Original Article

Inorganic arsenic induces apoptosis through downregulation of Ube2d genes and p53 accumulation in rat proximal tubular cells

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ABSTRACT — Ube2d ubiquitin-conjugating enzymes promote p53 ubiquitination and proteasomal degradation. We previously showed that cadmium induced p53-dependent apoptosis through the suppression of expression of Ube2d family genes (*Ube2d1*, *Ube2d2*, *Ube2d3* and *Ube2d4*) in normal rat proximal tubular cells. Here we examined the effects of inorganic arsenic and inorganic mercury, which induce apoptosis in proximal tubular cells, on cellular protein level of p53 and gene expression of Ube2d family. Inorganic arsenic induced apoptosis with p53 accumulation, and suppressed *Ube2d1*, *Ube2d2* and *Ube2d4* expression, but not *Ube2d3*. On the other hand, although apoptosis was induced in response to inorganic mercury in proximal tubular cells, protein level of p53 was not elevated by inorganic mercury. These results suggest that inorganic arsenic, but not inorganic mercury, may induce p53-dependent apoptotic pathways through downregulation of gene expression of Ube2d family in proximal tubular cells.

Key words: Inorganic arsenic, Inorganic mercury, p53, Apoptosis, Ube2d family

INTRODUCTION

Inorganic arsenic and inorganic mercury are environmental pollutants that adversely affect tissues such as the kidney. In addition, both toxicants are known to induce apoptotic cell death in several types of tissues and cells (Duncan-Achanzar *et al.*, 1996; Homma-Takeda *et al.*, 1999; Jimi *et al.*, 2004; Peraza *et al.*, 2006; Kanda *et al.*, 2008; Yadav *et al.*, 2010). However, the precise molecular mechanism by which apoptotic cell death is induced remains to be elucidated.

Cadmium, which exerts renal toxicity, induces apoptosis in proximal tubular cells *via* several pathways, including the endoplasmic reticulum (ER)-mediated pathway *via* ER stress and calcium release and the mitochondria-mediated pathway *via* direct and indirect activation of the mitochondria (Fujiwara *et al.*, 2012). Our recent

study demonstrated that cadmium induced p53-dependent apoptotic cell death in proximal tubular cells both *in vitro* and *in vivo* (Tokumoto *et al.*, 2011b; Fujiwara *et al.*, 2012). The p53 tumor suppressor functions as an inducer of apoptosis (Sharpless and DePinho, 2002; Mills, 2005). p53 is a short-lived protein, and under normal conditions p53 protein is immediately degraded by the ubiquitin-proteasome system (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). In MCF7 human breast carcinoma cells, ubiquitination of p53 was shown to be mediated by Ube2d2 and Ube2d3, which are members of the Ube2d family (Saville *et al.*, 2004). We found that cadmium suppressed the gene expression of all Ube2d family members (*Ube2d1*, *Ube2d2*, *Ube2d3* and *Ube2d4*), and induced accumulation and phosphorylation of p53, followed by p53-dependent apoptotic cell death (Tokumoto *et al.*, 2011a, 2011b; Fujiwara *et al.*, 2012). Together our

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data showed that cadmium-induced renal toxicity results from accumulation of p53 through the suppression of expression of the Ube2d family genes.

Similar to cadmium, the environmental pollutants inorganic arsenic and inorganic mercury also induce apoptosis in proximal tubular cells (Duncan-Achanzar *et al.*, 1996; Homma-Takeda *et al.*, 1999; Jimi *et al.*, 2004; Peraza *et al.*, 2006; Kanda *et al.*, 2008). Therefore, we speculated that these two toxicants cause apoptosis in proximal tubular cells through the p53-dependent apoptotic pathway. In the present study, we aimed to clarify the involvement of intracellular accumulation of p53 and downregulation of Ube2d family gene expression in inorganic arsenic- and inorganic mercury-induced apoptosis in NRK-52E normal rat proximal tubular cells.

MATERIALS AND METHODS

Cell culture and treatments

NRK-52E cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum solution (FBS; Life Technologies, Carlsbad, CA, USA), 29.2 g/l L-glutamine (Nacalai Tesque, Kyoto, Japan), 50 IU/ml penicillin, 50 µg/ml streptomycin (Dainippon Sumitomo Pharma, Osaka, Japan), and 1.5 g/l sodium bicarbonate (Wako Pure Chemical Industries, Osaka, Japan). NRK-52E cells were maintained in 100-mm dishes at 37°C in a humid atmosphere of 5% CO₂. Cells were plated for experiments at a density of 1.2×10^4 cells/cm² and cultured until confluence. Culture medium was then discarded and cells were washed twice with serum-free DMEM. The cells were incubated with 50 µM mercuric chloride (HgCl₂), 20 µM sodium arsenite (NaAsO₂) or 10 µM cadmium chloride (CdCl₂) (Wako Pure Chemical Industries) for 6 or 24 hr before harvest.

Cytotoxicity assay

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; DOJINDO Laboratories, Kumamoto, Japan) assay was used to estimate cell viability. After treatment, cultured medium was changed to fresh 10% FBS-DMEM containing MTT and incubated for another 4 hr at 37°C. After removing the medium, dimethyl sulfoxide (Wako Pure Chemical Industries) was added to MTT formazan. Absorbance at 540 nm was measured by an iMark microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA, USA).

Extraction of total RNA and real-time RT-PCR

After treatment, the cells were washed twice with calcium- and magnesium-free phosphate buffered saline [PBS(-); Nissui Pharmaceutical]. Total RNA was extracted with the Quick Gene RNA cultured cell kit S (Fujifilm, Tokyo, Japan), including an on-column DNase I (Takara Bio, Shiga, Japan) treatment step, and QuickGene-810 (Fujifilm). cDNA was generated from total RNA using the PrimeScript reverse transcription (RT) Reagent Kit (Perfect Real Time) (Takara Bio). Real-time PCR was performed with the SYBR Premix Ex TaqII (Perfect Real Time) (Takara Bio) and Thermal Cycler Dice Real Time System (Takara Bio). PCR primers used are as follows: 5'-cgggaccgctgggagatgactgtt-3' (sense) and 5'-gactccaccttgataggcgtctcg-3' (antisense) for *Ube2d1*; 5'-gcacatgtttacctgacacagca-3' (sense) and 5'-cggagaactggcttctcacac-3' (antisense) for *Ube2d2*; 5'-gacggttttcagggttctgg-3' (sense) and 5'-tcttcttctcacaccggtct-3' (antisense) for *Ube2d3*; 5'-agccttctccaggcataacc-3' (sense) and 5'-taggttggggacctttacc-3' (antisense) for *Ube2d4*; 5'-taattgaagggtcatgcctaagtgt-3' (sense) and 5'-gctgctccgacttaactgc-3' (antisense) for *Mdm2*; 5'-cctatccggtcagtgttga-3' (sense) and 5'-ttgcagagtggaggaaatgg-3' (antisense) for *p53*; 5'-catggcctccaaggagtaag-3' (sense) and 5'-ggtctgggatggaattgtga-3' (antisense) for *Gapdh*.

Detection of DNA fragmentation

After treatment, cells were harvested by scraping with a rubber policeman in ice-cold PBS(-) and centrifuged at $250 \times g$ for 10 min. Cell pellets were dissolved into 100 µl cell lysis buffer [50 mM Tris-HCl buffer, pH 6.8, 2% sodium dodecyl sulfate (SDS) and 10% glycerol] at 4°C. After 10 min, dissolved samples were centrifuged at $16,000 \times g$ for 5 min and the supernatants were transferred to new tubes. Two microliters of 20 mg/ml RNase A solution (Life Technologies) was added and samples were incubated at 37°C for 1 hr. Two microliters of 10 g/l Proteinase K solution (Life Technologies) was added and samples incubated at 50°C for 30 min. Samples were mixed with 20 µl of 5 M NaCl and 120 µl isopropanol, and cooled at -20°C for over 20 min. The mixed solutions were centrifuged at $16,000 \times g$ for 15 min and pellets were resuspended with 10 µl TE buffer (10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA). DNA fragments were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Western blot analysis

Anti-p53 rabbit polyclonal antibody and anti- β -actin mouse monoclonal antibody were obtained from Cell Signaling Technology (Danvers, MA, USA) and American Research Products (Waltham, MA, USA), respectively. After treatment, cells were washed twice with ice-cold PBS(-) and harvested by cell lysis SDS buffer. Cell lysates were boiled at 95°C for 15 min and protein concentrations were measured by BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Samples (50 μ g) were mixed with a fifth volume of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and separated by SDS-PAGE. Electrophoresed proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk in TTBS (20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 hr. The membrane was washed briefly with TTBS and incubated with appropriate antibodies overnight at 4°C. Peroxidase-conjugating secondary antibodies (GE Healthcare, Tokyo, Japan) were probed and immunoreactive proteins were detected by enhanced chemiluminescence using Chemi-Lumi One L (Nacalai Tesque).

Statistical analysis

The data are presented as mean $\pm$ standard deviation (S.D.) for each experimental group ($n = 3-4$). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni-type multiple t -test (parametric). Differences between the groups were considered significant at $P < 0.05$.

RESULTS

Inorganic arsenic and inorganic mercury induce apoptotic cell death through different pathways

To elucidate whether p53 is involved in NRK-52E cell apoptosis induced by other nephrotoxic substances, particularly toxic metal(loid)s, we examined the effects of inorganic arsenic and inorganic mercury on the induction of apoptosis and p53 protein level. Sodium arsenite at 20 μ M caused cytotoxicity only in the 24 hr treatment group (Fig. 1A). The viability of NRK-52E cells after treatment with 50 μ M mercuric chloride for 6 and 24 hr decreased to 77.3% and 61.6%, respectively (Fig. 1B). Under these conditions, the following analyses were conducted.

We next examined the effects of mercuric chloride and sodium arsenite on the induction of apoptosis using the DNA ladder assay. After 24 hr treatment, DNA fragmentation in mercuric chloride- and sodium arsenite-treated cells, as well as cadmium chloride treated cells, was

Fig. 1. Effects of inorganic arsenic and inorganic mercury on cytotoxicity and intracellular p53 accumulation. (A) Viability of NRK-52E cells treated with 20 μ M sodium arsenite [As(III)] for 6 and 24 hr. (B) Viability of NRK-52E cells treated with 50 μ M mercuric chloride [Hg(II)] for 6 and 24 hr. Values are means $\pm$ S.D. for 3-4 samples. * Significant difference from corresponding control, $P < 0.05$. (C) DNA fragmentation in NRK-52E cells treated with 50 μ M mercuric chloride [Hg(II)], 20 μ M sodium arsenite [As(III)] and 10 μ M cadmium chloride (Cd) for 24 hr. DNA fragments were separated by 2% agarose gel electrophoresis.

markedly observed (Fig. 1C). In addition, sodium arsenite treatment induced p53 protein level, as did cadmium chloride, whereas no significant changes in p53 were observed in response to mercuric chloride treatment (Fig. 2).

Fig. 2. Effects of inorganic arsenic and inorganic mercury on the protein level of p53 in NRK-52E cells. Western blot analysis of intracellular p53 protein levels in NRK-52E cells treated with 50 μ M mercuric chloride [Hg(II)], 20 μ M sodium arsenite [As(III)] and 10 μ M cadmium chloride (Cd) for 24 hr. β -actin was used as a control to confirm equal loading.

These results indicate that inorganic arsenic and inorganic mercury induce apoptotic cell death in NRK-52E cells through different mechanisms.

Effects of inorganic arsenic and inorganic mercury on the expression of Ube2d family genes

To test whether the expression levels of the Ube2d family genes are affected by inorganic arsenic, NRK-52E cells were incubated with sodium arsenite for 6 hr and mRNA levels of Ube2d family genes were examined. Real-time RT-PCR data revealed that sodium arsenite significantly reduced *Ube2d1*, *Ube2d2* and *Ube2d4* mRNA levels, whereas *Ube2d3* mRNA level remained unchanged (Fig. 3A). Conversely, mercuric chloride caused a decrease of only *Ube2d1* mRNA (Fig. 3B). These results indicate that inorganic arsenic, as well as cadmium, downregulates the expression of the Ube2d family genes, except for *Ube2d3*.

Effect of inorganic arsenic on the gene expressions of Mdm2 and p53

The stability of p53 protein is regulated by Mdm2, which is the ubiquitin-ligase enzyme specific to p53 (Kubbutat *et al.*, 1997). To examine whether the gene expression of Mdm2 is affected by inorganic arsenic, NRK-52E cells were incubated with sodium arsenite for 6 hr and the mRNA level of *Mdm2* and transcription level of *p53* were examined. The results showed that *Mdm2* mRNA and *p53* mRNA remained unchanged in response to sodium arsenite (Fig. 4). These results suggest that

Fig. 3. Effects of inorganic arsenic and inorganic mercury on the gene expression of Ube2d family members in NRK-52E cells. (A) NRK-52E cells were treated with 20 μ M sodium arsenite [As(III)] for 6 hr. mRNA levels of each indicate gene were determined by real-time RT-PCR. (B) NRK-52E cells were treated with 50 μ M mercuric chloride [Hg(II)] for 6 hr. mRNA levels of each indicate gene were determined by real-time RT-PCR. Ube2d family mRNA levels were normalized to *Gapdh* mRNA levels. Values are means $\pm$ S.D. for 3-4 samples. * Significant difference from corresponding control, $P < 0.05$.

intracellular accumulation of p53 induced by inorganic arsenic is controlled by the ubiquitin-conjugating system rather than transcriptional regulation.

p53 accumulation by arsenic in kidney cells

Fig. 4. Effect of inorganic arsenic on *Mdm2* and *p53* gene expression in NRK-52E cells. NRK-52E cells were treated with 20 μ M sodium arsenite [As(III)] for 6 hr. *Mdm2* and *p53* mRNA levels were determined by real-time RT-PCR and normalized to corresponding *Gapdh* mRNA levels. Values are means $\pm$ S.D. for 3-4 samples.

DISCUSSION

Our previous study demonstrated that cadmium induced apoptosis accompanied with an increase in intracellular p53 accumulation in NRK-52E cells (Tokumoto *et al.*, 2011b). The present study shows that, similar to cadmium, inorganic arsenic also increased intracellular p53 protein level and induced apoptosis in NRK-52E cells. In addition, inorganic arsenic caused a decrease in mRNA levels of *Ube2d1*, *Ube2d2* and *Ube2d4* without significant changes in *p53* and *Mdm2* gene expression. Because the ubiquitin proteasome system plays a major role in the degradation of p53, these findings suggest that inorganic arsenic as well as cadmium suppresses the expression of the Ube2d family genes, resulting in induction of p53 accumulation and p53-dependent apoptosis in NRK-52E cells.

Although a previous study showed that inorganic arsenic increased p53 protein level in murine embryonic fibroblasts through the suppression of p53 degradation (Yu *et al.*, 2013), the underlying mechanisms were unclear. In the present study, our results suggest that inorganic arsenic-induced suppression of Ube2d family gene expression may contribute to the suppression of p53 degradation. Further studies are required to determine whether inorganic arsenic induces downregulation of Ube2d

family genes in other cell lines.

In contrast to inorganic arsenic, inorganic mercury did not affect p53 protein level, even though apoptosis was induced drastically by mercury treatment. Inorganic mercury is known to induce apoptosis through the mitochondria-mediated pathway in various cell lines (Stacchiotti *et al.*, 2009; Chen *et al.*, 2010). In addition, oxidative stresses are known to induce mitochondria-mediated apoptosis in NRK-52E cells (Xu *et al.*, 2006; Taneda *et al.*, 2010; Kim *et al.*, 2012). Therefore, inorganic mercury may induce apoptosis through the mitochondria-mediated pathway, but not the p53-dependent pathway, in NRK-52E cells.

Inorganic arsenic decreased mRNA levels of *Ube2d1*, *Ube2d2* and *Ube2d4*, and increased p53 protein level in NRK-52E cells, with no change in *Ube2d3* mRNA level. In contrast, inorganic mercury did not increase in intracellular p53 protein level, and only *Ube2d1* gene was downregulated by inorganic mercury. These observations suggest that *Ube2d2* and *Ube2d4* may contribute to the ubiquitination and degradation of p53 in NRK-52E cells. In MCF7 cells, a previous report showed that *Ube2d2* and *Ube2d3* are associated with the ubiquitination of p53 (Saville *et al.*, 2004). Together this shows that specific ubiquitin-conjugating enzymes promote p53 ubiquitination, depending on the various cell types.

Saville *et al.* (2004) reported that suppression of expression of Ube2d family genes causes increased p53 protein level with continuous transcription of p53 and *Mdm2* in MCF7 cells. p53 induces apoptosis by promoting the transcription of pro-apoptotic factors (Fricker *et al.*, 2010). *Mdm2* not only acts as a ubiquitin-ligase enzyme for p53 but also represses transcription activity of p53, therefore high level of *Mdm2* result in reduced transcriptional activity of p53 (Saville *et al.*, 2004). Cadmium was found to decrease the expression of the *Mdm2* gene as well as Ube2d family genes (Tokumoto *et al.*, 2011b). Therefore, in NRK-52E cells, cadmium induces p53-dependent apoptosis through enhanced p53 transcriptional activity that results from accumulation of p53 protein caused by downregulation of not only Ube2d family genes, but also of the *Mdm2* gene. In the present study, inorganic arsenic did not decrease *Mdm2* mRNA level in NRK-52E cells. Although inorganic arsenic caused downregulation of *Ube2d1*, *Ube2d2* and *Ube2d4*, and induction of p53 accumulation and apoptosis, the molecular mechanisms in inorganic arsenic- and cadmium-exposed cells may be different.

In conclusion, our findings show that inorganic arsenic as well as cadmium may induce p53-dependent apoptotic pathways through the downregulation of Ube2d family

genes in proximal tubular cells. However, inorganic mercury-induced apoptosis in proximal tubular cells is independent of p53.

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