Significance of Nuclear Glutathione S-Transferase $\pi$ in Resistance to Anti-cancer Drugs

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Recent study has shown that nuclear glutathione S-transferase (GST) $\pi$ accumulates in cancer cells resistant to doxorubicin hydrochloride (DOX) and may function to prevent nuclear DNA damage caused by DOX (Goto et al., FASEB J., 15, 2702–2714 (2001)). It is not clear if the amount of nuclear GST$\pi$ increases in response to other anti-cancer drugs and if so, what is the physiological significance of the nuclear transfer of GST$\pi$ in the acquisition of drug-resistance in cancer cells. In the present study, we employed three cancer cell lines, HCT8 human colonic cancer cells, A549 human lung adenocarcinoma cells, and T98G human glioblastoma cells. We estimated the nuclear transfer of GST$\pi$ induced by the anti-cancer drugs cisplatin (CDDP), irinotecan hydrochloride (CPT-11), etoposide (VP-16) and 5-fluorouracil (5-FU). It was found that: (1) Nuclear GST$\pi$ accumulated in these cancer cells in response to CDDP, DOX, CPT-11, VP-16 and 5-FU. (2) An inhibitor of the nuclear transport of GST$\pi$, edible mushroom lectin (Agaricus bisporus lectin, ABL), increased the sensitivity of the cancer cells to DOX and CDDP, and partially to CPT-11. Treatment with ABL had no apparent effect on the cytotoxicity of VP-16 and 5-FU. These results suggest that inhibitors of the nuclear transfer of GST$\pi$ have practical value in producing an increase of sensitivity to DOX, CDDP and CPT-11.

Key words: Glutathione S-transferase $\pi$ — Doxorubicin — Cisplatin — Nuclear transfer — DNA damage

Human glutathione S-transferase (GST, EC 2.5.1.18) $\pi$, one of a family of GSTs, has been reported to accumulate in various human cancer tissues or pre-cancer tissues, and is employed in cancer research as a tumor marker.1–5 An increase in GST$\pi$ was also found in cancer cell lines resistant to doxorubicin hydrochloride (DOX), cis-diamminedi-chloroplatinum (II) (cisplatin, CDDP),6–8 and alkylating agents.9 GST$\pi$ in the nucleus has been reported in uterine cancer cells10 and glioma cells,11 suggesting a negative correlation between the existence of GST$\pi$ in the nucleus of cancer cells and patient survival. However, there has been no report on the mechanisms responsible for the nuclear accumulation of GST$\pi$ or on the physiological role of nuclear GST$\pi$.

Quite recently, we found evidence of a change in the amount of nuclear GST$\pi$ in human cancer cells exposed to DOX.12 The sensitivity to DOX was greater in nuclear GST$\pi$-negative than -positive cells, implying a role for nuclear GST$\pi$ in the acquisition of drug-resistance to DOX.

Wheat germ agglutinin (WGA) inhibits the nuclear transfer of proteins when it enters cells.13–17 However, plasma membranes are not permeable to WGA, and WGA can not be used in intact cells. Yu et al. reported that edible mushroom lectin (Agaricus bisporus lectin, ABL) was efficiently internalized into the cytoplasm of cultured cells, localized around the nucleus, and inhibited the nuclear transfer of proteins.18 This suggests that artificially induced modifications of the cell membrane are required for the internalization of WGA, but not ABL.

A previous report presented evidence that ABL inhibits the nuclear transfer of GST$\pi$ and increases the sensitivity of HCT8 human colonic cancer cells to DOX.12 However, several questions remain to be answered. First, does nuclear GST$\pi$ have a similar effect on the cytotoxicity of DOX in other cancer cells? Second, does the nuclear transfer of GST$\pi$ have any effect on the acquisition of resistance to other anti-cancer drugs, and if so, does ABL have the same effect? Third, are other GSTs transferred to the nucleus in response to these anti-cancer drugs? To find some answers, we estimated the change in the levels of nuclear GST in response to DOX, CDDP, irinotecan hydrochloride (CPT-11), etoposide (VP-16) and 5-fluorouracil (5-FU) in three human cancer cells in which GST is expressed.

Transfection of the GST$\pi$ gene into cancer cells to over-express the enzyme resulted in an enhancement of drug-resistance to DOX and CDDP,19–22 though another report did not examine the effect of transfection of GST$\pi$ into cancer cells on drug-resistance.23 These studies did not examine the nuclear localization of GST$\pi$ either. In this study, we found that the amount of GST$\pi$ in the nucleus seemed to correlate with drug resistance to DOX and

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CDDP, but not to CPT-11, VP-16 and 5-FU. The effect of an inhibitor of nuclear transport of GST\textpi, ABL, on the sensitivity of cancer cells to these drugs was also studied.

**MATERIALS AND METHODS**

**Materials** CDDP was a gift from Nihon Kayaku Co. (Tokyo). CPT-11 was from Yakult Honsha (Tokyo). DOX and ABL were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). RPMI 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen Corp. (Carlsbad, CA). 5-Chloromethylfluorescein diacetate (CMFDA) was from Molecular Probes, Inc. (Eugene, OR). Rabbit polyclonal antibodies against human GSTA1-1 and GSTM1-1 were purchased from Calbiochem-Novabiochem. Co. (Darmstadt, Germany). Horseradish peroxidase-labeled anti-rabbit IgG was from DAKO A/S (Glostrup, Denmark). The Enhanced Chemiluminescence (ECL) kit was obtained from Amersham Biosciences (Buckinghamshire, UK). All other chemicals and reagents, including VP-16 and 5-FU, were purchased from Sigma Aldrich (St. Louis, MO).

**Preparation of cells** We used the human cancer cell lines HCT8 (colonic carcinoma), A549 (lung adenocarcinoma), and T98G (glioblastoma). Dr. K. J. Scanlon (Berlex Biosciences, CA) donated the HCT8 cells and Dr. H. Isobe (Hokkaido University School of Medicine, Sapporo) the A549 cells. T98G cells were purchased from American Type Culture Collection (Rockville, MD). T98G cells were maintained in DMEM, and other cells in RPMI 1640. They were supplemented with 10% FBS at 37°C in 5% CO\textsubscript{2} with 100% humidity. Six hours before treatment with anti-cancer drugs or ABL, the cells were cultured in medium with 1% FBS. About 2\times10\textsuperscript{6} cells were harvested with trypsin and washed with phosphate-buffered saline (0.137 M NaCl, 2.68 mM KCl and 10 mM Na\textsubscript{2}HPO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} in pH 7.4, PBS) twice at 4°C. The pellets were stored at −80°C before use. The doses of anti-cancer drugs used in this study were based on the results of MTT assay and trypan blue dye exclusion. Cells were treated with various concentrations of anti-cancer drugs for 24 h. The doses that suppressed cell growth by 30%, 50%, and 70% of cells were determined.

**Preparation of proteins** The cytoplasmic and nuclear proteins were prepared as described by Dignam et al.\textsuperscript{24} Proteins in the whole cells were prepared as described previously.\textsuperscript{25}

**Preparation of GST\textpi antibody** GST\textpi was purified from human placenta and polyclonal antibody against human GST\textpi was obtained by immunizing rabbits as described previously.\textsuperscript{12}

**Immunological estimation** Immunological levels of GST\textpi, GSTA1-1 and GSTM1-1 in the cells were estimated by western blotting. Lysate from the extract of 1\times10\textsuperscript{7} cells was separated by SDS-polyacylamide gel electrophoresis (SDS-PAGE) in a 12.5% gel, transferred to a nitrocellulose membrane, and immunologically stained using rabbit IgG against human GST\textpi, GSTA1-1, and GSTM1-1 as the primary antibody, and then with horseradish peroxidase-labeled anti-rabbit IgG as the secondary antibody. Blots were developed by enhanced chemiluminescence using the ECL kit and the relative immunological activity was analyzed by NIH Image. The protein concentration was determined according to Redinbaugh and Turley,\textsuperscript{20} with bovine serum albumin as the standard.

**Histochemical analysis** For the histochemical analysis, HCT8 and A549 cells were maintained with RPMI 1640 medium, and T98G cells with DMEM, containing 10% FBS in a four-well Lab Tec Chamber (Nalge Nunc International, Naperville, IL). After treatment with the anticancer drug, cell chambers were washed 3 times with medium and treated with 10 \mu M CMFDA for 30 min to assess the formation of GSH (reduced form of glutathione) S-conjugate or with 10 \mu M Hoechst 33342 for 30 min to estimated the extent of nuclear condensation. They were then washed again with PBS. Fluorescence intensity was examined using an Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany), and the findings were analyzed using a CCD camera and AxioVison software. To calculate the apoptotic cell ratio, apoptotic cells revealing nuclear condensation were counted in one thousand cells selected at random. Fluorescence intensity corresponding to the GSH S-conjugate was analyzed in the nuclear area in one hundred cells randomly selected using NIH Image software.

**Cell viability** Cell number and viability were determined by the trypan blue dye exclusion method and MTT assay. Cells in PBS were treated with an equal vol. of 0.4% trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min. The chambers of the hemocytometer were filled with trypan blue and allowed to stand at room temperature for 5 min.
RESULTS

Nuclear transfer of GSTπ induced by anti-cancer drugs

Fig. 1 shows immuno-blots for GSTs in the human HCT8, A549 and T98G cancer cell lines. GSTπ was detected in the cytoplasm and nucleus, but no other GSTs, such as GST A1-1 and M1-1, were detected in these cells (A). Treatment with DOX (10 µM), CDDP (50 µM), CPT-11 (20 µM), VP-16 (20 µM) or 5-FU (20 µM) for 6 h resulted in an increase in the level of GSTπ in the nucleus in every cell line (B and D). After the treatment of these cells with five kinds of anti-cancer drugs, the levels of cytoplasmic GSTπ showed an increase within 6 h, though the increase was not statistically significant. Neither GST A1-1 nor M1-1 was detected in the nucleus of these cells following treatment with these anti-cancer drugs for 6 h (data not shown). Previously, we found that induction of GSTπ in the nucleus by DOX is independent of the level of GSTπ in the cytoplasm.12 Then, the effect of nuclear GSTπ on the cytotoxicity of anti-cancer drugs was estimated.

The effect of ABL on the cytotoxicity of DOX and CDDP ABL is a kind of lectin that is known to be internalized into intact cells, where it interferes with the trans-
fer of proteins including GSTπ.12,28) Cancer cells pre-
treated with 40 μg/ml of ABL for 10 h were further incu-
bated with 50 μM CDDP and apoptotic change was
observed morphologically. As shown in Fig. 2 (A and B),
CDDP alone or ABL alone caused no apparent nuclear
condensation. Treatment of cancer cells with ABL fol-
lowed by CDDP promoted nuclear condensation. Table I
shows the effect of ABL on the apoptosis induced by anti-
cancer drugs. Apoptotic change was estimated as nuclear
condensation in cancer cells pre-treated with ABL for 10 h
and then treated with anti-cancer drugs for 24 h. Fifty
micromolar CDDP, 10 μM DOX, 20 μM CPT-11, 20 μM
VP-16 and 20 μM 5-FU had no apparent effect on nuclear
condensation (data not shown). Nuclear condensation was
observed in HCT8, A549 and T98G cells pre-treated with

Table I. The Effect of ABL on Apoptotic Change by Anti-
cancer Drugs

| Cell line | Drug | | | | |
|-----------|------|---|---|---|---|
|           | CDDP| DOX| CPT-11| VP-16| 5-FU|
| HCT8      | +++ | ++ | +  | −  | −  |
| A549      | ++  | ++ | −  | −  | −  |
| T98G      | +++ | +++| −  | −  | −  |

Cells pre-treated with ABL (40 μg/ml) for 10 h were treated
with various concentrations of anti-cancer drugs for 24 h, then
the appearance of aggregated nuclei stained by Hoechst 33342
was estimated by using fluorescence microscopy. Concentra-
tions of anti-cancer drugs were: 50 μM CDDP, 10 μM DOX, 20
μM CPT-11, 20 μM VP-16, and 20 μM 5-FU. Apoptosis rate
was expressed as −, almost no apoptosis; +, 10–15%; ++,
16–30%; ++++, above 31%.

Fig. 2. Nuclear condensation. For the estimation of nuclear condensation, cells
were incubated in a four-well Lab Tec Chamber. After treatment with anti-cancer
drugs for 24 h, cells were treated with 10 μM Hoechst 33342 for 30 min for the
estimation of nuclear condensation. A, The observation of fluorescence intensity
was done using an Axioskop2 fluorescence microscope, and the findings were ana-
alyzed using a CCD camera and AxioVison software. Bar, 10 μm. B, To calculate
the apoptotic cell ratio, apoptotic cells revealing nuclear condensation were counted
in one thousand cells selected at random. Data are the means of three independent
analyses; bars, SD. *, P<0.01 compared with each cell with CDDP treatment. +,
P<0.01 compared with each cell with ABL pre-treatment.
Fig. 3. The effect of ABL on the sensitivity to anti-cancer drugs. A, The effect of change in nuclear GST\(\pi\) on the cell viability was examined using cells pre-treated with ABL (40 \(\mu\)g/ml) for 10 h followed by treatment with various concentrations of CDDP, DOX, 5-FU, or VP-16 for 24 h. The cell viability was estimated by MTT assay. Data in each cell line are expressed as percent of that before the treatment with anti-cancer drugs. B, HCT8 cells pre-treated with ABL on a 60-mm dish followed by treatment with CDDP or DOX were used for the colony assay. Each lane corresponds to that in the cells before the treatment with anti-cancer drugs. Open symbol, without pre-treatment with ABL; closed symbol, pre-treatment with ABL. *, \(P<0.05\) compared with HCT8 cells without ABL pre-treatment. **, \(P<0.01\) compared with HCT8 cells without ABL pre-treatment.
ABL for 10 h and treated with CDDP and DOX for 24 h. Such changes, although amounting to less than 15%, were observed on pre-treatment with ABL and further treatment with CPT-11 in HCT8 cells, but not in A549 or T98G cells. VP-16 and 5-FU did not affect the nuclear change in the cells pre-treated with ABL.

The effect of ABL on cell survival was estimated by MTT assay. Fig. 3A shows the effects of pre-treatment with ABL on the survival of cells treated with DOX, CDDP, 5-FU, or VP-16. Cells were incubated with various concentrations of CDDP, DOX, 5-FU, or VP-16 for 24 h. These anti-cancer drugs decreased cell survival compared to non-treated cells. Co-administration of ABL caused a significant augmentation of the cytotoxicity of CDDP and DOX, but no such influence of ABL was observed on the cytotoxic effect of VP-16 or 5-FU. Fig. 3B shows the effect of ABL on the cytotoxicity of DOX and CDDP as estimated by colony assay. Both DOX and CDDP decreased cell survival in a dose-dependent manner.

**Formation of GSH S-conjugate by nuclear GSTπ** The activity of GSTπ in the nucleus was estimated from the formation of GSH S-conjugate using CMFDA as a substrate. As shown in Fig. 4 (A and B), an increase in GSH S-chloromethylfluorescein was observed in the nucleus of cells treated with 50 μM CDDP for 6 h, and such formation was inhibited by the pre-treatment with ABL. Formation of the GSH S-conjugate was observed upon treatment of the cells with DOX (data not shown).

**DISCUSSION**

Previously, we reported that there are differences in the levels of nuclear GSTπ in human cancer cells, that inhibition of the nuclear transfer of GSTπ increased the DNA intercalation by DOX, and that the DNA was cross-linked by CDDP in HCT8 human colonic cancer cells, suggesting a role for nuclear GSTπ in the protection of cancer cells against anti-cancer drugs. However, there still remain several questions to be answered. (1) Does nuclear GSTπ protect against DNA damage by anti-cancer drugs in other cancer cells? (2) What is the function of nuclear GSTπ? (3) Does inhibition of the nuclear transfer of GSTπ increase apoptotic change caused by anti-cancer drugs?

In the present study, we found that: [1] Treatment of cells with DOX and CDDP increased the amount of nuclear GSTπ, but not other GSTs in HCT8 as well as
A549 and T98G human cancer cells. Treatment of cells with other anti-cancer drugs such as CPT-11, VP-16 and 5-FU also increased the amount of nuclear GST\(\pi\). Pre-treatment of these cancer cells with ABL promoted apoptosis induced by CDDP and DOX, and partially by CPT-11, but not by other anti-cancer drugs.

Levels of cytoplasmic GST\(\pi\) are shown in Fig. 2B for CDDP and DOX, and Fig. 1D for VP-16, 5-FU and CPT-11. Although there is no direct evidence that the nuclear GST\(\pi\) is independent of cytoplasmic GST\(\pi\), the data suggest that cytoplasmic GST\(\pi\) is induced by treatment of cancer cells with these anti-cancer drugs, and the increase in the levels of cytoplasmic GST\(\pi\) was not parallel to that in the levels of the nuclear GST\(\pi\). These data also suggest that the nuclear transfer of GST\(\pi\) is not regulated by cytoplasmic GST\(\pi\). Treatment with ABL inhibited the accumulation of GST\(\pi\) in the nucleus, but did not influence the levels of cytoplasmic GST\(\pi\). This suggested that ABL inhibits the transfer of GST\(\pi\) to the nucleus, but does not suppress the induction of GST\(\pi\) protein. ABL inhibited the nuclear transfer of GST\(\pi\), which does not possess the nuclear localization signal (NLS), but it did not inhibit the nuclear transfer of p53, which possesses the NLS, ruling out the possibility that the inhibitory effects of ABL on the nuclear transfer of proteins are non-specific. Pre-treatment of the cells with ABL decreased the steady-state level of nuclear GST\(\pi\), and furthermore, ABL inhibited the accumulation of nuclear GST\(\pi\) on exposure to DOX. These findings suggest that GST\(\pi\) is transferred to the nucleus through a protein transfer system both in the steady state and following exposure to anti-cancer drugs.

In the present study, the cytotoxicity assay revealed that nuclear GST\(\pi\) protects cells against the cytotoxicity of DOX and CDDP (Figs. 2, 3).

The accumulation of GST\(\pi\) in response to DOX and CDDP may due to the increase in nuclear transfer or may be due to inhibition of the nuclear export system, similar to the inhibition under oxidative stress observed in fission yeast. In the present study, the precise mechanism was not clarified. Further study is needed.

DOX is an anti-cancer drug that inhibits topoisomerase II. DOX interferes with the topoisomerase II/DNA complex, leading to the formation of double-strand breaks of DNA or direct intercalation with DNA, which in turn inhibits DNA duplication and transcription to mRNA. Production of reactive oxygen species (ROS) is also thought to be involved in the cytotoxic effect of DOX on cancer cells. Awasthi et al. and Maeda et al. reported that GST\(\pi\) is involved in the detoxification of DOX, though how is not clear.

CDDP, a platinum-containing drug, binds to guanine residues on DNA and forms cross linkages inside or among the DNA chains. The cross linkage of DNA by CDDP brings about a change in the structure of the DNA and inhibits the transcriptional activity to form mRNA. The DNA coupled with CDDP is recognized by proteins with high-mobility-group domains. Repair enzymes are then unable to bind to and repair the injured DNA, and finally these changes lead to cancer cell death.

The GSH is thought to be associated with the efflux system of DOX and CDDP through ATP-binding cassette transporters (ABCs), such as canalicular multispecific organic anion transporter (cMOAT), and multidrug resistance-associated protein 1 (MRP1). Depletion of intracellular GSH using buthionine sulfoximine (BSO) causes a decrease in the efflux activity of DOX and CDDP, reducing the drug resistance of cancer cells. We previously reported that GST\(\pi\) forms a CDDP-GSH adduct which is transported outside the cells, and the efflux activity of CDDP is elevated in cancer cells resistant to CDDP. It has been reported that DOX forms an adduct with GSH inside the cells, but the mechanism of the formation of the DOX-GSH adduct is unknown.

There have been many reports on the elevation of the intracellular GSH concentration in cancer cells resistant to DOX and CDDP. GSH is synthesized via two ATP-requiring steps, by \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS) and GSH synthetase. Increases in \(\gamma\)-GCS mRNA and protein have been reported to correlate with the acquisition of resistance to DOX and CDDP. Treatment of cancer cells with BSO, a specific inhibitor of \(\gamma\)-GCS, decreases the level of GSH and the sensitivity to anti-cancer drugs.

In the present study, pre-treatment of cancer cells with ABL caused apoptosis (Figs. 2, 3 and Tables I). Since the pre-treatment decreased the nuclear transfer of GST\(\pi\), a decrease in nuclear GST\(\pi\) may be a factor in the increase in apoptosis induced by DOX and CDDP.

CPT-11 targets topoisomerase I and SN-38, its active form, forms a conjugate with glucuronide. Increases in the level of lipid peroxidation by CPT-11 have been reported in the nucleus of cells from rat liver, but the involvement of GST\(\pi\) in the metabolism of CPT-11 has never been reported. In the present study, CPT-11 increased the level of nuclear GST\(\pi\) (Fig. 1), and the survival of HCT8 cells treated with CPT-11 was partially dependent on nuclear GST\(\pi\) (Table I). The nuclear GST\(\pi\) may, in part, play a role in the scavenging of ROS generated by CPT-11 in the nucleus.

VP-16 targets topoisomerase II and is metabolized in the cells to glucuronosyl etoposide, then transported out of the cells dependent on intracellular GSH. In the present study, VP-16 increased the levels of nuclear GST\(\pi\) (Fig. 1). However, the nuclear GST\(\pi\) showed no apparent effect on the sensitivity of cells to VP-16 (Table I). The formation of ROS by VP-16 was not observed in the present study (data not shown), suggesting that nuclear GST\(\pi\) is not important in the protection of DNA against damage by...
VP-16 and that other mechanisms affect the metabolism of this anti-cancer drug.

5-FU inhibits DNA synthesis. Fujishima et al. reported the pre-treatment of cancer cells with 5-FU decreases the induction of glutathione synthesis in response to CDDP, suggesting a role of 5-FU in the down-regulation of the defense system in cancer cells. However, involvement of the nuclear GST by ABL is efficient in increasing the cytotoxicity of CDDP and DOX, but not that of VP-16 or 5-FU (data not shown). The data, together with those in Fig. 3A indicating suppression of GST by ABL in cancer cells treated with VP-16 and 5-FU, suggest that GST may not be important in the intracellular metabolism of VP-16 and 5-FU. GST is known to form GSH-conjugates with anti-cancer drugs. The nuclear GST may prevent DNA-intercalation by DOX or cross-linking by CDDP. There still remain several questions: [1] What is the molecular mechanism by which GST is transferred to the nucleus? [2] What is the mechanism by which the nuclear GST is up-regulated by anti-cancer drugs? [3] What is the physiological significance of the increase in the levels of nuclear GST induced by VP-16 and 5-FU? These questions should be clarified.

In summary, inhibition of the nuclear transfer of GST by ABL is efficient in increasing the cytotoxicity of CDDP and DOX in cancer cells. The development of molecules targeting the nuclear transfer of GST may be useful in the treatment of human cancers.

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