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Characterization of multidrug resistance-associated protein mRNAs expression profiles in Caco-2 and HT-1080 cell lines induced by methotrexate

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An efflux function induced by multidrug resistance-associated proteins (MRPs) contributes to the mechanisms of resistance to methotrexate (MTX). Since it has been reported that the human Caucasian colon adenocarcinoma cell line (Caco-2) shows high MRPs expression, it is assumed that Caco-2 cells are resistant to MTX. Alternatively, the human fibrosarcoma cell line (HT-1080) has been reported to be highly sensitive to MTX. In this study, the difference in the growth-inhibitory activity of MTX in these cell lines was compared as a characteristic of sensitivity. Subsequently, the characterization of MRP (1–8) mRNAs expression profiles before and after MTX exposure in these cell types was examined with the multiplex reverse-transcription-polymerase chain reaction (RT-PCR) method. It was demonstrated that the expressions of *MRP2*, *MRP3*, *MRP6*, and *MRP8* mRNAs in the HT-1080 cells were lower than those in the Caco-2 cells and that only *MRP5* mRNA expression was induced in the Caco-2 cells after MTX treatment. The MRP mRNAs expression profiles between these two cell lines could be differentiated widely. The characterization of MRPs profiles might be associated with resistance to MTX in cells. Additionally, this multiplex RT-PCR method would be beneficial for further time-dependence investigations of the MRP mRNAs expression profiles.

1. Introduction

Methotrexate (MTX) is one of the antineoplastic drugs that can be used in high doses for chemotherapy against malignant tumors. The possibility of tumor tissues developing resistance to MTX is one of the reasons for the administration of high-dose MTX chemotherapy despite the risk of MTX-induced toxicity to normal tissues. Resistance to MTX is caused by several factors such as MTX polyglutamation (Chabner et al. 1985); reduced dihydrofolate reductase (Srimatkandada et al. 1989); and efflux caused by P-glycoprotein (encoded by the *MDR1* gene, also known as *ABCB1*), multidrug resistance-associated proteins (MRPs; encoded by the *MRP1–MRP9* genes, also known as *ABCC1–ABCC*6 and *ABCC10–ABCC12*), and breast cancer-resistance protein (*BCRP*; encoded by the *BCRP* gene, also known as *ABCG2*). In addition, it has also been reported that MTX is a substrate for MRPs, which transport it to the outside of the cell (Assaraf 2006, 2007; Choudhuri and Klaassen 2006). Furthermore, some studies have reported that MRPs and BCRP were expressed in certain antifolate-resistant cell lines and could restrict the intracellular transport of antifolates (Hooijberg et al. 1999; Xia et al. 2005).

It is well known that after exposure to MTX for a few days, the growth of MTX-sensitive tumor cells is inhibited, whereas that of MTX-resistant (insensitive) tumor cells is poorly inhibited. It has been reported that the growth-inhibitory activity of MTX differs among various malignant tumor cell types. The human fibrosarcoma cell line HT-1080 is highly sensitive to MTX, i.e., the growth-inhibitory activity of MTX against this cell line is high (Lesuffleur et al. 1990; Li et al. 2001). Moreover, the expression of efflux transporters is considered to be one of the resistance mechanisms of MTX-resistant tumor cells. It has been reported that the human Caucasian colon adenocarcinoma cell line Caco-2 shows high expression of MRPs (Prime-Chapman et al. 2004). However, almost no information is available regarding the expression profiles of MRPs before MTX treatment and changes in the expression profiles after MTX treatment in malignant tumor cell lines. MRPs belong to the same ATP-binding cassette (ABC) gene superfamily as membrane transporters such as P-glycoprotein, and have been observed to transport a diverse range of substrates. BCRP has also been described as an MTX polyglutamate transporter (Chen et al. 2003; Choudhuri and Klaassen 2006; Volk and Schneider 2003). P-glycoprotein has wide substrate specificity, and these membrane transport proteins have been observed to facilitate the management of a wide spectrum of xenobiotics. Further, it is known that MTX is mainly transported into the cytoplasm via the reduced folate carrier (encoded by

the *RFC1* gene) (Assaraf 2006). We believe that the mechanism underlying resistance to MTX can be clearly revealed by comparing the expression levels of MRP, MDR1, BCRP, and RFC1 mRNA in cell lines that show different sensitivities to MTX.

Sulfotransferase mRNA induction in MTX-treated Caco-2 cells (Chen et al. 2005) and P-glycoprotein induction in interferon-gamma-treated Caco-2 cells have been reported (Dixit et al. 2005). These findings indicate that the expression levels of MRP mRNA in Caco-2 cells may also be modulated by MTX treatment.

First, for verifying the difference in the growth-inhibitory activity of MTX between sarcoma and carcinoma cell lines, we investigated its activity in Caco-2 cells, HT-1080 cells, and human umbilical vein endothelial cells (HUVECs) after MTX treatment. It is assumed that the growth rate of these cultured cells declined after they reached confluence, which is a condition wherein growth is inhibited due to contact among cells. Generally, it is considered that cell-to-cell contact is present in malignant tumor tissues. In this study, the growth-inhibitory activity of MTX was investigated in two cell groups: the group that attained confluence, and the group that attained subconfluence (the condition present before confluence). When a drug is administered intravenously, blood or endothelial cells are the first to be exposed to the drug. In this investigation, we used HUVECs as the normal cell line for comparison with malignant tumor cells. In order to simultaneously identify the relationship between MTX resistance (reflected by low growth-inhibitory activity) and the mRNA expression profiles of MRPs (considered as one of the mechanisms of MTX resistance), Caco-2 cells, which are known to show high expression of MRPs, were examined to determine the difference in these expression profiles with and without MTX treatment. To determine these profiles, we used the semiquantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) method. Additionally, a semiquantification method with multiplex RT-PCR was used to assess the expressions of MDR1, MRPs, and BCRP mRNA. Moreover, this method was confirmed to be more efficient for determining mRNA expression levels when multiple cell lines were used and for time dependence investigation. Subsequently, for determining the relationship between MTX resistance and variation in the MRP mRNAs expression profiles, the difference in the growth-inhibitory activity of MTX in each cell line

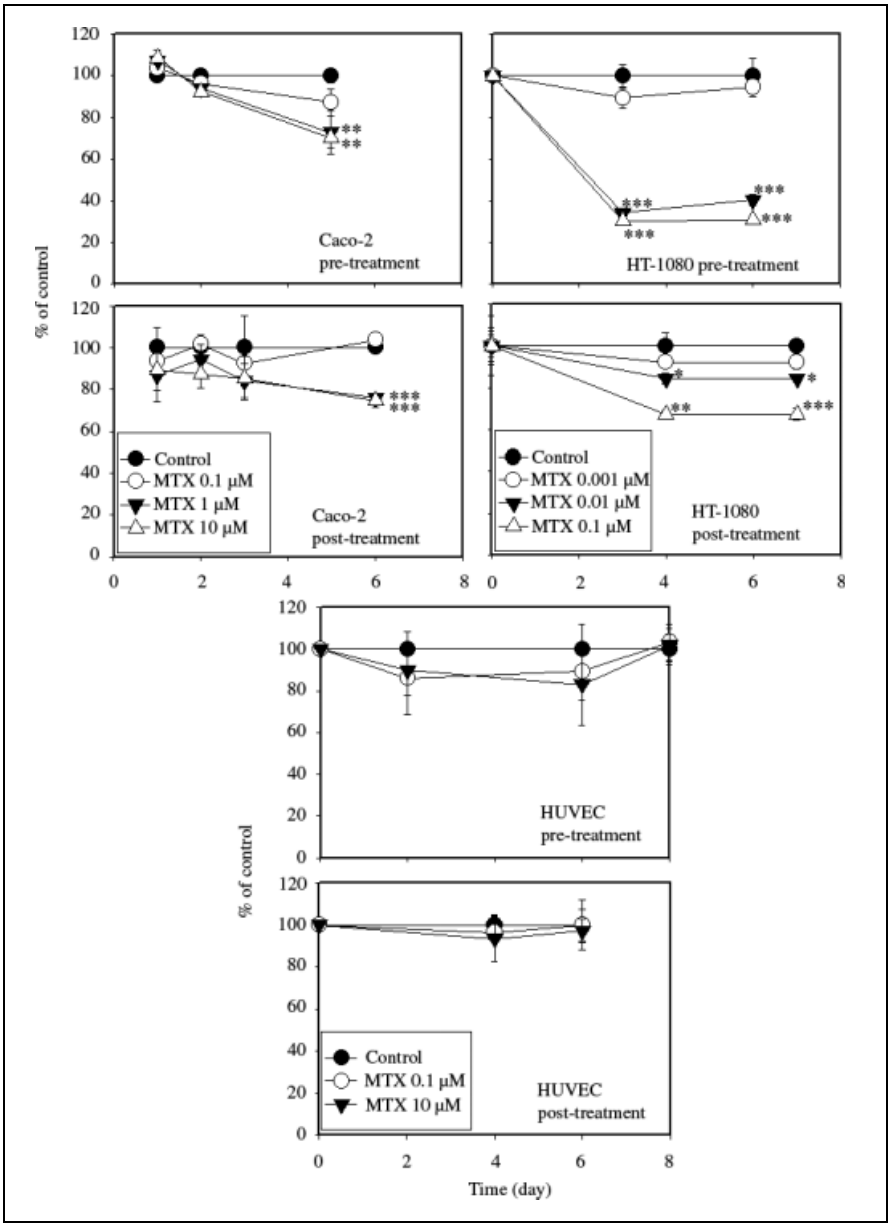

Fig. 1:
Growth inhibition of the Caco-2 cells (adenocarcinoma cell line), HT-1080 cells (fibrosarcoma cell line), and HUVECs by the MTX treatment. For the pre-treatment study, the cells were cultured without (control) or with MTX for 5–8 d after seeding at 5000 cells/well on a 96-well culture plate. Further, for the post-treatment study, the cells were cultured without (control) or with MTX for 6–7 d after subconfluence was attained. The cell number measured by the WST-8 cell counting kit was calculated as a percentage of the number of control cells. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; $n = 5$.

was compared to the alteration in the mRNA expression profiles in the 3 cell lines.

2. Investigations and results

2.1. Growth inhibition of the Caco-2 cells, HT-1080 cells, and HUVECs by MTX

Both pre-treatment and post-treatment of Caco-2 cells with MTX resulted in significant growth inhibition at a final concentration of more than 1 μM at 3 d after exposure, while in the MTX-treated HT-1080 cells, growth inhibition was significantly induced at a final concentration of more than 0.01 μM. The growth inhibition as a result of pre-treatment of HT-1080 cells with MTX was considerably high. However, in the HUVECs, growth inhibition was not noted even 6 d after exposure (Fig. 1). On day 3 after pre-treatment exposure, 50% arrest in cell growth was observed in the HT-1080 cells treated with MTX at a concentration of 0.0036 μM. This result suggested that the growth inhibitory activity in the HT-1080 cells was higher than that in the Caco-2 cells and HUVECs. The difference in the MTX-induced growth inhibition among these two cell lines was also demonstrated in the post-treatment. To collect total RNA from these cells for further investigations, a cell number of greater than that in subconfluence would be required. The post-treatment study design was then used for further investigations. The MTX concentrations of the prepared MTX solutions, stock solution, diluted and filtrated solution, and the final medium solution were determined with high-performance liquid chromatography. These concentrations did not decrease due to stocking at 4 °C or by filtration carried out for sterilization (data not shown).

2.2. MRP mRNAs expression profiles in the Caco-2 cells, HT-1080 cells, and HUVECs by MTX

The ratio of MRP1–MRP8 (MRPs) expression and MDR1 mRNA expression to ACTB mRNA expression were compared among cells that attained subconfluence (SC condition), confluence (C condition at day 5 after subconfluence was attained), and confluence following MTX addition (CM condition; 10 μM (for Caco-2 cells and HUVECs) and 0.1 μM (for HT-1080) of MTX). In the SC condition of Caco-2 cells, the MDR1 expression level was the highest, followed by the MRP2 mRNA expression level. In the C condition, the expression levels of the MRPs were generally higher than those in the SC condition. The expressions of all the MRPs except MRP5 were decreased in the CM condition, as compared to that in the C condition. The expression levels of MRP mRNAs in the HT-1080 cells markedly contrasted with those in the Caco-2 cells. Further, in the HT-1080 cells and HUVECs, the expression profiles almost did not change in the 3 conditions (Fig. 2).

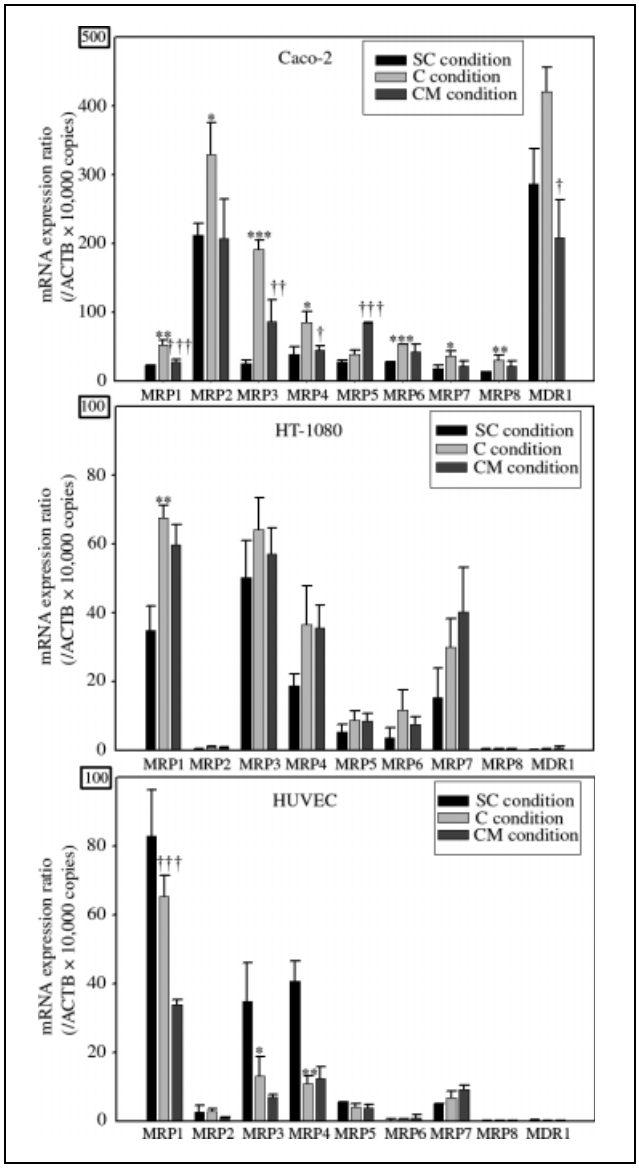

Fig. 2: The changes in the expression profiles of all the MRP mRNAs of the Caco-2 cells, HT-1080 cells, and HUVECs as determined by real-time RT-PCR. The cells were seeded on a 6-well culture plate and cultured without MTX for 1 d (subconfluence, SC condition), without MTX for 6 d (confluence for 6 d, C condition), and with the addition of 10 μM (for Caco-2 cells and HUVECs) and 0.1 μM (for HT-1080 cells) of MTX (confluence with MTX for 6 d, CM condition) after subconfluence was attained. The total RNA was isolated from the lysed cell solution. First strand cDNA templates were synthesized from 1 μg of the total RNA. The expressions of MRP1–MRP8, ACTB, and MDR1 were analyzed by semiquantitative real-time RT-PCR. Each mRNA expression ratio was calculated as the ratio to 10 000 copies of ACTB. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs. the SC condition and †: $p < 0.05$; ††: $p < 0.01$; †††: $p < 0.001$ vs. the CM condition; n = 3–5.

2.3. Multiplex RT-PCR

The aims of this investigation were to simplify the simultaneous measurement of the expression levels of the MRP mRNAs for comparison among the many cell groups and treatment groups; to compare the mRNA expression profiles of MDR1, MRPs, BCRP, and RFC1 in the normal condition among the three cell types; and to compare the normal condition with the MTX treatment condition. Further, the experimental errors that occur with the use of reaction tubes for different targets during real-time RT-PCR were expected to be negligible because of the presence of multiple mRNA targets in each PCR reaction tube. Each standard dsDNA fragment of *MDR1*, *MRPs*, *BCRP*, and *RFC1* was transferred from the total RNA and amplified by the target PCR. The standard dsDNA template fragments (1 fg/reaction tube) obtained by one target RT-PCR of the MRPs were compared with the standard multiplex PCR product (1 fg, 10,000 copies, and 5,000 copies of the standard dsDNA fragments in 50 μL PCR reaction solution); *MDR1*, *RFC1*, and *BCRP* (10 fg by one-target RT-PCR) were compared with the standard mul-

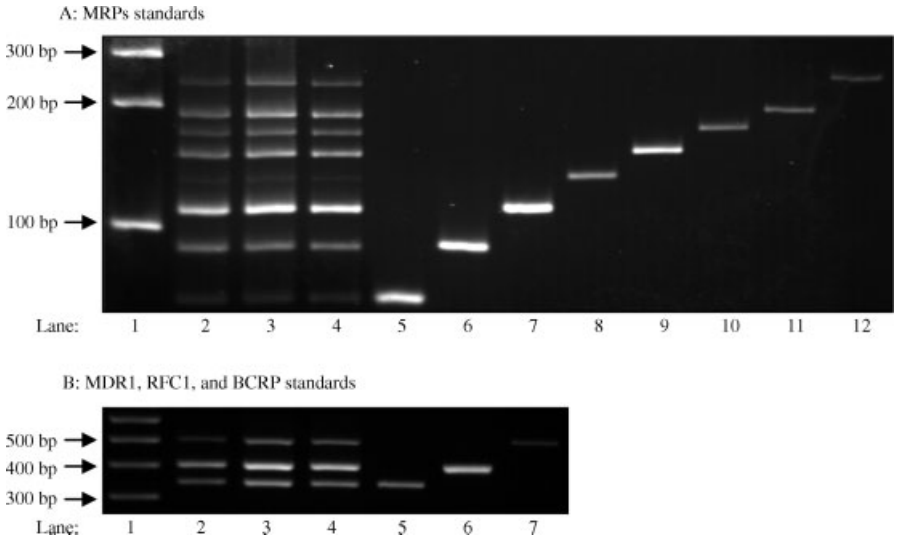

Fig. 3: Standard electrophoresis of cloning dsDNA fragments (MRPs, MDR1, RFC1, and BCRP) amplified by semiquantitative multiplex RT-PCR. Electrophoresis of the MRPs was performed using 4% Metaphor and 2% agarose gel. A: lane 1, – ladder; lane 2, – approximately 1 fg each of the dsDNA fragments (all MRPs) in 50 µL of the PCR reaction mixture; lane 3, – approximately 10 000 copies of the standard dsDNA fragment; lane 4, – approximately 5 000 copies; and lane 5–12, – approximately 1 fg of each dsDNA fragment of MRP1–MRP8. B: lane 1, – ladder; lane 2, – approximately 10 fg each of the dsDNA fragments (MDR1, RFC1, and BCRP) in 50 µL of PCR reaction mixture; lane 3, – approximately 100 000 copies of the standard dsDNA fragment; lane 4, – approximately 50,000 copies; and lane 5–7, – approximately 10 fg of each dsDNA fragment of MDR1, RFC1, and BCRP.

tiplex PCR product (10 fg, 100 000 copies and 50 000 copies of the standard dsDNA fragments) (Fig. 3). The electrophoresis bands obtained with multiplex PCR of the target fragments were quantitatively similar to the bands of the single-target RT-PCR products. Subsequently, the expression levels of the MRP mRNA in 1 µL of cDNA (50 µL) transferred from 1 µg total RNA were compared with 5 000 copies of the standard dsDNA fragments in a 50 µL PCR reaction solution; *MDR1*, *RFC1*, and *BCRP* were compared with 50,000 standard dsDNA fragment copies; and *ACTB* was compared with 500 000 standard dsDNA fragment copies. The expression profiles of the MRP mRNA in the Caco-2 cells obtained with the multiplex PCR method were similar to those obtained with the real-time PCR method. The growth of HT-1080 cells was highly inhibited by the MTX treatment, and the MRP2,

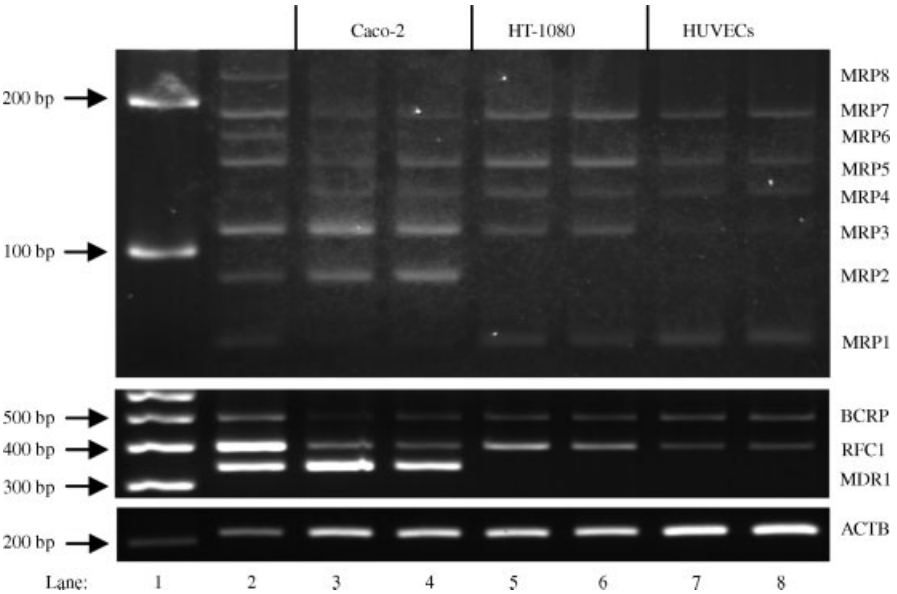

Fig. 4: Expression ratio of the MRPs in the MTX-treated Caco-2 cells, HT-1080 cells, and HUVECs by semiquantitative multiplex RT-PCR. The cells were seeded on a 6-well culture plate and were cultured without or with 10 µM (Caco-2 cells and HUVECs) or 0.1 µM (HT-1080 cells) of MTX for 6 d after subconfluence was attained. The total RNA (n = 3) was isolated from the lysed cell solution. First strand cDNA templates were synthesized from 1 µg of the total RNA. The expressions of MRP1–MRP8, ACTB, and MDR1 were analyzed using the semiquantitative multiplex RT-PCR method. Lane 1, – ladder; lane 2, – 5 000 copies (all MRPs), 50 000 copies (MDR1, RFC1, and BCRP), and 500 000 copies (ACTB) of each dsDNA fragment in 50 µL of the PCR reaction mixture; lane 3, – Caco-2 cells (no MTX treatment); lane 4, – Caco-2 cells (MTX treatment); lane 5, – HT-1080 cells (no MTX treatment); lane 6, – HT-1080 cells (MTX treatment); lane 7, – HUVECs (no MTX treatment); and lane 8, – HUVECs (MTX treatment).

MRP3, and MRP6 mRNA expression levels in the C condition were lower for the HT-1080 cells than for the Caco-2 cells. The MRP2 and MRP5 mRNA expression levels in the Caco-2 cells were induced in the CM condition, as compared to that in the C condition. On the other hand, the expression levels in the HUVEC cells were not detected, and MTX-induced growth inhibition was found to be lower in the HUVECs than in the HT-1080 cells (Fig. 4).

3. Discussion

Overall, in the Caco-2 cells, the MRP mRNA expressions were induced by a continuous confluence environment. This result and the results of the MRP mRNA expression profiles obtained using real-time RT-PCR were consistent with those obtained using the multiplex RT-PCR method. We demonstrated that the semiquantitative multiplex RT-PCR method was as useful as real-time RT-PCR and that it could visually confirm the expression profiles of the MRP mRNAs.

The Caco-2 cell line growth was eventually inhibited by an MTX concentration greater than 1 μM. However, the 50% inhibitory concentration in HT-1080 cells was achieved at an MTX concentration of 0.036 μM. This indicated that MTX-induced growth inhibition in the HT-1080 cells was more than 1,000 times higher than that in the Caco-2 cells. HT-1080 cells exhibited higher sensitivity to MTX than the Caco-2 cells and HUVECs.

The MRP mRNA expression levels in the Caco-2 cells were apparently higher than those in the HT-1080 cells. It was considered that the low growth-inhibitory activity of MTX in the Caco-2 cells could be associated with the high expression levels of MRP mRNAs. Further, MTX treatment induced the expression of 2 MRP mRNAs, namely, MRP2 and MRP5, in the Caco-2 cells. Zhang et al. (2006) demonstrated a higher *MRP5* gene expression level in mouse MTX-resistant cells. Wielinga et al. (2005) demonstrated a high level of resistance to antifolates with MRP5 overexpression in human embryonic kidney cells (HEK293). These reports and our results are in good agreement, and it was demonstrated that MRP5 mRNA expression in the Caco-2 cells was apparently induced by MTX exposure. Moreover, the high mRNA expression in the Caco-2 cells with regard to the MRP genes – *MRP2*, *MRP3*, and *MRP6* – suggested that these genes were primarily responsible for the efflux of MTX.

However, the MRP mRNA expression levels, except for that of MRP1, in the HUVECs were lower than those in the HT-1080 cells despite the low growth-inhibitory activity in HUVECs. It is suggested that the sensitivity of HUVECs to MTX does not depend on the function of these efflux transporters. Further studies conducted using umbilical cord HUVECs may reveal that in the fetal body, these cells have other defensive functions against xenobiotics.

In this study, we have shown that the MRP mRNAs expression profiles between these two cell lines could be differentiated and that unique variations in the expression profiles of MRP mRNAs in the two cell lines – Caco-2 and HT-1080 – might determine the sensitivity to MTX. In particular, it was suggested that the change in the *MRP2*, *MRP3*, and *MRP5* gene expression profiles might be mainly different between the MTX non-sensitive Caco-2 cells and MTX- sensitive HT-1080 cells; only *MRP5* mRNA expression was induced in the Caco-2 cells on MTX exposure.

In conclusion, the MRP mRNAs profile may indicate the characterization in a condition of the cells that are exposed to MTX. Then, the lack of cell growth inhibitory activity by MTX would represent a condition wherein the cells express drug resistance to MTX. Characterization of the MRP mRNAs profiles in the condition wherein MTX growth inhibitory activity is absent might indicate the role of MRPs in the drug resistance mechanisms against MTX. Our future aims will be to investigate the intimate time-dependent changes in the MRP mRNAs expression profiles with regard to MTX, the time-dependent changes in potential resistance of the cells to MTX, and the expression profiles in several other cell lines with regard to the differences in the growth inhibitory activity of MTX among the cell lines. We anticipate that this multiplex RT-PCR method with MRPs is beneficial for time dependence investigations of MRP mRNAs expression profiles.

4. Experimental

4.1. Materials

The Caco-2 cells, HUVECs, HT-1080 cells, fetal bovine serum, and CS-C medium for HUVECs were obtained from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). The apoptosis assay kit and specific oligonucleotide primers were obtained from Invitrogen Japan K.K. (Tokyo, Japan). The SYBR green real-time PCR master mix plus was obtained from Toyobo Co., Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and MTX were purchased from Sigma-Aldrich (St. Louis, MO). The RNeasy® protect cell mini kit was obtained from Qiagen Inc. (Valencia, CA). The EZLoad™ molecular ruler (100 bp) and the iScript™ cDNA synthesis kit were obtained from Bio-Rad Laboratories, Inc. (Tokyo, Japan). Cell culture plates and flasks were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). The multiplex PCR assay kit was purchased from Takara Bio Inc. (Shiga, Japan). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

4.2. Cell culture

The Caco-2 cells (passage number, 55–60) and HT-1080 cells (passage number, 25–30) were cultured in DMEM with 4.5 g/L glucose supplemented with 10% (v/v) fetal bovine serum, penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively), and 1% (v/v) nonessential amino acids (Chen et al. 2005; Li et al. 2001; Prime-Chapman et al. 2004). HUVECs (passage number, 3–5) were cultured in the CS-C medium (Ikeda et al. 1999). These cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air and subcultured weekly. MTX was dissolved in a 5 mM NaOH solution. For the control groups, the cells were used in the growth assay and collected for mRNA semiquantification after the MTX treatment and diluted NaOH solution treatment. Total RNAs for RT-PCR were extracted with the RNeasy® protect cell mini kit and used for subsequent studies.

4.3. Growth inhibition assays

The cells were seeded in 100 μL of medium at an initial density of 1×10^4 cells/cm^2 in individual wells of a 96-well plate. Different concentrations of MTX were added on day 1 after seeding (pre-treatment) or on the day the cells achieved subconfluence (post-treatment) (Fig. 5). The viability of the cells was determined by the cell count reagent SF (Nacalai Tesque) (Horie et al. 2006). In brief, 10 μL of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium and monosodium salt (WST-8) solution were added to each well. The cells to which the WST-8 solution was added were incubated for 2 h at 37 °C. The absorbance at 480 nm (reference at 620 nm) was determined by a microplate reader, i.e., Powerscan® (Dainippon Sumitomo Pharma, Co., Ltd., Osaka, Japan). The viabilities were calculated as the percentage of the number of control cells (5 mM NaOH treatment).

4.4. Real-time RT-PCR for MRP1–MRP8 and MDR1

The RNeasy® protect cell mini kit was used for total RNA extraction, and the iScript™ cDNA synthesis kit was used for the generation of the first-strand cDNA templates. The primer pairs that have been described previously (Chen et al. 2005; König et al. 2005; Prime-Chapman et al. 2004) were used for real-time RT-PCR. The cells were seeded on a 6-well culture plate and treated with MTX for 6 d after subconfluence was attained. The cells were lysed with the addition of the RNAprotect® cell reagent (Qiagen). The lysed cell solution was centrifuged for 5 min at 5,000 × *g*, and the total RNA was isolated with the RNeasy® mini protection kit. Subsequently, the first strand cDNA templates were synthesized from 1 μg of

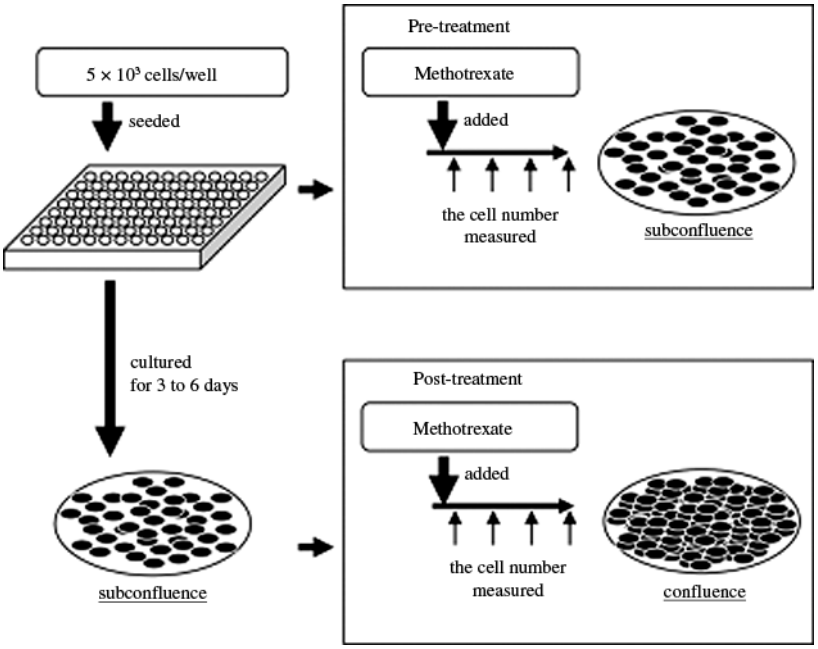

Fig. 5:
The study design for MTX treatment of the cultured cells.

the total RNA by the iScript™ cDNA synthesis kit and diluted to a total volume of 50 µL with Tris-EDTA (TE) buffer. The mRNA expression levels of MRP1–MRP8, β-actin (encoded by the *ACTB* gene), and MDR1 were analyzed with the real-time PCR detector, Opticon®2 (Bio-Rad) using the SYBR green real-time PCR master mix plus. PCR amplification comprised 30 cycles of denaturation at 94 °C for 30 s, annealing at 55.4–65.3 °C (primer specific) for 30 s, and extension at 72 °C for 30 s. The PCR products were separated by gel electrophoresis on 1% agarose, stained with ethidium bromide, and imaged under UV light. The expression ratios of MRP1–MRP8 and MDR1 were normalized to that of the housekeeping gene *ACTB*.

4.5. Multiplex RT-PCR

The primer pairs for multiplex RT-PCR are shown in the Table. Using the GeneAmp® PCR System 9700 (Applied Biosystems, CA), we performed multiplex PCR with the multiplex PCR assay kit in a total volume of 50 µL using 1 µL of each cDNA template. Multiplex PCR amplification consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 90 s (Schwarzenbach 2002). Subsequently, for confirming the standard expression profiles of MRP mRNAs (low expression level), we prepared 1 fg, approximately 10 000 copies, and 5 000 copies of the standard cloning double- stranded DNA (dsDNA) fragments in 50 µL of PCR reaction mixture. For *MDR1*, *RFC1*, and *BCRP* (intermediate expression level) expressions, we prepared 10 fg, approximately 100 000 copies, and 50 000 copies of the standard dsDNA fragments in 50 µL of the PCR reaction mixture. For ACTB (high expression level), multiplex PCR amplification was performed for 25 cycles and the standard dsDNA fragment was prepared as 500 000 copies in 50 µL of the PCR reaction mixture. Electrophoresis of the low-expression- level PCR products was performed using 4% Metaphor® agarose (Cambrex Bio Science Rockland Inc., ME), and electrophoresis of the other PCR products was performed using a 2% agarose gel. The gels were stained with ethidium bromide and imaged under UV light.

Table: Positions and amplified fragment length of the primer sequences used for multiplex RT-PCR

Gene			Sequence (Position)	NCBI GenBank Accession No.	Product Size (bp)
MRP1	Sense	1978	GGTCATCAGCAGCATCGTG	NM_004996	71
	Antisense	2048	GCTCCTCATGGGAGAGAAAG		
MRP2	Sense	2071	CTCCTTGATATCAGCCATGC	NM_000392	91
	Antisense	2161	GGACTGCTGTGGGACATAGG		
MRP3	Sense	4315	GGACTTCCAGTGCTCAGAGG	NM_003786	110
	Antisense	4424	CCTCGTCTAAAACCAGGATGC		
MRP4	Sense	930	GGATCAGGACCATGAATGAAG	NM_005845	130
	Antisense	1059	CAGGAACTTCTCAGAATCTTGG		
MRP5	Sense	543	GGAGCTCTCAATGGAAGACG	NM_005688	149
	Antisense	691	GGCAGAAGATCCACACAACC		
MRP6	Sense	1694	GGCCGAGAATGCTATGAATG	NM_001171	170
	Antisense	1863	CACCAGGGTCAACTTCTTCC		
MRP7	Sense	4139	CCTGTTGTTGGTGCTCTTCC	NM_033450	190
	Antisense	4328	GGCCCTGTCCTTATGTAGGC		
MRP8	Sense	1654	CAACCTGGTGGTGTCCAAG	NM_032583	251
	Antisense	1904	GATTCAGGGAGCAGCAGTG		
MDR1	Sense	2094	CAGCCTTGGACACAGAAAGC	NM_000927	349
	Antisense	2442	GATCCACGGACACTCCTACG		
BCRP	Sense	773	GCTGCAAGGAAAGATCCAAG	NM_004827	501
	Antisense	1273	GGCCAATAAGGTGAGGCTATC		
RFC1	Sense	983	GCACATCCTGTGGAACGAG	NM_194255	400
	Antisense	1382	CACGTCCGAGACAATGAAAG		
ACTB	Sense	888	GTGGCATCCACGAAACTACC	NM_001101	210
	Antisense	1097	CGATCCACACGGAGTACTTG		

4.6. Statistical analysis

Student's *t*-test was performed to calculate the statistical significance of the difference between the means of the control and MTX-treated Caco-2 cells, HT-1080 cells and HUVECs. The data indicated in the figures denote mean ± SD values.

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References

Assaraf YG (2006) The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. Drug Resist Updat 9: 227–246.

Assaraf YG (2007) Molecular basis of antifolate resistance. Cancer Metastasis Rev 26: 153–181.

Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, Drake JC, Jolivet J (1985) Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest 76: 907–912.

Chen X, Baker SM, Chen G (2005) Methotrexate induction of human sulfotransferases in Hep G2 and Caco-2 cells. J Appl Toxicol 25: 354–360.

Chen ZS, Robey RW, Belinsky MG, Shchaveleva I, Ren XQ, Sugimoto Y, Ross DD, Bates SE, Kruh GD (2003) Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res 63: 4048–4054.

Choudhuri S, Klaassen CD (2006) Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol 25: 231–259.

Dixit SG, Zingarelli B, Buckley DJ, Buckley AR, Pauletti GM (2005) Nitric oxide mediates increased P-glycoprotein activity in interferon-{gamma}-stimulated human intestinal cells. Am J Physiol Gastrointest Liver Physiol 288: G533–G540.

Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res 59: 2532–2535.

Horie T, Li T, Ito K, Sumi S, Fuwa T (2006) Aged garlic extract protects against methotrexate-induced apoptotic cell injury of IEC-6 cells. J Nutr 136: 861S–863S.

Ikeda K, Utoguchi N, Makimoto H, Mizuguchi H, Nakagawa S, Mayumi T (1999) Different reactions of aortic and venular endothelial cell monolayers to histamine on macromolecular permeability: role of cAMP, cytosolic Ca2+ and F-actin. Inflammation 23: 87–97.

König J, Hartel M, Nies AT, Martignoni ME, Guo J, Büchler MW, Friess H, Keppler D (2005) Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. Int J Cancer 115: 359–367.

Lesuffleur T, Barbat A, Dussaulx E, Zweibaum A (1990) Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. Cancer Res 50: 6334–6343.

Li WW, Takahashi N, Jhanwar S, Cordon-Cardo C, Elisseyeff Y, Jimeno J, Faircloth G, Bertino JR (2001) Sensitivity of soft tissue sarcoma cell lines to chemotherapeutic agents: identification of ecteinascidin-743 as a potent cytotoxic agent. Clin Cancer Res 7: 2908–2911.

Prime-Chapman HM, Fearn RA, Cooper AE, Moore V, Hirst BH (2004) Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells. J Pharmacol Exp Ther 311: 476–484.

Schwarzenbach H (2002) A diagnostic tool for monitoring multidrug resistance expression in human tumor tissues. Anal Biochem 308: 26–33.

Srimatkandada S, Schweitzer BI, Moroson BA, Dube S, Bertino JR (1989) Amplification of a polymorphic dihydrofolate reductase gene expressing an enzyme with decreased binding to methotrexate in a human colon carcinoma cell line, HCT-8R4, resistant to this drug. J Biol Chem 264: 3524–3528.

Volk EL, Schneider E (2003) Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. Cancer Res 63: 5538–5543.

Wielinga P, Hooijberg JH, Gunnarsdottir S, Kathmann I, Reid G, Zelcer N, van der Born K, de Haas M, van der Heijden I, Kaspers G, Wijnholds J, Jansen G, Peters G, Borst P (2005) The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. Cancer Res 65: 4425–4430.

Xia CQ, Liu N, Yang D, Miwa G, Gan LS (2005) Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. Drug Metab Dispos 33: 637–643.

Zhang CY, Feng YX, Yu Y, Sun WJ, Bai J, Chen F, Fu SB (2006) The molecular mechanism of resistance to methotrexate in mouse methotrexate-resistant cells by cancer drug resistance and metabolism SuperArray. Basic Clin Pharmacol Toxicol 99: 141–145.