Enhanced transport of anticancer agents and leukotriene C_4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2)

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Abstract **We established stable human canalicular multispecific organic anion transporter (*cMOAT*/*MRP2*) cDNA transfectants, CHO/cMOAT from non-polarized Chinese hamster ovary (CHO)-K1 and LLC/cMOAT from polarized pig kidney epithelial LLC-PK1. Human cMOAT was mainly localized in the plasma membrane of CHO/cMOAT and in the apical membrane of LLC/cMOAT. The ATP-dependent uptake of leukotriene C_4 (LTC_4) into CHO/cMOAT membrane vesicles was enhanced compared with empty vector transfectants. K_m values in CHO/cMOAT membrane vesicles were 0.24 μM for LTC_4 and 175 μM for ATP. Drug sensitivity to vincristine and cisplatin in human *cMOAT* cDNA transfectants decreased, but not to etoposide. Cellular accumulation of vincristine and cisplatin in human *cMOAT* cDNA transfectants decreased, but not of etoposide. The uptake of LTC_4 into CHO/cMOAT membrane vesicles was inhibited by exogenous administration of vincristine or cisplatin, but not that of etoposide. Moreover, this inhibition was more enhanced in the presence of glutathione. These consequences indicate that drug resistance to vincristine or cisplatin appears to be modulated by human cMOAT through transport of the agents, possibly in direct or indirect association with glutathione.**

Key words: cMOAT; MRP family; ABC transporter; Multidrug resistance; Anticancer agent

1. Introduction

Both P-glycoprotein (Pgp) and multidrug resistance protein 1 (MRP1) belong to the ATP binding cassette (ABC) transporter superfamily, and mediate multidrug resistance through altered membrane transport of various anticancer agents in cancer cells [1–4]. Human canalicular multispecific organic anion transporter (cMOAT/MRP2) also belongs to the ABC transporter superfamily and shows a 49% amino acid identity to human MRP1 [5,6]. Rat [7] and rabbit [8] cMOAT transport a GSH conjugate, leukotriene C_4 (LTC_4), which is a high affinity endogenous substrate for MRP1 [9–12]. This structural and functional similarity between cMOAT and MRP1 suggests that overexpression of cMOAT may confer multidrug resistance. Human cMOAT is substantially overexpressed in several cell lines, and human cMOAT levels correlate with cisplatin resistance in a subset of resistant cell lines [5,6]. A potent anticancer agent, cisplatin, which forms glutathione (GSH) conjugates, is predicted to be effluxed through the GS-X pump [13,14]. In our previous study, introduction of the human *cMOAT* antisense cDNA into human hepatic cancer HepG2 cells overexpressing human cMOAT results in increased sensitivity to cisplatin and vincristine, but not to etoposide [15]. In the human *cMOAT* antisense cDNA transfectants, cellular accumulation of cisplatin and vincristine increases, but cellular accumulation of etoposide does not change [15]. Transport of another anticancer agent, vinblastine, is also altered in polarized Madin-Darby canine kidney cells transfected with human *cMOAT* cDNA [16]. However, whether human cMOAT itself is involved in the membrane transport of any specific anticancer agent like MRP1 or Pgp remains unclear.

In this study, we established stable human *cMOAT* cDNA transfectants from non-polarized Chinese hamster ovary (CHO)-K1 and polarized LLC-PK1 cells, and examined if the drug sensitivity and cellular accumulation are altered in the transfectants. We further examined if human cMOAT transports LTC_4 which is a substrate for MRP1 [9–12] by using the membrane vesicles from human *cMOAT* cDNA transfectants, and if human cMOAT modulates the membrane transport of anticancer agents in association with GSH.

2. Materials and methods

2.1. Drugs and chemicals

Cisplatin was donated by Bristol-Myers Squibb K.K. (Kanagawa, Japan). Vincristine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Etoposide was obtained from Nippon Kayaku Co. (Tokyo, Japan). Lipofectamine and geneticin (G418) were purchased from Life Technologies, Inc. (Bethesda, MD, USA). [14,15,19,20-^{3}H(N)]-LTC_4 was obtained from DuPont NEN (Boston, MA, USA).

2.2. Construction of human cMOAT expression vector

Previously described cDNA overlapping clones (L3, AL28-33, 4-1, 1-1, AK1-8) [5] were reconstructed into a full-length cDNA, which was inserted into the *Not*I site of the pCI-neo mammalian expression vector (Promega).

2.3. Cell culture and transfection with human cMOAT expression vector

CHO-K1 and LLC-PK1 cells were cultured in Eagle's MEM (Nissui, Tokyo) containing 10% newborn calf serum and Medium 199 (Nissui, Tokyo) containing 10% fetal bovine serum, respectively. Transfection of human cMOAT expression vector was performed using lipofectamine according to the manufacturer's protocol. The cells were incubated in a selection medium containing 800 μg/ml G418 for 3–4 weeks. The stable transfectants expressing human cMOAT were selected using Western blot analysis among G418-resistant clones.

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2.4. Production of human cMOAT antibody

Antibody against human cMOAT was elicited by synthetic peptide DYGLISSVEEIPEDAAS that corresponded to the unique amino acid sequence at the linker region of human cMOAT (amino acids 884–900). The amino acid sequence of the linker region was unique when we compared it with the amino acid sequences of the corresponding regions of MRP1 and MRP3-MRP6. The human cMOAT antibody was affinity purified with the glutathione S-transferase human cMOAT fusion proteins. This antibody did not cross-react with Pgp or MRP1 [15]. Double immunofluorescence using this antibody and anti-DPPIV antibody, and confocal laser scanning microscopy, showed localization of human cMOAT in the canalicular membrane domain of normal human hepatocytes but not in patients with Dubin-Johnson syndrome [17].

2.5. Western blot analysis and indirect immunofluorescence

An immunoblot analysis of human cMOAT was made as described by Koike et al. [15]. Aliquots of whole cell lysates (200 μg) were electrophoresed on 6% SDS-polyacrylamide gels and were analyzed by immunoblotting with human cMOAT antibody. Protein-antibody interaction was detected using the enhanced chemiluminescence technique.

Indirect immunofluorescence analysis was performed as described by Koike et al. [18].

2.6. Membrane vesicle preparation and [^{3}H]LTC_4 uptake in membrane vesicles

Membrane vesicles were prepared by the nitrogen cavitation method as described by Cornwell et al. [19] from CHO/CMV and CHO/cMOAT-1 cells.

LTC_4 uptake in membrane vesicles was measured by a rapid filtration technique essentially as described by Ishikawa et al. [13,20]. Briefly, the standard incubation medium contained membrane vesicles (50 μg of protein), 1.368 nM [^{3}H]LTC_4, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 4 mM ATP, 10 mM phosphocreatine and 100 μg/ml creatine phosphokinase in a final volume of 50 μl. The reaction was carried out at 37°C. The samples were passed through Millipore filters (GVWP, 0.22 μm pore size) under a light vacuum to measure the trapped [^{3}H]LTC_4 in the vesicles. The filters were washed and placed in scintillation fluid. The level of radioactivity was measured using a liquid scintillation counter. ATP-dependent accumulation was calculated from the difference in the radioactivity incorporated into the vesicles in the presence or absence of 4 mM ATP.

2.7. Chemosensitivity and cellular accumulation of vincristine, cisplatin and etoposide

A colony formation assay was made as described by Koike et al. [15]. The 50% lethal dose (IC_{50}) for each cell line was calculated from the dose-response curve. The relative resistance of human *cMOAT* cDNA transfectants was determined by dividing the IC_{50} value for each transfectant by that of the empty vector transfectant. The accumulation of etoposide, vincristine and cisplatin was determined as described by Koike et al. [15].

3. Results

We established cell lines overexpressing human cMOAT by introducing the human *cMOAT* expression vector into non-polarized CHO-K1 and polarized LLC-PK1 cells. Western blot analysis with human cMOAT antibody showed the presence of human cMOAT of molecular weight 190–200 kDa in the human hepatic cancer HepG2 cells. Human *cMOAT* cDNA transfectants (CHO/cMOAT-1, 2 and LLC/cMOAT-1, 2 cells) expressed human cMOAT of molecular weight of 180–190 kDa, while cells transfected with empty vector alone (CHO/CMV and LLC/CMV cells) and parental cells (CHO-K1 and LLC-PK1 cells) did not express human cMOAT (Fig. 1).

The cellular distribution of human cMOAT was determined by indirect immunofluorescence with the human cMOAT antibody (Fig. 2). The photomicrographs of the horizontal

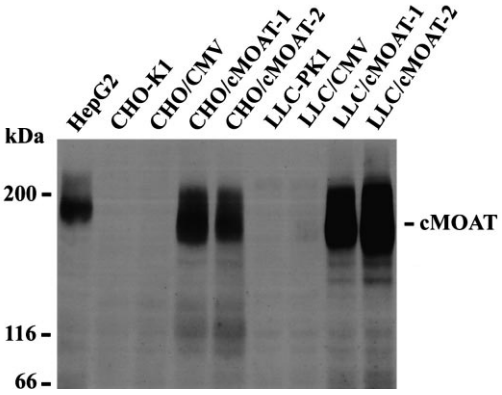

Fig. 1. Expression of human cMOAT in human *cMOAT* cDNA-transfected CHO-K1 and LLC-PK1 cells by Western blot analysis.

section of CHO/CMV (Fig. 2A), CHO/cMOAT-1 (Fig. 2B) and CHO/cMOAT-2 (Fig. 2C) cells show that the human cMOAT expression was observed in human *cMOAT* cDNA transfectants, and that human cMOAT was mainly localized in the plasma membrane. The photomicrographs of the top views of LLC/CMV (Fig. 2D), LLC/cMOAT-1 (Fig. 2E) and LLC/cMOAT-2 (Fig. 2F) cells show that the human cMOAT expression was also observed in human *cMOAT* cDNA transfectants. The photomicrograph of the vertical section of LLC/cMOAT-2 cell layer shows that the human cMOAT expression was mainly observed in the apical plasma membrane (Fig. 2G).

By using the membrane vesicles of human *cMOAT* cDNA transfectants, we examined if human cMOAT also transports LTC_4 like MRP1. Fig. 3A shows the time course of [^{3}H]LTC_4 uptake into membrane vesicles from CHO/CMV and CHO/cMOAT-1 cells in the absence or presence of ATP. We found a dramatic difference in LTC_4 uptake between CHO/CMV and CHO/cMOAT-1 vesicles when incubated in the presence of ATP. By contrast, any apparent LTC_4 uptake could not be observed between CHO/CMV and CHO/cMOAT-1 vesicles in the absence of ATP. The uptake was linear with incubation

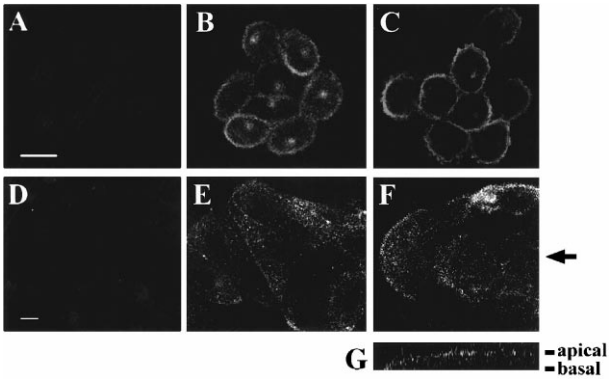

Fig. 2. Immunolocalization of human cMOAT in human *cMOAT* cDNA-transfected CHO-K1 and LLC-PK1 monolayers by confocal laser scanning microscopy. The panels of CHO/CMV (A), CHO/cMOAT-1 (B) and CHO/cMOAT-2 (C) show horizontal sections of the cell layers. The panels of LLC/CMV (D), LLC/cMOAT-1(E), LLC/cMOAT-2 (F) show the top views of the monolayers. The vertical section (G) of the cell layer indicated by arrow in panel F shows appearance of human cMOAT in the apical plasma membrane. The positions of apical and basal plasma membrane are indicated on the right of panel G. Bar = 25 μm.

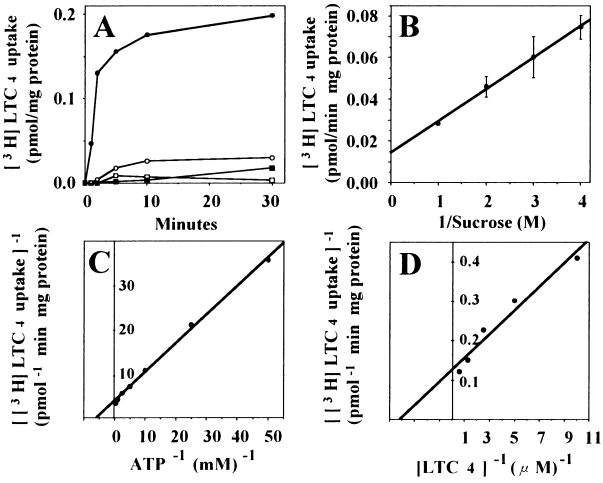

Fig. 3. Transport studies of [^{3}H]LTC$_4$ by membrane vesicles. A: Time course of [^{3}H]LTC$_4$ accumulation in membrane vesicles from CHO/CMV and CHO/cMOAT-1 cells in the presence or absence of 4 mM ATP. CHO/CMV−ATP (□), CHO/CMV+ATP (■), CHO/cMOAT-1−ATP (○) and CHO/cMOAT-1+ATP (●). B: Effect of osmolarity on the ATP-dependent [^{3}H]LTC$_4$ accumulation in membrane vesicles from CHO/cMOAT-1 cells (●). The [^{3}H]LTC$_4$ accumulation was measured at increasing concentrations of sucrose (0.25–1 M) in the presence or absence of 4 mM ATP for 2 min. C: Effect of ATP concentration on the rate of [^{3}H]LTC$_4$ uptake in membrane vesicles from CHO/cMOAT-1 cells (●). The [^{3}H]LTC$_4$ uptake was measured at various concentrations of ATP for 2 min. D: Effect of LTC$_4$ concentration on the rate of [^{3}H]LTC$_4$ uptake in membrane vesicles. Membrane vesicles (25 μg of protein) from CHO/cMOAT-1 cells (●) were incubated at various concentrations of LTC$_4$ in the presence or absence of 4 mM ATP for 2 min. A, C, D: Each point represents the average of two separate experiments. B: Each point represents the average of three separate experiments; bars, S.E.

time for 2 min (Fig. 3A). To confirm that [^{3}H]LTC$_4$ uptake reflects the transport of substrate into the vesicle lumen rather than the surface or intramembrane binding, we analyzed the osmotic sensitivity of [^{3}H]LTC$_4$ uptake. High osmotic pressure reduces the intravesicular space by shrinking the membrane vesicles, resulting in reduced uptake capacity. Osmotic sensitivity was examined by analyzing ATP-dependent [^{3}H]LTC$_4$ uptake by CHO/cMOAT-1 vesicles incubated in various concentrations of sucrose. The amounts of [^{3}H]LTC$_4$ accumulated in CHO/cMOAT-1 vesicles decreased proportionally to the increasing osmolarity of the extravesicular medium, indicating that a significant amount of [^{3}H]LTC$_4$ was actually transported into the intravesicular lumen of the CHO/cMOAT-1 vesicles (Fig. 3B). We further examined the ATP concentration dependence of [^{3}H]LTC$_4$ uptake. Lineweaver-Burk analysis indicated that [^{3}H]LTC$_4$ uptake was saturable with respect to ATP concentration. Kinetic analysis revealed that the K_m value for ATP in CHO/cMOAT-1 vesicles was 175 μM (Fig. 3C). The LTC$_4$ concentration dependence of [^{3}H]LTC$_4$ uptake was analyzed. Lineweaver-Burk analysis indicated that [^{3}H]LTC$_4$ uptake was saturable with respect to LTC$_4$ concentration. Kinetic analysis revealed that the K_m value for LTC$_4$ in CHO/cMOAT-1 vesicles was 0.24 μM (Fig. 3D).

We examined if the drug sensitivity to vincristine, cisplatin and etoposide is altered in human *cMOAT* cDNA transfectants. Dose-response curves were assayed by colony formation assay in the presence of various doses of each drug. CHO/cMOAT-1, 2 cells showed about 2-fold higher resistance to vincristine and to cisplatin, respectively (Fig. 4A and B) than CHO/CMV cells. LLC/cMOAT-1, 2 cells showed about 7-fold higher resistance to vincristine (Fig. 4D) and about 2-fold higher resistance to cisplatin (Fig. 4E) than LLC/CMV cells. However, no change appeared in the drug sensitivity to etoposide between empty vector transfectants and human *cMOAT* cDNA transfectants (Fig. 4C and F).

We examined if human cMOAT changes cellular accumulation of vincristine, cisplatin and etoposide. Cellular accumulation of [^{3}H]vincristine in both human *cMOAT* cDNA-transfected CHO-K1 (Fig. 5A) and LLC-PK1 (Fig. 5B) cells decreased greatly compared with empty vector transfectants. Cellular accumulation of cisplatin in both human *cMOAT* cDNA transfectants also decreased significantly compared with empty vector transfectants. The decreased cellular levels of [^{3}H]vincristine and cisplatin in both human *cMOAT* cDNA transfectants were about 50% and 80%, respectively, compared with those in empty vector transfectants. However, no

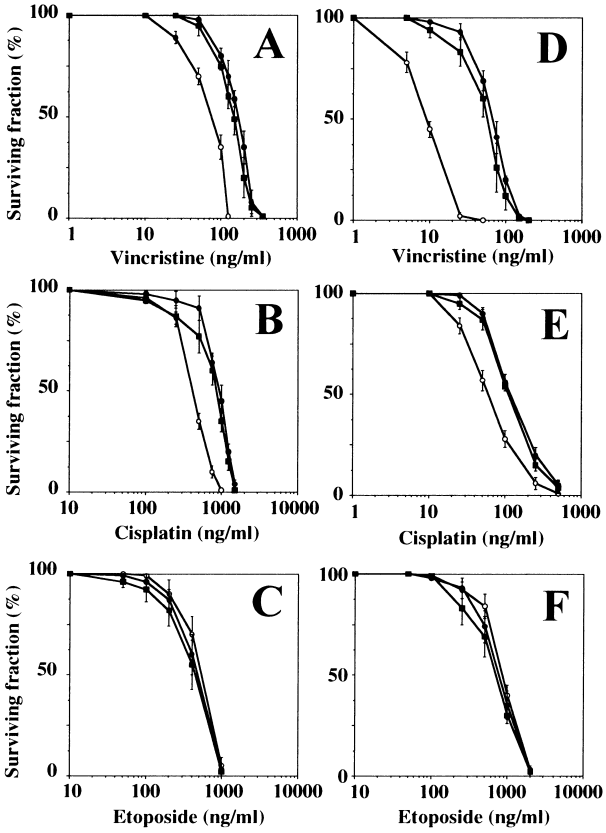

Fig. 4. Comparison of dose-response curves to vincristine (1–350 ng/ml), cisplatin (1–1500 ng/ml) and etoposide (10–2000 ng/ml) in human *cMOAT* cDNA transfectants and empty vector transfectants in CHO-K1 (A, B, C) and LLC-PK1 (D, E, F) cells. IC$_{50}$ values (ng/ml) for vincristine were 78 ± 7 (CHO/CMV) and 9 ± 1 (LLC/CMV); those for cisplatin were 425 ± 5 (CHO/CMV) and 62 ± 4 (LLC/CMV); and those for etoposide were 580 ± 8 (CHO/CMV) and 880 ± 10 (LLC/CMV). IC$_{50}$ values were almost the same between empty vector transfectants and parental cells (data not shown). Vincristine (A, D); cisplatin (B, E); and etoposide (C, F). CHO/CMV and LLC/CMV (○), CHO/cMOAT-1 and LLC/cMOAT-1 (●), CHO/cMOAT-2 and LLC/cMOAT-2 (■). 100% corresponds to the number of colonies in the absence of any drug. Each point indicates the average of three separate experiments; bars, S.D.

change appeared in the cellular accumulation of [^{3}H]etoposide between empty vector transfectants and human *cMOAT* cDNA transfectants (Fig. 5A and B).

Loe et al. [13] have reported that exogenous addition of vincristine and vinblastine which are substrates for MRP1 inhibit the MRP1 mediated ATP-dependent transport of LTC_4, and that the inhibition is significantly enhanced by physiological concentration of GSH. We then examined if human cMOAT modulates the membrane transport of anticancer agents in association with GSH. We examined the effect of exogenous addition of vincristine, cisplatin and etoposide on the human cMOAT mediated ATP-dependent LTC_4 uptake in the absence (Fig. 6A) or presence (Fig. 6B) of 3 mM GSH. The administration of vincristine and cisplatin at 100 μM significantly blocked the ATP-dependent uptake of LTC_4 in the absence or presence of GSH. The administration of vincristine and cisplatin at 10 μM also significantly ($P < 0.05$) blocked the ATP-dependent uptake of LTC_4 in the presence of GSH (Fig. 6B). In contrast, there appeared no such inhibition of the ATP-dependent uptake of LTC_4 by exogenous addition of etoposide at 10 and 100 μM even in the presence of GSH.

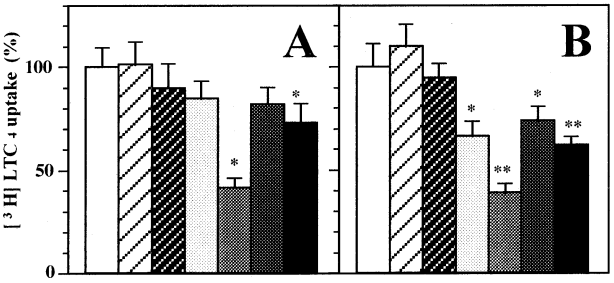

Fig. 6. Effect of vincristine, cisplatin and etoposide on ATP-dependent [^{3}H]LTC_4 uptake in the absence (A) or presence (B) of 3 mM GSH. Membrane vesicles from CHO/cMOAT-1 cells were incubated with three different anticancer agents at 10 or 100 μM for 2 min. Control (□); etoposide (10 μM) (light dashed box); etoposide (100 μM) (dark dashed box); vincristine (10 μM) (light gray box); vincristine (100 μM); cisplatin (10 μM) (dark gray box); cisplatin (100 μM) (■). 100% corresponds to ATP-dependent [^{3}H]LTC_4 uptake in each control. Bars represent means (±S.D.) of triplicate determinations. *, $P < 0.05$ compared with control. **, $P < 0.01$ compared with control.

4. Discussion

In this study we isolated transfected cell lines expressing human cMOAT predominantly on the plasma membrane. The size of the transfected human cMOAT protein is smaller than the endogenous human cMOAT protein in HepG2 cells. This difference may correspond to a different extent of glycosylation because of a species difference.

Our study revealed that human cMOAT had a high affinity K_m value of 0.24 μM for LTC_4. Human MRP1 also transports LTC_4 and had K_m values of 0.035 μM to 0.105 μM for LTC_4 [9,11,12,21]. The K_m values for LTC_4 of human cMOAT appear to be about 2-fold to 7-fold higher than those of human MRP1. Rat cMOAT shows K_m values of 0.25–0.32 μM for LTC_4 [22,23], which appear to be comparable to that of human cMOAT. Thus, the transport of LTC_4 was also modulated by human cMOAT like as MRP1.

This study indicated that human *cMOAT* cDNA transfectants showed about 2-fold to 7-fold higher resistance to vincristine and about 2-fold higher resistance to cisplatin, but a similar sensitivity to etoposide compared with empty vector transfectants. The growth rate is the same between human *cMOAT* cDNA transfectants and empty vector transfectants (data not shown) and is not likely contributing to the change of resistance observed. In this study we also observed that cellular accumulation of vincristine and cisplatin in human *cMOAT* cDNA transfectants decreased, but not of etoposide. Moreover, the cMOAT mediated ATP-dependent LTC_4 uptake by membrane vesicles was inhibited by exogenous addition of vincristine and cisplatin, but not that of etoposide. Although human cMOAT is reported not to be expressed on plasma membrane in non-polarized cells transfected with human *cMOAT* cDNA [16], our data clearly show that human cMOAT is expressed on plasma membrane in non-polarized CHO-K1 as well as polarized LLC-PK1 cells transfected with human *cMOAT* cDNA. Forced expression of human cMOAT on plasma membrane is expected to enhance export of drugs through plasma membrane, resulting in reduction of cellular level of drugs. These consequences suggest that vincristine and cisplatin show some affinity to human cMOAT, and that human cMOAT confers drug resistance to vincristine and cisplatin by the export of them. Thus, human cMOAT might be involved in the membrane transport of some anticancer agents and confer drug resistance similar to MRP1, but with some differences. Drug sensitivity to vincristine and etoposide is markedly altered, but not for cisplatin in *MRP1* cDNA-transfected cells or *mrp*(−/−) cell line [3,4,24]. The transport of etoposide might be rather specifically mediated through MRP1 than through human cMOAT. During the preparation of this report, Cui et al. have reported that forced expression of human cMOAT confers drug resistance to cisplatin and vincristine [25], consistent with our present study. However, their human *cMOAT* cDNA transfectants are also resistant to etoposide [25]. It remains unclear why drug sensitivity to etoposide is altered in their human *cMOAT* cDNA transfectants, unlike ours. Further study should be required to examine if any difference in experimental assay system, cell lines employed, and also cellular distribution of human cMOAT is responsible for the discrepancy.

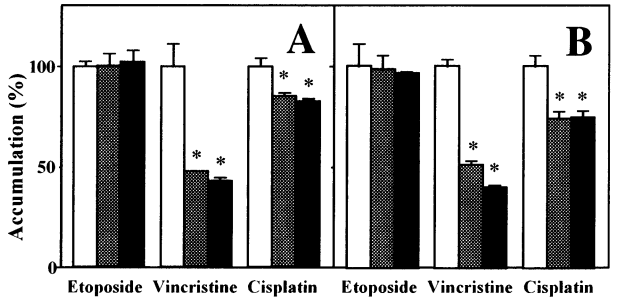

Fig. 5. Comparison of cellular accumulation of [^{3}H]etoposide, [^{3}H]vincristine, and cisplatin in CHO/cMOAT transfectants (A) and LLC/cMOAT transfectants (B). A: CHO/CMV (□), CHO/cMOAT-1 (gray box) and CHO/cMOAT-2 (■) cells. B: LLC/CMV (□), LLC/cMOAT-1 (gray box) and LLC/cMOAT-2 (■) cells. All results were normalized to cellular protein. 100% corresponds to the accumulation of each drug in each empty vector transfectant. Each value represents the average of three separate experiments; bars, S.D. *, $P < 0.01$ compared with CHO/CMV cells or LLC/CMV cells.

The human cMOAT mediated LTC_4 uptake by membrane vesicles was more greatly inhibited by each vincristine or cisplatin in the presence of GSH than in the absence of GSH. Loe et al. [12] have reported that GSH significantly enhances the ability of vincristine and vinblastine to inhibit MRP1 mediated ATP-dependent transport of LTC_4, suggesting that either efflux of certain drugs requires activation of MRP1 by GSH or a co-transport mechanism with GSH is involved in the MRP1-dependent drug transport [26]. Such co-transport mechanism might be also partly operated in the human cMOAT-dependent transport of vincristine or cisplatin with GSH.

In conclusion, human cMOAT, a family of MRP, shows a high affinity to LTC_4 like MRP1. Drug resistance to vincristine or cisplatin appears to be modulated by human cMOAT through transport of the agents, possibly in direct or indirect association with GSH.

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