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Contribution of aquaporin 9 and multidrug resistance-associated protein 2 to differential sensitivity to arsenite between primary cultured chorion and amnion cells prepared from human fetal membranes

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Abstract

Arsenic trioxide (arsenite, As^{III}) has shown a remarkable clinical efficacy, whereas its side effects are still a serious concern. Therefore, it is critical to understand the effects of As^{III} on human-derived normal cells for revealing the mechanisms underlying these side effects. We examined the effects of As^{III} on primary cultured chorion (C) and amnion (A) cells prepared from human fetal membranes. A significant dose-dependent As^{III}-mediated cytotoxicity was observed in the C-cells accompanied with an increase of lactate dehydrogenase (LDH) release. Higher concentrations of As^{III} were required for the A-cells to show cytotoxicity and LDH release, suggesting that the C-cells were more sensitive to As^{III} than the A-cells. The expression levels of aquaporin 9 (AQP9) were approximately 2 times higher in the C-cells than those in the A-cells. Both intracellular arsenic accumulation and its cytotoxicity in the C-cells were significantly abrogated by sorbitol, a competitive AQP9 inhibitor, in a dose-dependent manner. The protein expression levels of multidrug resistance-associated protein (MRP) 2 were downregulated by As^{III} in the C-cells, but not in the A-cells. No significant differences in the expression levels of MRP1 were observed between C- and A-cells. The protein expression of P-glycoprotein (P-gp) was hardly detected in both cells, although a detectable amount of its mRNA was observed. Cyclosporine A, a broad-spectrum inhibitor for ABC transporters, and MK571, a MRP inhibitor, but not PGP-4008, a P-gp specific inhibitor, potently sensitized both cells to As^{III}-mediated cytotoxicity. These results

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Conflict of interest statement

The authors declare that there is no conflict of interest.

suggest that AQP9 and MRP2 are involved in controlling arsenic accumulation in these normal cells, which then contribute to differential sensitivity to As^{III} cytotoxicity between these cells.

Keywords

Arsenite; Aquaporin 9; Multidrug resistance protein 2; P-glycoprotein; Fetal membranes

Introduction

Administration of arsenic trioxide (arsenite, As^{III}), an arsenic derivative, has demonstrated a remarkable efficacy in the treatment of relapsed and refractory acute promyelocytic leukemia (APL) patients. The successful clinical efficacy in the treatment of APL patients has led to investigations exploring potential treatment applications for other malignancies, including solid tumors (Dilda and Hogg, 2007; Litzow, 2008). In order to understand the mode of action of As^{III} and provide an effective treatment protocol for individual APL patients, studies have been conducted on the pharmacokinetics of As^{III} in APL patients using biological samples such as urine, blood and cerebrospinal fluid (Shen et al., 1997; Fujisawa et al, 2007; Yoshino et al., 2009; Kiguchi et al., 2010). In fact, we recently demonstrated that not only inorganic arsenic but also methylated arsenic metabolites accumulated in red blood cells during the consecutive administration of As^{III} to APL patients (Yoshino et al., 2009). Furthermore, we have demonstrated for the first time that these arsenic metabolites also existed in cerebrospinal fluid (Kiguchi et al., 2010), in which the concentrations of arsenic reached levels necessary for differentiation induction (Chen et al., 1997; Soignet et al., 1998). These findings on the pharmacokinetics of As^{III} in APL patients provide a new insight into clinical applications of As^{III}, and may contribute to better therapeutic protocols (Yuan et al., 2011).

Although a remarkable clinical efficacy of As^{III}-based regimens against APL has been reported (Shen et al., 1997; Soignet et al., 1998), and As^{III} has been suggested as a promising candidate for the treatment of refractory solid tumors (Dilda and Hogg, 2007; Litzow, 2008), side effects of As^{III} are still a serious concern and hamper its clinical applications. It is thus critical to investigate the effects of As^{III} on normal cells and/or tissues for clinical implications. However, very few studies to date have been conducted to investigate the effects of As^{III} on normal cells, because of difficulty in obtaining human-derived normal cells (Chattopadhyay et al., 2002; Ferrario et al., 2009).

Recently, we have established a unique in vitro system, comprising the primary cultured chorion (C−) cells and amnion (A−) cells prepared from human fetal membranes obtained at the month of normal parturition, for studying biological responses to external stimuli in normal cells (Yuan et al., 2006, 2008, 2009). So far, we have demonstrated that the C-cells are more vulnerable to oxidative stress than the A-cells (Yuan et al., 2006, 2008, 2009), suggesting that the in vitro system is a good model system to study the role of oxidative stress induced by various external stimuli including anticancer drugs. It is well known that oxidative stress is involved in the mechanisms underlying the therapeutic efficacy of As^{III} and plays a major role in the toxicity of As^{III} (Ninomiya et al., 2006). In fact, the model system has been proposed to be used for studying the toxicological as well as pharmacological relevance of As^{III} (Yuan et al., 2011).

It is quite logical to consider that intracellular arsenic accumulation (As[i]) is critical for the control of various biological functions and that these levels are tightly associated with As^{III} uptake and efflux (Liu et al., 2002; Leslie et al., 2004; Lee et al., 2006; Leung et al., 2007; Shinkai et al., 2009). Accumulating evidence indicates that aquaglyceroporins (AQPs),

which are members of the aquaporin superfamily and responsible for transporting small uncharged molecules such as glycerol and urea as well as water, play a pivotal role in the uptake of As^{III} (Lee et al., 2006; Leung et al., 2007; Shinkai et al., 2009). In particular, a close correlation between the expression levels of AQP9 and As^{III} efficacy has been established in leukemia samples (Leung et al., 2007). Regarding efflux, several adenosine triphosphate (ATP) binding cassette (ABC) transporters, including multidrug resistance-associated proteins 1 and 2 (MRP1/2), multidrug resistance protein 1 (MDR1; also known as P-glycoprotein, P-gp) are involved in the efflux of As^{III} (Liu et al., 2002; Leslie et al., 2004; Lee et al., 2006). All of the evidence for As^{III} transport was obtained from studies using human-derived malignant cells and/or primary mouse hepatocytes as well as in vivo studies on mice. However, the correlation between the effects of As^{III} and the expression levels of these transporters in human-derived normal cells has not yet been conducted.

In this study, we investigated the effects of As^{III} on C- and A-cells by assessing cell viability, apoptosis induction and lactate dehydrogenase (LDH) release. Furthermore, we investigated the role of As^{III}-associated transporters in these cells using various inhibitors, such as sorbitol and phloretin, both of which are inhibitors for AQP9 (Tsukaguchi et al., 1998; Shinkai et al., 2009); cyclosporine A (CsA), a broad-spectrum inhibitor for ABC transporters (Qadir et al., 2005); MK571, a MRP inhibitor (Matsson et al., 2009); and PGP-4008, a P-gp specific inhibitor (Lee et al., 2003). Results demonstrated for the first time that AQP9 and MRP2 are involved in controlling As[i] in primary cultured normal cells, which then contribute to differential sensitivity to As^{III} cytotoxicity between these cells.

Materials and Methods

Materials

Sodium arsenite (As^{III}) was purchased from Tri Chemical Laboratories (Yamanashi, Japan). LDH cytotoxicity detection kits were purchased from Roche (Mannheim, Germany). An RNA extraction kit, ISOGEN was obtained from Wako Pure Chemical Industries (Osaka, Japan). CsA, a broad-spectrum inhibitor for ABC transporters, was kindly provided by Novartis Pharma Co. (Basel, Switzerland). 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfan yl] propionic acid (MK571), an inhibitor of MRPs, was purchased from Merck (Darmstadt, Germany). N-(1-benzyl-2,3-dihydro-1H-pyrrolo[2,3-b]quinolin-4-yl)-2-phenylacetamide (PGP-4008), a specific inhibitor for P-gp, was obtained from Sigma (MD, USA). Sorbitol and phloretin, inhibitors for AQP9, were purchased from Sigma and Wako Pure Chemical Industries, respectively.

Preparation of primary cultured C- and A-cells from human fetal membranes

Fetal membranes were prepared aseptically from placenta obtained during normal parturition by Cesarean section as described previously (Ohyama et al., 1998). Primary cultured C- and A-cells were prepared from fetal membranes, which were freshly isolated according to the methods described previously (Ohyama et al., 1998). This study has been approved by the IRB committee of Tokyo University of Pharmacy and Life Sciences. An informed consent was obtained from patients at the time of surgery.

XTT assay

The cytotoxicity of As^{III} to C- and A-cells was investigated by XTT dye-reduction assays according to the method previously described with slight modifications (Scudiero et al., 1988). Briefly, the cells were seeded in 96-well plates coated with type I collagen (Iwaki, Tokyo, Japan) at a density of 5×10^4 cells per well in 0.1 mL cell culture medium and allowed to become confluent. Cultures in triplicate were treated with various concentrations

of As^{III} in the presence or absence of inhibitors for transporters at the concentrations indicated. In experiments using transporter inhibitors, both cells were preincubated with transporter inhibitors at the indicated concentrations for 30 min. After treatment with As^{III} for an additional 48 h, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) -5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (Sigma, MD, USA) and phenazine methosulfate (Wako Pure Chemical Industries, Osaka, Japan) were added into each well at final concentrations of 0.2 mg/mL and 1 mM, respectively. After incubation at 37 °C for 2 h, the plates were mixed, and the absorbance at 450 nm was measured with a microplate reader (Safire, Tecan, Switzerland). The relative cell viability was expressed as the ratio of the absorbance of each treatment group against those of the corresponding untreated control group. Data are shown as means and SD from three independent experiments.

Lactate dehydrogenase (LDH) assay

After treatment with various concentrations of As^{III} for 48 h, LDH leakage from both C- and A-cells was measured using a LDH cytotoxicity detection kit. Twenty-five microliters of culture supernatants were collected by centrifugation at 450×g for 5 min at 4 °C, and transferred into wells of a 96-well plate. After discarding the remaining culture supernatants, the cells were lysed with 200 μL of 0.1% Triton-X100. Twenty-five microliters of cell lysates were subsequently loaded into the 96-well plate. LDH activities in both the culture supernatants and the cell lysates were determined by adding 50 μL of reaction reagent from the kit and 25 μL of PBS to each well, followed by incubation at 37 °C for 15 min. The reaction was stopped by the addition of 100 μL of 1 N HCl, and the absorbance at 490 nm was measured with a microplate reader (Safire, Tecan, Switzerland). Cell damage was calculated as a percentage of LDH leakage from damaged cells using the following formula: LDH leakage (%) = (Sup)/(Sup + Cell) × 100 where Sup and Cell refer to absorption of culture supernatant and cell lysate, respectively. Data are shown as means and SD from three independent experiments.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA and complementary DNA were prepared as described previously (Yuan et al., 2006). Total RNA was extracted from cells using an RNA extraction kit, ISOGEN. Complementary DNA was synthesized from 1 g of RNA using 100 pmol random hexamers and 100 U Moloney murine leukemia virus reverse transcriptase (Invitrogen, CA, USA) in a total volume of 20 μL, according to the manufacturer's instructions. PCR was performed according to the method described previously (Andus et al., 1993) using a Takara Thermal Cycler MP (TAKARA SHUZO, Osaka, Japan). DNA sequence of PCR primers and optimal conditions for PCR were shown in Table 1. PCR primers were purchased from Sigma-Aldrich (Tokyo, Japan). PCR products and Ready-Load™ 100 bp DNA Ladder (Invitrogen, CA, USA) marker were electrophoresed, respectively, on a 2.0% UltraPure™ agarose gel (Invitrogen, CA, USA), and visualized by ethidium bromide staining, followed by viewing under UV Light Printgraph (ATTO Corp, Tokyo, Japan). The signal intensity of the specific mRNAs was calculated from the density of the gray level on a digitized image using a program, NIH ImageJ 1.43u.

Western blot analysis

For preparation of protein samples, cells were washed twice with cold PBS, and lysed in lysis buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 mM PMSF) on ice for 30 min. After centrifugation at 13, 000 g for 15 min at 4 °C, the supernatants were collected and mixed with 4×SDS buffer (50 mM Tris-HCl pH 6.8, 8% sodium dodecyl sulfate, 24% 2-mercaptoethanol, 40% glycerol, and 0.8% bromphenol blue) in a 1:3 ratio at 4 °C for

overnight. Protein concentrations were determined by Bradford's method using the protein assay dye reagent (Bio-Rad, CA, USA) according to the manufacturer's instructions, and using BSA as standard. Protein samples were separated on a SDS polyacrylamide gel electrophoresis, followed by transferring to a nitrocellulose membrane according to the method described previously (Yuan et al., 2009). Protein bands were detected using the following primary antibodies: mouse anti-human AQP9 (G-3), mouse anti-human MRP1 (QCRL-1) (Santa Cruz Biotechnology, CA, USA); mouse anti-human MRP2 (M2-III6), mouse anti-human P-gp (C219) (Abcam, MA, USA); and mouse anti-human β-actin (AC-15) (Sigma, MO, USA). Blotted protein bands were detected with respective horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK) and an enhanced chemiluminescence (ECL) Western bolt analysis system (GE Healthcare, Buckinghamshire, UK). The signal intensity of the immunoreactive proteins was calculated from the density of the gray level on a digitized image using a program, NIH ImageJ 1.43u.

Analysis of intracellular arsenic accumulation (As[i])

After exposure of C- and A-cells to nontoxic concentrations of 17 μM As^{III} in the presence or absence of inhibitors for transporters at the concentrations indicated, cells were washed three times with PBS. Cells were preincubated with transporter inhibitors at concentrations indicated for 30 min. Then, cells were harvested, and the weight of cell pellets were measured and used to normalize cellular arsenic concentrations. The As[i] was normalized by cell weights and given as nanomolar of arsenic per gram of cell weights. The analysis of total arsenic was performed by inductively coupled plasma-mass spectrometry (ICP-MS) (PerkinElmer SCIEX, ON, Canada) according to the methods reported previously (Yoshino et al., 2009).

Statistical analysis

Data were analyzed using Student's t-test and $p<0.05$ was considered as statistically significant.

Results

As^{III}-induced cytotoxicity in primary cultured C- and A-cells

Cytotoxic effects of As^{III} were observed in the C-cells after exposure to As^{III} ranging from 5 to 80 μM for 48 h accompanied with an increase of LDH leakage in a dose-dependent manner (Fig. 1A and B). When the concentrations of As^{III} increased up to 20 μM, statistically significant differences were observed between As^{III}-exposed group and control group (Fig. 1A). On the other hand, in the A-cells, the cytotoxicity of As^{III} was only observed when treated with 80 μM of As^{III} (Fig. 1A and B).

Expression profile of HO-1 gene in primary cultured C- and A-cells treated with As^{III}

After exposure of these primary normal cells to nontoxic concentrations of As^{III} (17 μM), we analyzed the expression profile of HO-1 gene by RT-PCR. An approximately 2-fold increase in the expression levels of HO-1 gene was observed in the A-cells after 0.5-h exposure. Furthermore, the expression levels of HO-1 gene markedly increased more than 8 times in As^{III}-treated A-cells compared to those in control cells at 2-h post-exposure, and the increase continued up to 24 h (Fig. 2). In contrast, the same phenomena were not observed in the C-cells, although a slight increase in HO-1 expression was detected in the cells (Fig. 2).

Intracellular arsenic accumulation (As[i]) in primary cultured C- and A-cells treated with As^{III}

After the exposure of these primary cells to 17 μM As^{III} for 0, 0.25, 0.5, 1, 2, 4, 6, 12 or 24 h, As[i] was measured by ICP-MS. As shown in Fig. 3, the levels of the As[i] increased with time in both cells, and almost reached a plateau at 6-h post-exposure. Furthermore, the levels of the As[i] were much higher in the C-cells than those in the A-cells, and statistically significant differences in the levels were confirmed at 1-h post-exposure and thereafter. These data clearly demonstrated that the accumulation of As^{III} in these cells almost reached a plateau at 6-h post-exposure, and that there was the largest difference in the levels of the As[i] between these cells at the same time point. Thus, the following experiments on arsenic accumulation were conducted by exposing these cells to 17 μM of As^{III} for 6 h.

Expression levels of As^{III}-associated transporters in primary cultured C- and A-cells treated with As^{III}

After the treatment of these primary cells with 17 μM As^{III} for 0, 6 or 48 h, the expression profiles of AQP9, ABCC1 (MRP1), ABCC2 (MRP2) and ABCB1 (P-gp) were assessed by RT-PCR and Western blot analysis. Since As[i] and cell viability were determined at 6-h and 48-h post-exposure, respectively, we focused on the expression profiles of As^{III}-associated transporter genes at the two time points. The levels of endogenous AQP9 gene expression were approximately 2 times higher in the C-cells than those in the A-cells at 0-time incubation and during the treatment with As^{III} (Fig. 4). Furthermore, the protein expression level of AQP9 was slightly but significantly upregulated by treatment with As^{III} in the C-cells at 48-h post-exposure, but not in the A-cells (Fig. 4C and D). Regarding ABCC1, no significant difference in its mRNA and protein expression level was confirmed between C- and A-cells, although a slight, but significant upregulation of its mRNA expression level was observed in both cells at 48-h post-exposure (Fig. 4). Intriguingly, an inductive effect of As^{III} on the expression of ABCC2 mRNA was observed and continued up to 48 h in the A-cells (Fig. 4A and B). However, in the C-cells, the expression levels of ABCC2 mRNA exhibited a transient, significant upregulation at 6-h, followed by a substantial decrease at 48-h post-exposure (Fig. 4A and B). Unlike ABCC2 mRNA expression pattern in both cells, a time-dependent decrease in its protein expression levels was observed in the C-cells, whereas no alteration was observed in the A-cells during the exposure period (Fig. 4C and D). Furthermore, the expression levels of endogenous ABCC2 protein were significant higher in the A-cells than those in the C-cells (Fig. 4C and D). Unexpectedly, the expression of ABCB1 protein was hardly detected in both cells regardless of As^{III} treatment, although a significant time-dependent increase in its mRNA expression was observed in these cells (Fig. 4).

Effects of sorbitol and phloretin on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells

Both primary cells were exposed to 17 μM As^{III} in the presence or absence of sorbitol (10, 30, 50 or 100 mM) and phloretin (50, 100, 200 or 500 μM), respectively, for 6 h, followed by the assessment of As[i]. In the C-cells, a significant, dose-dependent decrease in the As[i] was observed in the presence of sorbitol (Fig. 5A). Moreover, the levels of the As[i] were reduced by 40% in the presence of 100 mM sorbitol compared to those treated with As^{III} alone. However, no such effects were observed in the A-cells (Fig. 5A). Consistent with the decreased As[i], As^{III}-induced cytotoxicity in the C-cells was significantly abrogated by the presence of sorbitol in a dose-dependent manner when the cells were exposed to As^{III} ranging from 5 to 80 μM in the presence of 30 and 100 mM sorbitol for 48 h (Fig. 5B). Again, no alterations of As^{III}-induced cytotoxicity were observed in the A-cells exposed to As^{III} ranging from 5 to 40 μM with or without 30 and 100 mM sorbitol for 48 h (Fig. 5B). Consistent with XTT results (Fig. 1), As^{III}-induced cytotoxicity was observed in the cells

treated with as high as 80 μM As^{III} regardless of the presence or absence of sorbitol (Fig. 5B). Unexpectedly, the levels of As[i] were hardly altered by the addition of phloretin in both cells, although the addition of 500 μM phloretin slightly but significantly reduced the level of As[i] only in C-cells (Fig. 5C). Furthermore, no alternations of As^{III}-induced cytotoxicity were observed in both cells when treated with As^{III} ranging from 5 to 80 μM for 48 h, regardless of the presence or absence of 100 μM and 200 μM phloretin (Fig. 5D). Moreover, it was confirmed that cell viability was not altered by sorbitol or phloretin alone in both cells.

Effects of CsA on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells

Both primary cells were exposed to 17 μM As^{III} in the presence or absence of CsA at various concentrations (1, 3, 10, 30 μM) for 6 h, followed by the assessment of As[i]. In both cells, the addition of CsA significantly increased the As[i] in a dose-dependent manner (Fig. 6A). Moreover, the As[i] increased from 40.2 to 68.8 nmol/g of cell weight and from 25.5 to 43.3 nmol/g of cell weight in the C-cells and the A-cells, respectively, by the addition of 30 μM of CsA (Fig. 6A). Consistent with the increased As[i] in both cells, As^{III}-induced cytotoxicity was significantly enhanced by the addition of CsA in a dose-dependent manner when treated with As^{III} ranging from 5 to 80 μM in the presence of 3 or 10 μM of CsA for 48 h (Fig. 6B). More potent enhancing effect of CsA were observed in the C-cells when compared with those in the A-cells. Furthermore, it was confirmed that cell viability was not altered by the presence of CsA alone in both cells.

Effects of MK571 on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells

Both primary cells were exposed to 17 μM As^{III} in the presence or absence of MK571 at various concentrations (10, 30, 50, 100 μM) for 6 h, followed by the assessment of As[i]. The levels of the As[i] were prominently increased by the addition of MK571 in a dose-dependent manner in the C-cells (Fig. 7A). In contrast, the levels of As[i] in the A-cells were only slightly, but significantly, increased by MK571 (Fig. 7A). The As[i] increased from 36.1 to 70.4 nmol/g of cell weight in the C-cells, and from 19.6 to 25.7 nmol/g of cell weight in the A-cells respectively, by the addition of 100 μM of MK571 (Fig. 7A). Consistent with the increased As[i] in the C-cells, As^{III}-induced cytotoxicity was significantly enhanced by MK571 in a dose-dependent manner when treated with As^{III} ranging from 5 to 80 μM in the presence of 10 or 50 μM of MK571 for 48 h (Fig. 7B). Intriguingly, the addition of MK571 did not alter cell viability of the A-cells when exposed to As^{III} ranging from 5 to 40 μM (Fig. 7B). However, the addition of 50 μM of MK571 slightly but significantly enhanced As^{III}-induced cytotoxicity in the cells exposed to As^{III} at a concentration of 80 μM (Fig. 7B). Moreover, it was confirmed that cell viability was not altered by MK571 alone in both cells.

Effects of PGP-4008 on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells

Both primary cells were exposed to 17 μM As^{III} in the presence or absence of PGP-4008 at various concentrations (5, 10, 20 μM) for 6 h, followed by the assessment of As[i]. The levels of As[i] were not altered by the addition of PGP-4008 in both cells (Fig. 8A). At the same time, no alteration of As^{III}-induced cytotoxicity was observed in both cells when treated with As^{III} ranging from 5 to 80 μM for 48 h, regardless of the presence or absence of 3 or 10 μM of PGP-4008 (Fig. 8B). In order to verify whether these concentrations of PGP-4008 could functionally inhibit P-gp function, we initially evaluated P-gp efflux function using rhodamine 123 in P-gp-expressing daunorubicin-resistant MOLT-4 cells, a human T-lymphoblastoid leukemia cell line. Results demonstrated that these concentrations are enough to inhibit P-gp function (data not shown). Furthermore, it was confirmed that cell viability was not altered by PGP-4008 alone in both cells.

Discussion

In this study, we demonstrated that a significant As^{III}-mediated cytotoxicity was observed in C-cells in a dose-dependent manner, but not in A-cells, suggesting that C-cells were more sensitive to As^{III} than A-cells. We also demonstrated that the levels of the intracellular arsenic accumulation (As[i]) were much higher in the C-cells than those in the A-cells, indicating that the sensitivity to As^{III} correlates with the As[i]. Furthermore, our results suggest that the immediate and marked upregulation of HO-1 expression levels observed only in the A-cells contribute to the maintenance of cellular homeostasis. This idea is supported by our previous observations that an increased level of HO-1 gene expression is responsible for higher tolerance to oxidative stress in the A-cells (Yuan et al., 2008). Of note, it has been reported that the expression of HO-1 plays an important role in arsenite-resistant human lung adenocarcinoma cells (Lee and Ho, 1994). We further demonstrated that no nuclear condensation and DNA fragmentation (data not shown) was observed in both cells, indicating no involvement of apoptosis induction in these cells. Collectively, our results suggested that As^{III}-induced cell death in primary cultured normal cells is predominantly associated with a necrosis-like phenotype as assessed by LDH release, and that As^{III}-mediated side effects can be partially attributed to the necrosis-like cell death induction.

Accumulating evidence suggests that AQP9 is a primary route of As^{III} uptake into leukemia cells, suggesting that monitoring its expression levels in APL patients before or during treatment with As^{III} may provide an index for therapeutic efficacy (Leung et al., 2007; Yuan et al., 2011). On the other hand, it has been established that MRP1/2 and P-gp are involved in the efflux of As^{III} (Liu et al., 2002; Leslie et al., 2004; Lee et al., 2006; Yuan et al., 2011). In order to investigate whether these transporters regulate the As[i] in normal cells, we characterized the expression profiles of these transporter genes, and the contribution of these genes to As[i] as well as As^{III}-triggered cytotoxicity in both C- and A-cells using inhibitors for these transporters. Our results clearly demonstrated that the levels of endogenous AQP9 gene expression were much higher in the C-cells than those in the A-cells. These findings coincide well with a previous study indicating that the expression levels of AQP9 were much stronger in C-cells than that in A-cells, in which AQP9 was proposed to be responsible for the regulation of intramembranous amniotic fluid (Wang et al., 2004). Furthermore, both As[i] and As^{III}-triggered cytotoxicity in the C-cells were significantly attenuated by sorbitol, a competitive AQP9 inhibitor (Shinkai et al., 2009), in a dose-dependent manner, but a similar phenomenon was not observed in the A-cells. Intriguingly, both As[i] and As^{III}-induced cytotoxicity were hardly altered by the addition of phloretin, another AQP9 inhibitor, in both cells, results of which are similar to those reported by other group (McDermott et al., 2010). Furthermore, our experimental data also demonstrated that the inhibitory efficiency towards AQP9 of sorbitol (100 mM) is much higher than that of phloretin (200 μM) in an APL cell line, NB4 cells (data not shown), in which AQP9 is highly expressed (Leung et al., 2007; Wang et al., 2008). These results might be attributed to different cell types used and could explain why the levels of As[i] and As^{III}-induced cytotoxicity were altered by the addition of sorbitol, but not phloretin in our experimental system. Collectively, our results suggest that AQP9 is more functionally implicated in As^{III} uptake and As^{III}-induced cytotoxicity in the C-cells rather than in the A-cells. Although the exact molecular mechanisms underlying As^{III} uptake in the A-cells are not clear at present, it is possible that the uptake is through an alternative pathway involving AQP3, another member of the aquaporin transporter superfamily, and glucose transporter 1. This hypothesis is based on the studies demonstrating that these two transporters have been identified in A-cells (Mann et al., 2002; Gude et al., 2005), and involved in arsenic uptake (Liu et al., 2004, 2006; Lee et al., 2006; Yuan et al., 2011). Understandably, further investigation of a contribution of the alterative pathway is need.

We also demonstrated that the expression level of ABCC2 mRNA was upregulated in the A-cells, whereas it was downregulated in the C-cells after 48-h post-exposure. Furthermore, Western blot analysis demonstrated that the expression levels of endogenous ABCC2 protein were significant higher in the A-cells than those in the C-cells, and that a time-dependent decrease in ABCC2 protein expression levels was observed in the C-cells, but not in the A-cells. These results thus suggested that a differential expression pattern of the gene may contribute to the differences in the As^{III} sensitivity between these two cell types. It should be noted that while the upregulation of ABCC2 mRNA expression level was observed in the A-cells, no alternation in its protein expression level was observed during the exposure period. It is possible that a post-transcriptional regulation plays a key role in the discordance between mRNA and protein expression. Details of the study are currently underway. We further demonstrated that As^{III}-triggered cytotoxicity as a result of As[i] in the C-cells was significantly augmented by CsA and MK571 in a dose-dependent manner, suggesting that MRP2 appear to be functionally implicated in As[i] and cytotoxicity in the cells. It is noteworthy that in the A-cells, an increase in the As[i] was much higher in the presence of CsA compared to MK571. Consequently, CsA was much more efficient in potentiating As^{III}-mediated cytotoxicity compared with MK571, implicating a close correlation between sensitivity to As^{III} and As[i]. Moreover, several previous reports have demonstrated that CsA not only increases the production of reactive oxygen species in rat glial cells and porcine renal endothelial cells (Mun et al., 2008; de Arriba et al., 2009), but also alters the expression levels of antioxidant enzymes by decreasing the activity of catalase and glutathione peroxidase in rabbit kidney tissue (Durak et al., 1998). On the basis of these results and current findings, we suggest that not only modulating effects on multidrug resistant transporters but also reactive oxygen species induction effects of CsA might sensitize the A-cells to the cytotoxic effects of As^{III}.

The current study also demonstrated that no significant differences in the expression levels of MRP1 were observed between C- and A-cells, indicating that MRP2, instead of MRP1, plays a critical role in As^{III}-efflux system in both cells. Unexpectedly, the expression of ABCB1 protein was hardly detected in both cells, yet the expression of mRNA was detected. While more detailed analysis of the molecular event is obviously needed, our results suggested that P-gp was not functionally involved in arsenic efflux in these normal cells. This is clearly supported by our experimental data showing that neither As[i] nor As^{III}-mediated cytotoxicity was augmented by the addition of PGP-4008, a specific inhibitor for P-gp.

In conclusion, we propose for the first time that AQP9 and MRP2 are functionally involved in controlling arsenic accumulation in these normal cells, which in turn contribute to differential sensitivity to As^{III} cytotoxicity between these cells. Although studies regarding the action of arsenic have been conducted using normal human fetal brain explants and human cord blood cells (Chattopadhyay et al., 2002; Ferrario et al., 2009), the correlation between biological functions of arsenic and these transporters has not yet been clarified in human-derived normal cells. To the best of our knowledge, this is the first correlation of As^{III}-associated transporters with cytotoxic effects of As^{III} in primary normal cells. Given that these arsenic transporters play a pivotal role in As^{III}-mediated cytotoxic effects on normal cells and/or tissues, monitoring expression levels of As^{III}-associated transporters has important implications for predicting not only clinical efficacy but also appearance of side effects in patients with cancer.

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Research highlights

- Examination of effect of As^{III} on primary cultured chorion (C) and amnion (A) cells
- Dose-dependent As^{III}-mediated cytotoxicity in C-cells, not in A-cells
- Intracellular arsenic accumulation and cytotoxicity regulated by AQP9 and ABCC2
- Prediction of As^{III} side effects by monitoring these transporters

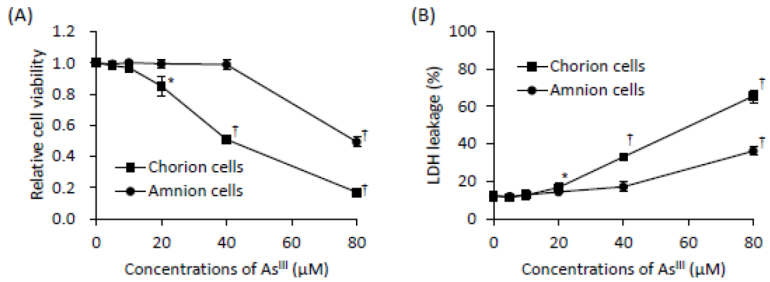


Fig. 1.

As^{III}-induced cytotoxicity in primary cultured C- and A-cells.

After treatment with various concentrations of As^{III} (0, 5, 10, 20, 40 or 80 μM) for 48 h, cell viability was determined by XTT assay (A), and LDH leakage (B) of C- and A-cells were analyzed, as described under Materials and Methods. Significant differences were observed between control group and As^{III} treatment group (*, $p<0.05$ and †, $p<0.001$). Data are shown as the mean and SD from three independent experiments.

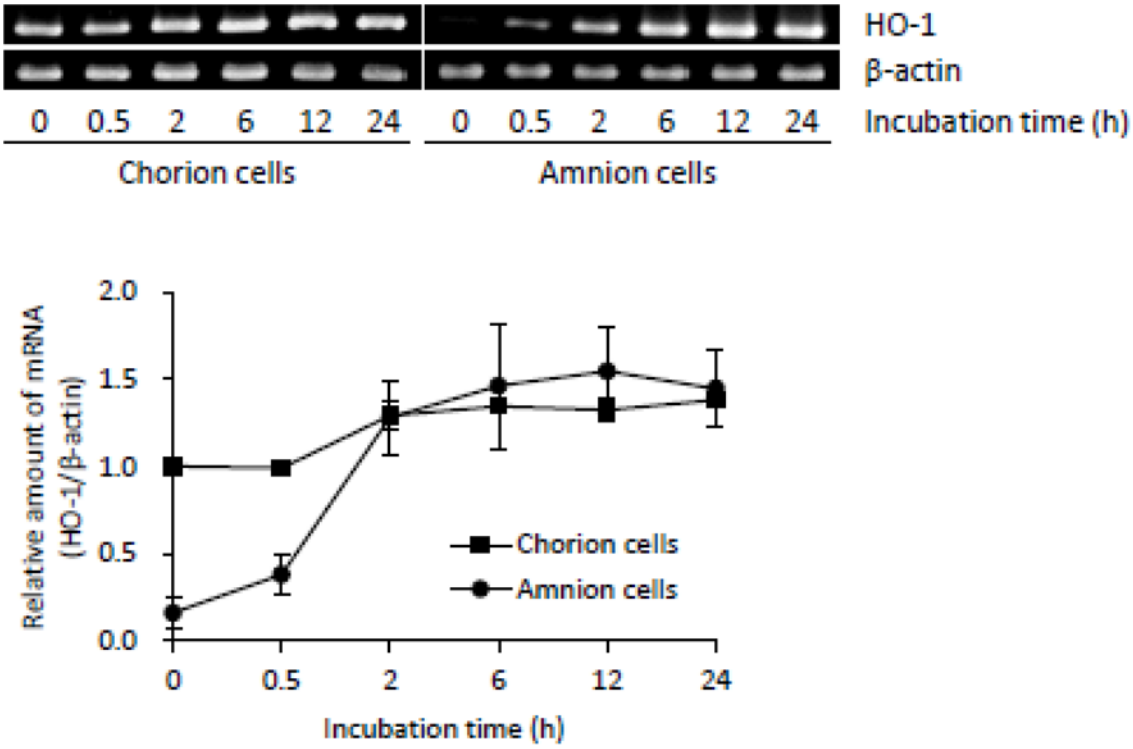


Fig. 2.

Expression profile of HO-1 mRNA in primary cultured C- and A-cells treated with As^{III}. After exposure to 17 μM As^{III} for 0, 0.5, 2, 6, 12, or 24 h, the expression level of HO-1 mRNA was analyzed by RT-PCR, followed by an agarose gel electrophoresis as described under Materials and Methods. The relative expression levels were expressed as the ratios of the expression levels of HO-1 against those of β-actin, and were compared with those in C-cells at 0-time incubation. Experiments were carried out in three independent experiments, and representative electrophoretic profiles are shown. Significant differences were observed between control group (0-time incubation) and As^{III} treatment group (from 2-h post-exposure in C-cells, $p<0.05$; from 0.5-h post-exposure in A-cells, $p<0.05$)

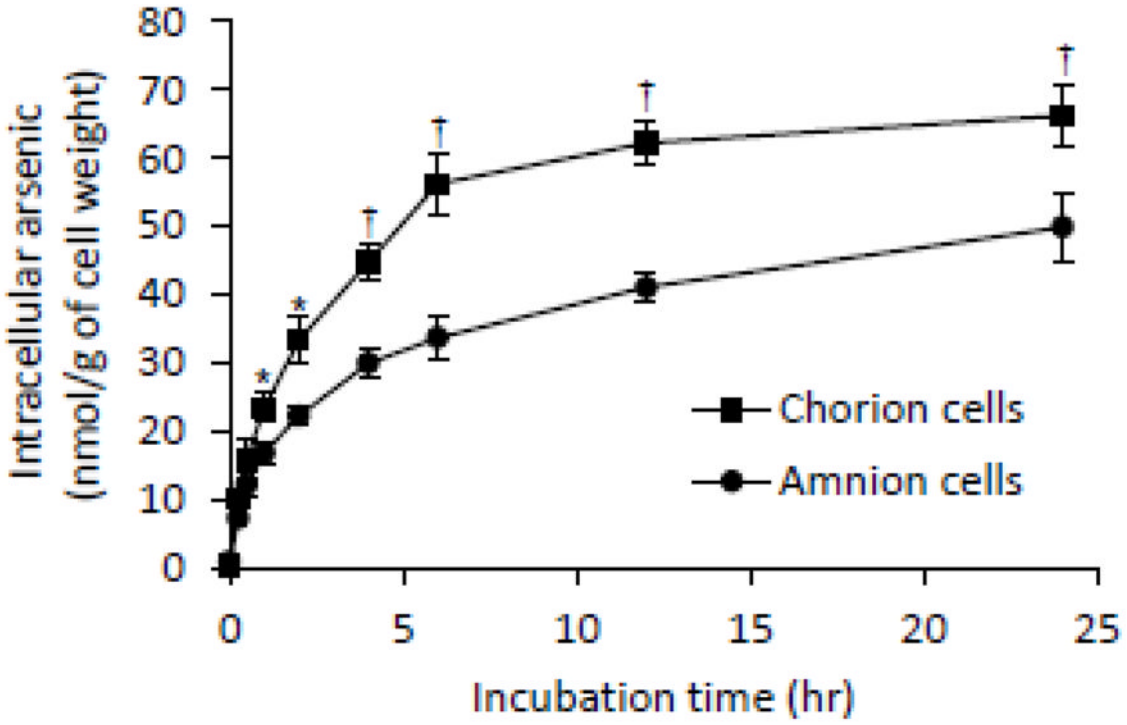
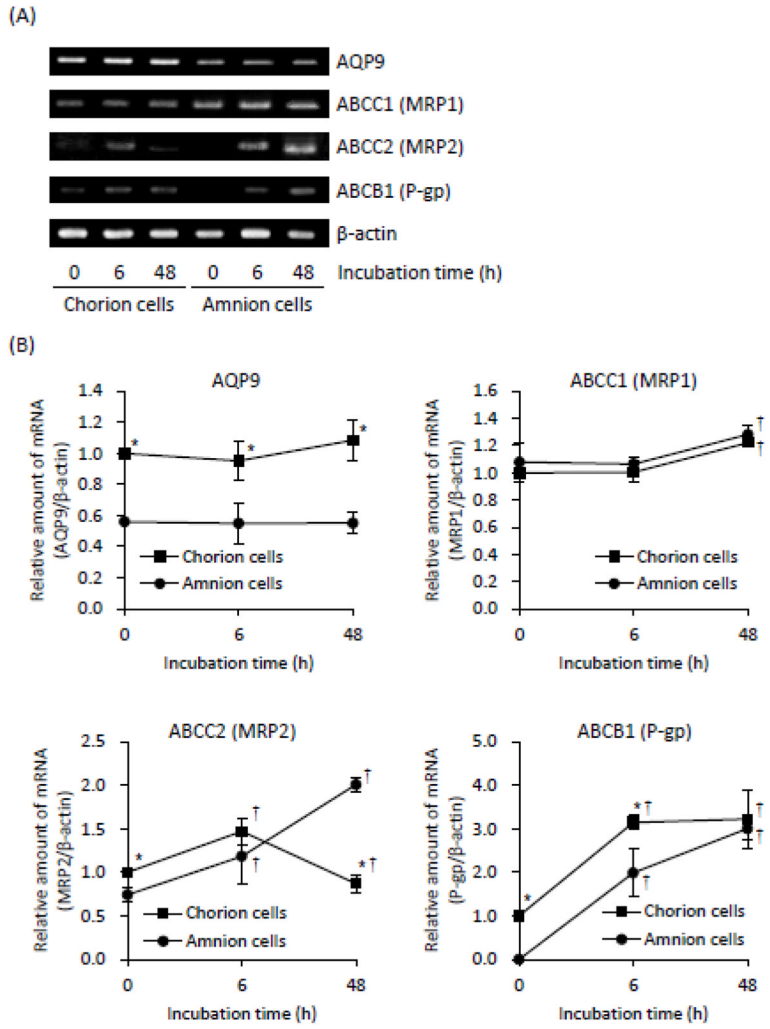


Fig. 3.

Intracellular arsenic accumulation (As[i]) in primary cultured C- and A-cells treated with As^{III}.

Both cells were treated with 17 μM As^{III} for 0, 0.25, 0.5, 1, 2, 4, 6, 12, or 24 h, followed by assessing the As[i] using ICP-MS. Data are shown as the mean and SD from three independent experiments. Significant differences between C- and A-cells are shown (*, $p<0.05$ and †, $p<0.001$).



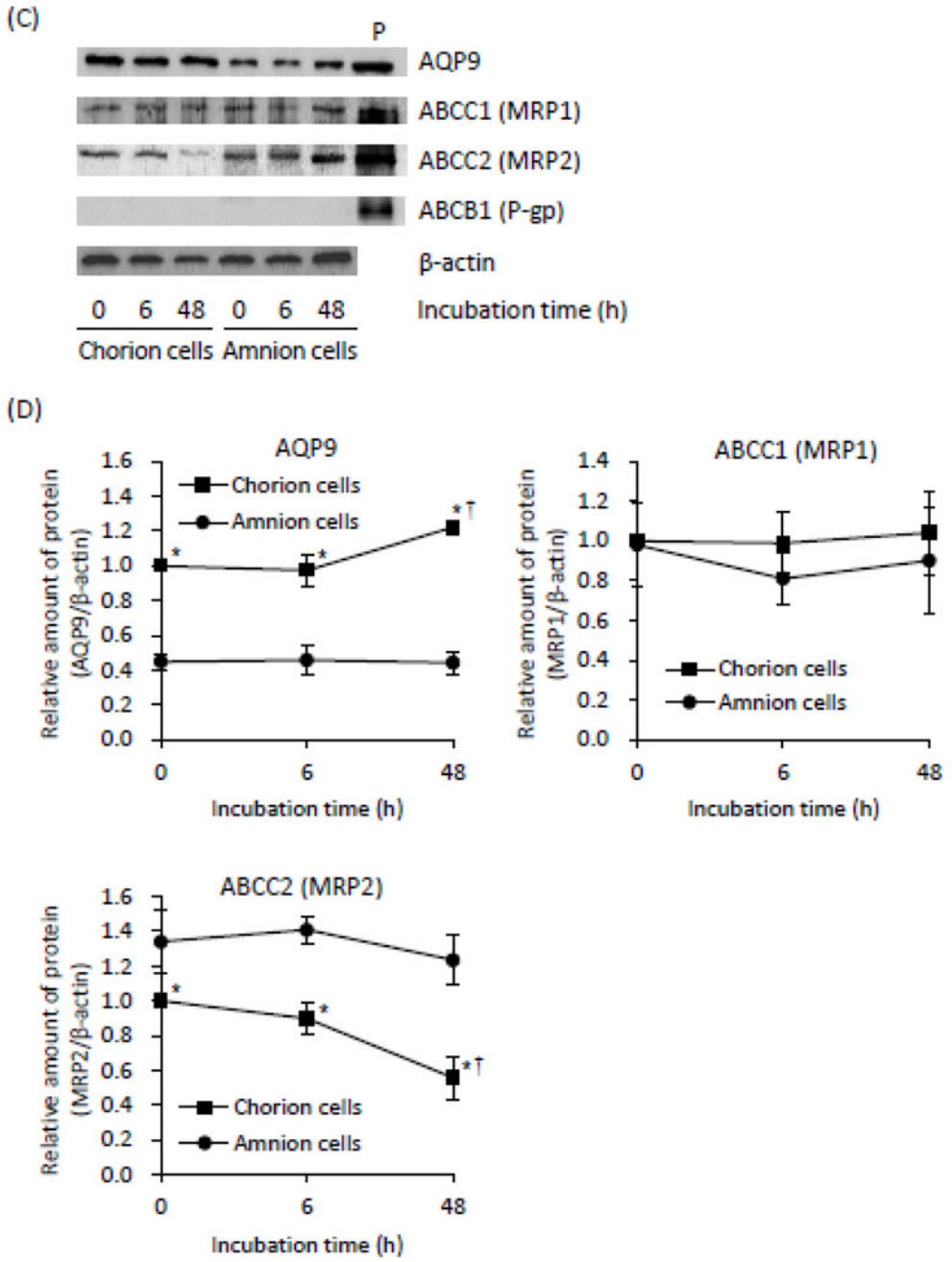


Fig. 4.

Expression levels of As^{III}-associated transporters in primary cultured C- and A-cells treated with As^{III}

Expression levels of mRNAs (A), the relative expression levels of each mRNA (B), the expression levels of proteins (C), and the relative expression levels of each protein (D) of As^{III}-associated transporters were analyzed, respectively, as described under Materials and Methods. The relative expression levels of each gene were expressed as described in Fig. 2. Both cells were treated with 17 μM As^{III}. A representative electrophoretic profile of three separate experiments is shown. P represents positive control used in this study, where NB4, an APL cell line, for AQP9; a MRP1-expressing cell line for MRP1; HEK293 cells stably

expressing MRP2 cDNA for MRP2; and P-gp-expressing daunorubicin-resistant MOLT-4 cells for P-gp protein. Data are shown as the mean and SD from three independent experiments. Significant differences in the expression levels of each gene are shown (* $p<0.05$ C-cell vs. A-cell; † $p<0.05$ vs. control)

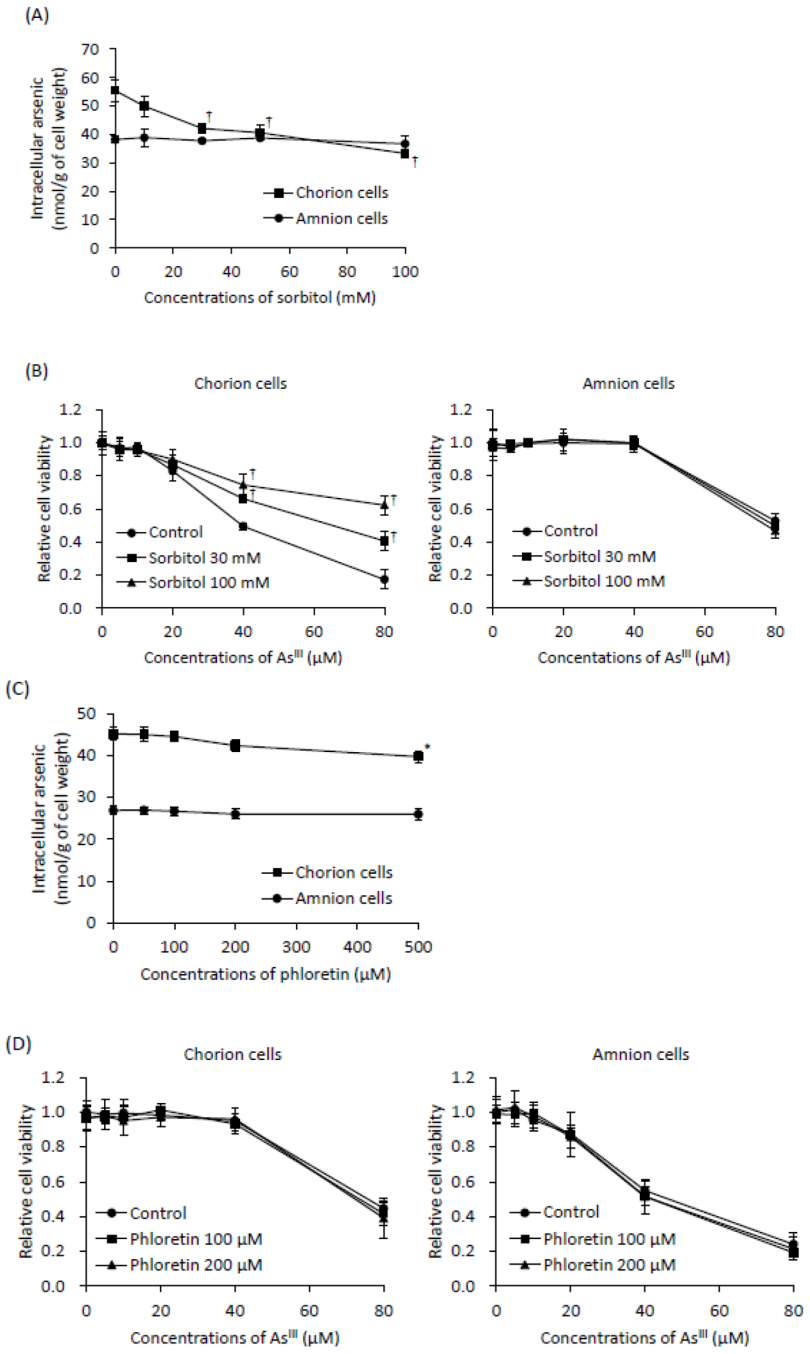


Fig. 5.

Effects of sorbitol on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells.

(A and C): Both cells were treated with 17 μM of As^{III} in the presence or absence of sorbitol (10, 30, 50 or 100 mM) and phloretin (50, 100, 200 or 500 μM), respectively, for 6 h, followed by the assessment of As[i] using ICP-MS. (B and D): Cell viability was determined by XTT assay after treatment with As^{III} ranging from 5 to 80 μM in the presence or absence of sorbitol (30 or 100 mM) and phloretin (100 or 200 μM), respectively, for 48 h. Data are shown as the mean and SD from three independent experiments. Significant differences

were observed between control groups (As^{III}-only treatment) and the treated groups (As^{III} plus sorbitol) (*, $p<0.05$ and †, $p<0.001$).

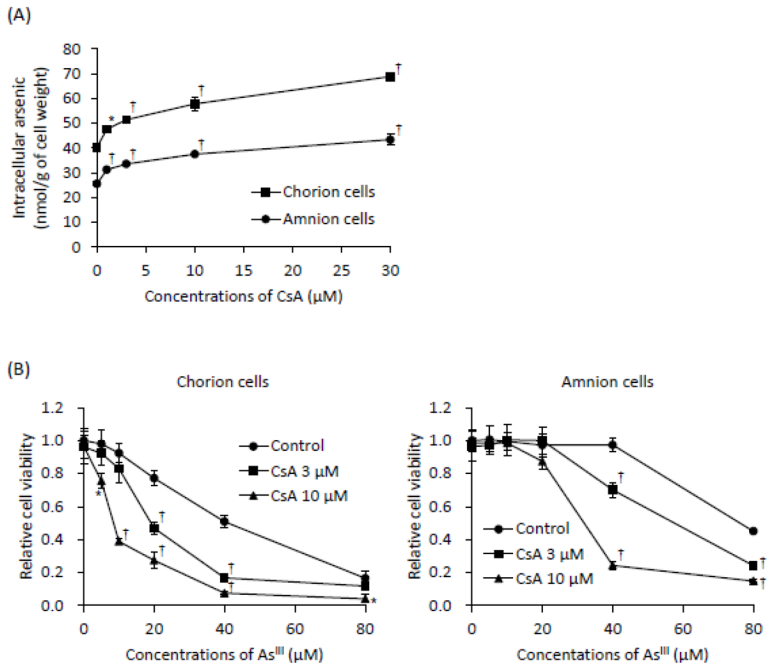


Fig. 6.

Effects of CsA on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells. (A) Both cells were treated with 17 μM of As^{III} in the presence or absence of CsA (1, 3, 10, or 30 μM) for 6 h, followed by the assessment of As[i] using ICP-MS. (B) Cell viability was determined by XTT assay after treatment with As^{III} ranging from 5 to 80 μM in the presence or absence of 3 or 10 μM CsA for 48 h. Data are shown as the means and SD from three independent experiments. Significant differences were observed between control groups (As^{III}-only treatment) and the other treatment groups (As^{III} plus CsA) (*, $p<0.05$ and †, $p<0.001$).

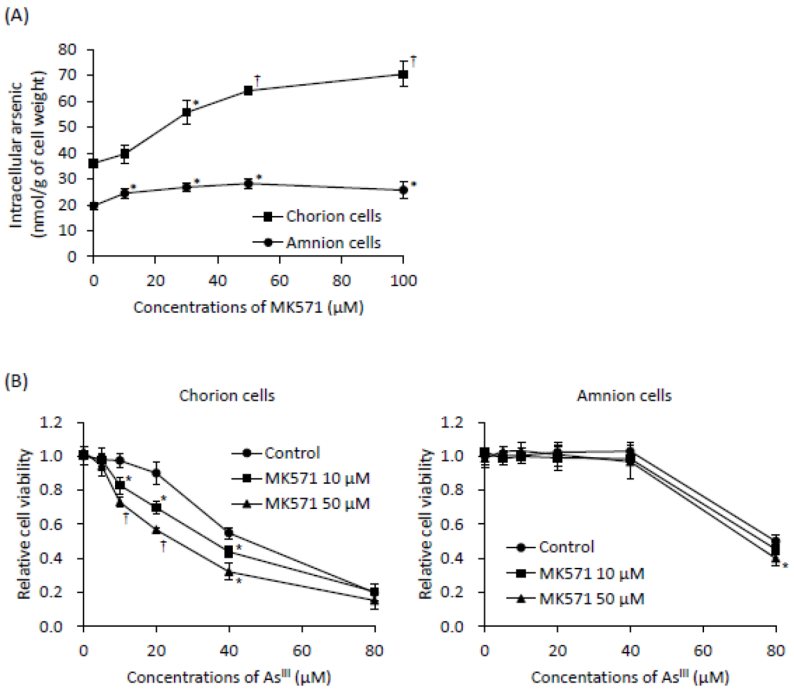


Fig. 7.

Effects of MK571 on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells.

(A) Both cells were treated with 17 μM of As^{III} in the presence or absence of MK571 (10, 30, 50, or 100 μM) for 6 h, followed by the assessment of As[i] using ICP-MS. (B) Cell viability was determined by XTT assay after treatment with As^{III} ranging from 5 to 80 μM in the presence or absence of 10 or 50 μM MK571 for 48 h. Data are shown as the means and SD from three independent experiments. Significant differences were observed between control groups (As^{III}-only treatment) and the other treatment groups (As^{III} plus MK571) (*, $p<0.05$ and †, $p<0.001$).

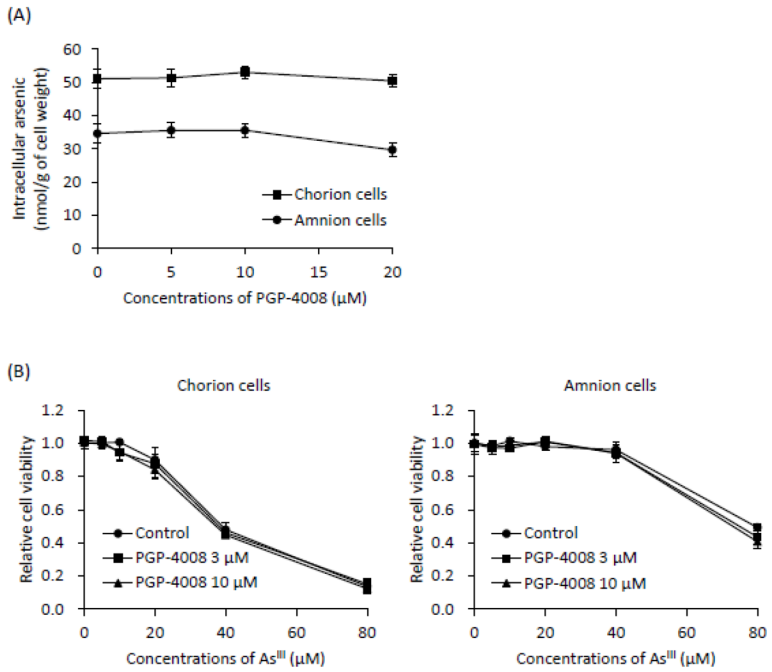


Fig. 8.

Effects of PGP-4008 on As[i] and As^{III}-induced cytotoxicity in primary cultured C- and A-cells.

(A) Both cells were treated with 17 μM of As^{III} in the presence or absence of PGP-4008 (5, 10, or 20 μM) for 6 h, followed by the assessment of As[i] using ICP-MS. (B) Cell viability was determined by XTT assay after treatment with As^{III} ranging from 5 to 80 μM in the presence or absence of 3 or 10 μM PGP-4008 for 48 h. Data are shown as the mean and SD from three independent experiments.

Table 1-1

PCR primers and optimal numbers of PCR cycle

Target mRNA		DNA sequence of PCR primer	Optimal cycles
HO-1	sense	5′-CCAGCAACAAAGTGCAAGATTC -3′	27
	antisense	5′-CTGCAGGAACTGAGGATGCTG -3′	
AQP9	sense	5′-CTCAGTG TCATCATGTAGTG-3′	39
	antisense	5′-GACTATCGTCAAGATGCCG-3′	
ABCC1 (MRP1)	sense	5′-CTGACAAGCTAGACCATGAATGT-3	30
	antisense	5′-TCACACCAAGCCGGCGTCTTT-3′	
ABCC2 (MRP2)	sense	5′-CTTCGGAAATCCAAGATCCTGG-3′	39
	antisense	5′-AGGCAAGTC CAGCATCTCTGG-3′	
ABCB1 (P-gp)	sense	5′-ATATCAGCAGCCCACATCAT-3′	39
	antisense	5′-GAAGCACTGGGATGTCCGGT-3′	
β-actin	sense	5′-CCTTCCTGG GCATGGAGTCCTG-3′	21
	Antisense	5′-GGAGCAATGATCTTGATCTTC-3′	

Table 1-2

Conditions for PCR

Target mRNA	Denaturation reaction		Annealing reaction		Extension reaction	
	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)
HO-1	94	60	65	60	72	60
AQP9	94	30	60	30	72	40
ABCC1 (MRP1)	94	60	66	60	72	90
ABCC2 (MRP2)	94	60	68	60	72	90
ABCB1 (P-gp)	94	60	55	30	72	30
β-actin	94	60	50	60	72	60

Abbreviations: HO-1, hemeoxygenase-1; AQP9, aquaporin 9; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein.

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