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ORIGINAL ARTICLE

Indomethacin overcomes doxorubicin resistance by decreasing intracellular content of glutathione and its conjugates with decreasing expression of γ-glutamylcysteine synthetase via promoter activity in doxorubicin-resistant leukemia cells

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Abstract Drug resistance continues to be a serious problem in cancer therapy. We investigated whether indomethacin, which inhibits cyclooxygenases, is able to overcome doxorubicin resistance in K562/ADR leukemia cells. Indomethacin at 10 μM increased the cytotoxicity of doxorubicin and vincristine in K562/ADR cells. Intracellular glutathione content was elevated in K562/ADR cells. Indomethacin treatment decreased glutathione content and glutathione-conjugates in K562/ADR cells. Increased expression of γ-glutamylcysteine synthetase (γ-GCS) was observed in K562/ADR cells, but this expression was decreased by indomethacin treatment. The activity of the γ-GCS promoter from K562/ADR cells decreased after indomethacin treatment in MDA231 cells. These data strongly suggest that the cyclooxygenase inhibitor indomethacin increases the cytotoxicity of doxorubicin by decreasing the intracellular contents of glutathione and its conjugates with decreasing expression of γ-GCS by inhibiting γ-GCS promoter activity.

Keywords K562 · Doxorubicin-resistant · MRP1 · Indomethacin · Promoter · γ-Glutamylcysteine synthetase · Glutathione

Introduction

Drug resistance remains a serious problem in cancer therapy and has been attributed to mechanisms such as overexpression of P-glycoprotein genes in doxorubicin-resistant leukemia cell lines and in leukemia patients [8]. Although several therapeutic approaches, including the use of reversal agents against P-glycoprotein export, have attempted to overcome doxorubicin resistance in leukemia patients, the results to date have been unsatisfactory [4, 9]. To overcome doxorubicin drug resistance, we recently investigated the gene expression profile of a doxorubicin-resistant myeloid leukemia cell line by microarray and confirmed cyclooxygenase-1 (Cox-1) overexpression in the doxorubicin-resistant cells [1]. We also found that indomethacin, which is an inhibitor of cyclooxygenases, sensitized the doxorubicin-resistant leukemia cells to doxorubicin and vincristine by decreasing the expression of multi-drug resistance protein 1 (MRP1) via inhibition of MRP1 promoter activity [14]. These results prompted us to investigate whether indomethacin is able to affect glutathione content, which is a conjugate of MRP1-mediated transport, in doxorubicin-resistant leukemia cells. In the present study, we found that indomethacin sensitized doxorubicin-resistant leukemia cells to doxorubicin and vincristine by decreasing the intracellular contents of glutathione and its conjugates and expression of γ-glutamylcysteine synthetase (γ-GCS) by inhibiting promoter activity.

Materials and methods

Reagents and drugs

RPMI 1640 medium, phosphate buffer saline (PBS), fetal calf serum (FCS) and Hanks Balanced Salt Solution (HBSS) were purchased from Invitrogen, Inc. (Tokyo, Japan). Adriamycin (doxorubicin), etoposide, vincristine, indomethacin, and other chemicals were purchased from Wako Pure Chemicals Co Ltd (Osaka, Japan). Monochlorobimane

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(Thyolite) was purchased from Calbiochem Corp (San Diego, CA, USA).

Cell lines

K562 cells (human erythroleukemia cell line), and MDA231 cells (human breast cancer cell line) were obtained from American Type Culture Collection (Manassas, VA, USA). Parent cells (K562/P) were incubated in RPMI 1640 containing 10% FCS (R10 media) under 5% CO_2 in a humidified incubator. A doxorubicin-resistant cell line (K562/ADR) was established by stepwise and continuous exposure to doxorubicin using the limiting dilution method [1]. Cell lines were confirmed free from *mycoplasma* organisms using the MycoFluor™ Mycoplasma detection kit (Molecular Probes, Eugene, OR, USA).

Cytotoxicity assay and indomethacin treatment

Cytotoxicity was measured by trypan blue dye exclusion assay, as described previously [1]. Briefly, 1×10^6 cells were incubated with various concentrations of doxorubicin for 72 h and viable cells were counted after trypan blue staining. Indomethacin treatment was carried out at various concentration for 72 h [10].

RT-PCR assay

RNA extraction was conducted as described previously [1]. RT-PCR was performed according manufacturer's instructions (Takara, Ootsu). Primers used were: for γ-glutamylcysteine synthetase (heavy subunit) (γ-GCS) 5′-GCTG CATCTCCCCTTTTACCGAG-3′, 5′-TGGCAACTGTCA TTAGTTCTCCAG-3′ yielding a PCR product of 880 bp [7], for Cyclooxygenase (Cox)-1, 5′-TGTTCGGTGTCCA GTTCCAAT-3′, 5′-CGCAACCGCATTGCCATGGAGT-3′, yielding a PCR product of 80 bp [19]; for Cox-2, 5′-GTTT GCATTCTTTGCCCAGC-3′, 5′-CAGGCACCAGACCA AAAGACC-3′, yielding a PCR product of 300 bp [24]; for multi-drug resistance protein 1 (MRP1), 5′-CCGTGTACT CCAACGCTGC-3′, 5′-CTGGACCGCTGACGCCGTGA C-3′, yielding a PCR product of 326 bp [17]; and for β-actin, 5′-GTGGGGCGCCCCAGGCACA-3′, 5′-CTCCT TAATGTCACGCACGATTTC-3′, yielding a PCR product of 548 bp [2].

Total cellular glutathione content measurement

The total cellular glutathione (GSH) content was measured using the colorimetric method of modified from that of Sedlak and Lindsay [20]. Cells (2×10^5) were cultured for 72 h with or without indomethacin, and then harvested. For experiments in the presence of exogenous GSH, cells were extensively washed by cold PBS, pelleted, and then lysed in 100 μL 0.2 M EDTA. Protein was precipitated by addition of 20 μL of ice-cold 20% (v/w) trichloroacetic acid (WAKO). The volume of this mixture was then made up to 200 μL with distilled water and the supernatant following centrifugation assay for GSH content. Assays were carried out in 96-well plates, the incubation mixture in each well consisting of 100 μL of protein-free supernatant, 160 μL of Tris buffer (0.4 M, pH 8.9), and 4 μL of 5,5-dithio-bis(2-nitrobenzolic acid; Ellman`s reagent, WAKO) dissolved in methanol at a concentration 3.4 mg/ml. The formation of 2-nitro-5-thiobenzoic acid (yellow product) was measured at 405 nm using a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Tokyo). Calibration curves of 0.5–500 μM GSH, dissolved in 0.2 M EDTA, were used as standard.

Intracellular glutathione conjugate accumulation using monochlorobimane

Monochlorobimane (Thyolite) is a nonfluorescent compound that is specifically conjugated with glutathione in the cell by the action of glutathione S-transferases and the resulting glutathione S-conjugate exhibits intense fluorescence [6]. Thyolite (20 μM) was incubated with K562/P or K562/ADR cells for 90 min at 37°C and cells were then washed with ice-cold HBSS three times. Cells were placed on glass slide plates and observed without fixation under a UV light microscope (Olympus IX70, Tokyo, Japan) and photographs were taken. Subsequently, the cells were incubated with Thyolite-free R10 media for 90 min at 37°C in 5% CO_2. Cells were washed ice-cold HBSS and observed in the same manner as described above. Fluorescent intensity was also measured with a fluorescence spectrophotometer, excitation at 370–385 nm; emission at 477–484 nm.

Western blotting

Protein blots were performed as described before [21]. Briefly, total cell lysates were made by lysing harvested cells in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). DNA was sheared by sonication and samples containing equal amount of protein were fractionated by 10% polyacrylamide gel. The proteins were transferred onto nitrocellulose filter (Bio-Rad Laboratory Inc., Hercules, CA, USA) by electroblotting. Non-specific binding sites were blocked for 2 h with Tris-buffered saline containing 0.2% Tween 20 and 10% non-fat skim milk. Rabbit polyclonal anti-γ-GCS antibody (1:200 dilution, Lab Vision Corp.) were incubated to the membrane for 2 h at room temperature. Following a second series of washes, a secondary horseradish peroxidase rabbit Ig antibody in Tris-buffered saline with 0.1% Tween 20 was

Table 1 IC_{50} in K562 parent cells and resistant cells

	Doxorubicin (nM)	Vincristine (nM)	Etoposide (nM)
K562/P	20 ± 3	2.0 ± 2.1	10 ± 4
K562/P with indomethacin	18 ± 3	1.8 ± 2.0	9 ± 5
K562/ADR	250 ± 20*	20.2 ± 8.1*	15 ± 10
K562/ADR with indomethacin	50 ± 50**	5 ± 3**	ND

ND not done

Leukemia cells were incubated with various doses of anti-cancer drugs for 72 h, and cytotoxicity was evaluated by trypan blue dye exclusion

Data are average ± standard deviation from three experiments

* $P < 0.05$; resistant cells versus parent cells; ** $P < 0.05$; cytotoxicity with indomethacin treatment versus cytotoxicity without indomethacin treatment

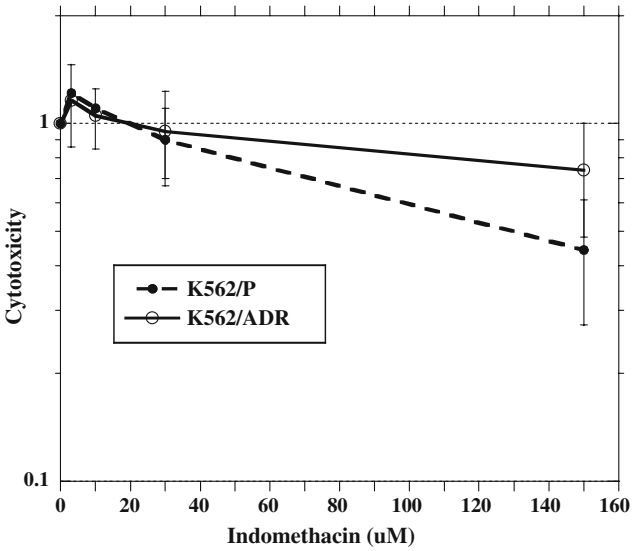

Fig. 1 Cytotoxicity with indomethacin in K562 cells. 0, 3, 10, 30, and 150 μM of indomethacin were incubated for 72 h in K562/P and K562/ADR cells. Then cytotoxicity against indomethacin was calculated. Representative data from three independent experiments

added (1:15,000 dilution) and incubated for 30 min at room temperature. After washing with Tris-buffered saline containing 0.2% Tween 20, the blot was visualized by chemiluminescence detection using the enhanced chemiluminescence (ECL) system (GE Healthcare, Tokyo) according to manufacture's instruction.

γ-Glutamylcysteine synthetase promoter activity

The promoter region of the γ-glutamylcysteine synthetase gene was amplified using primer set 5′-(-1088) GGA GGCGCAGGCAGAAGACCGA-3′ and 5′-(+225) CAG CCAGACCTTGGGTATTCATG-3′ (yielding a 1,313-bp product) [15] from K562/ADR cells cloned into a pSLO vector (Toyobo, Osaka). Inserted gene was verified PCR analysis using SLGOR-F primer, 5′-CAATGTATCT TATCATGTCTGGATC-3′. Reporter genes (2 μg) were transfected into 2×10^6 of MDA231 cells cultured under various conditions using Lipofect AMINE (Invitrogen) for 48 h. The cells were lysed and assayed using a Tripluc Luciferase Assay Reagent (Toyobo) according to the manufacturer's instructions. Protein concentration was measured using a Bradford protein assay kit (BioRad) and luciferase strength was adjusted with protein concentration.

Results

Cytotoxicity against doxorubicin and vincristine were partially restored with indomethacin treatment in doxorubicin-resistant leukemia cells

As shown in Table 1, the doxorubicin-resistant cell line (K562/ADR) was strongly resistant to doxorubicin and vincristine, but not to etoposide, when compared with the parent cell line (K562/P). Next, we determined optimal concentration of indomethacin (Fig. 1) in K562 cells and we used a 10 μM indomethacin treatment in the following experiments. Indomethacin treatment significantly increased the cytotoxicity of doxorubicin and vincristine in K562/ADR cells ($P < 0.05$, Table 1).

Cox-1, and MRP1 RNA expression were increased in K562/ADR cells and decreased with indomethacin treatment

We then examined whether indomethacin treatment decreased expression of cyclooxygenases and MRP1 in K562/ADR cells. Cox-1 and MRP1 expression was increased in K562/ADR cells when compared with K562/P cells. Indomethacin treatment decreased Cox-1, Cox-2 and MRP1 expression in K562/ADR cells, but had little effect in K562/P cells (Fig. 2).

Intracellular glutathione content was increased in K562/ADR cells and decreased with indomethacin treatment

We next measured the intracellular content of glutathione in K562/P and K562/ADR cells with or without indomethacin treatment. The accumulation of intracellular glutathione was significantly increased in K562/ADR cells when compared to K562/P cells (Table 2, $P < 0.02$). Incubation of K562/ADR cells with 10 μM indomethacin resulted in significantly decreased accumulation of glutathione (Table 2, $P < 0.02$). These data suggest that the doxorubicin-resistant cells contained higher levels of glutathione and that indomethacin blocked this process.

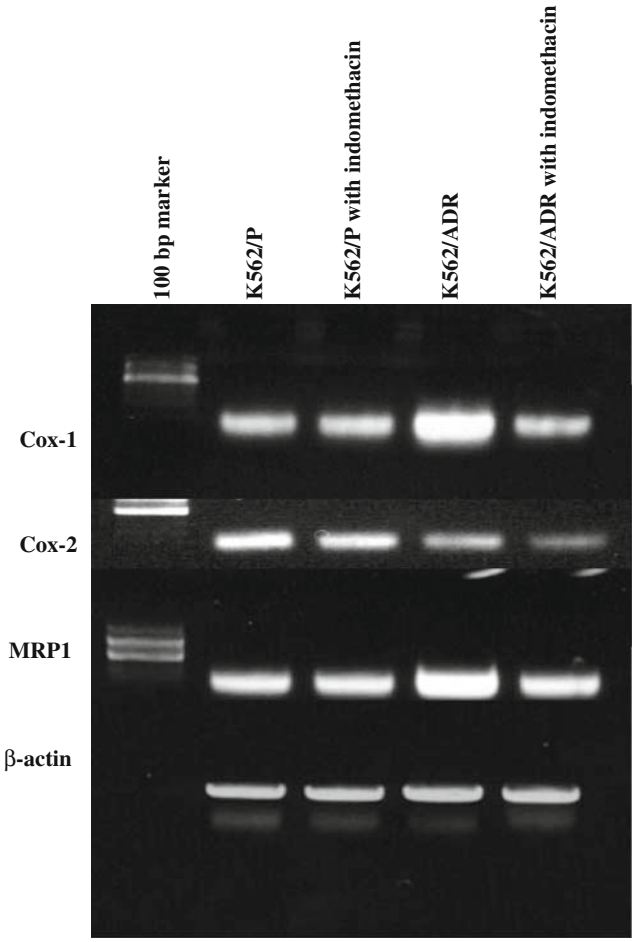

Fig. 2 Cox-1, 2 and MRP1 mRNA expression in K562/P and K562/ADR cells with or without indomethacin treatment. Increased expression of Cox-1 and MRP1 were observed in K562/ADR cells when compared with K562/P cells. Indomethacin treatment decreased expression in K562/ADR cells. Representative data from three independent experiments

Intracellular glutathione S-conjugate was increased in K562/ADR cells and decreased with indomethacin treatment

MRP1 has substrate specificity toward a variety of glutathione S-conjugates, and was thus used to investigate MRP1 pump-mediated transport by observing fluorescence by Thyolite under the microscope. The accumulation of fluorescence was more prominent in K562/ADR cells than in K562/P cells (Fig. 3a, b). The fluorescence intensity of intracellular vesicles in K562/ADR rapidly decreased after 90 min of incubation in MCB-free R10 medium and became almost identical to that in the parent cells (Fig. 3a, b). Incubation of K562/ADR cells with 10 μM indomethacin resulted in decreased accumulation of fluorescence, as was seen in K562/P cells (Fig. 3a, b). These data suggest that doxorubicin-resistant cells excrete the glutathione S-conjugate more effectively than the sensitive parent cells, and that indomethacin blocked this process.

γ-Glutamylcysteine synthetase mRNA and protein expression were increased in K562/ADR cells and decreased with indomethacin treatment

Because GSH content in K562/ADR cells is significantly higher when compared to K562/P cells, we investigated the expression of γ-glutamylcysteine synthetase (γ-GCS), which is the major synthetase of glutathione, in both cell types, as well as the effects of indomethacin on γ-GCS expression. Significant increases in mRNA and protein expression of γ-GCS were observed in K562/ADR cells when compared with K562/P cells (Fig. 4). Indomethacin treatment significantly decreased the expression of γ-GCS in K562/ADR cells (Fig. 4).

γ-Glutamylcysteine synthetase gene promoter activity in K562/ADR cells was decreased with indomethacin treatment

Actinomycin D is a substrate for MRP1 [16] and MDR1, and thus we were unable to perform stability assay using actinomycin D in K562/ADR cells overexpressing MRP1 and MDR1 proteins [14]. Instead, we performed promoter assay for the γ-GCS gene from K562/ADR cells. Indomethacin treatment significantly decreased the activity of the γ-GCS promoters (isolated from K562/ADR cells) in MDA231 cells ($P < 0.01$, Fig. 5).

Table 2 Relative intracellular glutathione content in K562 cells

Cell lines and treatment	K562/P	K562/P treated with indomethacin	K562/ADR	K562/ADR treated with indomethacin
Relative GSH content	1	1.5 ± 0.7 ($P = 0.13$)[a]	2.5 ± 0.7 ($P < 0.02$)[b]	1.2 ± 0.9 ($P < 0.02$)[c]

The relative GSH content was calculated the mean fluorescence (fluorescence arbitrary unit/ml) divided by the mean fluorescence in K562/P cells (fluorescence arbitrary unit/ml)

Data are average ± standard deviation from three experiments

[a] K562/P without indomethacin versus K562/P treated with indomthacin

[b] K562/P versus K562/ADR

[c] K562/ADR without indomethacin versus K562/ADR treated with indomethacin

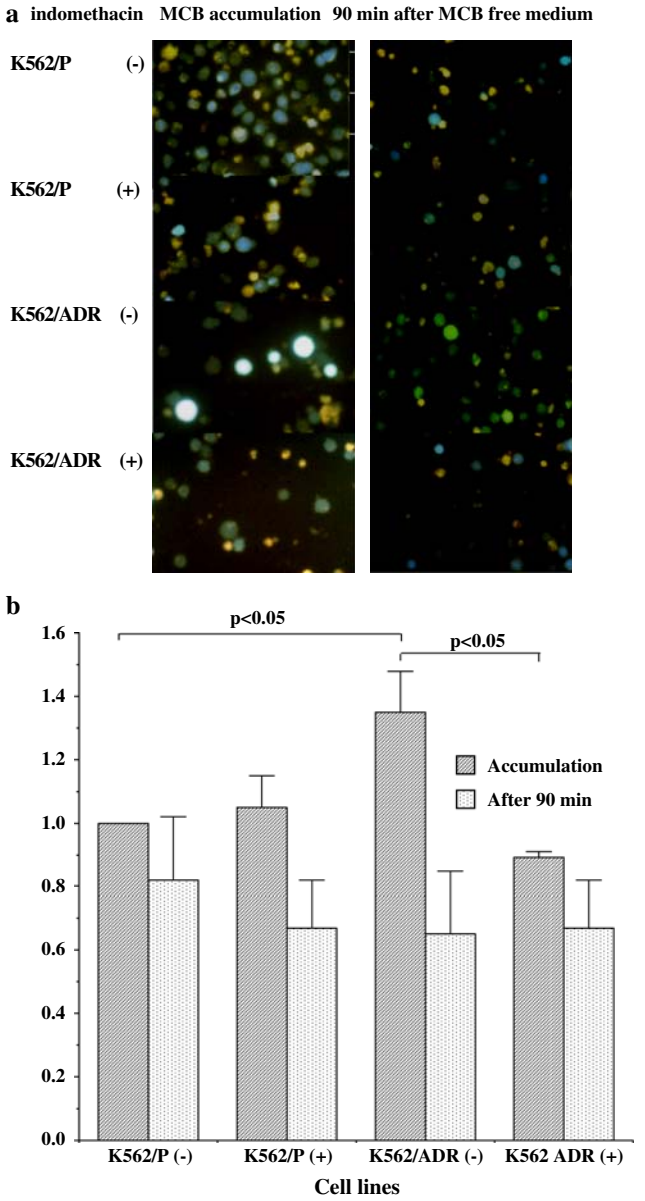

Fig. 3 **a** Intracellular glutathione conjugate in K562/P and K562/ADR cells with or without indomethacin treatment. Significant accumulation of glutathione–bimane conjugate was seen in K562/ADR cells when compared with K562/P cells. After 90 min of incubation in R10 media without MCB, glutathione-bimane content decreased significantly in K562/ADR cells (After 90 min). Indomethacin treatment significantly decreased accumulation of glutathione conjugate in K562/ADR cells, but not in K562/P cells. Represent data from five independent experiments. **b** Fluorescent intensity of intracellular glutathione conjugate in K562/P and K562/ADR cells with or without indomethacin treatment. MCB fluorescence intensity was measured with a UV spectrophotometer, as described in Materials and Methods. Significant accumulation of glutathione-bimane conjugate was seen in K562/ADR cells when compared with K562/P cells ($P < 0.05$). Indomethacin treatment significantly decreased accumulation of glutathione conjugate in K562/ADR cells ($P < 0.05$), but not in K562/P cells. Results are presented as ratio accumulation or efflux, as compared to K562/P cells in the absence of indomethacin. Data are means ± standard deviation from five independent experiments

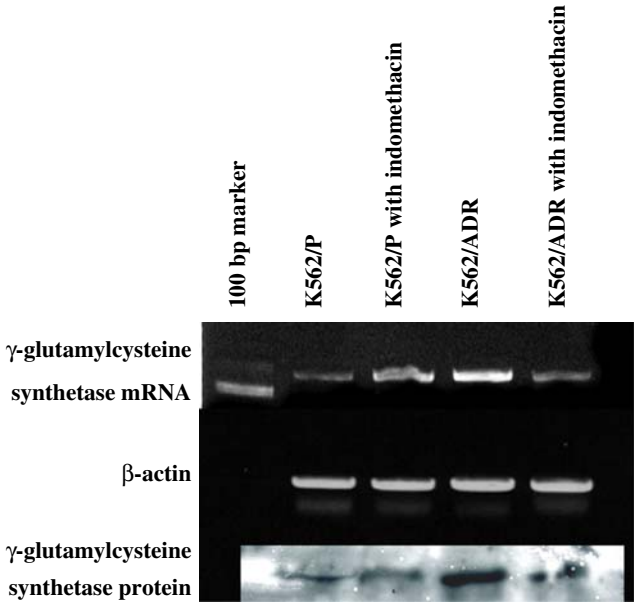

Fig. 4 γ-Glutamylcysteine synthetase (heavy subunit) mRNA and protein expression in K562/P and K562/ADR cells with or without indomethacin treatment. Increased mRNA and protein expression of γ-glutamylcysteine synthetase was observed in K562/ADR cells when compared with K562/P cells. Indomethacin treatment decreased expression of γ-glutamylcysteine synthetase in K562/ADR cells. Representative data from three independent experiments

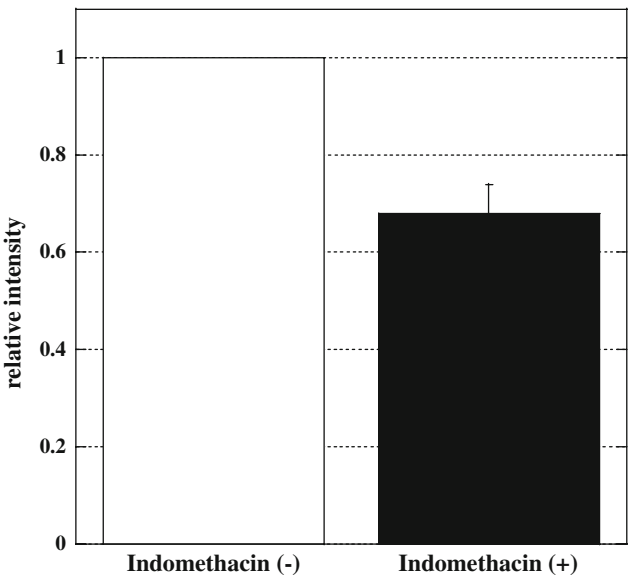

Fig. 5 γ-Glutamylcysteine synthetase (heavy subunit) promoter activity in MDA231 cells with or without indomethacin treatment. The promoter region of the γ-glutamylcysteine synthetase gene was amplified and cloned and then trasfected into MDA231 cells with various conditions in "Materials and methods". Indomethacin treatment significantly decreased promoter activity ($P < 0.01$). Results are presented as intensity relative to MDA231 cells without indomethacin treatment. (−) indicates absence of indomethacin and (+) indicates presence of indomethacin. Data are means ± standard deviation from three independent experiments

Discussion

Drug resistance is a serious problem in cancer therapy. Using indomethacin, a cyclooxygenase inhibitor, we were able to overcome doxorubicin resistance in K562/ADR cells by decreasing expression of γ-GCS and by decreasing the intracellular contents of glutathione and its conjugates. K562/ADR cells also exhibited MRP1 overexpression [14]. The MRP1 pump exports glutathione-conjugates from cells [11]. Overexpression of MRP1 in GLG4 cells is associated with a significant increase in intracellular GSH levels [18, 22]. In GLC4/ADR cells, drug transport (daunorubicin/GSH 1:1 co-transportation) in MRP1-overexpressing tumor cells can be regulated via intracellular GSH levels, as MRP1-mediated transport of daunorubicin requires the presence of GSH [18]. In our experiments, K562/ADR cells showed increased intracellular glutathione content along with increased efflux of glutathione-conjugates.

Incubation with indomethacin at 50 μM for 1 h reduced cellular glutathione content to 69% in CCRF-CEM/E1000 MRP1-overexpressing leukemia cells ($P < 0.01$), while having no effect on parental CCRF-CEM cells [3]. However, γ-GCS expression was not examined in that study. In mice, indomethacin decreased γ-GCS activity and GSH levels in intestines, but up-regulated it in liver [13]. Iida et al. [5] reported that a hammerhead ribozyme against γ-GCS sensitized human colonic cancer cells to cisplatin by down-regulating GSH and MRP1. In addition, we demonstrated that indomethacin decreases expression of MRP1 and γ-GCS mRNA in K562/ADR cells (present study, [14]), thus indicating that indomethacin is a novel sensitizer that acts via two mechanisms; decreasing doxorubicin export pump expression and decreasing doxorubicin conjugate substrate levels.

Intracellular glutathione is mainly regulated by γ-GCS gene expression and several reports, including the present study, have shown γ-GCS gene over-expression in resistant cells when compared with non-resistant cells [12, 23]. Based on the absence of changes in mRNA stability and a direct relationship (nuclear run-on assay) between steady-state mRNA levels for the heavy subunit of c-GCS, transcriptional activation is the major mechanism of up-regulating γ-GCS expression in resistant cells [23]. The promoter region, which we used in our experiments, contains an AP-1 sites [23]. Yao et al. [23] reported that nuclear extract binding activity to the AP-1 response element was closely associated with γ-GCS gene expression levels, which supports the present results.

In conclusion, the cyclooxygenase inhibitor indomethacin increased the cytotoxicity of doxorubicin by decreasing intracellular glutathione content and expression of γ-GCS via inhibition of γ-GCS promoter activity.

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