Enhanced Expression of Multidrug resistance-associated Protein 2 and Reduced Expression of Aquaglyceroporin 3 in an Arsenic-resistant Human Cell Line

Te-Chang Lee1, I-Ching Ho1, Wen-Jen Lu3, and Jin-ding Huang5

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Arsenic-resistant cells (R15), derived from a human lung adeno-carcinoma cell line (CL3), were 10-fold more resistant to sodium arsenite (As(III)). Because R15 cells accumulated less arsenic than parental CL3 cells, this arsenic resistance may be due to higher efflux and/or lower uptake of As(III). We therefore compared expression of the multidrug resistance-associated proteins MRP1, MRP2, and MRP3 in these two cell lines. MRP2 expression was 5-fold higher in R15 cells than in CL3 cells, whereas MRP1 and MRP3 expression levels were similar. Furthermore, verapamil and cyclosporin A, inhibitors of multidrug resistance transporters, significantly reduced the efflux of arsenic from R15. Thus, increased arsenic extrusion by MRP2 may contribute to arsenic resistance in R15 cells. We also examined the expression of several aquaglyceroporins (AQPs), which mediate As(III) uptake by cells. Little AQP7 or AQP9 mRNA was detected by reverse transcription-PCR in either cell line, whereas AQP3 mRNA expression was 2-fold lower in R15 cells than in CL3 cells. When AQP3 expression in CL3 cells was knocked down by RNA interference, CL3 cells accumulated less arsenic and became more resistant to As(III). Conversely, overexpression of AQP3 in human embryonic kidney 293T cells increased arsenic accumulation, and the cells were more susceptible to As(III) than 293T cells transfected with vector alone. These results suggest that AQP3 is involved in As(III) accumulation. Taken together, our results suggest that enhanced expression of MRP2 and lower expression of AQP3 are responsible for lower arsenic accumulation in arsenic-resistant R15 cells.
AQP3 and Reduced AQP3 in Arsenic-resistant Cells

Increased Arsenic Extrusion in Arsenic-resistant R15 Cells—Arsenic-resistant R15 cells were derived by growing CL3 cells in medium while progressively increasing the As(III) concentration to 4 pairs and TaqMan probes for MRP1, MRP2, and MRP3 are summarized in Table 1.

One-step RNA PCR Analysis of AQP mRNAs—Total cellular RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH). AQP3, AQP7, and AQP9 mRNA levels were estimated using the GeneAmp Gold RNA PCR reagent kit (Applied Biosystems, Foster City, CA). The amplified products were visualized on a 2% agarose gel. The primers for AQP3, AQP7 and AQP9, listed in Table 1, were prepared according to those reported in Liu et al. (26). GAPDH mRNA was used as a loading control.

Western Blot Analysis—Expression of MRP1, MRP2, MRP3, and AQP3 proteins was determined by Western blotting (37). In brief, the proteins in an aliquot of cell lysate (10–25 μg of protein) were electro- phoretically separated on a sodium dodecyl sulfate-polyacrylamide gel (7 or 10% acrylamide) and then transferred onto a polyvinylidene difluoride membrane using a semidry electrotransfer system (ATTO, Tokyo Japan). The membrane was blocked in 5% bovine serum albumin in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% (v/v) Tween 20) for 1 h at room temperature and then incubated with primary antibody in 2.5% bovine serum albumin in TBS-T overnight at 4 °C. After five washes with TBS-T, the membrane was incubated with secondary antibody for 60 min followed by another five washes with TBS-T. Immunopositive bands were detected using ECL Western blotting detection reagents (Amersham Biosciences). α-Tubulin or β-actin was used as an internal control. Protein concentrations were determined by Bradford analysis using bovine serum albumin as the standard (38).

Overexpression of AQP3 in 293T Cells—pFLAG-CMV22-AQP3 was constructed by inserting the reverse transcription-PCR-amplified full-length human AQP3 cDNA (sequence verified) into the EcoRI site of pFLAG-CMV22 (obtained from Dr. W. Y. Hu, The Salk Institute, La Jolla, CA). The amplified products were visualized on a 2% agarose gel. Recombinant plasmid and AQP3 cDNA were electrotransfected into 293T cells using FuGENE 6 (Roche Diagnostics). In brief, 2 × 10^6 cells were plated in a 6-well plate 1 day prior to transfection. Plasmid DNA (1 μg) in FuGENE 6 reagent (3 μl) was incubated with 200 μg/ml G418 (Geneticin, Invitrogen) were selected by incubation in the presence of 200 μg/ml G418 for 3 weeks. The accumulation of As(III) and sensitivity of CL3 cells (transfected with AQP3 siRNA or vector alone) to As(III) for 6 h was determined using AAS and the colony-forming assay, respectively.

RESULTS

Increased Arsenic Extrusion in Arsenic-resistant R15 Cells—Arsenic-resistant R15 cells were derived by growing CL3 cells in medium while progressively increasing the As(III) concentration to 4

EXPERIMENTAL PROCEDURES

Materials—As(III), trivalent sodium arsenite, was obtained from Merck (Darmstadt, Germany). The media and chemicals used for cell culture were purchased from Invitrogen. Fetal bovine serum was obtained from HyClone Laboratories, Inc. (Logan, UT). VP was purchased from Sigma, and CSA was from Novartis (Basel, Switzerland).

Antibodies against AQP3, α- tubulin, β-actin, MRP1 (QCRL-3), MRP2 (H-300), and MRP3 (C-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines—All cells were grown in media supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO2, CL3, a human lung adenocarcinoma cell line, was grown in F12 medium (15). Arsenic-resistant R15 cells were maintained in F12 medium containing 4 μM As(III). HepG2 cells were grown in Earle’s minimum essential medium, and human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium.

Cytotoxicity Assay—The survival rate of cells after drug treatment was determined with a colony-forming assay (36). In brief, 2–3 × 10^5 CL3 or R15 cells were plated on a 35-mm dish 1 day prior to chemical treatment. The cultures were treated with various concentrations of tested compounds for 6 h. At the end of treatment, the cells were replated at 150 cells per 60-mm dish, in triplicate, and incubated in fresh medium supplemented with 0.1 μg/ml insulin and 5 μM hydrocortisone for 9 days. The medium was refreshed once at day 5. The colonies (>50 cells) were fixed, stained, and counted as described previously (36). Under certain circumstances, the cell viability immediate after treatment was determined by dye exclusion assay. In brief, the cell suspension was mixed with an equal volume of trypan blue stain solution (Invitrogen), and the viable cells were counted under a microscope using a hemocytometer.

Arsenic Determination—Cellular levels of arsenic were determined by atomic absorption spectrophotometry (AAS, Hitachi Z-8000, Tokyo) as described previously (36). In brief, cells were plated on a 100-mm dish and treated with As(III) using protocols described in the figure legends. After treatment, the cells were washed five times with phosphate-buffered saline containing 1 mM EDTA, trypsinized, digested with nitric acid, and subjected to arsenic determination by an atomic absorption spectrophotometer equipped with a hydride formation system.

Real-time Quantitative PCR Analysis of MRP mRNA—Total cellular RNA was extracted from CL3 and R15 cells using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. MRP1, MRP2, and MRP3 mRNA levels were determined by real-time quantitative PCR on the LightCycler (Roche Diagnostics, Mannheim, Germany) using TaqMan probes. Real-time PCR was performed in glass capillaries with an initial denaturation step of 10 min at 95 °C followed by 50 cycles of 95 °C for 10 s and 60 °C for 30 s in a total volume of 10 μl containing 1 μl of 10X reaction buffer, 150 nM TaqMan probe, 500 nM each primer, 3 mM MgCl2, and 2.5 μl of reverse transcription product. The mRNA levels of MRP1, MRP2, and MRP3 were quantified using LightCycler analysis software, version 3.5, and expressed as concentrations relative to GAPDH mRNA. The sequences of the primer

One-step RNA PCR Analysis of AQP mRNAs—Total cellular RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH). AQP3, AQP7, and AQP9 mRNA levels were estimated using the GeneAmp Gold RNA PCR reagent kit (Applied Biosystems, Foster City, CA). The amplified products were visualized on a 2% agarose gel. The primers for AQP3, AQP7 and AQP9, listed in Table 1, were prepared according to those reported in Liu et al. (26). GAPDH mRNA was used as a loading control.

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RESULTS

Increased Arsenic Extrusion in Arsenic-resistant R15 Cells—Arsenic-resistant R15 cells were derived by growing CL3 cells in medium while progressively increasing the As(III) concentration to 4
Enhanced MRP2 and Reduced AQP3 in Arsenic-resistant Cells

Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Reference</th>
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<tr>
<td>MRP1-forward</td>
<td>5'-TAC CTC CTG TGG CTT GAT CTG-3'</td>
<td>61</td>
</tr>
<tr>
<td>MRP1-TaqMan probe</td>
<td>5'-ATG GCG ATG AAG ACC AAG AC-3'</td>
<td>61</td>
</tr>
<tr>
<td>MRP2-forward</td>
<td>5'-TGC AGC CTC CAT AAC CAT-3'</td>
<td>62</td>
</tr>
<tr>
<td>MRP2-reverse</td>
<td>5'-GAT CTG TGG CTG CTG-3'</td>
<td>62</td>
</tr>
<tr>
<td>MRP3-forward</td>
<td>5'-CTT AAG ACT TTC CTT GGC-3'</td>
<td>61</td>
</tr>
<tr>
<td>MRP3-reverse</td>
<td>5'-GTC ACC ACC ACC ACC-3'</td>
<td>62</td>
</tr>
<tr>
<td>MRP4-forward</td>
<td>5'-TGG TGC GCC ATC ACC-3'</td>
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<tr>
<td>MRP4-reverse</td>
<td>5'-GGG GCC ATC-3'</td>
<td>62</td>
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<td>AQP3-forward</td>
<td>5'-TGG CTT GCC GTC-3'</td>
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</tr>
<tr>
<td>AQP3-reverse</td>
<td>5'-ATT GCC ACC-3'</td>
<td>62</td>
</tr>
<tr>
<td>AQP7-forward</td>
<td>5'-ATT GTP CCA GCC-3'</td>
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<td>5'-GGA-3'</td>
<td>62</td>
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<tr>
<td>AQP9-forward</td>
<td>5'-TCC ATT CAT GAT GAC ACT GAG-3'</td>
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<tr>
<td>AQP9-reverse</td>
<td>5'-GAC TCA-3'</td>
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FIGURE 1. Sensitivity of CL3 and R15 cells to As(III). Logarithmically growing CL3 and R15 cells were treated with various concentrations of As(III) for 6 h. Relative survival was analyzed by a colony-forming assay as described under “Experimental Procedures.” The average colony-forming efficiencies for untreated CL3 and R15 cells were 73.3 ± 13.4 and 56.4 ± 12.0, respectively. Bars, S.D. of three independent experiments.

The resistance of R15 cells was confirmed by a colony-forming assay (Fig. 1). Using a 6-h exposure, the IC50 values were estimated to be 10 and 100 μM for CL3 and R15 cells, respectively. To understand whether altered arsenic transport was involved in resistance in R15 cells, we used AAS to determine the intracellular accumulation of arsenic in CL3 and R15 cells. We first assessed the viability of the cells under the experimental uptake conditions. Immediately after treatment of CL3 and R15 cells with 20 μM As(III) for up to 8 h, the cell viability examined by a dye exclusion assay was 88.3 ± 4.5% and 97.7 ± 6.7%, respectively. The rate of arsenic accumulation in R15 cells was much slower than in CL3 cells after incubation with 20 μM As(III) for up to 8 h (Fig. 2A). In the parental CL3 cells, arsenic accumulation increased throughout the 8-h incubation period; in fact, arsenic accumulation did not reach a steady state even after 24 h (data not shown). In contrast, arsenic accumulation in R15 cells reached a steady state after 60 min and did not increase significantly thereafter (Fig. 2A). Next, we compared the rate of arsenic extrusion in CL3 and R15 cells after treatment with 100 μM As(III) for 30 min. As shown in Fig. 2B, the half-life of arsenic elimination in R15 cells was ~15 min, whereas the half-life in CL3 cells was ~45 min. These kinetic data suggest that the activity of an efflux transporter was enhanced in R15 cells. The dye exclusion assay confirmed that the cells maintained their integrity (>95%) after treatment with 100 μM As(III) for 30 min.

Overexpression of MRP2 in Arsenic-resistant R15 Cells—We evaluated the mRNA and protein levels of efflux transporters in CL3 and R15 cells using real-time quantitative PCR and Western blotting. Fig. 3A shows that the relative concentration of MRP2 mRNA was 5.1 ± 0.4-fold higher in R15 cells than in CL3 cells, whereas the relative MRP1 and MRP3 mRNA levels were similar, with ratios of 1.3 ± 0.1 and 1.0 ± 0.2, respectively. Western blot analysis confirmed the higher level of MRP2 protein in R15 cells as compared with CL3 cells (Fig. 3B). There was no significant difference in MRP1 and MRP3 protein levels between cell lines, consistent with their relative mRNA levels analyzed by real-time PCR. When the vinblastine-resistant leukemia cell line CEM and its parental counterpart CEM were used as the respective negative and positive controls, little P-glycoprotein (MDR1) expression was detected in either CL3 or R15 cells by either reverse transcription and PCR amplification or Western blotting (data not shown). To investigate the possible correlation between MRP2 overexpression and arsenic resistance in R15 cells, we treated CL3 and R15 cells with As(III) in the presence or absence of the MRP inhibitors VP and CSA and subsequently examined arsenic accumulation and cellular sensitivity. The addition of either VP or CSA significantly increased the sensitivity of R15, but not CL3 cells, to As(III) (Fig. 4, A and B). Furthermore, VP and CSA each increased arsenic accumulation in R15 cells in a dose-dependent manner but did not change the arsenic content of CL3 cells (Fig. 4, C and D). Similar to other reports (22, 39), our results suggest that MRP2 has a crucial role in eliminating arsenic from R15 cells.

Lower Arsenic Uptake Rate and Lower Expression Levels of AQP3 in R15 Cells—The lower arsenic accumulation in R15 cells during the first 60 min (Fig. 2A) may additionally imply the down-regulation of an influx transporter. We therefore examined the expression of several influx channels or transporters, AQP3, AQP7, and AQP9, in CL3 and R15 cells. Neither AQP7 nor AQP9 mRNA expression was detected in either CL3 or R15 cells by GeneAmp Gold RNA PCR (Fig. 5A). However, the AQP3 mRNA expression level in R15 cells was approximately half that of CL3 cells (Fig. 5B). Western blot analysis confirmed the lower protein levels of AQP3 in R15 cells (Fig. 5B).

To validate the importance of AQP3 in As(III) uptake, RNA interference was used to suppress AQP3 gene expression (40). Transfection of the AQP3 siRNA expression vector into CL3 cells decreased AQP3 expression by approximately half (Fig. 6A) and decreased both arsenic...
accumulation (Fig. 6) and sensitivity to As(III) (Fig. 6). The IC50 values for a 6-h exposure were 9.7 ± 2.9 and 20.3 ± 3.2 μM for CL3 transfected with control vector and AQP3 siRNA, respectively (p < 0.001 by Student’s t test). Furthermore, transfection of an expression vector encoding FLAG-tagged AQP3 into human embryonic kidney 293T cells increased expression of AQP3 (Fig. 7A) and enhanced the initial arsenic uptake following a 10-min incubation with As(III) (Fig. 7B). We also observed decreased As(III) resistance in AQP3-transfected 293T cells (Fig. 7C). The IC50 values for a 6-h exposure were 20.0 ± 3.2 and 10.0 ± 0.9 μM for 293T cells transfected with control vector and FLAG-AQP3, respectively (p < 0.001 by Student’s t test). These results imply that AQP3 is involved in As(III) uptake in CL3 cells. Real-time quantitative PCR and Western blotting revealed no difference in the expression of OATP-C, an arsenic transporter that mediates the uptake of GSH conjugates of arsenite, in CL3 and R15 cells (31) (data not shown).

**DISCUSSION**

Arsenic-resistant R15 cells, derived from the human lung adenocarcinoma cell line CL3, have several distinct characteristics (15). We previously demonstrated that HO-1 is up-regulated and cyclooxygenase-2 (COX-2) is down-regulated in R15 cells (15, 41). The lower level of COX-2 renders R15 cells more resistant to benzo(a)pyrene (41). The mechanism of arsenic resistance in R15 cells, however, remained unexplained despite the fact that these cells have been established for some
time. However, recent advances in our knowledge of arsenic transporters and channels enabled us to examine the mechanisms of arsenic resistance in this regard. By examining the kinetics of arsenic accumulation in CL3 and R15 cells, we demonstrated that the amount of arsenic accumulation during the first 60 min was apparently lower in R15 cells than in the parental CL3 cells (Fig. 2A). Furthermore, in R15 cells, the accumulated arsenic had a short half-life and quickly reached a lower steady state than in the parental CL3 cells, in which arsenic accumulation did not plateau for at least 24 h. These kinetic data imply that R15 cells exhibit fast extrusion and/or slow uptake of arsenic.

Our observation that the half-life for arsenic elimination in R15 and CL3 cells was 15 and 45 min, respectively (Fig. 2B), suggests that R15 cells extrude arsenic more efficiently than CL3 cells. We examined the expression levels of several possible arsenic efflux transporters, and our results showed that CL3 and R15 cells did not differ in their expression levels of MRP1 and MRP3; the level of MRP2, however, was 5-fold higher in R15 cells. As(III) up-regulates MRP2 gene expression via activation of the c-Jun NH$_2$-terminal kinase (JNK) pathway in primary rat and human hepatocytes (39). It is not clear, however, why As(III) specifically enhanced the expression of MRP2, but not MRP1 and MRP3, in R15 cells. The enhanced arsenic efflux in R15 cells is therefore likely due to the higher expression of MRP2, which pumps arsenite triglutathionate out of cells (22, 39). Arsenite triglutathionate is generally transported by MRP1 and MRP2 (19). As reported previously, R15 cells have higher levels of GSH than CL3 cells (15). VP and CSA, usually used to inhibit multidrug resistance transporters including P-glycoproteins and MRPs (42, 43), significantly inhibited arsenic extrusion and thus decreased arsenic resistance in R15 cells (Fig. 3). These results are consistent with our previous study showing that VP and CSA inhibit arsenic efflux in arsenic-resistant Chinese hamster ovary cells (16). The inhibitory effects of VP and CSA on arsenic efflux support the idea that MRP2-mediated arsenic extrusion is one of the important mechanisms of arsenic resistance in R15 cells.

Similarly, P-glycoprotein and MRPI levels have been found to be elevated in As(III)-treated cells (44). Numerous reports have shown an association between P-glycoprotein and MRPI expression and arsenic toxicity (20, 45–47). In contrast, no cross-resistance to As$_2$O$_3$ has been observed in human leukemia MOLT-4 cells (48) or HL60 cells (49) overexpressing P-glycoprotein. Takeshita et al. (44) demonstrated that arsenic-resistant NB4 cells, an acute promyelocytic leukemia cell line, express higher levels of P-glycoprotein and MRPI than the parental cells but have similar levels of arsenic accumulation. We found little P-glycoprotein in either CL3 or R15 cells and no difference in the amount of MRPI (Fig. 3). The increased susceptibility of R15 cells to VP and CSA is apparently not due to their inhibitory effects on P-glycoprotein or MRPI. However, we cannot rule out an indirect mechanism, for example, that an inhibitor-mediated decrease in GSH efflux via MRP1 may increase cellular GSH levels and thus facilitate efflux of arsenic-GSH complexes via MRP2. This putative mechanism
Enhanced MRP2 and Reduced AQP3 in Arsenic-resistant Cells

**A**

- **B** and MRP2 were 45.3

- AQP3- and pFLAG-CMV22-transfected cells were exposed to various concentrations of As(III) for 10 min. Initial arsenic (As(V)) uptake by MRPs (25). In this study, we observed a 10-fold increase in the IC50 in R15 cells as compared with CL3 cells. This change can be explained by a 2-fold decrease in uptake and a 5-fold increase in elimination. This study does not rule out mechanisms other than the role of AQP3 and MRP2, important factors that regulate arsenic levels in cells, could be modulated to enhance the efficacy of the arsenic trioxide regimen during cancer treatment.

**C**

- **REFERENCES**

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Three primer sequences shown in Table 1 were incorrect. The corrected sequences are shown below:

AQP3-forward: 5’-ATG GGT CGA CAG AAG GAG CTG-3’ (addition of GAG)
AQP9-forward: 5’-ATG CAG CCT GAG GGA GCA GAA AAG GG-3’ (with the first 3 residues deleted)
AQP9-reverse: 5’-CTA CAT GAT GAC ACT GAG TTC-3’ (with the first 3 residues deleted)
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