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Characterization of the ATP-Dependent LTC_4 Transporter in Cisplatin-Resistant Human KB Cells

Yutaka Chuman,*,† Zhe-Sheng Chen,* Tomoyuki Sumizawa,* Tatsuhiko Furukawa,* Misako Haraguchi,* Yuji Takebayashi,† Kiyoshi Niwa,† Kazutaka Yamada,† Takashi Aikou,† and Shin-ichi Akiyama*

**The Institute for Cancer Research and †The First Department of Surgery, Faculty of Medicine, Kagoshima University, Kagoshima, Japan*

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An active efflux pump for *cis*-diamminedichloroplatinum(II) (cisplatin) has been identified in cisplatin-resistant KCP-4 cells isolated from human epidermoid carcinoma KB-3-1 cells. The adenosine triphosphate(ATP)-dependent transport of leukotriene C_4 (LTC_4), an endogenous substrate for the glutathione S-conjugate export pump(GS-X pump), has been found in membrane vesicles prepared from KCP-4 cells. Multidrug resistance-associated protein (MRP) has also been identified as an ATP-dependent LTC_4 transporter. To examine whether the GS-X pump expressed in KCP-4 cells is MRP, we investigated the expression of MRP in KCP-4 cells and compared the LTC_4 transporting activity of GS-X pump expressed in KCP-4 cells with that of MRP. The level of *MRP* gene expression in KCP-4 cells was low and similar to that in KB-3-1 cells. MRP was not detected in membrane vesicles prepared from KB-3-1 and KCP-4 cells by immunoblot analysis. The ATP-dependent transport of LTC_4 in KCP-4 and C-A120 vesicles showed saturable kinetics with an apparent Km of 0.18 μM and 0.25 μM, respectively. [^{3}H]LTC_4 transport in KCP-4 vesicles was more inhibited by 2,4-dinitrophenyl-S-glutathione(DNP-SG), bis-(glutathionato)-platinum(II) (GS-platinum) complex and glutathione disulfide(GS-SG) and less by LTD_4 compared with that in C-A120 vesicles. The character of the LTC_4 transporter expressed in KCP-4 vesicles is similar but not identical to that of MRP. Our results suggest that a glutathione S-conjugate export pump which is different from MRP exists in cisplatin-resistant KCP-4 cells.

The drug *cis*-Diamminedichloroplatinum(II) (cisplatin) is a widely used antitumor compound and it is highly effective against several malignancies, such as testicular, ovarian and small cell lung cancers(1). However intrinsic or acquired resistance to cisplatin reduces efficiency of treatment(2). To analyze the mechanisms of cisplatin-resistance, we isolated cisplatin-resistant KCP-4 cells from KB human cancer cells and showed that an active efflux pump for cisplatin is expressed in KCP-4 cells(3). Ishikawa and Ali-Osman have reported the adenosine triphosphate (ATP)-dependent transport of the bis-(glutathionato)-platinum(II) (GS-platinum) complex in plasma membrane vesicles prepared from mouse L1210 leukemia cells(4). Their data suggested that the ATP-dependent glutathione S-conjugate export pump (GS-X pump) plays a role in eliminating the GS-platinum complex from tumor cells. However, the molecular identity of the GS-X pump has not been established. We also found that cisplatin-resistant KCP-4 cells had an ATP-dependent transporter for leukotriene C_4(LTC_4), a substrate for the GS-X pump(5). The LTC_4 transport was inhibited by GS-platinum complex, but not significant by cisplatin or glutathione(GSH). These results suggested that the GS-X pump is involved in the decreased accumulation of cisplatin in KCP-4 cells.

The complementary DNA (cDNA) for the 190 kDa multidrug resistance(MDR)-associated protein (MRP) has been cloned from a multidrug resistant, doxorubicin-selected human lung cancer cell line, H69AR, which does not overexpress P-glycoprotein(P-gp)(6). MRP is a member of the ATP-binding cassette transmembrane transporter superfamily and transfection of full-length *MRP* cDNA into drug sensitive human cancer cells results in MDR(7,8). MRP

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seems to confer MDR by transporting anticancer agents outside the cells. LTC_4 is transported in the presence of ATP in membrane vesicles from cells expressing MRP(9-11). Since the transport of anticancer agents by MRP has not been proven, this phenomenon should help clarify the mechanisms for MRP-mediated MDR. Meanwhile the LTC_4 transporter in the membrane vesicles prepared from cisplatin-resistant KCP-4 cells may be identical to MRP. We isolated non-P-glycoprotein mediated MDR C-A120 cells from KB-3-1 cells(12), and found that the *MRP* gene is overexpressed in the MDR cells. To investigate whether the LTC_4 transport in the membrane vesicles prepared from cisplatin-resistant KCP-4 cells resulted from MRP, we examined MRP expression in KCP-4 cells and compared the activity of LTC_4 transport in membrane vesicles prepared from KCP-4 cells with those from C-A120 cells.

MATERIALS AND METHODS

Cell culture and cell lines. Human epidermoid carcinoma KB-3-1 cells, and multidrug resistant KB-C2 cells which overexpress *MDR1* gene were obtained from Dr. M. M. Gottesman (National Cancer Institute, Bethesda, MD). The KB-3-1 cells were cultured in minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) with 1 mg/ml of bacto-peptone(Difco), 0.292 mg/ml of glutamine and penicillin (100 u/ml), supplemented with 10% newborn calf serum (Flow Laboratories, Inc., McLean, VA). KB-C2 cells were maintained in the same medium containing 2 μg/ml of colchicine.

Cisplatin-resistant KCP-4 cells were isolated by culturing the KB-3-1 cells with increasing concentrations of cisplatin following ethyl methanesulfonate (EMS) induced mutagenesis, then incubated in selection medium with 7 μg/ml cisplatin(3). Multidrug resistant C-A120 cells, which overexpressed *MRP* mRNA, were isolated from KB-3-1 cells and maintained in the medium with 1μg/ml cepharanthine, 100 nM mezerein and 120 ng/ml doxorubicin, as described(12). C-AR, a complete revertant, was isolated by culturing C-A500 cells in the medium without ADM, cepharanthine and mezerein for 6 months(12). The KCP-4 cells were 25 times more resistant to cisplatin than the parental KB-3-1 cells. The C-A120 cells were 139-fold and 10-fold and KB-C2 cells were 258 and 3500-fold more resistant to vincristine and doxorubicin than KB-3-1 cells respectively, as described(3,12).

Slot blot analysis. Total cellular RNA was extracted from the cells in a single step using ISOGEN(Nippon Gene Co., Tokyo, Japan) and polyadenylated RNA was purified from the total RNA using the DYNABEADS mRNA Purification Kit (Dynal A.S., Oslo, Norway). Nylon membranes were moistened with 10×SSC(1×SSC=0.15 M Nacl plus 15mM sodium citrate, pH 7.0) before use and 0.3, 0.1, 0.03 and 0.01 μg of polyadenylated RNAs from the cells were applied to a slot blotter (Hybri-Slot TM manifold; Bethesda Research Laboratories, Gaithersburg, Md) under vacuum. The membranes were dried at room temperature then cross-linked by UV irradiation in a stratalinker (STRATAGENE, La Jolla, Calif.). Hybridization was performed using a DIG-labeled riboprobe prepared using a DIG-RNA labeling kit(Boehringer). The 1kb EcoRI fragment of MRP cDNA, was provided by Drs. Susan Cole and Roger Deely (Queen's University, Kingston, Ontario, Canada). The blot was detected using alkaline-phosphatase labeled anti-DIG antibody and a chemiluminescent substrate according to the manufacturer's instruction.

Membrane vesicle preparation. Membrane vesicles were prepared by nitrogen cavitation as described(13) from KB-3-1, KCP-4, C-A120 cells and KB-C2 cells grown in 24 × 24 cm dishes (Nunc) under standard growth conditions (14). Cell monolayers(10^9cells) were washed once and scraped into phosphate buffered saline (PBS) containing 1% aprotinin (Sigma). The cells were washed by centrifugation (4000 × g, 10 min) in PBS, then in buffer A (10m M Tris-HCl, pH 7.5, 0.25 M sucrose, 0.2 mM $CaCl_2$) and equilibrated at 4 ℃ under a nitrogen pressure of 25 kg/cm^2 for 15 min. EDTA was added to the lysed cell suspension to a final concentration of 1 mM. The lysed cell suspension was then diluted 1/4 with buffer B (10m M Tris-HCl, pH 7.5, and 0.25 M sucrose), and centrifuged at 1000 × g for 10 min. The supernatant was layered onto a 35% sucrose cushion (10m M Tris-HCl, pH 7.5, 35 % sucrose, 1 mM EDTA) and centrifuged for 30 min at 16,000 × g. The interface was collected and diluted 1/5 in buffer B, then centrifuged for 45 min at 100,000 × g. The vesicle pellet was resuspended in buffer B using a 25 gauge needle. Vesicles were stored at −70 ℃ prior to use. The vesicle preparations were enriched 3- to 4-fold in Na^+ K^+-ATPase and contained about 10% of the total cellular protein. The percent protein yield was equivarent for the parental and drug resistant cell lines. Na^+ K^+-ATPase activity in the vesicles was measured as described by Dickson et al.(15). Sialidase accessibility for the determination of inside-out vesicles was examined as described(16). The percentages of inside-out membrane vesicles were 48.4, 49.8, 50.6, 51.4, and 45.9% for KB-3-1, CA-R, KCP-4, C-A120 and KB-C2 vesicles, respectively.

Immunoblotting. To detect MRP, we prepared a polyclonal antibody against a synthetic peptide with the sequence KEDTSEQVVPVLVKN, selected from a region of MRP (amino acids 246-260)(17). A monoclonal antibody against Chinese hamster P-gp (C219), which was originally isolated by Kartner et al.(18) was obtained from Centocor (Malvern, Pennsylvania). Membrane vesicles (2 or 5 μg of protein) were mixed with an equal volume of SDS sample buffer consisting of 125 mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol and 0.005 % bromophenol blue, resolved by

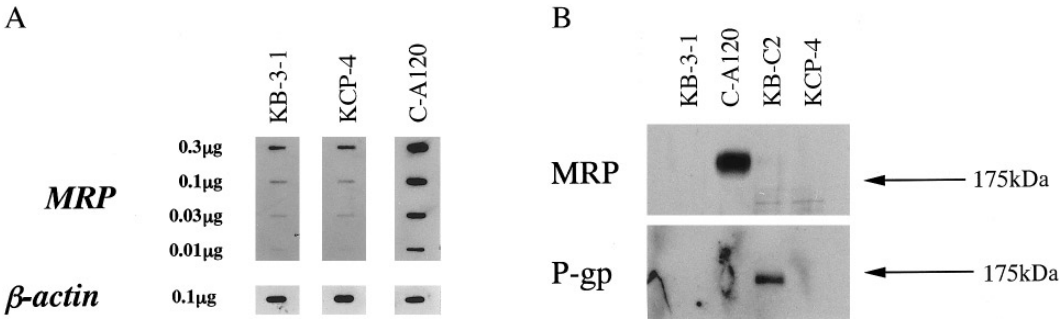

FIG. 1. A. Slot blot analysis of *MRP* mRNA extracted from KB-3-1, KCP-4 and C-A120 cells. Polyadenylated RNA(0.3, 0.1, 0.03 and 0.01μg) was applied to wells and hybridized with the *MRP* probe as described in ''Materials and Methods''. Hybridization with β-actin probe was performed to normalize the amount of RNA loaded. **B.** Immunoblots for MRP in KB-3-1, KCP-4, C-A120 and KB-C2 cells. Membrane vesicles (2μg of protein for MRP and 5μg of protein for P-gp) were resolved by SDS-PAGE and transferred to a PVDF membrane. The transferred proteins were immunoblotted with the anti-MRP polyclonal antibody or the monoclonal antibody against Chinese hamster P-gp (C219) as described in ''Materials and Methods''.

electrophoresis on SDS 7.5%(w/v) polyacrylamide minigels and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) using a Transblot SD apparatus(Bio-Rad, Richmond, CA). Thereafter, the membranes were blocked with 3% skim milk in Buffer A (0.35 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Tween 20) for 1 h at room temperature, followed by a 4 h incubation with C219 at a concentration of 1 μg/ml or with 2000-fold diluted polyclonal antibody against MRP in Buffer A containing 3% skim milk. The membrane was washed three times with Buffer A, then incubated for 1 h with 1000-fold diluted anti-mouse Ig whole antibody labeled with HRP (Amersham, UK) or HRP-conjugated anti-rabbit immunoglobulin for the detection of P-gp or MRP, respectively. PVDF membranes were rinsed once for 15 min and 4 times for 5 min with buffer A, then evenly coated using the ECL Western blotting detection system (Amersham, UK) for 1 min. The membrane was immediately exposed to Kodak X-OMAT AR film at room temperature for various periods in a film cassette.

[^3H] *LTC_4 uptake in membrane vesicles.* LTC_4 uptake in the vesicles was measured by filtration essentially as described by Ishikawa et al.(16,19). The standard incubation medium contained membrane vesicles (50 μg of protein), 1.34 nM [^{3}H]LTC_4 , 0.25 M sucrose, 10 mM Tris-HCl, pH7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, and 100 μg/ml creatine kinase in a final volume of 100 μl. The reaction that proceeded at 37°C was stopped with 1 ml of ice cold stop solution (0.25 M sucrose, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.4). The diluted samples were passed through Millipore filters (GVWP, 0.22 μm pore size) under a light vacuum. The filters were washed with 4 ml of ice cold stop solution, then oven-dried at 50 °C for 10 min. Each filter was placed in scintillation fluid and the level of radioactivity was measured by liquid scintillation counting. ATP-dependent uptake was obtained from the difference in the radioactivities incorporated into the vesicles in the presence and absence of ATP.

Chemicals. [14,15,19,20-^{3}H(N)]-LTC_4(150 Ci/mmol) was obtained from DuPont NEN. Cisplatin was a gift from the Bristol-Myers Research Laboratory. Other drugs and chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Statistical analysis. Statistical analysis was performed by using Student's *t* test. Significance was attributed to $P<0.05$.

RESULTS

Expression of MRP and P-gp

We investigated the expression of *MRP* mRNA in KB-3-1, KCP-4 and C-A120 cells by means of slot blotting(Fig. 1 A). *MRP* mRNA was overexpressed in C-A120 cells compared with that in KB-3-1 cells as described (12). Although *MRP* mRNA was detected in KCP-4 cells, the level was similar to that expressed in KB-3-1 cells. The 190kDa MRP was detected in the membrane vesicles prepared from C-A120 cells but not in those from parental KB-3-1 and cisplatin-resistant KCP-4 cells by immunoblotting analysis(Fig. B). P-gp was detected in KB-C2 , but not in other cells.

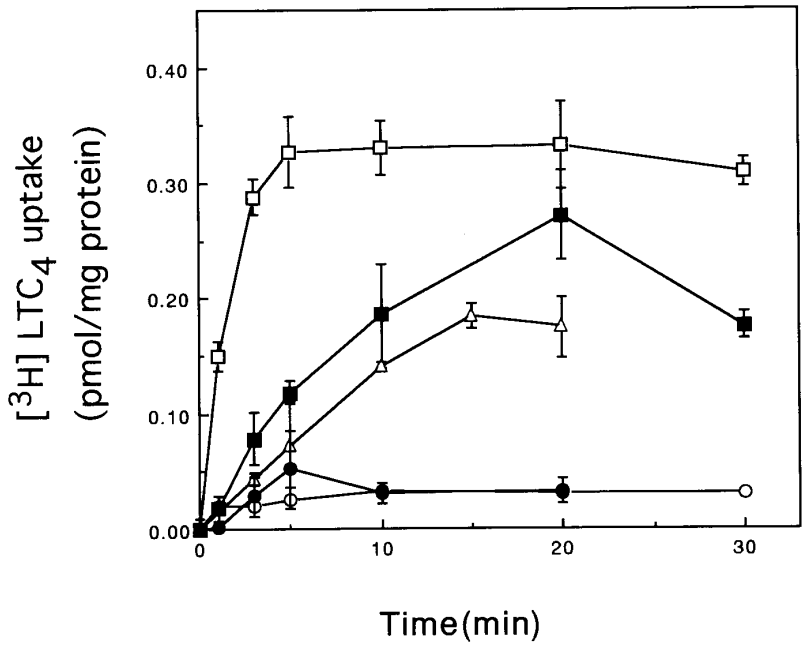

FIG. 2. Time course of[^{3}H]LTC_4 accumulation in membrane vesicles. Membrane vesicles (100 μg of protein) prepared from KB-3-1 (○), C-AR (●), KCP-4 (■), C-A120 (□) and KB-C2 (△) cells were incubated at 37°C in 200 μl transport buffer (0.01 M Tris-HCl, pH7.5, 0.25 M Sucrose, 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase) containing 1.34 nM [^{3}H] LTC_4 in the presence or absence of 1 mM ATP, and ATP-dependent [^{3}H]LTC_4 transport in each membrane vesicles was determined as described in ''Materials and Methods''. At the indicated times, aliquots of 20 μl were removed from the assay mixture and diluted to 1 ml with ice cold stop solution (0.25 M sucrose, 100 mM NaCl, 10 mM Tris-HCl, pH7.5). Vesicles were collected and washed by filtration and the radioactivity levels on the dried filters was measured by liquid scintillation counting. Data are expressed as means±SD (n=3).

[^{3}*H*] *LTC_4 Uptake in Membrane Vesicles*

Figure 2 shows the time course of ATP-dependent [^{3}H]LTC_4 uptake in membrane vesicles prepared from KB-3-1, KCP-4, C-A120 and KB-C2 cells. We calculated the ATP-dependent uptake by subtracting the vesicle-associated radioactivity in the absence of ATP from that in the presence of 1mM ATP. The ATP-dependent uptake of [^{3}H]LTC_4 in C-A120 vesicles occured rapidly and the vesicle-associated amount reached a plateau in 5 min. The uptake in KCP-4 and KB-C2 vesicles was slower than that in C-A120 vesicles and reached a peak at 15 and 20 min, respectively. In contrast, only a small amount of [^{3}H]LTC_4 was taken up in KB-3-1 and C-AR vesicles. At 20 min incubation, the amount of [^{3}H]LTC_4 uptaken by KCP-4, C-A120 and KB-C2 vesicles was 9 ,12 and 5 fold higher than that by KB-3-1 vesicles, respectively.

The Effect of Osmolarity on the [^{3}H]LTC_4 Uptake

To determine whether the differences in [^{3}H]LTC_4 uptake among the membrane vesicles from the four cell lines were due to increased binding to or increased transport into the vesicles, we examined the osmotic sensitivity of the ATP-dependent [^{3}H]LTC_4 uptake in the vesicles(Fig. 3). The amounts of [^{3}H]LTC_4 that accumulated in C-A120 and KCP-4 vesicles decreased proportionally to the increase in osmolarity of the extravesicular medium, indicating that a considerable amount of [^{3}H]LTC_4 was actually transported into the intravesicular space of the

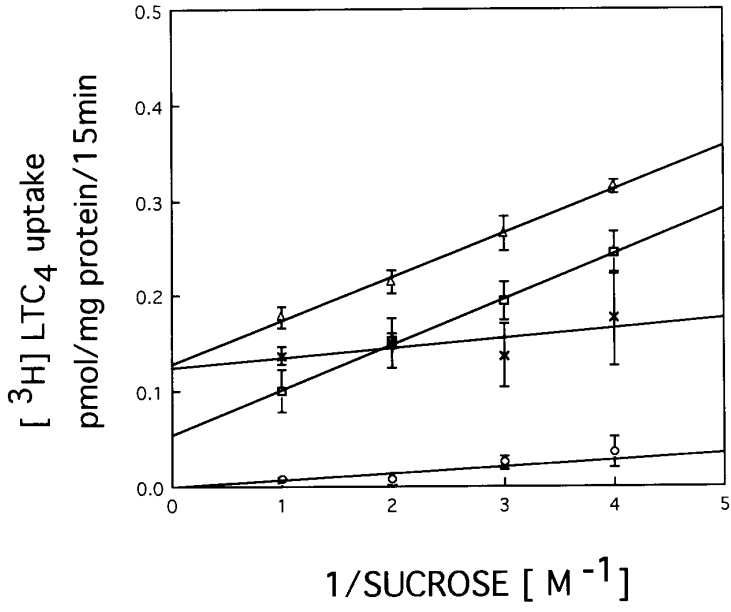

FIG. 3. Effect of osmolarity on the ATP-dependent [^{3}H]LTC_4 accumulation in membrane vesicles. Membrane vesicles (50 μg of protein) prepared from KB-3-1 (○), KCP-4 (□), C-A120 (△), KB-C2 (×) cells were incubated at 37°C in increasing concentrations of sucrose in transport buffer containing 1.34 nM [^{3}H]LTC_4 in the presence or absence of 1 mM ATP. The accumulation of [^{3}H]LTC_4 was measured at 15 min by filtration as described in ''Materials and Methods''. The osmolarity data were inversed and plotted. Data are expressed as means±SD (n=3). The accumulations of [^{3}H]LTC_4 in the absence of ATP were 0.029±0.002, 0.034±0.003, 0.020±0.002 and 0.028±0.007 pmol/mg protein/15min for KB-3-1, C-A120, KCP-4 and KB-C2 vesicles, respectively.

C-A120 and KCP-4 vesicles. The increased external sucrose concentration had only a slight effect, if any, on the [^{3}H]LTC_4 uptake in KB-3-1 and KB-C2 vesicles. These data suggest that the [^{3}H]LTC_4 uptake in KB-3-1 and KB-C2 vesicles reflects binding, rather than the transport of [^{3}H]LTC_4.

Km for LTC_4 and ATP

The effect of the LTC_4 concentration on the rate of ATP-dependent LTC_4 uptake is shown in Fig. 4. The apparent Km values for LTC_4 were estimated to be 0.18 and 0.25 μM for KCP-4 and C-A120 vesicles, respectively. The effect of the ATP concentration on the rate of ATP-dependent [^{3}H]LTC_4 uptake was also studied (data not shown). The apparent Km values for ATP were 155 and 80 μM for KCP-4 and C-A120 vesicles, respectively.

Effect of Glutathione S-Conjugates, Leukotrienes and Verapamil on [^{3}H]LTC_4 Uptake

The specificity of ATP-dependent [^{3}H]LTC_4 uptake in KCP-4 vesicles was investigated with regard to the effect of various agents and compared with that in C-A120 vesicles (Fig. 5). DNP-SG(10 μM), GS-platinum complex(100 μM) and GS-SG(100 μM) reduced the [^{3}H]LTC_4 uptake in KCP-4 vesicles by 86, 67 and 70%, respectively. However, they reduced the [^{3}H]LTC_4 uptake in C-A120 vesicles only by 27%. In contrast, LTD_4 more efficiently reduced the [^{3}H]LTC_4 uptake in C-A120, than in KCP-4 vesicles. Verapamil, a substrate for P-gp, which is responsible for *MDR1* mediated MDR, was not inhibitory.

DISCUSSION

The mechanisms of resistance to cisplatin have been widely studied using cisplatin-resistant cell lines and some possibilities such as reduced drug accumulation, increased detoxification

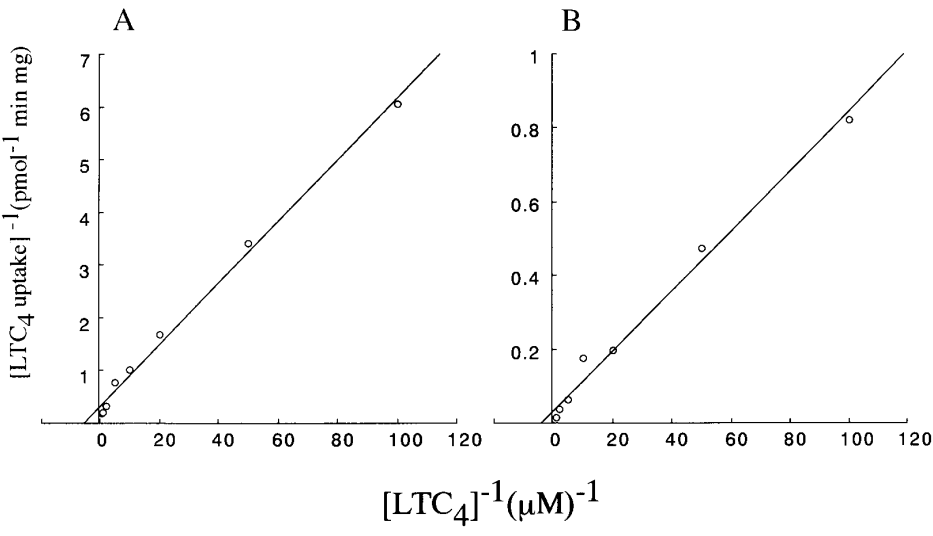

FIG. 4. Effect of LTC_4 concentrations on the rate of LTC_4 uptake in membrane vesicles. Membrane vesicles (50 μg of protein) prepared from KCP-4 cells(A) and C-A120 cells(B) were incubated at 37°C in 100 μl transport buffer containing indicated concentrations of LTC_4 in the presence or absence of 1 mM ATP. For initial rate determinations, 2 and 4 min time points were used for C-A120 and KCP-4 vesicles, respectively. ATP-dependent LTC_4 uptake in the vesicles was determined as described in ''Materials and Methods''. Data are means of triplicate determinations.

of cisplatin in the cellular cytoplasm, decreased DNA accessibility and increased DNA repair have been reported(2,20-26). We described the decreased accumulation of cisplatin in KCP-4 cells and the ATP-dependent efflux of cisplatin from KCP-4 cells(3). We demonstrated ATP-dependent [^{3}H] LTC_4 transport in membrane vesicles prepared from KCP-4 cells that was inhibited by GS-platinum complexes but not significantly by cisplatin and glutathione(5). Decreased accumulation of cisplatin in KCP-4 cells is thus supposed to be caused by an efflux of GS-platinum complex through a GS-X pump that appeared similar to that reported by Ishikawa et al.(4,16,19,27). They reported that the GS-X pump expressed in human leukemia HL-60 cells contributed to the vesicle-mediated excretion of the cytotoxic GS-platinum complex from the cells(4). Although the GS-X pump expressed in KCP-4 cells is idendified only by its function and its molecular identity has not been established, our data and reports from other laboratories suggest that the GS-X pump is associated with the decreased accumulation of cisplatin in some cisplatin-resistant cell lines.

Jedlitschky and Leier have reported that LTC_4 and DNP-SG are transported by MRP(9-11). We thus investigated whether the [^{3}H] LTC_4 transporter in the membrane vesicles prepared from KCP-4 cells is MRP. The expression of *MRP* mRNA in KCP-4 cells was similar to that in KB-3-1 cells. MRP was not detected in membrane vesicles prepared from KCP-4 cells as well in those from KB-3-1 cells. These results are compatible with the findings that KCP-4 cells are not cross-resistant to vincristine and doxorubicin, and indicate that MRP is not concerned in the LTC_4 transport in membrane vesicles prepared from KCP-4 cells. The transport of LTC_4 in membrane vesicles from KCP-4 cells was thus compared with that from C-A120 cells. The apparent Km values for LTC_4 and ATP of the KCP-4 vesicles differed from those of C-A120 vesicles. The GS-X pump expressed in KCP-4 vesicles had higher affinity for LTC_4 than that expressed in C-A120 vesicles. LTC_4 uptake in KCP-4 vesicles was 86% inhibited by 10 μM DNP-SG, but that in C-A120 vesicles was 33% inhibited by the same concentration. The inhibition of LTC_4 uptake in KCP-4 vesicles by GS-platinum complex and GS-SG was

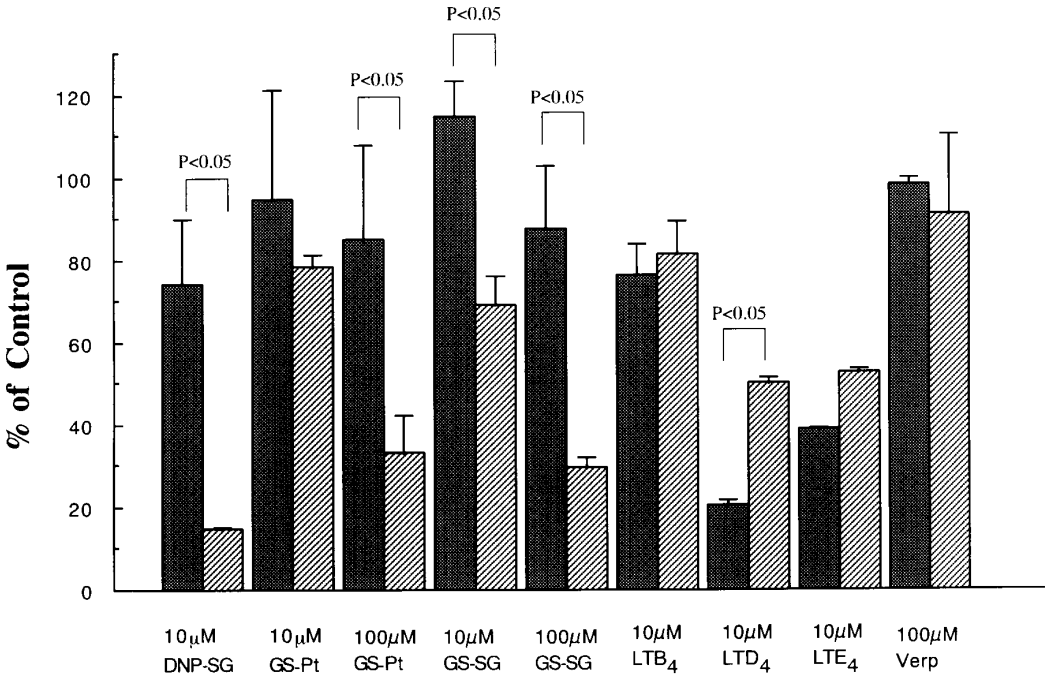

FIG. 5. Effect of various agents on the ATP-dependent [^{3}H]LTC_4 uptake in KCP-4 (▨) and C-A120 (▨) membrane vesicles. KCP-4 and C-A120 membrane vesicles (50 μg of protein) were incubated with 1.34 nM [^{3}H]LTC_4 at 37°C in 100 μl of 0.25 M sucrose containing 10 mM Tris-HCl, pH7.4, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 mg/ml creatine kinase and the indicated concentrations of each agent in the presence or absence of 1 mM ATP. Data are expressed as a percentage of [^{3}H]LTC_4 uptake in the vesicles in the absence of agents. Values are means±SE of triplicate experiments. GS-Pt, GS-platinum complex ; LTB_4, leukotriene B_4 ; LTD_4, leukotriene D_4 ; LTE_4, leukotriene E_4 ; Verp, verapamil.

also higher than that in C-A120 vesicles. In contrast to these results, the inhibition of LTC_4 uptake by 10 μM LTD_4 (63%) in the KCP-4 vesicles was significantly less than that (80%) in the C-A120 vesicles. These results suggested the LTC_4-binding site of the GS-X pump in KCP-4 vesicles has higher affinity for DNP-SG, GS-platinum complex and GS-SG than that in C-A120 vesicles. The active transport of LTC_4 in C-A120 vesicles seemes to be due to MRP since the transport was correlated with the expression level of MRP in the KB cells. Overexpression of MRP in lung cancer cells stably transfected with *MRP* cDNA resulted in the increased activity of a glutathione S-conjugate carrier(11). Further, MRP expressed in human leukemia cells(HL60/ADR) and the MRP-transfected HeLa cells was photolabeled with LTC_4 (9,10). Although the possibility that other pump(s) than MRP operate(s) in C-A120 cells to transport LTC_4 is not completely excluded, we prefer the idea that MRP transports LTC_4 because it provides the simplest and compatible explanation for the above evidence. GS-X pump expressed in KCP-4 cells and MRP, but not P-gp transported LTC_4 and the transport was not inhibited by verapamil, a substrate for P-gp. These data indicate that the property of the GS-X pumps expressed in KCP-4 and C-A120 cells are different from that of P-gp. The results of this study suggest that the GS-X pump expressed in KCP-4 cells is similar, but not identical to MRP. Recently the canalicular multispecific organic anion transporter (cMOAT) cDNA of rat was isolated and sequenced (28). cMOAT protein was identified as a member of the ABC transporter family with highest overall identity to hMRP1(47.6%). cMOAT is supposed to transport bilirubin glucuronides and LTC_4 (28). Human cMOAT or a protein

similar to MRP that transports LTC_4 may be overexpressed in KCP-4 cells. Futher study is needed to determine the structure and function of the GS-X pump expressed in KCP-4 cells and whether it is related to cisplatin-resistance.

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