BrdU was incorporated to cellular DNA in an interrupted form, suggesting an incomplete DNA synthesis. The time-course of iAsIII effects (10 μM) showed an increase in p53 protein content and a transient increase in p21 protein levels accompanying the changes in S-phase. These effects were correlated with iAs concentrations inside the cells, which were not able to metabolize inorganic arsenic. Our findings suggest that p21 was not able to block CDK2–cyclin E complex activity and was therefore unable to arrest cells in G1 allowing their progression into the S-phase. Further studies are needed to ascertain the mechanisms underlying the effects of iAsIII on the G1 to S phase transition in bladder cells.

Keywords: Arsenic; Bladder; Cell cycle; p53; p21

1. Introduction

Epidemiological studies have indicated that ingestion of inorganic arsenic via drinking water results in increased risks of internal cancers, particularly bladder

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and lung cancer (Tseng et al., 1968). Bladder cancer is particularly important in view of its high prevalence and mortality rate. More recently, Smith et al. (1998) observed an increased mortality due to bladder cancer in Chileans exposed chronically to iAs in drinking water containing 470 μg/l (6.1 μM).

Although little is known about the molecular mechanisms by which iAs exposure causes bladder cancer, the existing evidence indicates that iAs increases cell proliferation in bladder epithelium. Simeonova et al. (2000) reported that mice C57BL/6 treated with sodium arsenite (0.76 nM; 5.8 ppb) in their drinking water for 4 or 16 weeks showed bladder hyperplasia accompanied with increased levels of proliferating cell nuclear antigen (PCNA). In vitro studies on fibroblasts (C3H 10T1/2) treated with iAsIII (6 μM) during 2 months and stimulated with EGF, showed an increase in both DNA synthesis and the proportion of cells entering into S phase followed by an increased expression of c-*myc* and E2F-1, indicating that arsenic enhanced cellular proliferation induced by EGF (Trouba et al., 2000).

Cell proliferation depends on an ordered and tightly regulated process known as the cell cycle, involving multiple checkpoints assessing extracellular growth signals, cell size, and DNA integrity (Ho and Dowdy, 2002). The cell cycle is regulated by complexes composed of regulatory cyclins and catalytic cyclin-dependent kinases (CDKs), whose formation and activation promote cell cycle progression (Weinberg, 1995). On the other hand, cyclin-dependent kinase inhibitors, act as negative regulators of the cell cycle by extinguishing the activity of CDKs (Cai and Dynlacht, 1998). Other important cell cycle progression regulators are tumor-suppressor proteins, such as p53 and retinoblastoma (pRb), which play important roles in the G1/S-phase transition (Lohez et al., 2003). p53 protein is a transcription factor that accumulates in response to DNA damage and other stress stimulus, increasing the expression of CKI p21 protein, which is able to silence CDKs essential for S-phase entry (those associated with cyclins E and A), thus inhibiting cell cycle progression (Sherr and Roberts, 1995). p21 is a cyclin kinase inhibitor that induces growth arrest by preventing phosphorylation of pRb in the G1/S transition (García del Muro et al., 2004). pRb regulates the G1/S transition depending on its phosphorylation status, which is modified by the CDKs–cyclin complexes (Barket and Lukas, 2001).

Bladder tumors from people chronically exposed to As showed mutations in *TP53* gene at codon 175 and transitions at points 9 and 10 (Shibata et al., 1994). In addition, iAsIII treatment (10 μM) has been reported to induce an accumulation of p53 protein in different cell lines having a wild or mutated gene, with a greater effect in cells having normal *TP53* than in those with mutated *TP53* (Salazar et al., 1997). Increases in p53 protein have also been reported in fibroblasts exposed to iAsIII (50 μM) and treated with 6 Gy ionizing radiation (Vogt and Rossman, 2001). Recent studies have shown that changes in p53 levels were associated with cell cycle arrest in fibroblasts and hepatocytes (Park et al., 2001; Oketani et al., 2002). Although bladder carcinoma is one of the more common tumors associated to iAs exposure, there is little information available on the effects of iAsIII treatment on cell cycle progression in bladder cells. Thus, our purpose was to evaluate the effects of iAsIII on cell cycle progression and the response of p53 and p21 on the human-derived epithelial bladder cell line HT1197, whose retinoblastoma (RB) and *TP53* genes are normal.

2. Materials and methods

2.1. Cell culture

HT1197 epithelial human bladder cancer cells were purchased from American Tissue Cell Culture (ATCC) (Manassas, VA). The HT1197 cell line was derived from a grade-4 transitional cell carcinoma obtained by resection from a 44-year-old Caucasian man (Rasheed et al., 1977). Cells were grown in culture dishes in minimal essential medium (MEM, SIGMA Chemical) supplemented with 10% fetal bovine serum, glutamine (100 mM), penicillin (50 μg/ml) and streptomycin (50 μg/ml). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C and maintained in exponential growth phase. Sodium arsenite was obtained from SIGMA Chemical (St. Louis, MO), and a stock solution was prepared at a final concentration of 100 mM, dissolved in deionized water, filter-sterilized and stored at −70 °C. The stock solution was diluted to working concentrations in phosphate buffer saline (PBS).

2.2. Cell viability

Logarithmically growing cells were incubated with varying concentrations of iAsIII (0, 1, 5, 10, 25 and 50 μM) for 24 h. Cultures were harvested and cell viability was assessed by the trypan blue exclusion assay. Briefly, cultures were washed with PBS, trypsinized for 3 min and neutralized by the addition of supplemented medium. After centrifuging (1000 rpm, 5 min), cells were suspended in 1 ml of PBS and a 50 μl aliquot was taken and suspended in 400 μl of PBS and 50 μl of trypan blue. Cells were counted using a Neubauer chamber.

2.3. Dose–response and time-course relationships

To study the dose–response relationships, logarithmically growing cells were incubated with iAsIII (0, 1, 5 and 10 μM). For the time-course study, logarithmically growing cells were incubated with 10 μM of iAsIII at different times (4, 8, 12, 16, 20 and 24 h). Cell cultures were harvested, and the cell cycle profile was determined.

2.4. Cell cycle analysis

To determine the cell cycle profile in the HT1197 cell line after iAsIII treatments, cell cultures were harvested and fixed with 1 ml of ice-cold ethanol 80% overnight. An aliquot of 250 μl of fixed cells was centrifuged (3000 rpm, 3 min), suspended in lysis buffer (100 mM sodium citrate and 0.5% Tween-20) and incubated during 15 min at room temperature. Cells were incubated with RNAse A (10 mg/ml) (SIGMA Chemical, St. Louis, MO) for 10 min at room temperature and DNA was stained with propidium iodide (50 μg/ml) for at least 1 h at 4 °C. DNA content was determined by flow cytometry using a FACSort (Beckton Dickinson) and ModFit software (Modfit Verity Software House).

2.5. Determination of arsenic and its metabolites in medium and cells

We determined intracellular As levels according to Styblo et al. (1999) with minor modifications. Briefly, 2.0×10^5 cells were seeded, and 36 h later, 10 μM of iAsIII was added to the medium during different times. After each incubation time (every 4 h until 24 h), cell culture medium was collected and stored at −20 °C, whereas cells were trypsinized and lysed with PBS-Triton 0.5% and stored at −20 °C until analysis. Arsenic levels were determined in three independent experiments run by duplicate by hydride generation-atomic absorption spectrophotometry (Perkin-Elmer 3100) according to Del Razo et al. (1997). As a positive control, As levels were determined in hepatocytes (HepG2 cell line) because of their high capacity to metabolize iAs.

2.6. BrdU incorporation assay

The percentage of HT1197 cells in S-phase was performed determinating the BrdU incorporation rate using the 5-bromo-2-deoxy-uridine Labeling and Detection Kit I (Roche, Indianapolis, IN). Briefly, 5×10^4 cells were seeded on coverslips and 24 h later, 10 μM of iAsIII was added. After 23 h of incubation, 10 μM BrdU was added and further incubated for 1 h. Then, cell cultures were fixed with 50 mM ethanol–glycine, pH 2.0 for 1 h at −20 °C. BrdU incorporation was detected according to manufacturer instructions. Cells were covered with an appropriate mounting medium and were examined in a fluorescence microscope. Confocal images were obtained with a MCR 1024 Bio-Rad laser scanning system equipped with an Ar Kr/Ar air-cooled laser attached to an inverted Nikon TMD 300 microscope.

2.7. Western blotting

Cells were washed with PBS and harvested in cell lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS), containing protease inhibitors (leupeptin, aprotinin, antipain, chemostatin, pepstatin and benzamidine) and phosphatase inhibitors (25 mM NaF, 1 mM orthovanadate). After 30 min of incubation at 4 °C, cell lysates were clarified at 12000 rpm for 10 min at 4 °C, and the supernatant was collected as the whole cell extract. Protein concentration was determined with the Bio-Rad (Hercules, CA) protein assay reagent, using bovine serum albumin (BSA) as standard. Same quantities of each sample were separated by electrophoresis on 12.5% SDS–polyacrylamide gels with pre-stained protein molecular weight standards. The separated proteins were then electroblotted on nitrocellulose membranes

(0.45 μm, Bio-Rad) and reacted with anti-p53, anti-p21 and anti-tubulin primary monoclonal antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). Immunodetection was followed by incubation with horseradish peroxidase coupled to secondary antibody (Zymed Laboratories, San Francisco, CA). Antigen–antibody complexes were visualized using chemiluminescent ECL reagents (Amersham Pharmacia Biotech UK Limited, UK). The intensity of the bands was evaluated by densitometric analysis and normalized with values of α-tubulin.

2.8. Statistical analysis

All data represent media ± standard deviation of at least three independent assays. Statistically significant differences were determined using a one-way ANOVA followed by multiple comparisons by Student–Newman–Keuls.

3. Results

3.1. Effects of arsenite on HT1197 cell viability

We determined cell viability after incubation with 0, 1, 5, 10, 25 and 50 μM of iAsIII during 24 h. iAsIII treatment reduced cell viability in a dose-dependent manner. Doses lower than 10 μM of iAsIII did not significantly reduce cell viability, whereas doses of 10, 25 and 50 μM reduced cell viability to 87, 64 and 32%, respectively (Fig. 1).

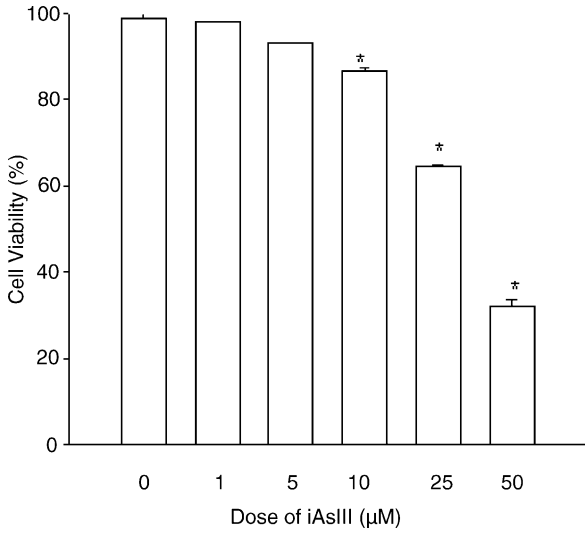

Fig. 1. Effects of sodium arsenite on the viability of HT1197 cells, as determined by trypan blue exclusion; $n = 4$ ($^{*}p < 0.05$).

3.2. Effect of arsenite on cell cycle progression in bladder cells

In order to analyze the effects of iAsIII sub-lethal doses on cell cycle regulation, we incubated sub-confluent HT1197 cell cultures with 1, 5 and 10 μM of iAsIII for 24 h. iAsIII treatment produced a dose-dependent increase (1–10 μM) in the proportion of cells in S-phase (Table 1). The highest proportion of cells in S-phase (65%) occurred at the highest dose of iAsIII used. In addition, incubation of cell cultures with varying doses of iAsIII showed a progressive reduction in cell proliferation and 10 μM completely inhibited cell proliferation. These data strongly suggest that iAsIII treatment arrested the cell cycle in S-phase. In order to confirm this effect, HT1197 cells were analyzed for BrdU uptake and examined for DNA content by propidium iodide staining. In cells treated with iAsIII, BrdU was incorporated to cellular DNA in an interrupted form, suggesting an incomplete DNA synthesis (Fig. 2).

To assess the temporal course of the cell cycle arrest produced by iAsIII treatment, HT1197 cells were incubated with 10 μM of iAsIII at different times (4, 8, 12, 16, 20 and 24 h). The increase in the proportion of cells in S-phase began after 16 h of incubation, reaching its highest level at 24 h of incubation (Table 2). Intra- and extra-cellular levels of iAs in HT1197 cells showed that the cellular intake of iAsIII was also time-dependent, reaching its highest concentration inside the cells after 16 h of incubation (Fig. 3). There was a correlation

Table 1
Effects of sodium arsenite treatment (24 h) on the cell cycle of HT1197 cells

Dose of iAsIII (μM)	Cell cycle phase		
	G1	S	G2/M
0	54 ± 1.2	31.9 ± 1.7	14.1 ± 1.5
1	51.1 ± 1.2	35.3 ± 1.1	14.0 ± 1.0
5	27.55 ± 1.8[a]	47.8 ± 1.3[a]	24.9 ± 1.2[a]
10	9.7 ± 0.5[a]	63.7 ± 2.1[a]	26.5 ± 1.4[a]

Data are means ± S.D., $n = 6$.

[a] Different with respect to control $p < 0.05$ (Student–Newman–Keuls test).

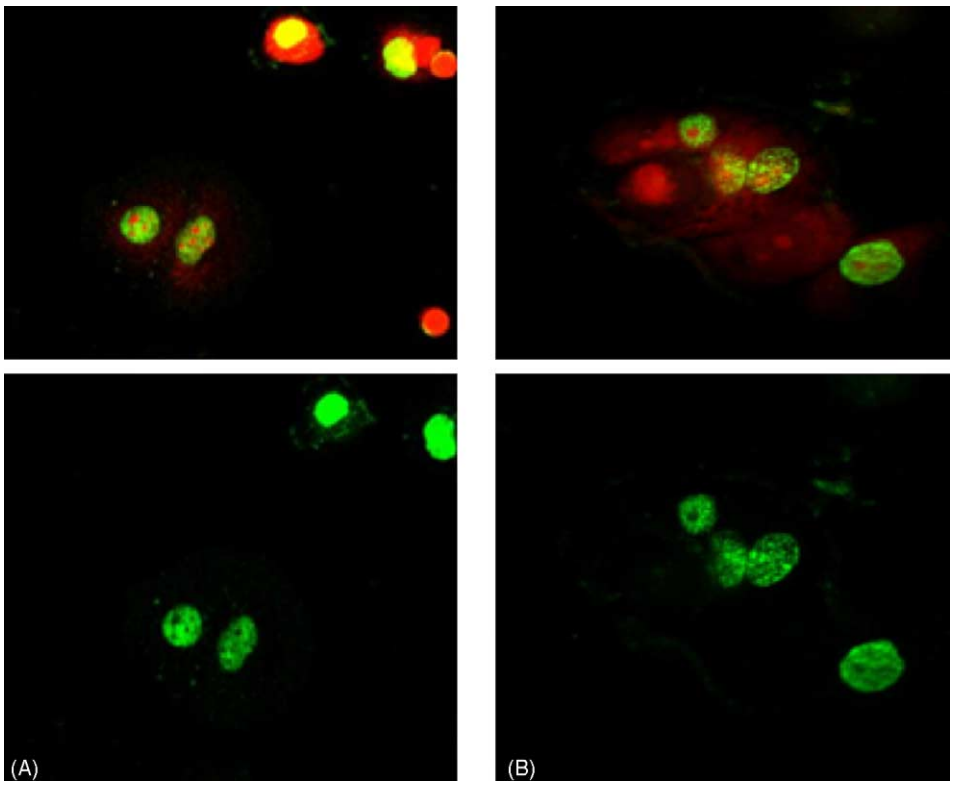

Fig. 2. Effects of sodium arsenite (10 μM, 24 h) on BrdU incorporation in HT1197 cells: (A) untreated cells and (B) treated cells; these cells were analyzed for BrdU uptake and examined for DNA content by propidium iodide staining.

between the concentration of iAsIII inside the cell and the percentage of cells in the S-phase. However, there were no detectable methylated As compounds.

In HT1197 cells treated with iAsIII (10 μM), a time-dependent p53 protein increase was observed. After 8 h of incubation, p53 levels began to increase, reaching its highest level at 24 h. These changes in p53 corresponded with an increase in p21 levels during the first 8 h, decreasing drastically to undetectable levels at 24 h (Fig. 4).

4. Discussion

The main findings of this study are that iAsIII produced a dose-dependent increase in the proportion of

Table 2
Time-course of sodium arsenite (10 μM) effects of on the cell cycle of HT1197 cells

Cell cycle phase	Time (h)						
	Control	4	8	12	16	20	24
G1	53.9 ± 1.8	48.6 ± 1.9	42.4 ± 2.0	35.6 ± 1.8[a]	23.5 ± 1.4[a]	23.7 ± 1.1[a]	21.3 ± 1.3[a]
S	30.7 ± 1.7	35.2 ± 1.6	37.62 ± 1.3	46.1 ± 1.6[a]	54.5 ± 1.7[a]	55.9 ± 1.8[a]	64.6 ± 1.2[b]
G2/M	15.4 ± 0.11	16.2 ± 1.3	20.0 ± 1.2	18.3 ± 1.4	21.0 ± 1.2[a]	20.7 ± 1.3[a]	14.1 ± 1.9[a]

Data are means ± S.D., $n = 4$.
[a] Different respect to control $p < 0.05$ (Student–Newman–Keuls).
[b] Different respect to control $p < 0.01$ (Student–Newman–Keuls).

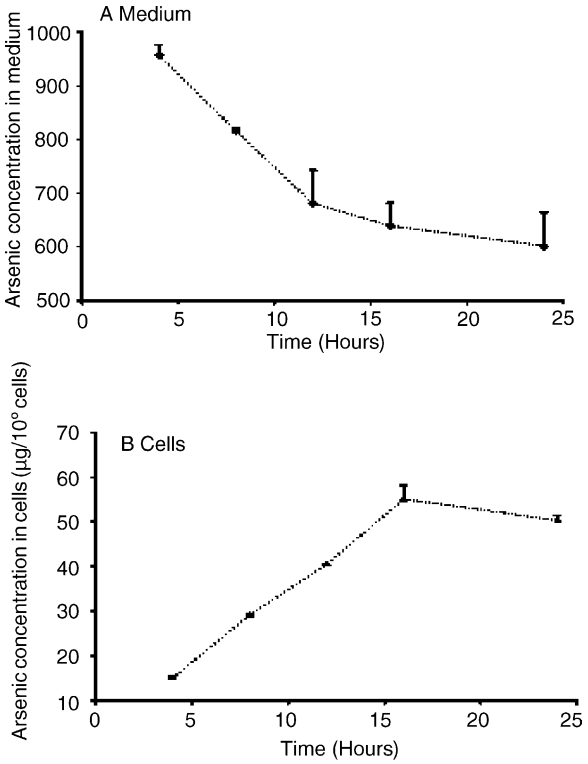

Fig. 3. Arsenic concentration in medium and HT1197 cells treated with 10 μM of sodium arsenite at different times ($n = 6$). All points were significantly different respect to control. The arsenic content in control cells was below detection limits (<1 μg/l).

HT1197 cells in S-phase associated with an increase in p53 protein content and a transient increase in p21 levels. The changes observed in cell cycle progression and p53 levels were correlated with iAs concentrations inside the cells. Our results were in agreement with studies reporting that urothelial bladder-derived cell lines do not methylate As (Styblo et al., 2000), suggesting that the effects observed in our study were due to iAs and not to its metabolites. Therefore, HT1197 bladder cells offer a good model for the study of the toxic effects of iAs without effects attributable to trivalent intermediates. On the other hand, it would be interesting to test the effects of the methylated compounds, since several reports indicate that DMA administered to mice or rats through drinking water or diet is able to cause bladder tumors (Wei et al., 1999, 2002; Arnold et al., 1999).

Our results were in partial agreement with the small increase (3%) in the proportion of cells in S-phase induced by iAsIII reported by Simeonova et al. (2000) in UROtsa cells, derived from urothelial bladder carcinoma, exposed to iAsIII (4 μM) for 72 h. The differences in the magnitude of the effect with respect to our study (50%) could be explained by differences in the genetic backgrounds of both models and the treatment schedule. UROtsa cells have their p53 function blocked by the presence of the SV40 T large antigen (Petzold et al., 1995), whereas HT1197 cells have a normal function of *TP53*, *RB*, *catenins* and *MDM2* genes (Rieger et al., 1995). However, it is possible that other genetic alterations could be present since this cell line has not yet been fully characterized. The iAsIII concentration used in our study was 2.5-fold higher than that used by Simeonova et al. (2000) even though time of exposure was shorter. In our study, doses lower than 10 μM of iAsIII did not significantly reduce cell viability, suggesting that HT1197 cells are less affected than non-neoplastic urothelial cells in vitro.

It is known that functional p53 plays an important role in the stress response of many cell types by activating p21 and arresting the cell cycle in G1-phase (Sherr and Roberts, 1995). The effects of As_2O_3 on the cell cycle have been studied in a variety of cellular types. In most cases, particularly in MC/CAR cells derived from myeloid lineage, an increased proportion of cells in G1 accompanied by increases in p53 and p21 proteins were observed (Park et al., 2000). In contrast, in our study, we observed a time-dependent p53 induction lasting for at least 24 h, accompanied by a transient increase (8 h) in p21 levels followed by a drastic decrease to background levels. Among the factors that could contribute to explain the differences between our results and those obtained by Park et al. (2000) are the genetic background of both models and the physical–chemical characteristics of the compounds.

Our findings in arsenic-treated HT1197 cells suggest that p21 was not able to block the activity of the CDK2–cyclin E complex and was therefore unable to arrest cells in G1, allowing their progression to the S-phase. Our results are also in agreement with studies reporting that p21 levels were lower or absent in human tumors, including bladder carcinoma, as compared with normal tissues (Pfister et al., 1999; el-Deiry et al., 1993). The mechanism by which HT1197 human bladder cells respond to As could involve thioredoxin reductase (TRx), a NADPH-dependent flavoenzyme, which catalyzes the reduction of many disulfide-containing substrates and plays an important role in the cellular

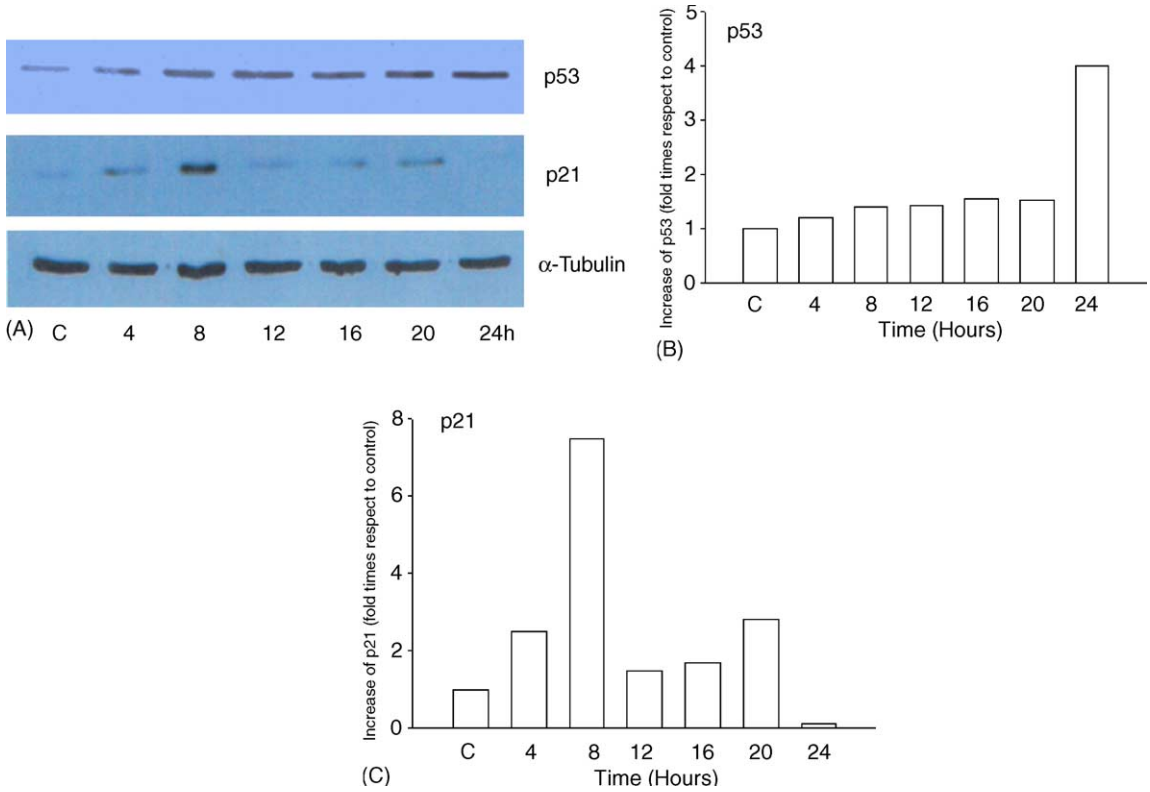

Fig. 4. Effects of sodium arsenite (10 μM) on cellular levels of p53 and p21 at different incubation times in HT1197 cells, C = control (A). Increase of p53 and p21 expressed as times respect to control (B) and (C). The figure depicts a representative blot.

response to oxidative stress (Lin et al., 2001b). Furthermore, sodium arsenite has been observed to decrease TRx activity and to induce ROS generation in hepatocytes and rabbit liver (Lin et al., 2001b; Nikaido et al., 2003). Although the mechanism(s) by which ROS induces arrest of the cell cycle is still not clearly defined, it is known that they are able to induce DNA damage, therefore activating cell cycle control points (Kurata, 2000; Lin et al., 2001a). More precisely, in our work, we observed that sodium arsenite induced a very efficient response of *TP53*, the main regulator of the G1/S transition, since p53 and p21 increased concomitantly. However, after a short time, p21 protein level sharply decreased even though p53 continued to increase, suggesting a degradation of p21. Xie et al. (2002) reported that hydrogen peroxide (200 μM) reduced p21 levels in human fibroblasts (GM00637) by increasing its degradation through the ubiquitin pathway. Thus, our findings suggest that iAsIII increases p21 degradation rate through the ubiquitin pathway, and that the low levels of p21 were not able to inhibit the activity of the cyclin E–CDK2 complex. As a consequence, Rb would be hyperphosphorylated allowing the transcription of genes regulated by the transcription factor E2F and the progression into the S phase, causing a deregulation in the cell cycle and promoting cancer development. Further studies are needed to ascertain the mechanisms underlying the effects of iAsIII on the G1 to S phase transition in HT1197 bladder cells.

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