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In vivo Cisplatin Resistance Depending upon Canalicular Multispecific Organic Anion Transporter (cMOAT)

Tomoyoshi Minamino,[1] Mitsuo Tamai,[2] Yoshie Itoh,[2] Yasuaki Tatsumi,[2] Masaaki Nomura,[2] Koichi Yokogawa,[2] Hiroshi Suzuki,[3] Yuichi Sugiyama,[3] Tohru Ohshima[1] and Ken-ichi Miyamoto[4, 5]

[1]Department of Legal Medicine, Kanazawa University School of Medicine, [2]Department of Pharmacology and Pharmaceutics, Graduate School of Natural Science & Technology, [4]Hospital Pharmacy, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934 and [3]Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

The *in vitro* sensitivities to cisplatin of AH66 and AH66F cells, a variant obtained from AH66 cells, were very similar, when assayed in a medium containing 5% fetal bovine serum (FBS), whereas in the *in vivo* experiments AH66F cells were sensitive and AH66 cells were highly resistant to cisplatin. In this study, we examined the mechanism of the *in vivo* cisplatin resistance of AH66 cells. The *in vitro* cisplatin sensitivity of AH66 cells was lowered by changing FBS to 5% ascites fluid (ASF) in the assay medium and the sensitivity in FBS by treatment with buthioninesulfoximine (BSO). The sensitivity of AH66F cells was not changed by these treatments. Moreover, after culture in 5% ASF for 48 h, the accumulation of cisplatin in AH66 cells was decreased and the efflux of cisplatin from the cells was accelerated. The accumulation of cisplatin in AH66 cells in ASF was increased by pretreatment with BSO, sodium azide or probenecid. Then, we examined the expression of the glutathione (GSH) conjugate efflux pump family. Among them, only the expression of canalicular multispecific organic anion transporter (cMOAT) in AH66 cells was decreased by culture in FBS and enhanced by ASF. These results suggest that some substances contained in ASF enhanced the expression of cMOAT in the plasma membrane of AH66 cells and this transporter actively extruded cisplatin-GSH conjugate from the cells. Consequently, AH66 cells afford a cisplatin-resistant tumor in the host.

Key words: Rat ascites hepatoma — AH66 cells — Cisplatin — *In vivo* resistance — cMOAT

Many investigators have developed *in vitro* chemosensitivity systems to predict the response to chemotherapy of malignancies in human. Generally there is a satisfactory correlation between the *in vitro* sensitivity and the clinical responsiveness to anticancer agents. However, there are examples of low or nonresponders *in vivo* among tumor specimens, which exhibited high sensitivity to anticancer agents in *in vitro* assay. It is very important to clarify the cause of the discrepancy.

We have reported that the rat ascites hepatoma cell line AH66 acquires multiple-drug resistance on account of overexpression of P-glycoprotein and glutathione S-transferase (GST)-placental form and low activity of topoisomerase II,[1–5] compared with the drug-sensitive variant line AH66F. Moreover, though the *in vitro* sensitivities to cisplatin of both cell lines were similar, cisplatin hardly prolongs the life-span of AH66-bearing rats, while the agent can achieve almost complete cure of AH66F-bearing rats.[6] In that paper, we showed that AH66 cells responded to ascites fluid (ASF) and zinc ion, resulting in an increased content of metallothionein in the cytosol. However, the *in vivo* unresponsiveness of the tumor to cisplatin could not be explained simply in terms of detoxification by metallothionein. It has been reported that cisplatin resistance was related to an increase in GST activity,[7, 8] an increase in DNA-repair ability,[9, 10] and a decrease in intracellular accumulation,[11–14] as well as the increase of metallothionein contents in the cells.[15, 16] Cisplatin resistance due to a decrease in intracellular accumulation has recently been reported to be based on overexpression of the ATP-dependent glutathione (GSH) conjugate efflux pump (GS-X pump).[17–20] The GS-X pump family in rats has many members, such as multidrug resistance-associated protein (MRP),[21] canalicular multispecific organic anion transporter (cMOAT),[22–24] MRP-like protein (MLP)-1 and MLP-2,[25] which are homologues of human MRP1, MRP2, MRP6, and MRP3, respectively. It is possible that these transporters are related to the cisplatin resistance of tumor, but whether their expression is changed *in vivo* is unclear.

This study shows that the *in vivo* cisplatin resistance of AH66 cells is partially reproducible by culture in a medium containing ASF. The cisplatin accumulation in the

[5] To whom requests for reprints should be addressed.
E-mail: miyaken@kenroku.ipc.kanazawa-u.ac.jp

cells is decreased by cMOAT expressed in the plasma membrane after culture in ASF.

MATERIALS AND METHODS

Chemicals Cisplatin, verapamil, probenecid, and *dl*-buthionine-[*S, R*]-sulfoximine (BSO) were obtained from Sigma Chemical Co., St. Louis, MO. 5,5′-Dithio-bis(2-nitrobenzoic acid) and sodium azide were from Wako Pure Chemical Industries, Ltd., Osaka. Monochlorobimane was purchased from Molecular Probes, Eugene, OR.

Cell lines The AH66 cell line was induced by dimethylaminoazobenzene and established as a transplantable tumor in 1954 and the AH66F cell line was a variant spontaneously obtained during intraperitoneal passage of AH66 cells.[26] These cell lines were donated by the Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University. Cells were maintained by intraperitoneal passage in female Donryu rats weighing 100 to 150 g (Nippon SLC, Hamamatsu) and harvested from the tumor-bearing rats 1 week after tumor transplantation.

***In vitro* sensitivity assay** Cells (5×10^5/ml) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) for 24 h, and further cultured with or without cisplatin in culture medium containing 5% FBS or 5% ASF from AH66-bearing rats at 37°C for 48 h in a CO_2 incubator. Cells were counted under a microscope, and the effect of cisplatin was expressed as the 50% growth-inhibitory concentration (IC_{50}).

Measurement of cisplatin After treatment and washing with phosphate-buffered saline (PBS), cells were suspended in 60% nitric acid, warmed at 120°C for 3 h in an oil bath, dried at 150°C, and resuspended in 1 *N* hydrochloric acid. The platinum content was analyzed as platinum using a flameless atomic absorption spectrophotometer (Hitachi, Z-8000, Tokyo).

Measurement of sulfhydryl compounds The content of non-protein sulfhydryl compounds in the microsomal fraction of the cells was colorimetrically measured using 5,5′-dithio-bis(2-nitrobenzoic acid), according to the report of Palamanda and Kehrer.[27]

Measurement of GS-X pump activity Cells harvested from tumor-bearing rats were suspended in Hanks' solution containing 5.5 m*M* glucose at a density of 5×10^6 cells/ml and incubated with 20 μM monochlorobimane at 4°C or 37°C for a suitable period. To determine the extruded bimane-GSH conjugate, the fluorescence intensity (E_x 380 nm; E_m 460 nm) in the culture supernatant was measured using a fluorospectrophotometer (Hitachi, F-4500). Data are given as the fluorescence intensity after subtracting the value at 4°C from the value at 37°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR) Poly A^+ mRNA was isolated from AH66, AH66F cells and rat hepatocytes by using a Quick Prep micro mRNA Purification Kit (Pharmacia Biotech, Tokyo). Synthesis of cDNA from the isolated mRNA was carried out using RNase H^- reverse transcriptase (GIBCO BRL, Rockville, MD). The RT mixture was amplified by PCR using *Taq* DNA polymerase (GIBCO BRL) in the presence of 0.2 μM of sense and anti-sense primers. Primers used for rat MRP were 5′-ATC ATC TCT CAC CCT GGG TT-3′ and 5′-CAC TCA TGG TTC AGC TTG TC-3′ (270 bp),[21] those for rat cMOAT were 5′-ATC CTC AGC TGC TGA AGT TG-3′ and 5′-CTG ATC TTG GAT GCC AGA AC-3′ (439 bp),[22] those for rat MLP-1 were 5′-CAC CAG TGA TGA CCT TGG AG-3′ and 5′-CAC GCA GGG CTG AAT GCA TC-3′ (363 bp),[25] those for rat MLP-2 were 5′-TCA AAG AGG AGA TCG CAG AG-3′ and 5′-AGC ATG AGG ATG GTG GGG GCC AG-3′ (439 bp),[25] and those for rat β-actin were 5′-TTC TAC AAT GAG CTG CGT GTG GC-3′ and 5′-CTC (A/G)TA GCT CTT CTC CAG GGA GGA-3′(456 bp), as previously reported by Waki *et al*.[28]

Immunoblotting Anti-rat cMOAT polyclonal antibody was prepared by immunizing rabbits with a synthetic peptide corresponding to the 12-amino-acid sequence at the carboxyl terminus of rat cMOAT, as reported by Buchler *et al*.[23] The plasma membrane was prepared by the Percoll sedimentation method, as previously reported.[29] The protein concentration in membrane fractions was determined by the Lowry-Folin method using bovine serum albumin as a standard. The membrane protein (50 μg) was electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Dassel, Germany). After having been blocked with 5% skim milk, the membrane was incubated overnight with 1 μg/ml anti-rat cMOAT polyclonal antibody and for 1 h with biotinylated anti-rabbit IgG.

Table I. *In Vitro* Effects of ASF Cisplatin Sensitivities of AH Cells[a]

Cells		IC_{50} (μM)	
		5% FBS	5% ASF[b]
AH66F	−BSO	1.56±0.21	1.25±0.39 (1.42±0.54)[c]
	+BSO	1.46±0.32	1.32±0.44
AH66	−BSO	1.14±0.54	3.98±0.78* (3.14±0.43*)
	+BSO	1.09±0.34	1.19±0.42

a) Cells were cultured in the absence or presence of 100 μM BSO for 24 h, then the *in vitro* sensitivity assay was done in culture medium containing 5% FBS or 5% ASF. Each value is the mean±SE of at least three experiments.
b) From AH66-bearing rats.
c) Value in parenthesis is IC_{50} for cisplatin in medium containing 5% ASF from AH66F-bearing rats.
∗ Significantly different from the 5% FBS at $P<0.05$.

Thereafter, the membrane was extensively washed with PBS containing 0.1% Tween-20. The immunopositive band was detected by a light-emitting nonradioactive detection system (Amersham International pcl, Little Chalfont, Buckinghamshire, UK) and exposure to a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY).

Data analysis Experiments were done at least three times. Statistical analysis was done using Student's *t*-test and Welch's *t*-test.

RESULTS

Effects of ASF on cisplatin sensitivity Cells harvested from tumor-bearing rats were incubated in 5% FBS-con-

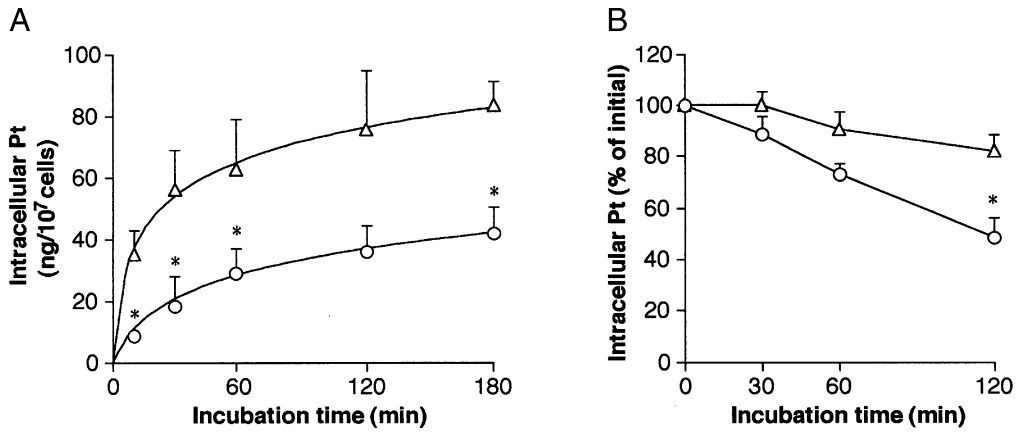

Fig. 1. Time-courses of accumulation and efflux of cisplatin in AH cells immediately after harvest from tumor-bearing rats. (A) Accumulation of cisplatin. Cells (5×10^6/ml) were incubated with 10 μM cisplatin in DMEM without FBS at 37°C. (B) Efflux of cisplatin. Cells (5×10^6/ml) were preloaded with 100 μM cisplatin in glucose-deprived Hanks' solution for 30 min and then incubated in DMEM without FBS at 37°C. * Significantly different from AH66F cells harvested from tumor-bearing rats at $P<0.05$. Data are the means±SE of at least three experiments. ○ AH66 cells, △ AH66F cells.

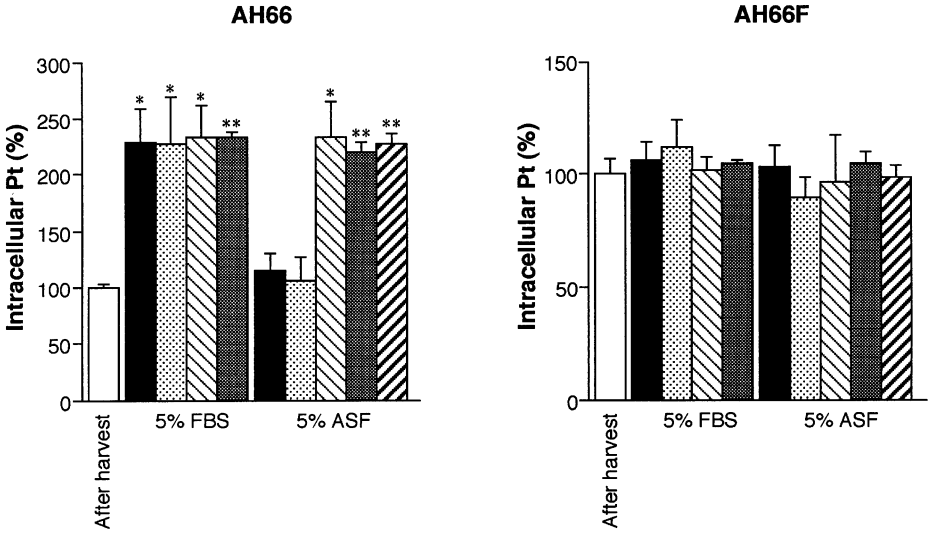

Fig. 2. Effects of inhibitors on cisplatin accumulation in AH cells. Harvested cells (5×10^6/ml) were incubated in 5% FBS-containing medium for 48 h (5% FBS) and further incubated in 5% ASF-containing medium at 37°C for 48 h (5% ASF). Cells were treated without or with 30 μM verapamil, 10 mM sodium azide, or 1 mM probenecid for 1 h or 100 μM BSO for 24 h, then incubated in DMEM containing 100 μM cisplatin without FBS and ASF for 1 h. Data are the means±SE of % accumulation (cells after harvest as 100%) in at least three experiments. *, ** Significantly different from AH66 cells after harvest from tumor-bearing rats at $P<0.05$ and 0.005, respectively. □ after harvest from tumor-bearing rats, ■ without agents, ▩ verapamil, ▨ sodium azide, ▩ BSO, ▨ probenecid.

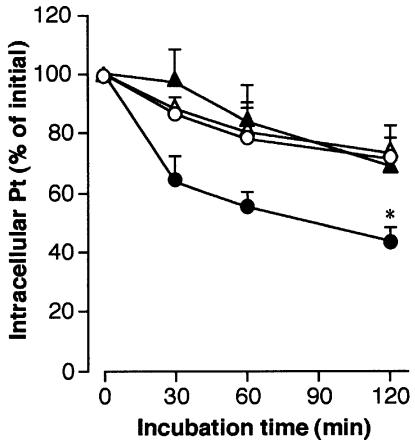

Fig. 3. Courses of cisplatin efflux from AH cells after incubation in 5% FBS or 5% ASF. Cells (5×10^6/ml) were incubated in 5% FBS or 5% ASF for 48 h and incubated with 100 μM cisplatin in glucose-deprived Hanks' solution for 1 h, then incubated in fresh DMEM without FBS and ASF at 37°C. Data are the means±SE of at least three experiments. * Significantly different from AH66 cells cultured in 5% FBS at $P<0.05$. ○ AH66 cells incubated in 5% FBS, ● AH66 cells incubated in 5% ASF, △ AH66F cells incubated in 5% FBS, ▲ AH66F cells incubated in 5% ASF.

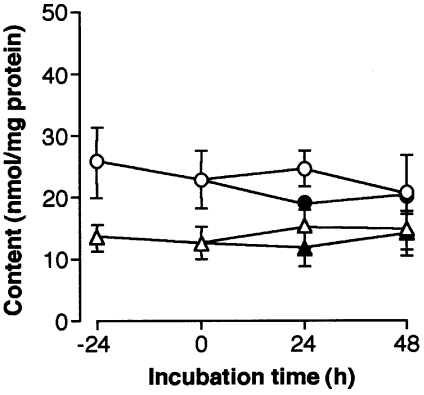

Fig. 4. Changes in the contents of sulfhydryl compounds in AH cells during incubation in 5% FBS or 5% ASF. Cells (3×10^7/ml) were incubated in DMEM with 5% FBS for 24 h, then incubated in DMEM with 5% FBS or 5% ASF for 48 h. Data are the means±SE of at least three experiments. ○ AH66 cells incubated in 5% FBS, ● AH66 cells incubated in 5% ASF, △ AH66F cells incubated in 5% FBS, ▲ AH66F cells incubated in 5% ASF.

taining medium for 24 h, then the cisplatin sensitivity assay was done in 5% FBS or 5% ASF, and the results are shown in Table I. The IC_{50} value of cisplatin for AH66 cells was significantly lowered by changing FBS to ASF (obtained from AH66-bearing rats) in the culture medium,

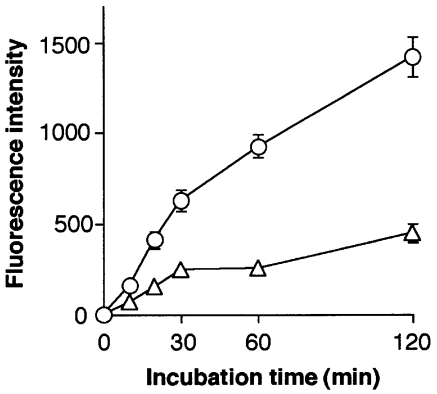

Fig. 5. Courses of bimane-GSH-conjugate efflux from AH cells. Cells (5×10^6/ml) were incubated with 20 μM monochlorobimane in Hanks' solution containing 5.5 mM glucose, and the fluorescence intensity of bimane-GSH conjugate in the medium was measured at the indicated times. Data are the means±SE of at least three experiments. ○ AH66 cells, △ AH66F cells.

but the sensitivity of AH66F cells was not changed. The lowered cisplatin sensitivity of AH66 cells was also observed even with ASF from AH66F-bearing rats.

Table I also indicates the effects of treatment with BSO on the cisplatin sensitivities. BSO did not affect the IC_{50} values for both cell lines in FBS, but completely reversed the IC_{50} value for only AH66 cells in ASF to the level in FBS.

Accumulation of cisplatin Fig. 1A shows the time courses of accumulation of cisplatin in the cells, just after harvest from tumor-bearing rats. AH66 cells accumulated much less cisplatin than AH66F cells. The cisplatin uptake of the cells in 1 h was measured after incubation in the medium containing FBS and ASF (Fig. 2). The cisplatin uptake of AH66 cells was increased after incubation in 5% FBS for 48 h, but on further incubation in 5% ASF for 48 h the uptake of AH66 cells returned to the level after harvest from the rats (before *in vitro* incubation). Fig. 2 also shows that the cisplatin uptake of AH66 cells after incubation in ASF was significantly increased by prior treatment with BSO, sodium azide, and probenecid, but not verapamil. On the other hand, in AH66F cells the cisplatin uptake was not changed after incubation in FBS and ASF or even on treatment with verapamil, BSO, sodium azide, or probenecid.

Efflux of cisplatin When the efflux assay was done in the cells just after harvest, AH66 cells extruded more cisplatin than AH66F cells (Fig. 1B). After incubation in 5% FBS for 48 h, the efflux rate of cisplatin in AH66 cells was slower, but was accelerated by further incubation in 5% ASF for 48 h. The efflux rate in AH66F cells was not changed after *in vitro* incubation (Fig. 3).

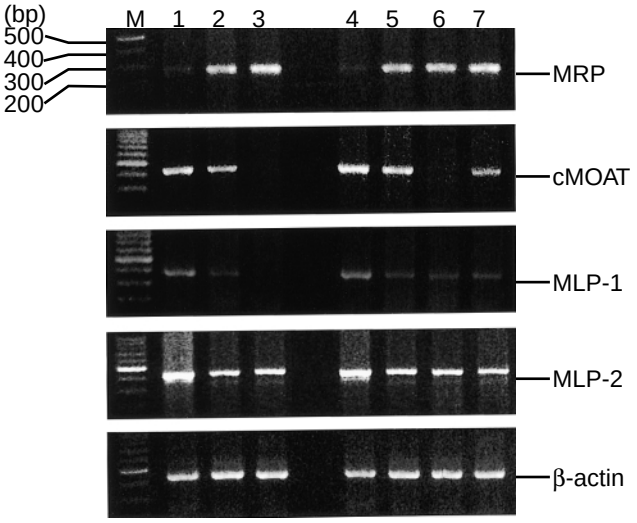

Fig. 6. Expression of GS-X pump superfamily members in AH cells. Lanes 1, 4, rat hepatocytes; lanes 2, 5, AH66 cells harvested from tumor-bearing rats; lane 3, AH66F cells harvested from tumor-bearing rats; lane 6, AH66 cells incubated in 5% FBS for 48 h; lane 7, AH66 cells harvested from tumor-bearing rats, which were inoculated with the cells after incubation in 5% FBS for 48 h.

Intracellular content of non-protein sulfhydryl compounds The content of non-protein sulfhydryl compounds in AH66 cells was about 2-fold higher than that in AH66F cells, and was unchanged after incubation in medium containing FBS or ASF (Fig. 4).

GS-X pump activity The fluorescence intensity of bimane-GSH conjugate in the culture medium of AH66 cells rapidly increased up to 120 min, but that in AH66F culture was very slow (Fig. 5). This indicated that the GS-X pump activity of AH66 cells was much higher than that of AH66F cells.

Expression of GS-X pump superfamily members The expression of MRP, cMOAT, MLP-1 and MLP-2 mRNAs in AH cells and rat hepatocytes, as a reference control, was measured by an RT-PCR method (Fig. 6). Hepatocytes expressed mRNAs of all these transporters. After harvest from tumor-bearing rats, AH66 cells also expressed these transporter mRNAs, and AH66F cells expressed only MRP and MLP-2 mRNAs. The expression of cMOAT mRNA among these transporters in AH66 cells was markedly decreased after incubation in 5% FBS-containing medium for 48 h and recovered after re-inoculation of the cells into rats. The intrinsic expression of cMOAT protein in the plasma membrane of AH66 cells was also observed by western blotting analysis (Fig. 7). This protein almost disappeared in the membrane after culture in FBS for 48 h, but when the cultured cells were re-inoculated into rats, the protein expression appeared again.

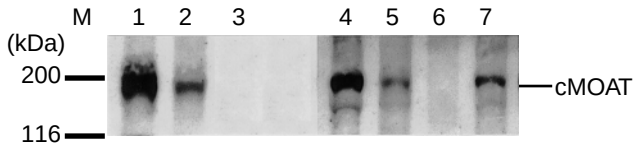

Fig. 7. Western blot of cMOAT protein in the plasma membrane of AH cells. Lanes 1, 4, rat hepatocytes; lanes 2, 5, AH66 cells harvested from tumor-bearing rats; lane 3, AH66F cells harvested from tumor-bearing rats; lane 6, AH66 cells incubated in 5% FBS for 48 h; lane 7, AH66 cells harvested from tumor-bearing rats, which were inoculated with the cells after incubation in 5% FBS for 48 h.

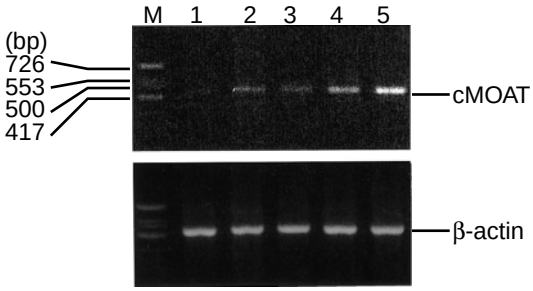

Fig. 8. Effect of ASF on the expression of cMOAT mRNA in AH66 cells. Cells were incubated in 5% FBS for 48 h (lane 1), 5% ASF (lane 2), 10% ASF (lane 3), or 20% ASF for 48 h (lane 4). Lane 5, AH66 cells from tumor-bearing rats.

Fig. 8 shows the expression of cMOAT mRNA as a function of ASF. The decreased expression of cMOAT mRNA in AH66 cells after incubation in FBS for 48 h recovered on incubation in ASF-containing medium for 48 h (intensity ratio of cMOAT vs. β-actin by NIH image; 0.07 in FBS, 0.25 in 5% ASF, 0.28 in 10% ASF, 0.37 in 20% ASF, 0.64 just after harvest from rat). Namely, the cMOAT expression in AH66 cells was decreased to about one-ninth during incubation in FBS and was increased over 3- to 5-fold by ASF in a concentration-dependent manner.

DISCUSSION

We previously found that AH66-bearing rats were unresponsive to treatment with cisplatin, but AH66F-bearing rats were sensitive to the antitumor drug.[6] In this study, the *in vitro* sensitivities to cisplatin of AH66 and AH66F cells were similar in a medium containing FBS, but when assayed in ASF, the sensitivity to cisplatin of AH66 cells was significantly lower than that of AH66F cells. Moreover, the cisplatin sensitivity of AH66 cells in ASF recov-

ered to the level in FBS on treatment of the cells with BSO, an inhibitor of GSH biosynthesis.[30] Cisplatin is conjugated with GSH either by GST or non-enzymatically in the cells. We have previously reported that the GSH content and GST activity in AH66 cells are much higher than in AH66F cells.[4] However, since the cisplatin sensitivities in FBS were similar in both cell lines, even after treatment with BSO (Table I), and the cellular contents of non-protein sulfhydryl compounds were not changed by incubation in FBS or ASF (Fig. 4), it may be difficult to explain the difference of cisplatin sensitivities of AH cells only in terms of the contents of non-protein sulfhydryl compounds or GSH.

This study indicated that AH66 cells expressed MRP, cMOAT, MLP-1, and MLP-2 mRNAs and AH66F cells expressed MRP and MLP-2 mRNAs, when examined immediately after harvest from tumor-bearing rats (Fig. 6). Then, we examined the GS-X pump activity of the cells using monochlorobimane, which is conjugated with GSH in the cells and extruded by MRP and cMOAT,[31, 32] and found that AH66 cells extruded bimane-GSH conjugate much more efficiently than AH66F cells (Fig. 5). This suggests that the difference of GS-X pump activities was based on the cMOAT function. Similarly, AH66 cells accumulated much less cisplatin and more rapidly extruded cisplatin than did AH66F cells (Fig. 1). The activity of AH66 cells to accumulate cisplatin was markedly increased after incubation in FBS, but this was reversed by incubation in ASF-containing medium. The decrease of cisplatin accumulation in AH66 cells after incubation in ASF was reversed by treatment with BSO, sodium azide, an energy metabolic inhibitor,[33] and probenecid, an inhibitor of the organic anion transporter.[34] However, the activity of AH66F cells was not influenced by these treatments and agents (Fig. 2). These results suggest that cisplatin as well as bimane was conjugated with GSH, then actively extruded by a GS-X pump such as cMOAT. Many investigators have reported, based on *in vitro* experiments, that cMOAT is closely related to the cisplatin resistance of tumor cells.[18–20] However, we observed that the expression of cMOAT mRNA and its protein in AH66 cells was markedly decreased by *in vitro* incubation in FBS (Figs. 6 and 7), and could be restored by addition of ASF instead of FBS into the culture (Fig. 8). The expression of transporters in AH66F cells was not changed by incubation in FBS and ASF (data not shown). On the other hand, although AH66 cells and AH66F cells expressed other anion transporters, such as MRP and MLP even after incubation in FBS, the cisplatin accumulation was never increased by sodium azide or probenecid (Fig. 2). This suggests that MRP and MLP proteins have less ability to transport cisplatin-GSH conjugate than cMOAT. These results strongly suggest that cMOAT in AH66 cells is inducible by ASF and is closely related to the *in vivo* cisplatin resistance of AH66 cells.

There are a few reports on inducible transporters of GSH-conjugates. It has recently been reported that cMOAT (MRP2) expression was increased by cisplatin, 2-acetylaminofluorene, phenobarbital, and ethinyl estradiol.[35, 36] In this study, AH66 cells responded to ASF, without cisplatin, and expressed cMOAT. Identification of the inducer of cMOAT in ASF is the next step.

AH66 cells, but not AH66F cells, have been shown to overexpress P-glycoprotein, an efflux pump of cationic and hydrophobic substances, in the plasma membrane and to exhibit multiple drug resistance.[2–4] In this study, the cisplatin accumulation in AH66 cells was not influenced by verapamil, an inhibitor of P-glycoprotein[37] (Fig. 2), and P-glycoprotein expression was unchanged after incubation in FBS and ASF (data not shown). Therefore, P-glycoprotein may not mediate the *in vivo* cisplatin resistance of AH66 cells.

In conclusion, we clarified in this study that the *in vivo* cisplatin resistance of AH66 cells is dependent upon cMOAT, a membrane efflux pump of cisplatin-GSH conjugate, inducible by ASF. Because this type of resistance, which is undetectable by the *in vitro* sensitivity test, is likely to be present in low-responsive tumors to cisplatin in the clinic, further studies are warranted.

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