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EXPRESSION OF ABC TRANSPORTERS IN HUMAN HEPATOCYTE CARCINOMA CELLS WITH CROSS-RESISTANCE TO EPIRUBICIN AND MITOXANTRONE

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Running title
ABC transporters in EPI- and MIT-resistant HCC cells

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Abstract

Background. To understand the cross-resistance between epirubicin (EPI) and mitoxantrone (MIT), we established EPI- and MIT-resistant cells and evaluated their cross-resistance. **Materials and Methods.** To clarify the degree of resistance, we measured the degrees of growth inhibition of EPI-resistant HLE-EPI cells and MIT-resistant HLE-MIT cells by anticancer drugs. Then we measured mRNA expression of multidrug resistance protein 1 (MDR1)/ABCB1 and breast cancer resistance protein (BCRP)/ABCG2 using quantitative real-time RT-PCR. Moreover, intracellular accumulation of EPI was investigated. **Results.** HLE-EPI cells were resistant to EPI, MIT and docetaxel. HLE-MIT cells were resistant to EPI, MIT and SN-38. HLE-EPI cells overexpressed MDR1 and HLE-MIT cells overexpressed BCRP. Intracellular accumulation of EPI was decreased in HLE-EPI and HLE-MIT cells. **Conclusions.** The results suggest that both MDR1 and BCRP can upregulate the efflux of EPI and that causes the resistance to EPI in HLE-EPI and HLE-MIT cells.

Introduction

Cell lines selected by exposure to anticancer drugs often show cross-resistance to other compounds, and these cells often overexpress multidrug resistance transporters, such as multidrug resistance protein 1 (MDR1)/ABCB1 (1), multidrug resistance-associated protein (MRP) family (2,3), and breast cancer resistance protein (BCRP)/ABCG2 (4). Epirubicin (EPI) is an anthracycline that is transported by both MDR1 (5) and BCRP (4). Mitoxantrone (MIT) is well known as a substrate of BCRP and can be a substrate of MDR1 (6). However, expression of these transporters is not considered in oncotherapy when first-line chemotherapy has failed. To clarify cross-resistance mechanisms caused by selection with EPI or MIT, we established two drug-resistant cell lines from HLE human hepatocellular carcinoma cells. This study showed that over-expression of a transporter affects cross-resistance to anticancer drugs.

Materials and Methods

Drugs. EPI hydrochloride was obtained from Pfizer Japan Inc. (Tokyo, Japan). MIT hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Docetaxel hydrate was from Aventis Pharma Japan Ltd. (Tokyo, Japan). 7-Ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of irinotecan, and cisplatin were from Yakult Honsha Co., Ltd. (Tokyo, Japan).

Cell culture. HLE cells, human hepatocellular carcinoma cells from JCRB Cell Bank (Osaka, Japan), were

cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (7). Resistant cells were developed by exposure to EPI hydrochloride or MIT hydrochloride at concentrations starting at 1 ng/mL with a gradual increase to 32 ng/mL over 4 months until 2 days before passage. The resulting HLE-EPI or HLE-MIT cells were maintained by exposing them to each drug at 32 ng/mL until 2 days before passage.

Estimation of drug resistance. Drug resistance was estimated using the collagen gel droplet embedded culture-drug sensitivity test (CD-DST) (8). Collagen solution was made by using a collagen gel culture kit (Nitta Gelatin Inc., Osaka, Japan). A cell suspension was added to the collagen solution at a final concentration of 2×10^5 cells/ml. The collagen-cell mixture was dropped into 6-well culture plates at a volume of 30 µl/droplet and was placed in a 5% CO_2 incubator at 37°C for 1 h to gel. Three ml of culture medium was added to each well. After 24 h, anticancer drugs were added.After 24-h incubation for drug exposure, each well was washed twice with 4 ml of phosphate-buffered solution (PBS) by slowly shaking in the incubator for 10 min. After removal of PBS, 4 ml of culture medium was added to each well and the cells were cultured for 6 days. At the end of the incubation, neutral red was added to each well to a final concentration of 50 µg/ml. Cells were fixed with 10% neutral-buffered formalin for 45 min and washed in water for 10 min. Then the collagen gel droplets were air-dried and quantified by Primage® (Nitta Gelatin Inc., Osaka Japan), an

imaging apparatus.

Quantification of mRNA. Real-time RT-PCR was performed using a LightCycler™ (Roche Diagnostics K.K., Japan) with hybridization probes. Primer sequences were as follows: MDR1 sense 5'-AAGAA GCCCT GGACA AAGCC-3', MDR1 antisense 5'-ACAGT CAGAG TTCAC TGGCG-3', BCRP sense 5'-ATCTT GGCTG TCATG GCT-3', BCRP antisense 5'-TGATT CTTCC ACAAG CCC-3', GAPDH sense 5'-GCCTC CTGCA CCACC TG-3' and GAPDH antisence 5'-CGACG CCTGC TTCAC CACCT TCT -3'. Hybridization probes for the LightCycler™ were designed and synthesized at Nihon Gene Research Laboratories Inc. (Sendai, Japan). Real-time PCR cycles started with 10 min at 95° C and then 45 cycles of 15 sec at 94° C, 15 sec at 56° C and 30 sec at 72° C for MDR1 and BCRP or 45 cycles of 10 sec at 94° C, 10 sec at 60° C and 20 sec at 72° C for GAPDH. Expression levels of MDR1 and BCRP were expressed as ratios (MDR1 or BCRP/GAPDH).

Uptake study. Cells were seeded on 12-well culture plates and incubated for 5 days. The cells were incubated in a neutral-pH buffered incubation medium containing a substrate (10 µg/ml EPI hydrochloride) at 37°C. The cells were solubilized in 1% of Tween 20. EPI was determined using a high-performance liquid chlomatography (HPLC) system equipped with a fluorescence spectrophotometer (9). Settings were as follows: column, TSK-GEL CN-80Ts (TOSOH); column temperature, 40°C; mobile phase, 50 mM NaH_2PO_4 (pH 4.0):acetonitrile=65:35; flow rate, 0.8 mL/min; λ_{ex}, 480 nm, λ_{em}, 560 nm; retention time, 4.8 min.

Results

HLE-MIT cells showed strong resistance to MIT and SN-38 (69- and 76-fold, respectively) and moderate resistance to EPI (6.9-fold). On the other hand, HLE-EPI cells showed 18-fold resistance to docetaxel and moderate resistance to EPI and MIT (5.9- and 3.4-fold, respectively). Neither cell line showed cross-resistance to fluorouracil and cisplatin (Table I). These spectra of multidrug resistance indicated that HLE-EPI and HLE-MIT overexpressed multidrug resistance transporters. To clarify the expression levels of multidrug resistance transporters, we measured MDR1 and BCRP mRNA levels by real-time RT-PCR. MDR1 mRNA was overexpressed in HLE-EPI cells (21-fold) but not in HLE-MIT cells. On the other hand, BCRP mRNA was overexpressed in HLE-MIT cells (18-fold) but not in HLE-EPI cells (Figure 1). To determine whether intracellular accumulation of EPI was changed in HLE-EPI and HLE-MIT cells, we evaluated the intracellular accumulation of EPI by using an HPLC system with a fluoroscence spectrophotometer. Accumulation of EPI in HLE-EPI and HLE-MIT cells was significantly less than that in HLE cells. The accumulation of EPI in HLE-EPI cells was the same as that in HLE-MIT cells from 15 min to 60 min (Figure 2).

Discussion

EPI is an anthracycline that is known as a substrate of MDR1 and BCRP (4,5). To understand cross-resistance and

expression status of ABC transporters in EPI-selected cell lines, we established EPI-selected sublines from human hepatocellular carcinoma HLE cells (7). HLE-EPI cells showed cross-resistance to MIT and docetaxel. HLE-MIT cells showed strong resistance to MIT and SN-38. MIT is a substrate of MDR1 and BCRP. Docetaxel is the substrate of MDR1 (10) but not of BCRP. On the other hand, SN-38 is a substrate of BCRP (11) but not of MDR1. These results strongly suggested that the multidrug resistance of HLE-EPI cells mainly depended on the over-expression of MDR1 and that of HLE-MIT cells mainly depended on the over-expression of BCRP.

To evaluate the over-expression of transporters, we measured mRNA levels of MDR1 and BCRP by the real-time RT-PCR and intracellular accumulation of EPI. HLE-EPI cells overexpressed MDR1 with no change in the BCRP mRNA expression level. Furthermore, accumulation of EPI in HLE-EPI cells was significantly less than that in HLE cells. These findings suggest that HLE-EPI cells acquire multi-drug resistance by over-expression of MDR1. On the other hand, HLE-MIT cells overexpressed BCRP with no change in the MDR1 mRNA expression level and with reduced accumulation of EPI. Although different transporters are overexpressed in these resistant cells, the degrees of accumulations of EPI were almost the same. That may be reason why the degrees of resistance of HLE-EPI and HLE-MIT to EPI are almost the same. Furthermore, Robey et al. showed that the relative resistance value of EPI was less than that of MIT or SN-38 in

codon 482 wild type (482R) and mutants (482G and 482T) (12). The sequence of BCRP codon 482 in HLE-MIT cells has not been revealed, but the cross-resistant status we examined is consistent with the results of their experiment. It is possible that cross-resistance beyond the analogs of an anticancer agent mainly depends on multidrug efflux transporters such as MDR1 or BCRP.

In conclusion, the EPI-selected human hepatocellular carcinoma cell line HLE overexpressed MDR1 with no change in BCRP mRNA. On the other hand, MIT-selected HLE cells overexpressed BCRP with no change in MDR1 mRNA. However, the degree of resistance and the intracellular accumulation of EPI were declined in both HLE-EPI and HLE-MIT cells. These findings suggest that both MDR1 and BCRP can upregulate the efflux of EPI and that causes the resistance to EPI in HLE-EPI and HLE-MIT cells.

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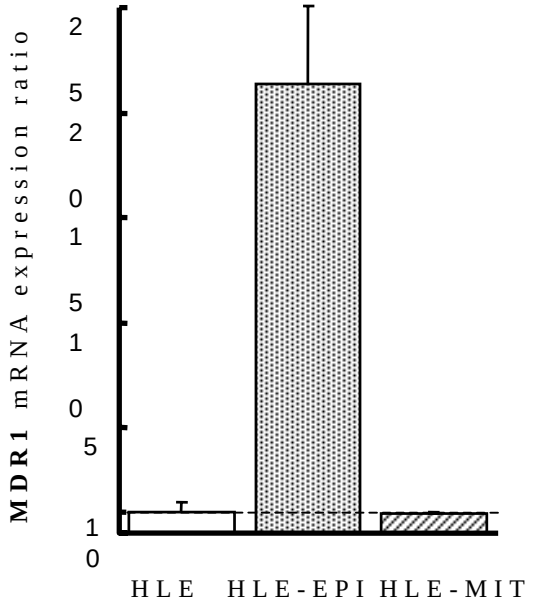
Figure 1. Quantification of mRNA expressions in HLE, HLE-EPI and HLE-MIT cells.

mRNA expression ratio was defined as the ratio to (A) MDR1 or (B) BCRP in HLE cells. HLE cells expressed MDR1 and BCRP at 6.56×10^{-3} and 1.26×10^{-3} (copies/GAPDH), respectively. HLE (blank column), HLE-EPI (dotted column) and HLE-MIT (slashed column) represent the mean with S.D. (n=3).

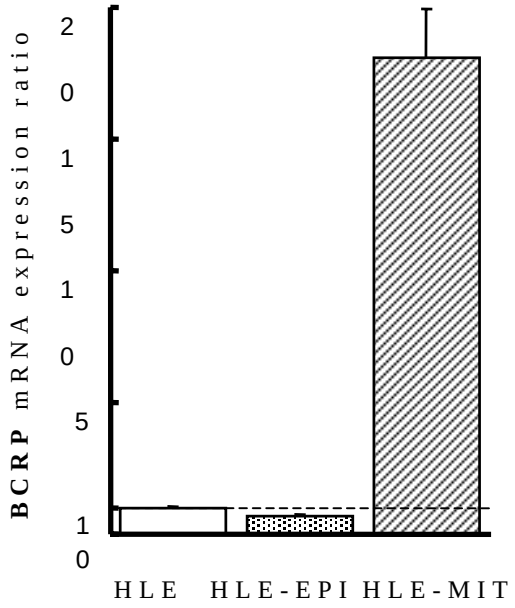
Figure 2. Intracellular accumulation of EPI in HLE, HLE-EPI and HLE-MIT cells.

Symbols represent HLE (blank squares and solid line), HLE-EPI (blank circles and solid line) and HLE-MIT (black circles and dotted line). Each point represents the mean ± S.D. (n=9-12). *, significantly different from HLE at $p<0.01$

(A)


MDR1 mRNA expression ratio
2
5
2
0
1
5
1
0
5
1
0
HLE
HLE-EPI
HLE-MIT

(B)


BCRP mRNA expression ratio
2
0
1
5
1
0
5
1
0
HLE
HLE-EPI
HLE-MIT

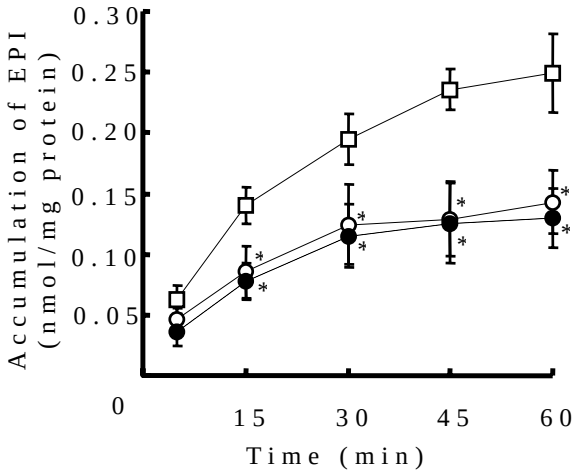

Accumulation of EPI
(nmol/mg protein)
0.30
0.25
0.20
0.15
0.10
0.05
0
15
30
45
60
Time (min)

Table I. IC_{50} (μM) and fold resistance

	HLE	HLE-EPI	HLE-MIT
Epirubicin	0.034 ±0.005	0.17 ±0.033 (**5.9**)	0.20 ±0.051 (**6.9**)
Mitoxantrone	0.006 ±0.002	0.019 ±0.003 (**3.4**)	0.39 ±0.35 (**69**)
Fluorouracil	40 ±5.7	40 ±2.6 (1.0)	40 ±10 (1.0)
Cisplatin	0.63 ±0.20	0.54 ±0.083 (0.86)	0.47 ±0.097 (0.74)
Docetaxel	0.002 ±0.0002	0.036 ±0.011 (**18**)	0.001 ±0.0003(0.69)
SN-38	0.008 ±0.003	0.010 ±0.002 (1.2)	0.62 ±0.22 (**76**)

Fifty percent inhibition concentration (IC_{50}) was calculated with sigmoid fitting.Fold resistance is shown in parenthesis.