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ORIGINAL ARTICLE

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Molecular changes to HeLa cells on continuous exposure to cisplatin or paclitaxel

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Abstract *Objective*: To achieve a reversal of multidrug resistance (MDR) in cancer chemotherapy, it is crucial to clarify the characteristics of MDR cells generated by various types of chemotherapeutic agents and to find novel targets. *Methods*: Cisplatin- and paclitaxel-resistant HeLa sublines (HeLa/CDDP and HeLa/TXL, respectively) were established by continuous exposure and their cellular changes were examined based on growth inhibition assays, the transport activity of P-glycoprotein/MDR1, and a RT-PCR analysis of MDR-related factors. *Results*: HeLa/CDDP cells showed cross-resistance to platinum derivatives, whereas HeLa/TXL cells were resistant to a variety of MDR1 substrates. Transport activity of MDR1 was reduced in HeLa/CDDP cells and the expression of MDR1 was significantly accelerated in HeLa/TXL cells, compared with HeLa cells. In addition, the expression levels of MDR-related transporters (MRP1–5 or BCRP), βtubulin which is a target for taxanes, and apoptosis-regulated factors were comparable among the three cell lines. On the other hand, the mRNA levels of γ-glutamyl transferase, but not γ-glutamyl cysteine synthetase, were higher in HeLa/CDDP cells than in HeLa and HeLa/TXL cells. *Conclusions*: HeLa/CDDP cells showed decreased activity and expression of MDR1 and overexpression of $\tilde{\gamma}$-GT but not $\tilde{\gamma}$-GCS, whereas the activity of MDR1 in HeLa/TXL cells was significantly enhanced. Thus, the molecular changes to HeLa cells caused by continuous exposure to cisplatin or paclitaxel were in part clarified, and therefore an understanding of the cellular changes induced by chemotherapeutic agents will be necessary to establish a strategy for reversing MDR.

Keywords Cisplatin · Paclitaxel · Multidrug resistance · MDR1/P-glycoprotein · HeLa cell

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Introduction

Cisplatin is one of the most potent chemotherapeutic agents, displaying clinical activity against various solid tumors. Therefore, the development of resistance to cisplatin is a major obstacle in clinical treatment. Possible mechanisms of acquired resistance to cisplatin have been reported [1–3], i.e., reduced intracellular accumulation of cisplatin, enhanced drug inactivation by metallothionine and glutathione, increased repair activity of DNA damage, and altered expression of oncogenes and regulatory proteins.

On the other hand, multidrug resistance (MDR) is also a serious problem [4] and a way of reversing it is urgently needed. MDR is defined as resistance to various types of chemotherapeutic agents which differ comprehensively in mechanisms of action and molecular structure, for example, paclitaxel, vinblastine, etoposide, doxorubicin. Some of the factors responsible for MDR [5–7] include: (1) acceleration of the efflux of chemotherapeutic agents, (2) decrease in uptake of chemotherapeutic agents, (3) changes in quality and/or quantity at target sites (topoisomerase, tubulin, etc.), (4) enhancement of detoxification , and (5) suppression of apoptosis. Among them, the overexpression of P-glycoprotein/MDR1, multidrug resistance-associated protein (MRP), breast cancer resistance protein (BCRP) in tumor cells has attracted attention [8–10]. These proteins are all of ATP binding-cassette (ABC) transporters, and considered as efflux pumps of chemotherapeutic agents.

Thus, a variety of investigations on modulators against drug resistance have been carried out in basic and clinical research [4]. Regrettably, such modulators are still not in clinical use. To develop a technique of reversing drug resistance, especially MDR, it is crucial to clarify the characteristics of resistant cells generated by various chemotherapeutic agents and to find a novel target.

In the present study, we concentrated on cisplatin and paclitaxel, as both are widely used in clinical practice, and cisplatin- and paclitaxel-resistant HeLa sublines have been established. Using these HeLa sublines, the molecular changes to HeLa cells following continuous exposure to cisplatin or paclitaxel were examined.

Materials and methods

Chemicals

Nedaplatin (lot. no. 1001) was a gift from Shionogi & Co. (Osaka, Japan). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). Rhodamine123 was purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other agents were obtained commercially and were of analytical grade requiring no further purification.

Cells and cell culture

The human cervical carcinoma cell line HeLa (passage no. 396–401) was maintained in a culture medium consisting of Dulbecco's modified Eagle's medium (D-MEM; Cat. no. 12800-017, Invitrogen, Corp., Carlsbad, CA, USA) supplemented with 10% FBS (lot. no. 99H2314, Sigma-Ardrich Chemical, Co., St Louis, MO, USA) and 100 mg/l kanamycin sulfate (Invitrogen). HeLa cells (4×10^4 cells/cm^2) were seeded into culture flasks, grown in a humidified atmosphere of 5%CO_2–95% air at 37°C, and subcultured with 0.05% trypsin–0.02% EDTA (Invitrogen).

Establishment of cisplatin- or paclitaxel-resistant HeLa sublines

Continuous exposure to cisplatin or paclitaxel was carried out as follows. In the case of cisplatin, HeLa cells were cultured in complete D-MEM including 1 μM cisplatin for about 3 months. The concentration of cisplatin to which cells were exposed was initially 1 μM. After that, a subline possibly tolerant to 1 μM cisplatin was isolated, and the clone was designated HeLa/CDDP cells. On the other hand, paclitaxel-resistant HeLa cells were isolated by continuous exposure to 20 nM paclitaxel followed by exposure of 10 nM paclitaxel for about 3 months. The clone obtained was named HeLa/TXL.

HeLa/CDDP or HeLa/TXL cells (4×10^4 cells/cm^2) were seeded into culture flasks, grown in a humidified atmosphere of 5%CO_2–95% air at 37°C, and subcultured with 0.05% trypsin–0.02% EDTA (Invitrogen). HeLa/CDDP and HeLa/TXL cells were maintained in D-MEM containing 1 μM cisplatin and 20 nM paclitaxel, respectively.

WST-1 colorimetric assay

The effects of chemotherapeutic agents on the growth of HeLa, HeLa/CDDP and HeLa/TXL cells were evaluated with the WST-1 assay [7, 11]. Cells (1,000 cells/well) were seeded into 96-well plates in 100 μl of D-MEM without any drugs. After a 24 h pre-culture, the medium was aspirated off, and exchanged for one containing a test chemotherapeutic agent at various concentrations. After incubation for 72 h at 37°C, D-MEM was exchanged for 110 μl of medium containing WST-1 reagent (10 μl WST-1 + 100 μl D-MEM); 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a Spectra Fluor microplate reader (Tecan Switzerland, Switzerland). The 50% growth inhibitory concentration (IC_{50}) of chemotherapeutic agents was estimated according to the sigmoid inhibitory effect model, $E = E_{max} \times [1\text{-}C^{\gamma}/(C^{\gamma} + IC_{50}^{\gamma})]$, using the non-linear least-squares fitting method (Solver, Microsoft® Excel 2001 for Macintosh). E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively; C and γ represent the concentration in the medium and the sigmoidicity factor, respectively.

Transport experiments with Rhodamine123

In the uptake experiments, HeLa cells (2×10^5 cells) were seeded into 24-well plates in 1 ml/well of D-MEM, and incubated for 48 h in a humidified atmosphere of 5% CO_2–95% air at 37°C [12–14]. After pre-culture, cells were washed three times with a warmed Hanks' balanced salt solution containing 25 mM HEPES (HBSS), and then the uptake experiments were started by the addition of fresh HBSS containing 10 μM Rhodamine123, a substrate for MDR1, and further incubated for specific periods at 37°C.

In the efflux experiments, cells were pre-cultured as described for the uptake experiments. They were then washed three times with warmed HBSS and incubated in fresh HBSS containing 10 μM Rhodamine123 for 60 min (loading time). After loading, HBSS was immediately removed from the wells, and cells were washed rapidly twice with ice-cold HBSS. Efflux experiments were started by the addition of fresh warmed HBSS, and further incubated for given periods at 37°C. In addition, 10 μM ciclosporin (CsA), which is a representative substrate/inhibitor for MDR1, was added to the reaction

buffer, in order to clarify the contribution of MDR1 in the uptake and efflux experiments.

Uptake and efflux experiments were stopped by aspirating the HBSS from the well, followed by washing three times with ice-cold phosphate buffered saline. After the experiments were stopped, cells were solubilized with 1 ml of 0.3 M NaOH, and aliquots (500 µl) were neutralized with 500 µl of 0.3 M HCl. Aliquots (200 µl) of the neutralized cell lysate solution were transferred to 96-well black plates, and the fluorescence intensity of Rhodamine123 was measured with an excitation wavelength of 485 nm and emission wavelength of 535 nm using Spectra Fluor (Tecan). Protein content was determined by the Lowry method [15], and bovine serum albumin was used as the standard.

RT-PCR

HeLa, HeLa/CDDP and HeLa/TXL cells (2×10^5 cells) were seeded on plastic culture dishes (60 mm in diameter) in 5 mL of D-MEM. Cells were precultured for 48 h, and then total RNA from the cells was extracted using a Mammalian Total RNA Miniprep kit (Sigma-Aldrich). RT-PCR was carried out using an RNA PCR kit (AMV) ver. 2.1 (Takara Bio, Inc., Shiga, Japan) and a thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, Inc., CA, USA) [12–14, 16]. The PCR primers used are listed in Table 1 [13, 16–24], and were synthesized by Proligo Japan K.K. (Kyoto, Japan). β_2-microglobulin (B2M) was used as internal standard. PCR amplification was initiated by one cycle of 94°C for 2 min followed by 22–34 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s. The number of PCR cycles is indicated in Table 1. PCR products were separated on Tris-acetate-EDTA 3% agarose gels containing 100 ng/ml ethidium bromide, and the band densities were measured using the computer program NIH Image ver. 1.63 (National Institutes of Health, Bethesda, MD), and the ratio of band density (target gene/B2M) was calculated.

Western blotting

HeLa and the resistant cells were harvested, lysed with CelLytic™-M (Sigma-Aldrich) and centrifuged at 10,000 rpm (9,100*g*) at 4°C for 15 min. The supernatant was collected. The total protein concentration was determined according to the Bradford method [25] using bovine γ-globulin as the standard (Bio-Rad Laboratories, Hercules, CA, USA). An aliquot of protein (50 or 20 µg) was loaded in each lane to detect the expression of MDR1 or β-actin (BA), electrophoresed on a 7.5% SDS-polyacrylamide gel by the method of Laemmli [26], and transferred to a polyvinylidene difluoride (PVDF) membrane Immun-Blot (pore size 0.2 µm, Bio-Rad). For immunoblotting, the membranes were blocked with 5% skim milk (Wako Pure Chemical Co., Osaka, Japan) in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 137 mM NaCl and 0.1% Tween 20) at 37°C for 1 h. The blots were incubated with anti-MDR1 monoclonal antibody C219 (1:200, Zymed Laboratories Inc., South San Francisco, CA, USA) or anti-BA monoclonal antibody (1:5,000, Sigma-Aldrich) for 2 h, and then with horseradish peroxidase (HRP)-linked whole sheep antibody to mouse IgG (NA931, Amersham Biosciences) as a secondary antibody for 1 h, and washed five times with

Table 1 Sequences of oligonucleotide primers used and the number of cycles for PCR

Gene	Forward (5′-3′ orientation)	Reverse (3′-5′ orientation)	Base pair[a]	Cycle
B2M	ACCCCCACTGAAAAAGATGA	ATCTTCAAACCTCCATGATG	305–418	22
MDR1	CCCATCATTGCAATAGCAGG	GTTCAAACTTCTGCTCCTGA	2,712–2,868	27
SXR	TCCGGAAAGATCTGTGCTCT	CACTCCCAGGTTCCAGTCTC	1,187–1,484	27
MRP1	ATCAAGACCGCTGTCATTGG	GAGCAAGGATGACTTGCAGG	1,379–1,559	27
MRP2	CTGCCTCTTCAGAATCTTAG	CCCAAGTTGCAGGCTGGCC	4,072–4,312	27
MRP3	GATACGCTCGCCACAGTCC	CAGCCGCTTCAGTTGCCGTG	3,198–3,459	27
MRP4	CCATTGAAGATCTTCCTGG	GGTGTTCAATCTGTGTGC	3,494–3,732	27
MRP5	GGATAACTTCTCAGTGGG	GGAATGGCAATGCTCTAAAG	4,100–4,480	27
BCRP	TGCCCAGGACTCAATGCAACAG	ACAATTTCAGGTAGGCAATTGTG	2,012–2,183	27
βtubulin class I	CCATACATACCTTGAGGCGA	GCCAAAAGGACCTGAGCGAA	103–390	25
βtubulin class II	CGCATCTCCGAGCAGTTCAC	TCGCCCTCCTCCTCCTCGA	1,338–1,525	30
βtubulin class III	CTGCTCGCAGCTGGAGTGAG	CATAAATACTGCAGGAGGGC	1,414–1,553	30
βtubulin class IVa	TCTCCGCCGCATCTTCCA	TCTGGGGACATAATTTCCTC	32–293	34
βtubulin class Ivb	CAACAGCACGGCCATCCAGG	CTTTCCCCAGTGACTGAAGG	1,181–1,434	25
γ-GCS*h*	AGAGAAGGGGGAAAGGACAA	GTGAACCCAGGACAGCCTAA	329–559	25
γ-GCS*l*	TCAGTCCTTGGAGTTGCACA	AAATCTGGTGGCATCACACA	596–852	25
γ-GT	AATGGACGACTTCAGCTCTCC	AGCCGAACCAGAGGTTGTAGA	1,627–1,843	30
BCL2	ATGTGTGTGGAGAGCGTCAACC	TGAGCAGAGTCTTCAGAGACAGCC	858–1,053	27
BAX	CTTTTGCTTCAGGGTTTCA	GATGGTCACGGTCTGCCAC	52–504	27

[a]Area amplified refers to sequences deposited at GenBank with accession numbers as follows: BC032589 (B2M), AF016535 (MDR1), AY091855 (SXR), L05628 (MRP1), U63970 (MRP2), Y17151 (MRP3), AY081219 (MRP4), U83661 (MRP5), AF098951 (BCRP), AF141349 (βtubulin class I), BC001352 (βtubulin class II), BC000748 (βtubulin class III), BC013683 (βtubulin class IVa), BC029529 (βtubulin class IVb), M90656 (γ-GCS*h*), L35546 (γ-GCS*l*), BC025927 (γ-GT), BC027258 (BCL2), and AJ417988 (BAX)

PBS-T. Except where stated specifically, all washing and incubation steps were performed at ambient temperature. MDR1 and BA were detected with the HRP chemiluminescent reaction (Immobilon™ Western Chemiluminescent HRP Substrate, Millipore Corp., Billerica, MA, USA) according to the manufacturer's instructions. Blots were then exposed to a Polaroid film using an ECL Mini-Camera (Amersham Biosciences), and the bands obtained were quantified with NIH Image ver. 1.63.

Statistical analysis

Comparisons between two and among more than three groups were performed with Student's unpaired *t* test (transport experiments) and the repeated one-way analysis of variance (ANOVA) followed by the Bonferroni test (RT-PCR analysis), and *P* values of less than 0.05 (two-tailed) were considered significant.

Results

Growth inhibitory effects of chemotherapeutic agents

The IC_{50} values for various chemotherapeutic agents and the relative resistance (R.R.) are summarized in Table 2. The IC_{50} value for cisplatin was higher in HeLa/CDDP cells than in HeLa cells, indicating ca. 2.6-fold greater resistance. In addition, resistance to carboplatin and nedaplatin was increased by about 2.4- and 2.8-fold, respectively. Although the sensitivity to daunorubicin, mitomycin C, bleomycin and paclitaxel was enhanced slightly in HeLa/CDDP cells, the cytotoxicities of other chemotherapeutic agents were comparable to those in HeLa cells.

In the case of HeLa/TXL cells, no resistance to platinum derivatives was observed, but the IC_{50} value for paclitaxel was higher than in HeLa cells, indicating ca. 15-fold greater resistance (Table 2). Furthermore, HeLa/TXL cells showed an increase in resistance to doxorubicin, daunorubicin, pirarubicin, mitomycin C, actinomycin D, aclarubicin, bleomycin, etoposide, vincristine and vinblastine of ca. 2- to 6.6-fold. No resistance to other chemotherapeutic agents was found.

Transport characteristics of Rhodamine123

The uptake of Rhodamine123 by HeLa and the resistant cells was dependent on time, with a steady state reached at 60 min in HeLa and HeLa/CDDP cells and at 30 min in HeLa/TXL cells (Fig. 1). The uptake by HeLa/CDDP cells was greater than that by HeLa cells (Fig. 1b), and the addition of CsA had no remarkable effect. On the other hand, the uptake of Rhodamine123 was significantly lower in HeLa cells than in HeLa/TXL cells (Fig. 1c). This decrease was reversed in the presence of 10 μM CsA, resulting in an uptake comparable to that in HeLa cells.

The efflux of Rhodamine123 from each cell line was time-dependent (Fig. 2), and the residual amount of Rhodamine123 was larger in HeLa/CDDP cells and lower in HeLa/TXL cells than in HeLa cells (Fig. 2b, c). The efflux from HeLa and the resistant cells was inhibited in the presence of 10 μM CsA.

Table 2 IC_{50} values for chemotherapeutic agents in HeLa, HeLa/CDDP and HeLa/TXL cells

Anticancer drug	HeLa	HeLa/CDDP		HeLa/TXL	
	IC_{50}	IC_{50}	R.R.[a]	IC_{50}	R.R.
Cisplatin	1.34 ± 0.32 μM	3.44 ± 0.89 μM	2.57	1.23 ± 0.50 μM	0.92
Carboplatin	23.5 ± 6.38 μM	56.7 ± 6.38 μM	2.41	24.3 ± 13.8 μM	1.03
Nedaplatin	5.56 ± 0.71 μM	15.5 ± 0.86 μM	2.78	4.19 ± 0.60 μM	0.75
Doxorubicin	33.1 ± 7.5 nM	33.4 ± 9.3 nM	1.00	102 ± 41.0 nM	3.08
Daunorubicin	39.3 ± 8.25 nM	27.7 ± 4.60 nM	0.70	83.0 ± 3.83 nM	2.11
Pirarubicin	62.4 ± 4.15 nM	88.8 ± 9.14 nM	1.42	231 ± 17.8 nM	3.70
Mitomycin C	0.16 ± 0.04 μM	0.13 ± 0.03 μM	0.83	0.39 ± 0.14 μM	2.51
Actinomycin D	0.21 ± 0.10 nM	0.24 ± 0.07 nM	1.15	1.13 ± 0.15 nM	5.36
Aclarubicin	1.48 ± 0.21 nM	1.43 ± 0.39 nM	0.97	3.02 ± 0.30 nM	2.06
Bleomycin	65.4 ± 13.7 μM	54.4 ± 13.6 μM	0.83	355 ± 217 μM	5.43
Etoposide	1.94 ± 0.19 μM	2.42 ± 0.58 μM	1.25	12.8 ± 5.50 μM	6.59
Paclitaxel	1.14 ± 0.36 nM	1.25 ± 0.48 nM	1.10	17.0 ± 5.08 nM	14.9
Vincristine	5.24 ± 0.48 nM	5.93 ± 2.06 nM	1.13	14.3 ± 3.17 nM	2.73
Vinblastine	0.93 ± 0.17 nM	1.75 ± 0.23 nM	1.88	3.86 ± 0.17 nM	4.13
Tamoxifen	6.79 ± 1.96 μM	8.80 ± 1.41 μM	1.30	8.61 ± 2.01 μM	1.27
5-Fluorouracil	4.33 ± 0.76 μM	4.15 ± 0.45 μM	0.96	3.52 ± 0.27 μM	0.81
Cytarabine	34.9 ± 11.2 nM	48.1 ± 31.3 nM	1.38	64.3 ± 34.9 nM	1.84
Cyclophosphamide	3.09 ± 1.13 mM	3.25 ± 0.20 mM	1.05	1.75 ± 0.64 mM	0.57

Each IC_{50} value represents the mean ± SE ($n = 4$)

[a] Relative resistance, the IC_{50} values for the chemotherapeutic agent in HeLa/CDDP or HeLa/TXL cells were divided by that in HeLa cells

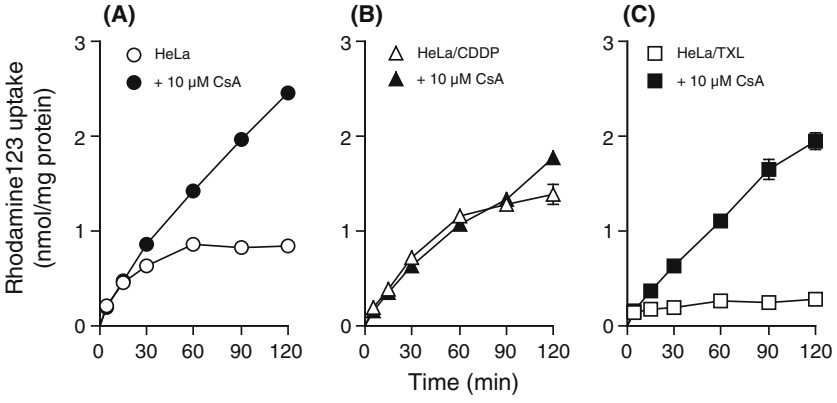

Fig. 1 Time course of Rhodamine123 uptake by HeLa (**a**), HeLa/CDDP (**b**) and HeLa/TXL (**c**) cells in the absence or presence of ciclosporin (CsA). Cells were incubated with 10 μM Rhodamine123 in the absence or presence of 10 μM CsA for the periods indicated at 37°C. *Each point* represents the mean ± SE ($n = 4$–8)

Expression profiles of MDR1 or steroid and xenobiotics receptor (SXR) mRNA

The level of MDR1 mRNA in HeLa/CDDP cells was about half of that in HeLa cells ($P > 0.05$, Fig. 3a). In addition, the level in HeLa/TXL cells was ca. threefold higher than that in HeLa cells (Fig. 3a). Western blotting also indicated that MDR1 expression in HeLa/CDDP and HeLa/TXL cells was 84 or 228%, respectively, that in HeLa cells (Fig. 3b). The expression of steroid and xenobiotics receptor (SXR) was also examined, because MDR1 expression was in part regulated by SXR [27]. However, it was not detected in HeLa, HeLa/CDDP or HeLa/TXL cells (data not shown).

Expression profiles of ABC transporter mRNAs

The expression profiles of MRP1 to 5 and BCRP, which participated in MDR along with MDR1, were evaluated (Fig. 4). The mRNAs of MRP1 to 5 and BCRP were found in HeLa cells. In addition to HeLa cells, MRP1 to 5 and BCRP mRNAs were expressed in HeLa/CDDP and HeLa/TXL cells, and expression levels were comparable among the three cell lines.

Expression profiles of glutathione-related enzyme mRNAs

Table 3 summarizes the expression levels of glutathione-related enzymes, which are involved in the detoxification of cisplatin, in HeLa, HeLa/CDDP and HeLa/TXL cells. The mRNAs of γ-glutamyl cysteine synthetase heavy unit (γ-GCS*h*), γ-glutamyl cysteine synthetase light unit (γ-GCS*l*) and γ-glutamyl transferase (γ-GT) were expressed in HeLa, HeLa/CDDP and HeLa/TXL cells, and the levels of γ-GCS*h* and γ-GCS*l* mRNAs were similar among the cells. However, the level of γ-GT in HeLa/CDDP cells was ca. 4.5- and 3-fold higher than that in HeLa and HeLa/TXL cells, respectively.

Expression profiles of βtubulin isotype mRNAs

The expression level of βtubulin, a target of taxanes and *vinca* alkaloids, was compared among HeLa, HeLa/CDDP and HeLa/TXL cells (Table 3). The mRNAs of βtubulin isotypes examined here were expressed in HeLa and the resistant cells. Although the levels of βtubulin class III and class IVa tended to increase and decrease, respectively, in both HeLa/CDDP and HeLa/TXL cells,

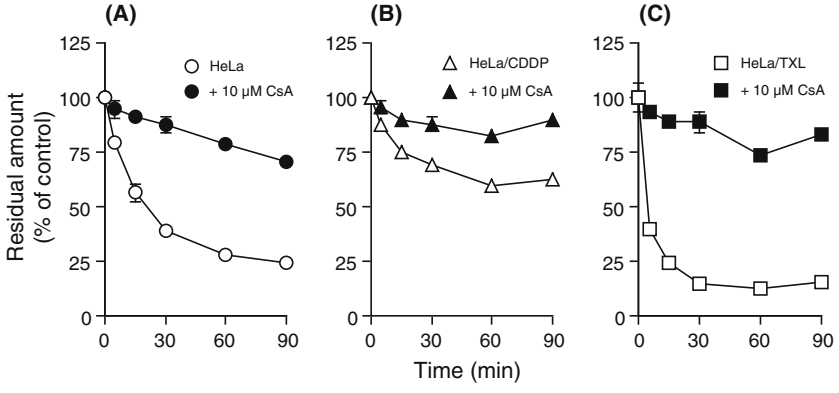

Fig. 2 Time course of Rhodamine123 efflux from HeLa (**a**), HeLa/CDDP (**b**) and HeLa/TXL (**c**) cells in the absence or presence of CsA. After cells were loaded with 10 μM Rhodamine123 in the absence or presence of 10 μM CsA for 60 min at 37°C, they were washed twice with ice-cold HBSS and incubated with warmed HBSS in the absence or presence of 10 μM CsA for the periods indicated. *Each point* represents the mean ± SE ($n = 4$)

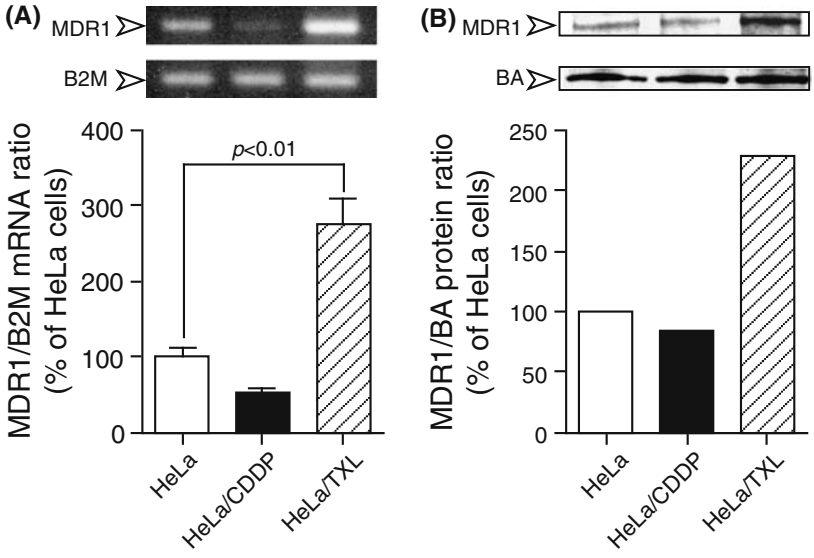

Fig. 3 Expression profiles of MDR1 in HeLa, HeLa/CDDP and HeLa/TXL cells. **a** Total RNA was extracted from HeLa and the resistant cells, and MDR1 and B2M mRNA expression was evaluated by RT-PCR. A representative electrophoretogram is presented. *B2M* represents an internal standard gene. Data are presented as a percentage of the MDR1/B2M level in HeLa cells. *Each bar* represents the mean ± SE ($n = 3$). **b** Total protein was extracted from HeLa and the resistant cells, and electrophoresed on a 7.5% SDS-polyacrylamide gel. For immunoblotting, the blots were incubated with anti-MDR1 (C219) or anti-BA antibodies. *BA* was used as the reference protein. Data are presented as a percentage of the MDR1/BA protein level in HeLa cells on a representative immunoblot

the expression levels of βtubulin isotypes were not significantly different among the three cell lines.

Expression profiles of apoptosis-regulated factor mRNAs

The mRNA levels of BCL2 and BAX, which are apoptosis-regulated factors, were compared among HeLa, HeLa/CDDP and HeLa/TXL cells (Table 3). Both mRNAs were expressed in all three cell lines and at comparable levels among the cells.

Discussion

The IC_{50} value for cisplatin was higher in HeLa/CDDP cells than in HeLa cells, indicating a ca. 2.6-fold increase in resistance (Table 2). In addition, the resistance to carboplatin and nedaplatin was increased by about 2.4- and 2.8-fold, respectively. Although the sensitivity to daunorubicin, mitomycin C, bleomycin or paclitaxel was slightly enhanced in HeLa/CDDP cells, the cytotoxicity of the other chemotherapeutic agents was comparable to that in HeLa cells. In the case of HeLa/TXL cells, no resistance to platinum derivatives was observed, but the IC_{50} value for paclitaxel was higher than in HeLa cells, showing a ca. 15-fold increase in resistance (Table 2). Also, HeLa/TXL cells showed an increase in resistance to doxorubicin, daunorubicin, pirarubicin, mitomycin C, actinomycin D, aclarubicin, bleomycin, etoposide, vincristine and vinblastine by about 2- to 6.6-fold. However, no resistance to other chemotherapeutic agents was found. Collectively, the spectrum of sensitivity to chemotherapeutic agents was different between HeLa/CDDP and HeLa/TXL cells, suggesting the existence of different cellular changes in them.

Focusing on MDR1, a major factor of MDR in cancer chemotherapy, the transport activity was compared using Rhodamine123 between HeLa and the resistant cells (Figs. 1, 2). Rhodmaine123 uptake in HeLa/CDDP cells under steady state conditions was greater than that in HeLa cells, and not affected by the presence of 10 μM CsA that is a representative substrate/inhibitor for MDR1 (Fig. 1). In addition, the efflux of Rhodamine123 from HeLa/CDDP cells decreased compared with that from HeLa cells (Fig. 2). On the other hand, the uptake of Rhodamine123 by HeLa/TXL cells under steady state conditions was about one-third of that by HeLa cells (Fig. 1). This reduced uptake was reversed by the addition of CsA, resulting in a level comparable to that in HeLa cells in the presence of CsA. The efflux from HeLa/TXL cells was also accelerated compared with that from HeLa cells (Fig. 2). In addition, MDR1 mRNA levels in HeLa/CDDP and HeLa/TXL cells were about half-fold and threefold the level in HeLa cells, respectively (Fig. 3a), and these results were confirmed by immunoblotting (Fig. 3b). To clarify why the change in MDR1 mRNA expression differed between HeLa/CDDP and HeLa/TXL cells, the expression profile of SXR mRNA [16, 27, 28] was examined. The mRNA was not detected in HeLa or the resistant cells (data not shown), suggesting no participation of SXR into MDR1 expression in the three cells. From these findings, it was clarified that

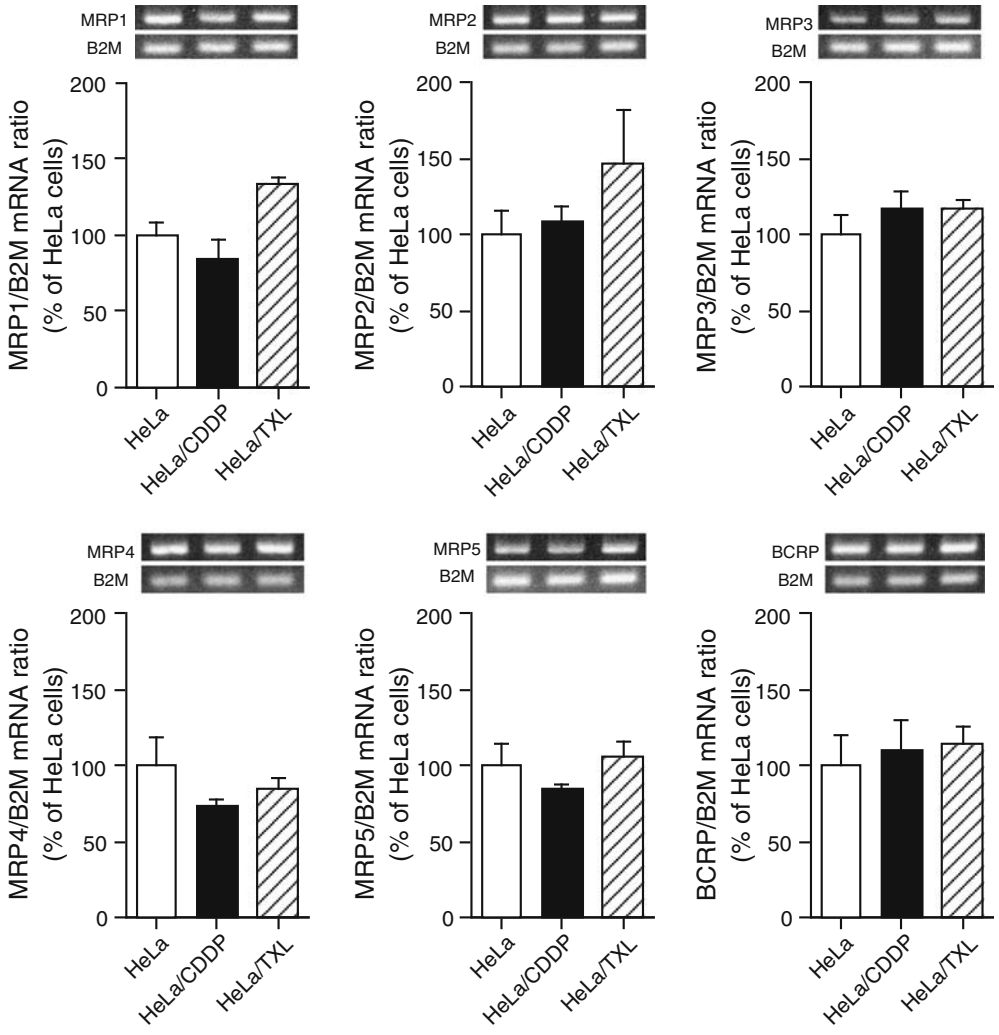

Fig. 4 Expression profiles of MRP1 to 5 and BCRP mRNAs in HeLa, HeLa/CDDP and HeLa/TXL cells. Total RNA was extracted from HeLa and the resistant cells, and the target gene and B2M mRNA expression was evaluated by RT-PCR. Representative electrophoretograms are presented. *B2M* represents an internal standard gene. Data are presented as a percentage of target gene/B2M levels in HeLa cells. *Each bar* represents the mean ± SE ($n = 3$)

the MDR1-mediated transport decreased in HeLa/CDDP cells, possibly via the downregulation of MDR1 mRNA expression but independent of SXR. Thus, it was demonstrated for the first time that the downregulation of MDR1 expression was induced by continuous exposure to cisplatin, implying that chemotherapeutic agents which are MDR1 substrates, may be effective against cisplatin-resistant tumors. On the other hand, the

Table 3 Expression profiles of target gene mRNAs in HeLa, HeLa/CDDP and HeLa/TXL cells

Target gene	HeLa	HeLa/CDDP Percentage of HeLa cells	HeLa/TXL Percentage of HeLa cells
Glutathione-related enzyme			
γ-GCS*h*	100 ± 12.2	145 ± 3.42	125 ± 15.1
γ-GCS*l*	100 ± 22.1	113 ± 15.7	92 ± 19.0
γ-GT	100 ± 23.9	453 ± 85.5*	180 ± 6.70
βtubulin isotype			
βtubulin I	100 ± 13.2	100 ± 5.44	91 ± 9.58
βtubulin II	100 ± 24.3	107 ± 2.65	102 ± 2.72
βtubulin III	100 ± 38.3	154 ± 21.7	159 ± 30.9
βtubulin IVa	100 ± 16.5	74 ± 6.95	64 ± 8.47
βtubulin IVb	100 ± 16.5	111 ± 17.8	99 ± 1.51
Apoptosis-regulated factor			
BCL2	100 ± 9.32	82 ± 7.29	81 ± 6.10
BAX	100 ± 5.61	112 ± 10.5	94 ± 6.80

Each value represents the mean ± SE ($n = 3$)
γ-GCS*h*: γ-glutamyl cysteine synthetase heavy unit; γ-GCS*l*: γ-glutamyl cysteine synthetase light unit; and γ-GT: γ-glutamyl transferase
*$P < 0.01$ significantly different from HeLa cells

MDR1-mediated transport in HeLa/TXL cells was accelerated, presumably via the upregulation of MDR1 mRNA expression but independent of SXR. Thus, continuous exposure to paclitaxel is considered to induce classical MDR, i.e., the overexpression of MDR1.

As the MRP family and BCRP are well-known multidrug transporters, the expression profiles of MRP1 to 5 and BCRP mRNAs were compared among HeLa, HeLa/CDDP and HeLa/TXL cells (Fig. 4). All of these transporters were expressed in the cells and the level of each was comparable among the cell lines, suggesting that MRP1 to 5 and BCRP did not participate in the resistance mechanism of either HeLa/CDDP or HeLa/TXL cells. Kool et al. [21] reported that MRP1, MRP2 and MRP5 were mainly overexpressed in various types of cisplatin-resistant cells, in contrast to the present findings. However, the expression of the MRP family was also reported to be unchanged or downregulated in some cisplatin-resistant cells in the same report [21], suggesting that the overexpression of the MRP family was dependent on the type of cell.

Next, the change in glutathione-related enzymes, which are known to participate in cisplatin resistance, was examined (Table 3). The expression of γ-GCS*h* and γ-GCS*l* was similar among HeLa, HeLa/CDDP and HeLa/TXL cells. It was reported that the overexpression or transduction of γ-GCS resulted in resistance to platinum derivatives [29–31], whereas Tipnis et al. [32] indicated that the overexpression of γ-GCS was not significant to resistance to cisplatin in HeLa cells. The present findings were in part supported by the latter. On the other hand, γ-GT mRNA expression was ca. 4.5 and 3-fold higher in HeLa/CDDP cells than in HeLa and HeLa/TXL cells, respectively (Table 3). $\tilde{\gamma}$-GT plays a role in the degradation of glutathione in the extracellular space and re-synthesis of glutathione, and thus its increase was reported to generate resistance to platinum derivatives [29, 33]. Also, a decrease in sensitivity to platinum derivatives was detected in $\tilde{\gamma}$-GT cDNA-transfected cells [34]. Consequently, the upregulation of γ-GT expression was considered to contribute to the resistance to platinum derivatives in HeLa/CDDP cells. Presumably, it was considered that the overexpression of $\tilde{\gamma}$-GT increased the intracellular concentration of glutathione, and the glutathione-mediated detoxification was accelerated.

Furthermore, changes in quality and/or quantity at the sites targeted by chemotherapeutic agents were evaluated in the HeLa and resistant cells (Table 3). βtubulin is a target of taxanes and *vinca* alkaloids, and has several isotypes [35]. The levels of βtubulin isotype mRNAs were comparable to those in HeLa, HeLa/CDDP and HeLa/TXL cells, suggesting no participation of them into the resistance.

Finally, the possibility of resistance to apoptosis was examined in HeLa, HeLa/CDDP and HeLa/TXL cells (Table 3). Although it was reported that apoptosis was suppressed in MDR1-overexpressing cells [36–38], the mRNA levels of BCL2 and BAX, which are apoptosis-regulated factors, were comparable between HeLa and the resistant cells. The present findings suggested that the induction of apoptosis did not change in these cells.

Summarizing the present findings, the upregulation of $\tilde{\gamma}$-GT but not $\tilde{\gamma}$-GCS expression was suggested to be part of the mechanism of resistance in HeLa/CDDP cells. Although numerous reports have demonstrated that glutathione-related enzymes play a role in cisplatin-resistance, Godwin et al. [29] indicated that the expression of $\tilde{\gamma}$-GT mRNA precedes that of $\tilde{\gamma}$-GCS mRNA, and established resistant cells overexpressing $\tilde{\gamma}$-GT. These resistant cells were isolated by exposure to cisplatin at 10–200 μM, but the concentration used in the present study was 1 μM, which is achievable in plasma under clinical conditions. That is, HeLa/CDDP cells showed the possibility of developing resistance mediated by the expression of $\tilde{\gamma}$-GT in the clinical setting, and thus it was suggested that HeLa/CDDP cells were useful as a model of novel $\tilde{\gamma}$-GT-expressing resistant cells. On the other hand, the expression of MDR1 was upregulated and the function of MDR1 enhanced only in HeLa/TXL cells, suggesting that the overexpression of MDR1 was one of the mechanisms of MDR. Although many resistant cells overexpressing MDR1 have been established, these cells were more resistant to chemotherapeutic agents than clinically isolated resistant cells. Therefore, HeLa/TXL cells were considered to be useful as a model of clinically achievable resistant cells.

In conclusion, newly established HeLa/CDDP cells showed decreased activity and expression of MDR1 and overexpression of $\tilde{\gamma}$-GT but not $\tilde{\gamma}$-GCS, whereas the activity of MDR1 was significantly enhanced in HeLa/TXL cells. Consequently, the molecular changes to HeLa cells on continuous exposure to cisplatin or paclitaxel were in part clarified. An understanding of the cellular change caused by chemotherapeutic agents will be necessary to establish a strategy for reversing MDR.

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