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Arsenite induced poly(ADP-ribosyl)ation of tumor suppressor P53 in human skin keratinocytes as a possible mechanism for carcinogenesis associated with arsenic exposure

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Abstract

Arsenite is an environmental pollutant. Exposure to inorganic arsenic in drinking water is associated with elevated cancer risk, especially in skin. Arsenite alone does not cause skin cancer in animals, but arsenite can enhance the carcinogenicity of solar UV. Arsenite is not a significant mutagen at non-toxic concentrations, but it enhances the mutagenicity of other carcinogens. The tumor suppressor protein P53 and nuclear enzyme PARP-1 are both key players in DNA damage response. This laboratory demonstrated earlier that in cells treated with arsenite, the P53-dependent increase in *p21^{WAF1/CIP1}* expression, normally a block to cell cycle progression after DNA damage, is deficient. Here we show that although long-term exposure of human keratinocytes (HaCaT) to a nontoxic concentration (0.1 μ M) of arsenite decreases the level of global protein poly(ADP-ribosyl)ation, it increases poly(ADP-ribosyl)ation of P53 protein and PARP-1 protein abundance. We also demonstrate that exposure to 0.1 μ M arsenite depresses the constitutive expression of *p21* mRNA and P21 protein in HaCaT cells. Poly(ADP-ribosyl)ation of P53 is reported to block its activation, DNA binding and its functioning as a transcription factor. Our results suggest that arsenite's interference with activation of P53 via poly(ADP-ribosyl)ation may play a role in the comutagenic and cocarcinogenic effects of arsenite.

Keywords

arsenite; keratinocytes; carcinogenesis; p53; p21; PARP

INTRODUCTION

Epidemiologic evidence strongly implicates exposure to arsenic in the causation of human cancer of the skin, lung, and bladder (NRC, 2000; IARC, 2004). Arsenite is the most likely environmental carcinogenic species (Tinwell et al., 1991). This laboratory established mouse model to study skin carcinogenesis associated with arsenite exposure and demonstrated that non-toxic (10mg/l) concentration of arsenite in drinking water enhances UV irradiation-induced skin carcinoma in the Skh1 hairless mice (Rossman et al., 2001; Burns et al., 2004). The mechanism of carcinogenicity of arsenite is unknown. Arsenite itself (as well as its trivalent

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methylated metabolites) is a very weak mutagen and only at toxic concentrations (Klein et al., 2007), but chronic exposure to sub- μ M (non-toxic) arsenite causes delayed mutagenesis and transformation of HOS cells to anchorage independent (Mure et al., 2003).

Inhibition of DNA repair and decreased genomic stability are likely to be important in arsenic cocarcinogenesis. Arsenite enhances the mutagenicity of other carcinogens such as UV and methylnitrosourea (reviewed in Rossman, 2003, 2007). The mechanism for the co-mutagenic effect of arsenite with methylnitrosourea appears to involve inhibition of base excision repair, particularly of the DNA ligation step (Li and Rossman, 1989). Nucleotide excision repair is also blocked in cells treated with arsenite (Okui and Fujiwara, 1986; Hartwig et al., 1997). However, the inhibition of DNA repair by arsenite does not appear to be via direct inhibition of DNA repair enzymes (Li and Rossman, 1989; Hu et al., 1998). Rather, it appears to affect DNA damage signaling events that indirectly affect DNA repair, such as those dependent on the tumor suppressor gene p53 (Vogt and Rossman, 2001; Tang et al., 2006; Huang et al., 2008) or on poly(ADP ribose) polymerase (Hartwig et al., 2003; Walter et al., 2003).

Tumor suppressor protein P53 plays a crucial role in the cellular response to DNA damage, functioning as a cell cycle checkpoint in maintaining genomic stability. Mutations in the *p53* gene have been detected in the majority of all human cancers and are the most common mutations in human tumors (Hofseth et al., 2004; Petitjean et al., 2007). Mutations in the *p53* gene occur in almost all skin carcinomas, and are early events (de Gruij and Rebel, 2008; Pfeifer and Besaratinia, 2009). P53 protein becomes activated by phosphorylation and other protein modifications in response to many DNA damaging agents including ultraviolet light (UV), ionizing radiation (IR) and many chemical carcinogens (reviewed in Braithwaite et al., 2005). P53 mediates cell cycle arrest after DNA damage, presumably to allow time for DNA repair or to allow the cell to undergo apoptosis if DNA damage proves to be irreparable, thus reducing mutations from being passed on to daughter cells (reviewed in Harris and Levine, 2005; Millau et al., 2009). Activated P53 acts as a transcription factor for numerous specific target genes (Smeenk et al., 2008; Millau et al., 2009). One of these is *p21^{WAF1/CIP1}* (hereafter referred to as *p21*), which is needed for cell cycle arrest in G1 after genotoxic insult (reviewed in Abbas and Dutta, 2009).

Earlier, this laboratory showed that a 14-day exposure to a non-toxic concentration (0.1 μ M) of arsenite suppressed the ionizing radiation (IR)-induced P53-dependent increase in P21 abundance in WI38 normal human fibroblasts, despite the increased abundance of P53 protein (Vogt and Rossman, 2001). This suggests that arsenite affects the activating modification of P53 protein. Similar results were reported by others (Tang et al., 2006; Huang et al., 2008). One such modification is poly(ADP-ribosyl)ation. Arsenite has been shown to suppress poly(ADP-ribosyl)ation in mammalian cells (Yager and Weincke, 1997; Hartwig et al., 2003; Qin et al., 2008). Most cellular poly(ADP-ribosyl)ation is catalyzed by poly(ADP-ribosyl) polymerase-1 (PARP-1), a protein involved in the DNA damage response. Poly(ADP-ribosyl)ation represents a major mechanism to regulate genomic stability both when DNA is damaged by exogenous agents and during cell division (Oei et al., 2005; Miwa and Masutani, 2007).

PARP-1 is an abundant nuclear enzyme that binds to, and is activated by, DNA single and double strand breaks (reviewed in Miwa and Masutani, 2007). PARP-1 catalyzes the sequential transfer of ADP-ribose monomers onto nuclear protein acceptors, including itself, using NAD⁺ as substrate. Poly(ADP-ribosyl)ation of proteins increases its negative charge and prevents interaction with other anionic molecules such as DNA. Poly(ADP-ribosyl)ation of transcription factors such as P53, NFkB, and Sp1 prevents their specific binding to DNA and formation of transcription complexes (Oei et al., 2005; Miwa and Masutani, 2007). Malanga et al (1998) identified two ADP-ribose polymer binding sites in the DNA binding domain and

one in the oligomerization domain of P53. The poly(ADP-ribosyl)ation of P53 resulted in the inhibition of its binding to its consensus DNA binding sequence, resulting in loss of activity as a transcription factor. Poly(ADP-ribosyl)ation also interfered with P53's DNA single strand end-binding (Malanga et al., 1998; Mendoza-Alvarez, 2001). On the other hand, there have been reports of poly(ADP ribosyl)ation of P53 resulting in its activation rather than its inactivation (Vaziri et al., 1997; Valenzuela et al., 2002; Weiler et al., 2003).

We hypothesize that arsenite might alter P53 functioning through its effect on PARP-1 activity in the cell. In this study we used human skin keratinocytes (HaCaT). Although spontaneously immortalized, HaCaT cells have similar properties to normal keratinocytes and retain a capacity for normal differentiation (Boukamp et al., 1988). In response to UVB irradiation, HaCaT cells show increased expression of P53 target genes such as *RhoE* and *p21* as well as increased P53 activation (serine 15 phosphorylation) (Harmand et al., 2003; Boswell et al., 2007), despite the fact that both *p53* alleles contain a mutation. One allele has a his to tyr mutation at codon 179 and the other has an arg to trp mutation at codon 282 (Lehman et al., 1993). The elevated P53 protein level in HaCaT cells (Lehman et al., 1993) makes it convenient to study post-translation modification of P53. Here, we report the effect of treatment of HaCaT cells with a nontoxic (0.1 μ M) concentration of arsenite on the level of poly(ADP-ribosyl)ated proteins, PARP-1 protein, modification of P53 by poly(ADP-ribosyl)ation, and the level of *p21*.

MATERIALS AND METHODS

Cell lines

Spontaneously immortalized human keratinocyte cell line HaCaT (Boukamp et al., 1988), was obtained from Dr. C. Huang (NYU Langone School of Medicine, Tuxedo, NY). The cells were maintained in monolayer culture in DMEM (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA) at 37°C in a 5% CO₂ atmosphere. Sodium arsenite (Sigma, St. Louis, MO) (using a freshly prepared stock solution) was added to the cells after attachment.

Clonal survival assay

HaCaT cells were plated at a density of 500 cells/60 mm dish. After attachment (4h), various concentrations (0.01–10 μ M) of arsenite were added to the medium. After 10 days of growth, the resulting clones were fixed with methanol and stained with 0.5% crystal violet (Sigma, St. Louis, MO) in 50% methanol. Clones were counted and expressed as the percent of control survival. All doses were assayed in triplicate.

Immunoprecipitation and immunoblotting (Western)

Cells were harvested and the lysates were prepared using RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate buffered saline, pH 7.4, supplemented immediately before use with 4mg/ml aprotinin, 2mM AEBSF, and 100mM sodium ortho-vanadate). Lysates were cleared of cellular debris by centrifugation at 10,000 $\times$ g for 10 min, and protein concentrations were determined with BioRad DC protein assay (BioRad, Hercules, CA).

Immunoprecipitation and immunoblotting were described elsewhere (Vogt and Rossman, 2001). Briefly, lysates containing equal protein concentrations were precleared of nonspecific antibody binding by incubating for 1h at 4°C with normal rabbit IgG (sc-2027) and protein G-plus agarose (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA). RIPA buffer with the normal rabbit IgG was used as negative control. Lysates were incubated with rabbit polyclonal antibody to P53 (sc-6243, Santa Cruz Biotechnology, Inc.) and

immunoprecipitated. Precipitates were resuspended in 50 μ l of 1.5 $\times$ gel sample buffer Laemmli (Sigma Chemical CO., St. Louis, MO), separated by SDS-polyacrylamide gel electrophoresis, and transferred to Hybond-P PVDF membranes (Amersham Biosciences Corp., Piscataway, NJ). Membranes were probed with mouse monoclonal antibody for PARP-1 (SA-250), poly (ADP-ribose) (PAR) (SA-216) (both from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), P53 (sc-126), or P21 (sc-817) (both from Santa Cruz Biotechnology, Inc.) followed by horseradish peroxidase-conjugated goat anti-mouse antibody (#474–1806, Kirkegaard-Perry Laboratories, Gaithersburg, MD). Protein bands were detected using enhanced chemiluminescence (ECL, Amersham Biosciences Corp.), followed by exposure on BioMax film (Eastman Kodak Company, Rochester, NY). Specific protein bands were quantitated on a ChemiImager 4400 (Alpha Innotech. Corp., San Leandro, CA).

All immunoprecipitation and Western blot experiments were performed with lysates from at least two separate batches of cells, with good reproducibility, and representative results are shown.

RT-PCR

Total RNA was isolated from cells with TRI Reagent according to manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, Ohio). cDNA synthesis and PCR amplification were performed in a single tube using SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's recommendations.

The primers for *p21* (5'-CCAAGAGGAAGCCCTAATCC-forward; 5'-CCCTAGGCTGTGCTCACTTC-reverse) and for β -actin (5'-CAGATCATGTTTGAGACCTTCAACAC-forward; 5'-TCTGCGCAAGTTAGGTTTTGTCAAG-reverse) were purchased from Sigma Genosys (The Woodlands, TX). PCR parameters were: for cDNA synthesis, 55 $^{\circ}$ C for 25 min; for denaturation, 94 $^{\circ}$ C for 2 min; for PCR amplification, 94 $^{\circ}$ C for 15 sec (denature), 54 $^{\circ}$ C for 30 sec (anneal), 68 $^{\circ}$ C for 1 min (extend); and for final extension, 68 $^{\circ}$ C for 5 min. PCR amplification was performed for 25 cycles. cDNA was tested in 1% agarose gel electrophoresis followed by quantitation on a ChemiImager 4400 (Alpha Innotech. Corp.)

All RT-PCR experiments were performed with RNA from at least two separate batches of cells, with good reproducibility, and representative results are shown.

RESULTS

Cytotoxicity of arsenite

The cytotoxicity of arsenite in HaCaT cells was determined by a clonal survival assay using continuous arsenite exposure (Figure 1). No reduction in clonal survival was seen with 0.1 μ M arsenite. Viability begins to decrease at 0.5 μ M, and there are no survivors at 5 μ M arsenite. The LC₅₀ of sodium arsenite is approximately 1.07 μ M. The non-toxic concentration of 0.1 μ M arsenite was chosen for further studies.

Effect of arsenite on PARP1 activity and PARP1 protein level in HaCaT cells

HaCaT cells were exposed to 0.1 μ M sodium arsenite for different times prior to protein isolation, and levels of total poly(ADP-ribose)ation of proteins were analyzed by Western blotting using a poly(ADP-ribose)-specific antibody that recognizes only poly(ADP-ribose) modified proteins independent of species source without cross reactivity with RNA, DNA, monomers of ADP-ribose or NAD (Menard and Poirier, 1987; Kupper et al., 1990). Figure 2 shows that growth in 0.1 μ M arsenite for 4 days or more resulted in decreases in total protein

poly(ADP-ribosyl)ation. Paradoxically, at the same time, PARP-1 protein levels increased up to 2.5 fold after 4 days exposure and remained high for at least 14 days (Figure 3).

P53 protein is poly(ADP-ribosyl)ated in HaCaT cells exposed to arsenite

To determine whether arsenite affects the poly(ribosyl)ation of P53 in HaCaT cells, P53 protein was precipitated with specific P53 antibody from the same cell lysates that were been used for the PARP-1 study. Then poly(ADP-ribosyl)ated P53 was detected by immunoblotting with antibody to PAR. Poly(ADP-ribosyl)ated P53 is absent in untreated HaCaT cells, but appears after 1 days growth in arsenite-containing medium. At 4 days and afterward up to 14 days, P53 remained poly(ADP-ribosyl)ated but at a level about 50% of that on day 1 (Figure 4). During this time, the abundance of P53 protein remained constant.

Expression of P21 transcript and protein in HaCaT cells exposed to arsenite

Figure 5 shows the effect of arsenite on expression of *p21*. RT-PCR with *p21*-specific primers shows that the *p21* gene is constitutively expressed in HaCaT cells. Even one day's exposure to 0.1 μ M arsenite resulted in a 2-fold decrease of the *p21* transcript.

P21 protein levels in the cell were analyzed by Western blot. As can be seen in Figure 6, untreated HaCaT cells contain detectable P21 protein. The constitutive P21 protein level was not altered after 1-day's growth in arsenite containing medium. However, long-term growth (14 days) resulted in a 3-fold decrease in P21 protein level. Taken together, the immunoblotting with antibody to P21 and RT-PCR with *p21*-specific primers show that a low, non-toxic concentration of arsenite suppress expression of *p21*.

DISCUSSION

Yager and Wiencke (1997) reported that treatment with μ M concentrations of arsenite for 24h decreased poly(ADP-ribosyl)ation in a dose-dependent manner in a human T-cell lymphoma-derived cell line Molt-3. Hartwig et al. (2003) found inhibition even in the nM range in HeLa cells treated with hydrogen peroxide. Low concentrations of arsenite exacerbate solar-UV-induced DNA strand breaks by inhibiting PARP-1 activity, possibly by interfering with its zinc-finger motif. Supplementation with zinc was able to block the effects of arsenite and restore PARP-1 activity (Qin et al., 2008). Here we observed that long-term (4 days and longer) exposure to 0.1 μ M arsenite, in the absence of a second agent, depressed total poly(ADP-ribosyl)ation of proteins in HaCaT cells while at the same time poly(ADP-ribosyl)ation of P53 protein was increased. Continuous exposure of HaCaT cells to 0.1 μ M arsenite for >25 weeks in the absence of a second agent induced malignant transformation (colony formation in soft agar and increased matrix metalloproteinase-9 secretion) (Sun et al., 2009). It is possible that progressive reduction or increases in poly(ADP-ribosyl)ation of proteins such as transcription factors alters gene expression that can lead to malignant transformation.

It was previously reported that there was no effect on PARP-1 expression after an 18 hour exposure of HeLa cells to 1 μ M arsenite (Walter et al., 2007). However, in this study PARP-1 protein abundance increased 2.5 fold after 4 day's exposure to 0.1 μ M arsenite, and remained high for at least 14 days (Fig 3). PARP-1 is reported to regulate its own transcription *via* PARP-1 auto-poly(ADP-ribosyl)ation (Sato and Lindahl, 1992; Soldatenkov et al., 2002). It is possible that arsenite prevented the auto-poly(ADP-ribosyl)ation of PARP-1, resulting in its up-regulation. Figure 2 shows decreased PAR antibody binding to bands around 116 kD (the size of PARP-1) after growth in arsenite, but this was not pursued further.

Measuring total cellular PAR is less informative than measuring protein-specific poly(ADP-ribosyl)ation. Despite a decrease in global PAR, our results show that growth of HaCaT cells

in 0.1 μ M arsenite for 24 hours resulted in poly(ADP-ribosyl)ation of P53 which lasted during the entire 14 day's growth in arsenite (Fig. 4). We did not observe any bands larger than 53 kDa, suggesting that only a few units of ADP-ribose were attached.

A number of studies have suggested a role for poly(ADP-ribosyl)ation in the P53-mediated response to DNA damage, although the nature and consequence of this interaction may be dependent on the type of DNA lesion and on the upstream pathway leading to the activation. PARP-1 deficient primary mouse embryo fibroblasts exhibit decreased P53 accumulation and activation following treatment with IR, but not with 2'-methyl-2'-nitrosourea (MNU) (Valenzuela et al., 2002), suggesting a positive role for PARP-1 in DNA damage-induced signaling where the protein kinase ATM (ataxia telangiectasia mutated) is involved in P53 activation (i.e. IR but not MNU). Inhibition of PARP1 activity with 1,5-dihydroxyisoquinoline (IQ), or by overexpression of its catalytically inactive DNA-binding domain, also suppressed the transactivation function of P53 and the ability of P53 to mediate G1 arrest in response to IR (Weiler et al., 2003). A positive role for PARP-1 in P53 activation in response to DNA damage induced by IR was also reported by Vaziri et al (1997). PARP-1-mediated poly(ADP-ribosyl)ation of P53 was reported to block the interaction of P53 with nuclear export receptor CRM1, causing nuclear accumulation and super-activation of P53 protein (Kanai et al., 2007).

On the other hand, poly(ADP-ribosyl)ation of P53 has also been reported to inhibit its transactivation activity (Malanga et al., 1998; Mendoza-Alvarez et al., 2001). We detect arsenite-induced poly(ADP-ribosyl)ation of P53, without a change in its molecular weight, and concomitant reduction of *p21* gene expression in HaCaT cells. These results are consistent with an inactivating role of small amounts of poly(ADP-ribosyl)ation of P53. This situation may be analogous to that of another protein modification, i.e. ubiquitination, where monoubiquitination and polyubiquitination of a protein have different consequences for its activity (Sun and Chen, 2004).

The *p21* transcript, constitutively expressed in HaCaT cells, decreased in abundance after only 1 day of exposure to 0.1 μ M arsenite (Fig. 5). In contrast, the P21 protein level took more than 1 day to decrease in abundance (Fig. 6), suggesting that some of this protein has a long half-life in these cells. Huang et al. (2008) report that arsenite causes a cytoplasmic accumulation of P53, thus blocking its nuclear transactivating activity, and Tang et al. (2006) report that arsenite blocks UVB-induced phosphorylation and DNA binding activity of P53, and decreased P53-dependant *p21* expression. Although the poly(ADP-ribosyl)ation state was not measured in these two studies, their results are consistent with ours. While *p21* expression following genotoxic insult is induced in a P53-dependent manner, basal expression of *p21* is controlled by both P53-dependent and P53-independent mechanisms (Li et al., 1994; Macleod et al., 1995).

Normal diploid cells generally contain much lower constitutive levels of P53 than do cell lines such as HaCat (Shaked et al., 2008), making this type of study impractical. This brings up the question whether the results reported here would be the same in normal cells. The *p21* promoter has 5 response elements that can bind P53 (Millau et al., 2009b). Nutlin 3, an inhibitor of MDM2, increases the constitutive level of P53 in normal cells in the absence of genotoxic (or other) stress, resulting in its binding to the -1,354 and the -2,241 base pair response elements of *p21* (Millau et al., 2009b). Future studies could address the mechanism of low level arsenite's inhibition of P53-dependant *p21* activation by using Nutlin 3 to increase constitutive P53 levels.

In conclusion, we confirm previous reports that exposure to a non-toxic low concentration arsenite causes global reduction in cellular PAR content, but find that it is also associated with a low level of poly(ADP-ribosyl)ation of P53. We suggest that this modification blocks its

activation and perhaps its cellular location. Exposure to nontoxic concentrations of arsenite enhances cell proliferation (Germolec et al., 1996; Burns et al., 2004; Komissarova et al., 2005). Thus, in addition to affecting DNA repair, the lack of normal P53 functioning might also be important in the lack of cell cycle control via reduction of *p21* expression. Increased cell proliferation as well as inhibition of DNA repair might result from inhibition of P53 function by low level poly(ADP-ribosyl)ation, and could contribute to arsenic cocarcinogenesis. It is also likely that growth in low concentrations of arsenite causes increased poly(ADP-ribosyl)ation of other proteins that could play a role in the malignant transformation of HaCat cells (Sun et al., 2009).

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Fig. 1. Toxicity of arsenite to HaCaT cells in clonal survival assay using continuous arsenite exposure

Cells were plated at a density 500 cells/60mm dish with different concentrations of arsenite added 4h later. After 10 days incubation with arsenite-containing medium, colonies were fixed and stained (see Materials and Methods). The results were obtained from three separate experiments with each assay in triplicate and expressed as the mean plus/minus standard error of the mean. The plating efficiency of untreated HaCaT cells was about 50%.

Fig. 2. Reduction in poly(ADP-ribosylation) of proteins in HaCaT cells exposed to arsenite
HaCaT cells were treated with 0.1 μ M arsenite for various periods prior to lysis. Poly(ADP-ribosylated) proteins were detected with PAR antibody.

Fig. 3. Treatment with arsenite results in up-regulation of PARP-1 protein

HaCaT cells were exposed to 0.1μM arsenite for various periods of time prior to lysis. PARP-1 and β-actin proteins were detected with specific mouse monoclonal antibodies.

Fig. 4. Poly(ADP-ribosylation) of p53 protein in HaCaT cells exposed to arsenite
HaCaT cells were exposed to 0.1 μ M arsenite for various periods of time before lysis. P53 protein was precipitated with P53 specific rabbit polyclonal antibody then detected in Western blot with P53 specific mouse monoclonal antibody. Poly(ADP-ribosyl)ated P53 protein was detected with PAR-specific antibody.

Fig. 5. Effect of arsenite on *p21* expression in HaCaT cells

Total RNA was isolated from HaCaT cells exposed to 0.1 μ M arsenite for various periods of time. *p21* transcript was detected by RT-PCR using *p21* specific primers. Housekeeping gene β -actin transcript was detected in the same samples with β -actin specific primers.

Fig. 6. Effect of arsenite on P21 protein level in HaCaT cells

Cells were exposed to 0.1 μ M arsenite for various periods of time before lysis. P21 and β -actin proteins were detected in Western blot with specific mouse monoclonal antibodies.