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Preclinical report

Glutathione-S-transferase-π expression regulates sensitivity to glutathione–doxorubicin conjugate

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We have reported that glutathione–doxorubicin conjugate (GSH–DXR) exhibited potent cytotoxicity against tumor cells and inhibited glutathione-S-transferase (GST) enzyme activity. In order to determine whether or not the expression of GST-π lowered the cytotoxicity of GSH–DXR, cytocidal activity of the conjugate was examined using tumor cells in which the level of GST-π expression was regulated by transfecting GST-π cDNA in the correct or reverse direction and comparing with that of DXR. Enhancement of GST-π expression by transfecting GST-π sense cDNA into human hepatoblastoma HepG2 cells in which GST-π expression was extremely low caused an increase in GST activity from 0.26 to 55.0 nmol/mg/min and a marked reduction in transfectant sensitivity to GSH–DXR to 1/120 (0.15–18 nM IC_{50}) although the sensitivity to DXR was slightly decreased to 1/2.6 (380–990 nM IC_{50}). By contrast, a high GST-π-expressing human colon cancer cell line, HT29, showed a decrease in GST enzyme activity from 72.0 to 45.9 nmol/mg/min after transfecting GST-π antisense cDNA and a marked improvement in transfectant sensitivity to GSH–DXR was observed (28–2.9 nM IC_{50}) compared with the transfectant sensitivity to DXR (1020–320 nM IC_{50}). Additionally, the expression of GST-π in HepG2 cells caused a decrease in GSH–DXR-induced activation of caspase-3, which was an apoptotic marker, whereas the suppression of GST-π in HT29 cells showed an increase in caspase-3 activation. These results suggested that the cytocidal efficacy of GSH–DXR, but not that of DXR, was controlled by the level of GST-π expression in the cells.

Key words: Doxorubicin, glutathione–doxorubicin conjugate, glutathione-S-transferase-π, human colon cancer cells HT29, human hepatoblastoma HepG2.

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Introduction

Several mechanisms, either alone or in combination, have been proposed to explain cellular drug resistance. These include overproduction of multidrug resistance (MDR)-related 170 kDa P-glycoprotein (P-gp),[1,2] increase in the glutathione (GSH) content,[3,4] enhanced expression of glutathione-S-transferase (GST)[3–8] and change in topoisomerase II activity[9,10] in the resistant cells. In particular, it has been reported that increased levels of GST-π, one of the GST isozymes, was frequently associated with the emergence of resistance to anticancer agents in some malignant cells.[5,11–14] As reported previously, the drug resistance was reversed by a variety of substances, such as inhibitors or anti-P-gp antibody for the P-gp efflux pump mechanism,[15–19] and inhibitors of GST or of GSH synthetase in the GSH/GST detoxification system.[20–22]

We have reported that a conjugate of doxorubicin (DXR) with bovine serum albumin reversed MDR and markedly increased cytotoxicity against several MDR cell lines.[23,24] Further study demonstrated that a conjugate of DXR with GSH (GSH–DXR, one of the simple forms of the conjugate) reversed MDR, and induced potent cytotoxic activity against DXR-sensitive and -resistant cells.[25–27] However, the mechanism of action of the conjugate in exhibiting the potent cytotoxic efficacy has not been elucidated.

In this study, in order to determine the involvement of GST-π in the cytotoxic mechanism of GSH–DXR, we investigated whether or not the level of GST-π expression regulated drug sensitivity to DXR and GSH–DXR using low GST-π-expressing human hepatoblastoma HepG2 cells and high GST-π-expressing human colon carcinoma HT29 cells transfected with GST-π sense and antisense cDNA, respectively.

Materials and methods

Materials

DXR was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Glutathione (GSH), RNase A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazorium bromide (MTT), 1-chloro-2,4-dinitrobenzene (CDNB) and *o*-phthalaldehyde were obtained from Sigma (St Louis, MO). Acetyl-Asp-Glu-Val-Asp-a-4-methyl-coumaryl-7-amide (DEVD-MCA) and 7-amino-4-methyl-coumarin (AMC) were purchased from Peptide Instrument (Osaka, Japan). All other chemicals were of analytical grade.

Cell lines

Human hepatoblastoma HepG2 cells and human colon carcinoma HT29 cells were cultured with DMEM and RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) (growth medium) under conventional conditions, respectively.[24–27]

Construction of expression vector

The plasmid pGpi-2 containing GST-π cDNA was digested with *Eco*RI and a 0.7 kb *Eco*RI–*Eco*RI fragment containing the whole coding region for GST-π was recovered. The pcDNA3 (origin) vector was linearized with *Eco*RI and dephosphorylated with calf intestine phosphatase. Both of these processed fragments were ligated with DNA ligation kit version 2 (Takara, Tokyo, Japan), and two kinds of clones in which the GST-π cDNA was inserted in the correct or reverse direction were selected. GST-π sense vector was digested into two fragments of 1.37 and 4.78 kb, and its antisense vector was digested into fragments of 1.19 and 4.96 kb by *Bgl*II treatment. The clones were named pcDNA3/GSTπ and pcDNA3/antiGSTπ, respectively.[28]

Transfection of GST-π in the correct and reverse direction into HepG2 cells and HT29 cells, respectively

The transfection of pcDNA3 and pcDNA3/GSTπ into HepG2 cells was performed using non-liposomal FuGENE6 transfection reagent. Briefly, 7.5 μl of FuGENE6 diluted with 250 μl of serum-free DMEM was mixed with 5 μg of pcDNA3 or pcDNA3/GSTπ. After incubation at room temperature for 15 min, the mixture was added to 10-cm culture dishes containing 2×10^5 dispersed cells and the cells were incubated for 48 h. After G418 selection (1 mg/ml), the G418-resistant clones of each cell line were obtained and some clones were designated HepG2/V as the transfectant of pcDNA3 and HepG2/GSTπ as the transfectant of pcDNA3/GSHπ.[28,29] On the other hand, HT29 cells were transfected with pcDNA3 and pcDNA3/antiGSTπ using TransFast Reagent. HT29 cells (2×10^5) were treated with the transfection mixture containing 10 μg of pcDNA3 or pcDNA3/antiGSTπ and 30 μl of TransFast Reagent in 3 ml of serum-free RPMI 1640. The cells were incubated for 1 h at 37°C in a humidified CO_2 incubator. The transfection mixture was replaced with RPMI 1640 containing 10% FBS and the cells were incubated for 48 h. After G418 selection (0.4 mg/ml), the G418-resistant clones of each cell line were obtained and some clones were designated HT29/V as the transfectant of pcDNA3 and HT29/antiGSTπ as the transfectants of pcDNA3/antiGSTπ.

Assay of GST activity

The scraped and washed cells were sonicated in 10 mM sodium phosphate buffer (pH 7.4) and the resultant suspension (50 μg protein) was used as the enzymatic source. GST activity was measured at 340 nm (ε=9600) in 100 mM CDNB, 100 mM GSH and 0.2 M sodium phosphate buffer (pH 6.5) at 37°C for 10 min in the presence or absence of test drugs.[30]

Measurement of cellular GSH concentration

The cell suspensions each gave 50 μg protein which was mixed with 9 M perchloric acid and centrifuged at 10 000 *g* for 15 min. The resultant supernatant was neutralized with sodium hydroxide and incubated with 2 ml of 0.1 M sodium phosphate buffer (pH 8.2) containing 50 μl of 0.1% *o*-phthalaldehyde in methanol at room temperature for 20 min. The mixture was measured by fluorospectrometry at an emission wavelength of 420 nm and an excitation wavelength of 350 nm.[31]

Conjugation of DXR with GSH

GSH–DXR was prepared as described previously.[25] In brief, the combination of 1 mg of GSH and 0.5 mg of DXR in 0.5 ml of 0.15 M NaCl containing 0.1% glutaraldehyde was incubated at room temperature for 30 min. After incubation, GSH–DXR was separated from GSH and DXR using Dowex 50Wx8. The concentration of DXR was measured by absorbance at 495 nm.

Cytotoxicity of DXR and GSH–DXR conjugate

To investigate the growth-inhibitory effect of GSH–DXR conjugate, cells were cultured continuously for 96 h in a 48-well culture plate (Corning Costar) with 0.5 ml of growth medium containing graded equivalent concentrations of DXR with different of GST-π levels in HepG2 and HT29. After incubation, viable cells were determined with the colorimetric assay using MTT as described previously,[32] and the results were expressed by the following equation: survival rate (%)=100 × (absorbance at 570 nm of the drug-exposed cells)/(absorbance at 570 nm of the non-treated control cells).

Assay of caspase-3 activity

Reaction mixtures, that contained 100 μM of DEVD-MCA, the appropriate protein concentration of cell extract, 50 mM HEPES–NaOH (pH 7.5), 10% glycerol and 2 mM dithiothreitol with or without 0.1 μM DEVD-CHO were monitored for AMC liberation at 37°C for 15 min in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.[33] The caspase-3 proteolytic activity was expressed as the difference between nmol AMC liberations in the presence and absence of the inhibitor/min/mg protein.

Results and discussion

To confirm that inhibition of GST-π by treatment with GSH–DXR might play a major role in severe cytotoxicity, conjugate cytotoxicity against GST-π transfectants in the cDNA sense or antisense direction was examined and compared with that of DXR. By transfecting GST-π sense cDNA into HepG2 cells, the expression of GST-π was recognized in HepG2/GSTπ cells as a specific band by Western blot analysis, although the expression of GST-π was not detected in HepG2 and HepG2/V cells (Figure 1a). Total GST activity was also increased in HepG2/GSTπ cells from 0.26 to 55.0 nmol/mg/min (Figure 1b). The IC_{50} value of GSH–DXR was 0.15 nM for HepG2 cells and 18.0 nM for HepG2/GSTπ cells, and the drug sensitivity of HepG2/GSTπ cells to GSH–DXR was markedly reduced to 1/120 by the increased expression of GST-π, although the sensitivity to DXR decreased slightly to 1/2.6 with the expression of GST-π (380–990 nM IC_{50} value) (Figure 1c and Table 1). By contrast, Western blot analysis demonstrated slight suppression of GST-π expression in HT29/antiGSTπ cells as compared with

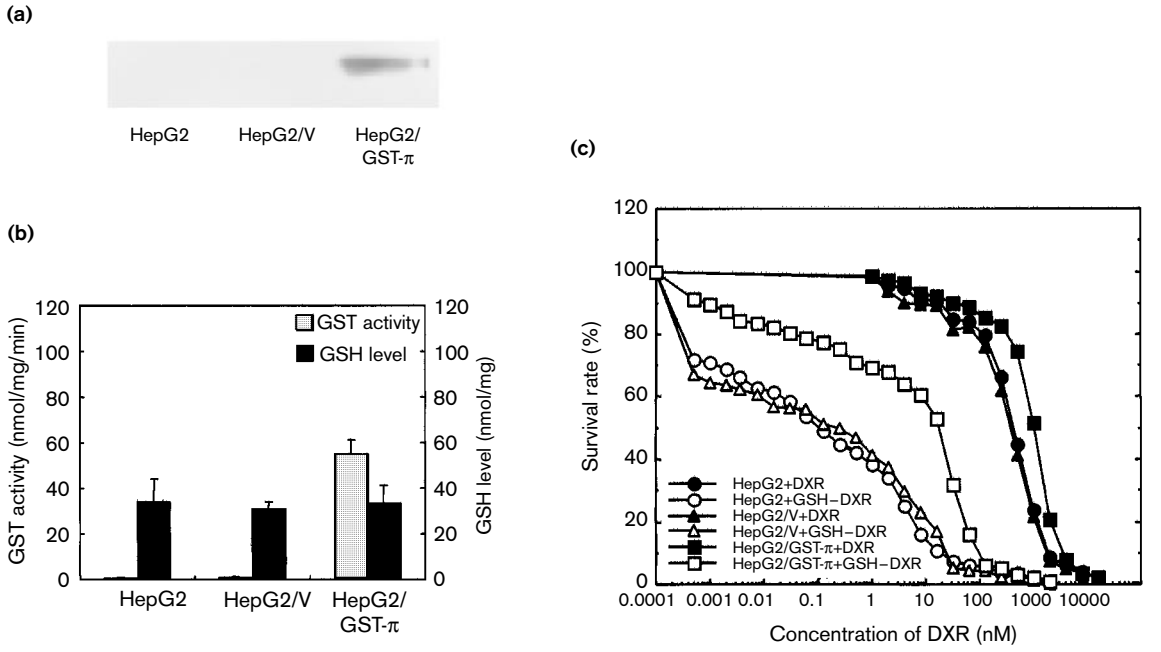

Figure 1. Expression of GST-π (a), and GST activity and GSH level (b) in HepG2 cells transfected using pcDNA3 (HepG2/V) or pcDNA3/GSTπ (HepG2/GSTπ). Cytotoxicity of DXR and GSH–DXR against HepG2, HepG2/V or HepG2/GSTπ cells (c). GST-π was detected by Western blot analysis using anti-human GST-π antibody. The IC_{50} value of each drug is shown in Table 1.

that in HT29 or HT29/V cells (Figure 2a). Total GST activity in HT29/antiGSTπ cells was decreased from 72.0 to 45.9 nmol/mg/min (Figure 2b). The suppression of GST-π expression in HT29/antiGSTπ cells increased remarkably in terms of sensitivity to GSH–DXR to 9.7-fold (28–2.9 nM IC_{50} value) compared with that to DXR (3.2-fold, 1020–320 nM IC_{50} value) (Figure 2c and Table 1). Cells of each transfectant, HepG2/GSTπ and HT29/antiGSTπ, were prepared without any effect on the GSH level (Figures 1b and 2b), showing that the fluctuation of GST-π by transfection with GST-π cDNA in either the sense or antisense direction into the cells caused no significant change in GSH level as reported previously.[12,14,29] Therefore, it was demonstrated that the sensitivity to GSH–DXR was controlled by the level of GST-π expression. Similarly, it has been reported that transfection with GST-π cDNA in the sense and antisense direction resulted in a decrease or an increase in the sensitivity of various cell lines to anticancer drugs, respectively.[12,14,29] Potent cytotoxicity of GSH–DXR was noticed against GST-π-expressed cells in comparison to that of DXR. The result was supported by the findings of our previous study, which showed that GST-π was strongly inhibited by GSH–DXR,[26] but not DXR, and the enzyme inhibition led to expression of potent cytotoxicity of the conjugate.[28] A mechanism proposed to explain the inhibitory effect of GSH–DXR on enzyme activity was that the conjugate interacted with the active center of the GST molecule via the L-cysteine residue of GSH, resulting in inhibition of the enzyme activity, whereas DXR itself did not interact with the GST molecule and did not inhibit the activity.[34] Moreover, a recent report showed that GST-P1-1 did not effectively catalyze the

Table 1. IC_{50} values of DXR and GSH–DXR for HepG2 transfected with GST-π cDNA and HT29 transfected with GST-π antisense cDNA.

Cell line	GST-π expression level (%)	IC_{50} (nM)	
		DXR	GSH–DXR
HepG2	ND	380 ± 67	0.15 ± 0.06
HepG2/V	ND	370 ± 64	0.37 ± 0.35
HepG2/GSTπ	90	990 ± 61	18 ± 2.9
HT29	100	1020 ± 28	28 ± 18
HT29/V	105	850 ± 64	18 ± 4.9
HT29/antiGSTπ	65	320 ± 84	2.9 ± 2.2

HepG2 cells were transfected using the pcDNA3 vector or pcDNA3/GSTπ. HT29 cells were transfected using the pcDNA3 vector or pcDNA3/antiGSTπ. The level of GST-π expression was measured in a densitometer by Western blot analysis of the GST-π band and the level in each transfectant was expressed as 100% of that in the non-transfected HT29 cells. Results are expressed as means ± SD (three independent experiments). ND, not detected.

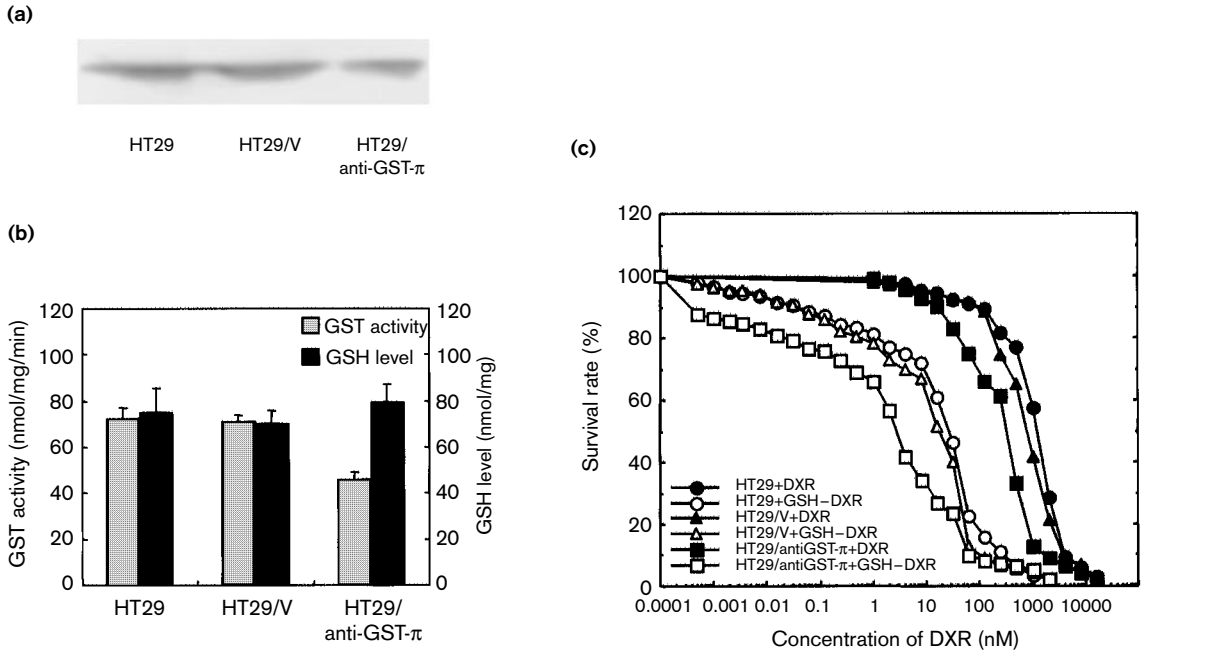

Figure 2. Expression of GST-π (a), and GST activity and GSH level (b) in HT29 cells transfected using pcDNA3 (HT29/V) or pcDNA3/antiGSTπ (HT29/antiGSTπ). Cytotoxicity of DXR and GSH–DXR against HT29, HT29/V or HT29/antiGSTπ cells (c). GST-π was detected by Western blot analysis using anti-human GST-π antibody. The IC_{50} value of each drug is shown in Table 1.

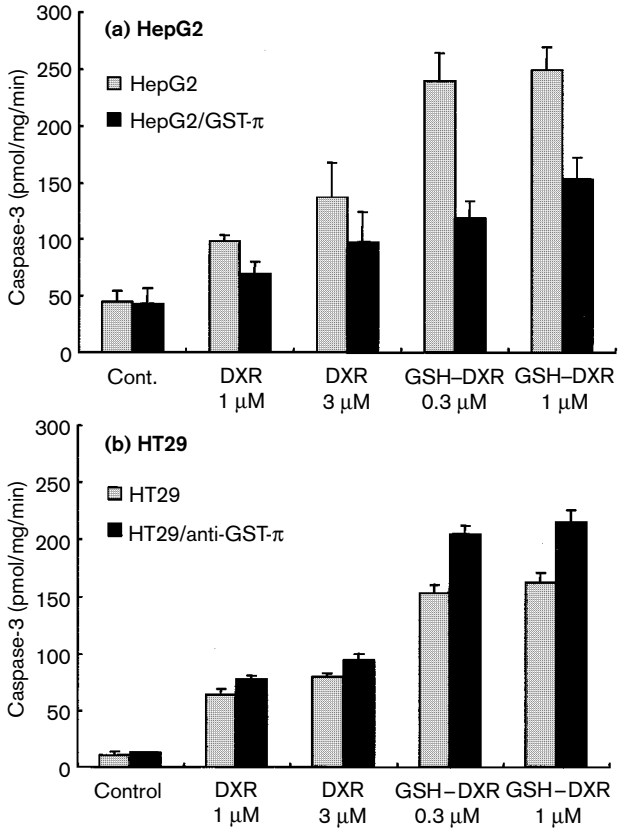

Figure 3. Caspase-3 activity in HepG2 or HepG2/GSTπ cells (a) and HT29 or HT29/GSTπ cells (b) treated with DXR and GSH–DXR for 24 h.

conjugation of DXR with GSH under physiological conditions, although conjugation behaved as a strong inhibitor of the enzyme.[35] Therefore, based on these findings it was suggested that treatment with DXR did not enhance cytocidal efficacy through the inhibition of GST-π.

Additionally, to confirm whether or not drug-induced apoptosis was also regulated by the level of GST-π expression, caspase-3 activity resulting from treatment of the GST-π transfectants with DXR or GSH–DXR was measured. Since the caspase-3 activation correlated well with the ability to induce DNA fragmentation,[27,33] the activity was used as an apoptotic marker. Although the enhancement of GST-π expression in HepG2/GSTπ cells slightly reduced caspase-3 activation by treatment with 3 μM DXR, enhanced expression caused a remarkable decrease in activation resulting from treatment with 0.3 μM GSH–DXR (Figure 3a). By contrast, the suppression of GST-π expression in HT29/antiGSTπ cells increased caspase-3 activity resulting from treatment with 0.3 μM GSH–DXR, whereas treatment of the transfectant with 3 μM DXR did not lead to any significant increase in the activity (Figure 3b). Therefore, it was confirmed that GSH–DXR-induced apoptosis also controlled the fluctuation of GST-π expression. Our previous report suggested that the suppression of GST-π in the cells treated with GSH–DXR must play an important role in the induction of apoptosis.[27] However, the regulatory mechanisms of caspase-3 activation by the expression of GST-π have not yet been clarified. Apoptosis-induced factors such as c-Jun N-terminal kinase,[36,37] mitogen-activated protein kinase,[37,38] Raf[38] and apoptosis signal-regulating kinase 1,[39] which interact with GST or GST-π, are predictable candidates for the downstream target molecule regulated by GST-π.

Further study will attempt to identify whether or not GST-π is a specific signaling molecule that plays a role in the cytotoxic mechanism of GSH–DXR.

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