J. Life Science. (1999), 9(1), 40~44

JOURNAL OF
Life Science
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Comparison of Glutathione S-transferase-π Content in Drug-resistant and -sensitive Cancer Cells

Sang-Han Lee* **and Soon-Duck Hong**[†]

Department of Microbiology, Kyung-pook National University, Taegu 702-701, Korea
**Samsung Biomedical Research Institute, Suwon 440-746, Korea*

Received: April 12, 1999

Abstract Glutathione S-transferase (GST) is a multifunctional protein that catalyzes the conjugation of glutathione with electrophilic compounds. It exists in a variety of isoenzymatic forms with a wide range of substrate specificity and plays a pivotal role in detoxification of various drugs. In order to elucidate the GST-π's involvement of multidrug resistance (MDR) in drug-resistant tumor cell lines, we determined GST-π content by "1 step sandwich method". Consequently, adriamycin resistant cells of MCF-7 (MCF-7/ADM) have 7-fold increase of GST-π content than that of MCF-7 cells, while its IC_{50} was 116-fold greater than parent cell line. By northern blotting, we compared whether MCF-7/ADM cells express GST-π mRNA. The GST-π mRNA expression in these cells was not inducible, but constitutive when treated for 24 h with a concentration of 0, 20, 200, and 2000 nM of adriamycin, respectively. Taken together, these results suggest that GST-π may not be directly associated with multidrug resistance in these human cancer cell lines.

Key words: GST-π, Multidrug resistance, anticancer drugs, mdr-1

Introduction

A major marker of early stages of neoplasia is glutathione S-transferase (GST, EC 2.5.1.18) which is a multifunctional protein that catalyzes the conjugation of glutathione with electrophilic compounds and endogenous substrate, resulting in the formation of a stable complex [4]. It exists in a variety of isoenzymatic forms, which have a wide range of substrate specificity and play a physiological role in detoxification of many drugs. In the majority of cases, the elevated expression of the GST-α family of isozymes has been correlated with increased resistance to alkylating anticancer drugs. Their deficiency of GST status may be a useful prognostic factor to determine the clinical outcome of chemotherapy [4]. It has reported that resistance in an adriamycin-resistant breast cancer cell line increased with a 45-fold increase in GST activity [2,15]. This increased activity was due to the appearance of an isozyme that is immunologically related to GST activity in human placenta between drug-resistant and -sensitive cells. Of more recent interest, the overexpression of GST-π in a multidrug-resistant cell line has served to identify a role for this enzyme in the detoxification of free redical-induced damage as caused by quinone metabolites of adriamycin. Interest in GST-π has been further emphasized by the numerous observations that it is the predominant GST isozymes formed in human cancer tissue and that it serves as a putative tumor marker in some instances of both rodent and human diseases [9].

We studied the content of GST-π in order to assess the role of GST-π as a potential marker of multidrug resistance in various human cancer cells. In addition, we examined whether or not the content of GST-π and expression of *mdr-1* in several drug-resistant and -sensitive cell lines correlated with multidrug resistance.

In this report, we show that the induction of multidrug resistance is not involved with GST-π mRNA expression.

Materials and Methods

Reagents

All reagents were of analytical-reagent grade and the solutions were prepared with deionized water. The antitumor agents were all commercially available.

Cells and cell culture

Human myelogeneous leukemia K562 cell line and its ADM-resistant subline (K562/ADM), human ovarian cancer cell line MCF-7 and its ADM-resistant subline (MCF-7/ADM), human colon cancer cell line HT-29 and its drug resistant subline (HT-29/ADM, HT-29/BLM, HT-29/MMC,

[†]Corresponding author
Phone: 82-331-299-6142, Fax: 82-331-299-6149
E-mail: shlee@medical.skku.ac.kr

HT-29/ACR, and HT-29/CDDP), human stomach cancer cell line ST-4 and its CDDP-resistant subline (ST-4/CDDP) were used in this study. A cloned human epidermoid carcinoma cell line KB3-1 and its colchicine-resistant subline (KBC-4) were provided by Dr. I. Pastan (National Cancer Institute, NIH) [13]. All cell lines were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Gibco Lab.), and penicillin-streptomycin mixture (100 IU/ml and 100 μg/ml, respectively) under 5% CO_2 humidified atmosphere. For all experiments cells were harvested at logarithmic growth stage.

Preparation of cell lysates

The cell lysis buffer was prepared with 50 mM HEPES buffer (pH 7.6) containing various concentrations of Triton X-100, 1 mM PMSF, and 1 mg/ml bacitracin. Cells were seeded at an initial concentration of 10,000 cells/ml, and cultured in RPMI 1640 growth medium for 72 h. At this point, the cells were 90% confluent condition. After washing twice with PBS, the cells (1×10^6 cells/ml) were collected in effendorf tubes by centrifugation. The cell lysates (500 μl) was added to the tubes before incubation for 1 h at 4℃, and recentrifuged for 10 min at 10,000 rpm. The resulting supernatants were diluted with PBS to 100-200 folds in order to determine GST-π content.

Drug sensitivity

Cells (2,000 cells/ml) were cultured at 37℃ for 24 h in 96-well flat-bottom plates (for adherent cells or for other cell lines) containing 100 μl growth medium in a humidified atmosphere comprising 5% CO_2-95% air. The cells were then treated with a graded concentration of each agent and reincubated for 72 h in the presence of the drugs. Three samples were used for each drug concentration. In the control cultures, tumor cells grew exponentially during the incubation period. The median concentration of the drug necessary to inhibit the growth of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of the drug concentration versus the growth rate (percentages of control) of the treated cells [16].

Protein determination

Protein was determined by the BCA protein assay reported by Smith *et al.* [12], using bovine serum albumin as a standard.

Determination of GST-π

The content of GST-π was carried out by 1 step sandwich method as a supplier's manual of Teijin, Tokyo. Briefly, the cell lysates (40 μl) were added to each 5 ml tube and the assay buffer was filled up to 240 μl. With vigorously vortexing, 200 μl of enzyme-labeled antibody was added and then fixed antibody beads were mixed with equal ratio and added to 400 μl of each tube. After 30 min of incubation at 37℃, the reaction was stopped by adding 1 ml of stop solution. The absorbancy was measured at 450 nm.

Nothern blotting analysis

Total mRNA was prepared by the acid thiocyanate-phenol-chloroform extraction method designed by Chomczynski *et al.* [3]. Hybridization was carried out as described previously [7]. 20 ug of total RNA was electrophoresed and transferred to nitrocellulose filters. Probes (1.2 kb fragment for human *mdr-1* cDNA and 1.1 kb pGP5 DNA for GST-π) [2,6] were labeled with [α-^{32}P]dCTP to a specific activity of 2×10^9 cpm/mg DNA using the multipriming DNA labeling system. Hybridization was performed for 15 h under stringent conditions (5 X SSC, 50% formamide, 42℃). After hybridization, the filters were washed three times in 0.1 X SSC containing 0.1% SDS at 65℃ for 15 min and then exposed to Kodak X-Omat films at -80℃ for 24 h.

Results and Discussion

Mechanism of drug resistance.

The mechanism of MDR have been classified as 9 categories: by-pass of target's function, decreased uptake of the drug, increased deactivation of the drug, increased efflux of the drug, increased repair, reduced delivery of the drug, reduced metabolic activation, sequestration of the drug and structural alteration of target site [8,11,13,14]. Recently, it has been reported that the expression of antiapoptotic proteins (for example, Bcl-2 or Bcl-X_L) may be one factor of multidrug resistance in cancer cells [5]. As many researchers have been published, the expression of topoisomerase II, P-glycoprotein, or DNA repair enzymes were also associated with MDR [10]. We, therefore, compared whether or not THE CONTENT (not THE ACTIVITY) of GST-π in drug-resistant and -sensitive tumor cells is associated with MDR by checking the *mdr-1* mRNA in these cells [17].

Effect of lysis buffer on determination of GST-π content

For determination of GST-π content, 1 step sandwich method for GST-π was used. First, we examined the optimal concentration of detergent. As GST-π is an intracellular enzyme, the cell membranes should be disrupted to determine GST-π. As shown in Fig. 1, the optimal concentration of Triton X-100 which solubilize the cell membrane is 0.005%. Upto 0.005% of Triton-X100 in the cell lysis buffer, the absorbancy was drastically reduced compared to control. Although the treatment of sonication in order to disrupt the cell membranes was effective (data not shown), the use of detergent took advantages of timesaving and reproducibility. As a positive control cell line, we used KATO III cells. The GST-π content of KATO III cells was 2015±45 ng/ml.

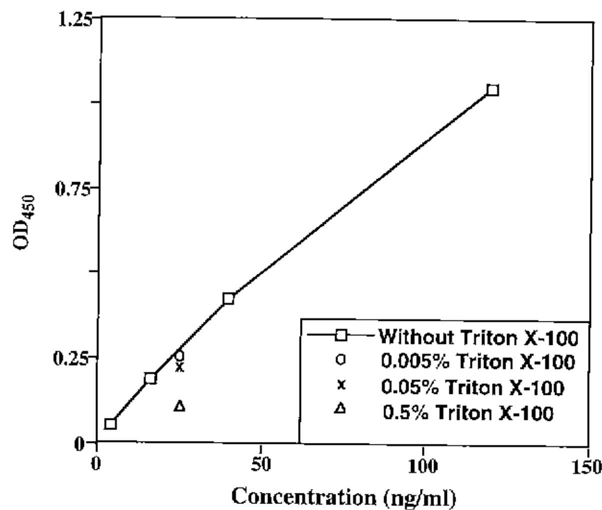

Fig. 1. Effect of cell lysis buffer for GST-π determination. The cell lysis buffer (CLB) was composed of 50 mM HEPES buffer (pH 7.6) containing various concentrations of Triton X-100. The cells were suspended with 100 ul of CLB buffer, and then centrifuged at 12,000 rpm for cleared cell lysates. The data showed the mean values of triplicate determinations. One of three representative experiments is shown.

GST-π content in various tumor cells.

There is no report that GST-π content of various tumor cells was determined by the quantitative method, which on update other related works mostly determined GST-π ACTIVITY, not GST-π CONTENT. We, first, tested whether the GST-π content in various cancer cells is associated with multidrug resistance or not. All tested HT-29 cell lines contained a great mount of GST-π compared to KATO III cell line as a positive control. Of them, HT-29/CDDP and HT-29/MMC showed about 1.8 to 1.9-fold increase of GST-π in compared with that of the parent cell lines, while HT-29/MMC cells showed 5-fold resistance to MMC as compared with the parental cell. Interestingly, MCF-7/ADM cells had a 7-fold increase of GST-π content than its parent cell line while MCF/ADM cells had 116-fold resistance to ADM than that of parent cells. It was reported that the levels of GST activity in human cancer cells Hattori and MCF-7 are significantly lower than those of other tumor cell lines. Although there are a limited number of breast cancer cell lines, the question whether lower GST activity is common to breast cancer is of great interest. Up to now, we have no evidence why the level of GST-π of the resistant cell line MCF-7/ADM (2.68±0.1) is greater than those of MCF-7 parent cell line (0.38±0.15). Other resistant variants (for example, MCF-7/CPT or MCF-7/ CDDP, if we can get the cell lines) will elucidate not only the role of GST-π but also the involvement of GST-π −related drug resistance in breast cancer cells.

Comparison of mRNA of GST-π and *mdr*-1 mRNA

To ascertain the mRNA expression of GST-π and *mdr-1* in MCF-7 and its resistant cells, we next examined whether

Table 1. Comparison of GST-π content and cytotoxicity to antineoplastic agents in various tumor cell lines.

Cell line	GST-π/protein (μg/ml)*[fold]	IC_{50} (nM)**[fold]#
K562	1.48±0.1	14.1±1.0
K562/ADM	1.30±0.1	2070.1±39.0 [146.8]
A2780	2.50±0.59	6.7±0.2
AD10	3.05±0.4	500.5±13.8 [83.3]
MCF-7	0.38±0.15	12.4±2.5
MCF-7/ADM	2.68±0.1 [7.05]	1440.3±13.0 [116.2]
ST-4	3.75±0.56	31.7±4.2
ST-4/CDDP	4.13±0.54	2990.5±118.2 [94.3]
KB3-1	0.39±0.19	11.2±1.3
KBC-4	0.47±0.21	972.0±45.1 [86.8]
HT-29	4.99±0.23	38.7±1.4
HT-29/ADM	3.46±0.71	ND##
HT-29/BLM	5.53±0.76	ND
HT-29/MMC	9.53±1.59 [1.90]	102.0±6.0 [2.6]
HT-29/ACR	5.17±0.12	ND
HT-29/CPT	5.61±0.1	ND
HT-29/CDDP	9.14±0.65 [1.83]	151.4±2.2 [3.9]

*Values in parentheses represent an average of three determinations.
**IC_{50} represents the concentration of a drug that limits the increase in cell population of a culture to 50% that of control.
#Numbers in parenthses represent the degree [X-fold] of resistance as compared with parental cells.
##ND, not determined.

or not the expression of GST-π mRNA is correlated with that of *mdr-1*. As shown in Fig. 2(A), in K562 and K562 adriamycin-resistant cells, we treated adriamycin with a concentration of 0, 20, 2000 nM, respectively, and tested GST-π and *mdr-1* mRNA expression. As a result, GST-π mRNA was highly expressed in both cell lines while *mdr-1* mRNA was only expressed in K562 adriamycin-resistant cells. On the other hand, in MCF-7 and MCF-7 adriamycin-resistant cell lines, we also treated adriamycin with a concentration of 0, 10, 1400 nM, respectively, and examined the mRNA expression level. GST-π and *mdr-1* expression were only expressed in MCF-7 adriamycin-resistant cells (Fig. 1B), which mean GST-π did not induce by 1400 nM of adriamycin. These data suggest that, in MCF-7 and its resistant cells, GST-π expression is not associated with *mdr-1*, which considered as a marker of multidrug resistance.

We, herein, compared the content of GST-π between drug-resistant and -sensitive tumor cells and examined the expression of *mdr-1* mRNA by northern blotting. GST-π may not play a role in multidrug resistance in used cell lines. Studies on the high levels of GST-π content in HT-29-sensitive and -resistant cells (especially HT-29/MMC and HT-29/CDDP sublines) may elucidate why colon cancer cells have high levels of GST-π.

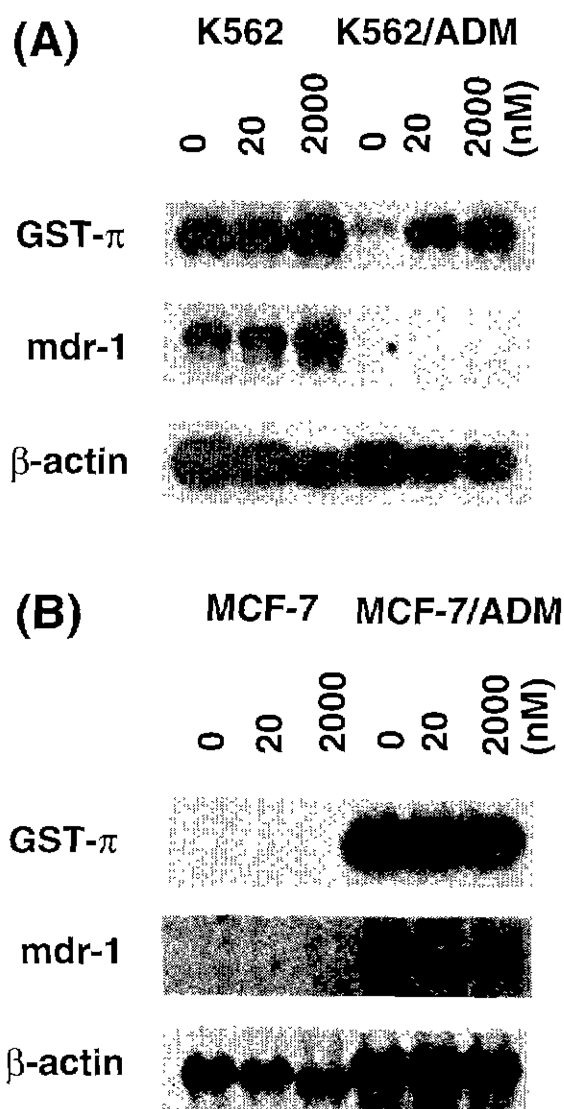

Fig. 2. Expression of GST-π and *mdr-1* mRNA in K562 and K562 adriamycin-resistant (K562/ADM) cells (A), and MCF-7 and MCF-7 adriamycin-resistant (MCF-7/ADM) cells (B) by Northern blotting analysis. Twenty μg of total RNA from various cells were loaded, and Northern blot analysis was carried out as described in "Materials and Methods".

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