Docetaxel enhances the cytotoxicity of cisplatin to gastric cancer cells by modification of intracellular platinum metabolism

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We have examined the combined anticancer effects of docetaxel (DOC) and cisplatin (CDDP) *in vitro* using the gastric cancer cell lines MKN-45, MKN-74, and TMK-1. Treatment of the cell lines with 30 μg/ml of DOC for 24 h followed by incubation with 3 or 10 μg/ml of CDDP for 24 h showed a clear synergistic effect. Sequence dependency of the agents was observed in these cell lines: DOC followed by CDDP (DC) showed a stronger antitumor effect than CDDP followed by DOC (CD) in all cell lines. To clarify the mechanism of action of the DC combination, total intracellular platinum (Pt) levels were evaluated after treatment with CDDP alone or combined with DC. For the MKN-45 and -74 cell lines, cells treated with DOC (10 μg/ml for 12 h) and then CDDP showed significantly increased intracellular Pt accumulation compared to cells treated with CDDP alone. We also investigated alterations in intracellular glutathione (GSH) concentration in response to DOC and CDDP. MKN-45 and -74 cells pretreated with DOC (10 μg/ml for 12 h) showed significantly increased intracellular GSH levels compared to cells administered CDDP only. To explain these findings, messenger RNA (mRNA) levels for multidrug resistance-associated protein-1 (MRP-1), the ATP-dependent pump for Pt-GSH complexes, were quantified in CDDP-treated MKN-45 cells with and without DOC pretreatment. While CDDP administration increased MRP-1 mRNA expression in MKN-45 cells, MRP-1 was not up-regulated after CDDP administration in DOC pretreated MKN-45 cells. Our results suggested that the enhanced CDDP toxicity due to DOC pretreatment may be related to the accumulation of intracellular Pt-GSH complexes, because DOC appears to suppress the MRP-1 up-regulation induced by CDDP exposure in gastric cancer cells. (Cancer Sci 2004; 95: 679–684)

Docetaxel (DOC) is derived from needles of the yew tree *Taxus baccata* as a semi-synthetic compound, and has been shown to be active against several kinds of cancer, including gastric cancer. Clinical studies using DOC alone against gastric cancer provided an efficacy rate of approximately 20%,[1)] and the antitumor spectrum of DOC was different from those of conventional anticancer agents such as mitomycin C, doxorubicin, and cisplatin (CDDP).[2, 3)] Therefore, it is thought that DOC may be useful as a potent agent in combination chemotherapies with conventional chemotherapy agents.

Taxoids such as DOC bind to free tubulin and promote the formation of stable microtubules, inhibiting microtubule depolymerization. Recently, a relationship between bcl-2 phosphorylation, Raf-1 (Ras) signaling, and apoptosis during M phase cell cycle arrest induced by DOC was proposed.[4)] A study that examined transcription factor levels found that DOC appeared to induce AP-1 activation, and that resistance to DOC was characterized by increased AP-1 levels.[5)] Thus, DOC may possess potent ability to alter cell biology through the modification of transcription factor expression levels. We consider that DOC used in combination with conventional anticancer agents may be of considerable benefit in cancer therapy.

While the combination chemotherapy of DOC and CDDP has already been applied to the clinical treatment of several types of cancer, with high efficacy rates and only moderate toxic effects in phase I and II studies,[6–9)] the mechanism of action of this therapy remains to be clarified. Elucidation of this mechanism, including investigations into synergistic or additive antitumor effects, biochemical modulation, and the importance of sequence dependency, will assist the further clinical application of such combination chemotherapy.

In the present study, we demonstrated the synergistic antitumor activity of the DOC and CDDP combination against three gastric cancer cell lines, MKN-45, MKN-74, and TMK-1. We also clarified the sequence dependency and the optimal time-schedule of the two agents, and analyzed the mechanism of DOC enhancement of CDDP antitumor activity in our cell line models by evaluating the amounts of intracellular platinum (Pt) and its intracellular scavenger, glutathione (GSH). Lastly, we examined the expression dynamics of multidrug resistance-associated protein-1 (MRP-1) in response to DOC and/or CDDP treatment, and we propose a possible mechanism that may underlie the enhancement of CDDP toxicity by DOC in gastric cancer cells.

Materials and Methods

Cell lines and culture. Three human gastric cancer cell lines (MKN-45, MKN-74, and TMK-1) were obtained from the Japanese Cancer Research Resources Bank (Tokyo). Cells were maintained in RPMI 1640 (Gibco BRL, Gaithersburg, MD) with 1% antibiotics and 10% fetal bovine serum (FBS; F4010, Sigma, St. Louis, MO). All cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Drugs. DOC was kindly provided by Aventis Pharma, Japan (Tokyo). Cisplatin (CDDP) was purchased from Nippon Kayaku, Co., Ltd., Tokyo.

Drug treatment schedule. Wells of 96-well microplates containing 3×10^3 MKN-45 cells or 5×10^3 TMK-1 or MKN-74 cells were cultured in RPMI 1640 medium including 10% FBS for 24 h. Cells were then incubated without or with various concentrations of DOC for 24 h and then with various concentrations of CDDP for 24 h each. Cells were then washed with phosphate-buffered saline (PBS) to give a total incubation time of 96 h for all treatments. For the other experiments, the cancer cells were incubated with or without 10 μg of DOC for 24 h and/or 3 μg of CDDP for 12 h or 24 h, then harvested, and stored at −80°C.

MTT assay. Cancer cell survival and viability *in vitro* were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) assay developed by Mosmann *et al*., with some

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modifications.[10, 11] This method evaluates viable cell numbers by measuring the activity of mitochondrial succinate dehydrogenase. At the end of the incubation period, 0.4% MTT and 0.1 *M* sodium succinate, each dissolved in 10 µl of PBS and filtered through a 0.45-µm membrane filter (Millipore, Bedford, MA), were added to each well, and plates were incubated for an additional 3 h at 37°C. Dimethyl sulfoxide (DMSO) was then added to make a final volume of 150 µl/well, and plates were mechanically shaken for 10 min on a mixer (Model 250, Sonifier, Branson, MO) to dissolve the MTT-formazan product. Absorbance (Abs) at 540–630 nm was determined on a model EAR 340 easy reader (SLT-Labinstruments, Salzburg, Austria). Experiments were repeated at least three times to calculate the mean and standard deviation values for survival ratios, using the following formula: (Abs of treated cells−Abs of the blank)/(Abs of control cells−Abs of the blank).

Quantification of intracellular total platinum. The amount of total Pt in 10^7 cancer cells (µg/10^7 cells) was quantified by atomic absorption spectrophotometry (AAS) using a Z-8000R polarized Zeeman atomic absorption spectrophotometer (Hitachi Co., Ltd., Hitachi, Japan). After incubation with DOC/CDDP for 0, 6, 12, or 24 h, cancer cells were collected, cell numbers counted in each group, and each sample was subjected to AAS. The analytical detection limit for total Pt was 0.02 µg.

Intracellular glutathione quantification. Total GSH level in 10^7 cells was quantified using the Total Glutathione Quantification Kit (Dojindo Molecular Laboratories, Kumamoto, Japan). After incubation with or without DOC and/or CDDP for 6, 12, or 24 h, 10^5 cells were collected and homogenized with 10 m*M* HCl. The homogenate was placed in a 96-well microplate and the absorbance at 412 nm was measured. Reaction between 5,5′-dithiobis(2-nitrobenzoic) acid (DNTB) and intracellular GSH was quantified by comparing the absorbance at 412 nm for each sample with that of a GSH standard solution (0–100 µ*M*). Absorbance was measured 10 min after the addition of DNTB.

RNA extraction and cDNA synthesis. Total RNA was isolated using an RNeasy mini kit (Qiagen, Inc., Chatsworth, CA), and DNase treatment was performed using RNase-Free DNase Set (Qiagen, Inc.) according to the manufacturer's instructions.

Reverse transcription of up to 10 µg of total RNA was carried out in a total volume of 100 µl containing 250 pmol of oligo(dT)18, 80 U of rRNasin ribonuclease inhibitor (Promega, Madison, WI), and 500 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) in 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, and 0.5 m*M* dNTPs. Total RNA and oligo(dT)18 solutions were initially heated at 70°C for 10 min and immediately chilled on ice. The other reagents were then added and incubated for 15 min at 30°C and then 60 min at 42°C.

Primers and *Taq*Man probes. Primers and *Taq*Man probes for MRP-1 were designed using Primer Express software (PE Biosystems, Foster City, CA). Primers and *Taq*Man probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from PE Biosystems. Probes were labeled with a reporter dye (FAM) at the 5′ end of the oligonucleotide and a quencher dye (TAMRA) at the 3′ end. Sequences of primers and probes used were: GAPDH-forward primer, GAAGGTGAAGGTCGGAGTC; GAPDH-reverse primer, GAAGATGGTGATGGGATTTC; GAPDH-probe, CAAGCTTCCCGTTCTCAGCC (from GenBank accession no. M33197); MRP-1-forward primer, CCTGCAGCAGAGAGGTCTTTTC; MRP-1-reverse primer, GGCATATAGGCCCTGCAGTTC; and MRP-1-probe, CAAAGACGCCGGCTTGGTGTG (from GenBank accession no. L05628).

PCR procedure. Quantification of target cDNA (MRP-1) and an internal reference gene (GAPDH) was conducted using a fluorescence-based real-time PCR method (*Taq*Man PCR on an ABI PRISM 7700 Detection System, PE Biosystems).[12] PCR was carried out in a 25-µl reaction volume containing cDNA equivalent to 1–10 ng of total RNA, 200 n*M* each primer, 100 n*M* probe, and 12.5 µl of *Taq*Man universal PCR Master Mix (containing 1× *Taq*Man buffer, 200 µ*M* dATP, dCTP, dGTP, and 400 µ*M* dUTP, 5 m*M* $MgCl_2$, 1.25 U of Ampli*Taq*Gold, and 0.5 U of AmpErase UNG) (PE Biosystems). Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification of gene expression was performed using the relative standard curve method. The standard curve was created automatically by the ABI PRISM 7700 Detection System software by plotting the threshold cycle (CT) against each input amount of control total RNA (16, 4, 1, 0.25, 0.063, 0.016, and 0.0039 ng of total starting RNA), prepared from A549 human lung cancer cells (American Type Culture Collection, Manassas, VA). The coefficient of linear regression (r) for each standard curve was more than 0.990. For each unknown sample, the relative amount was calculated using linear regression analysis from the respective standard curve. A relative target gene expression value was obtained by division of the target gene value by the GAPDH value (internal reference gene).

Protein evaluation by western blotting. MRP-1 protein expressions in MKN-45 were evaluated at 0 and 24 h after the start of incubation with 3 µg/ml of CDDP, and compared with the MRP-1 protein expression of MKN-45 incubated with CDDP after preincubation with DOC. The cells were harvested and lysed with lysis buffer (50 m*M* Tris HCl pH 7.4, 250 m*M* NaCl, 0.1% Triton X-100, 2 m*M* EDTA, 1 m*M* DTT, 1 m*M* PMSF, 1.5 m*M* $MgCl_2$) containing protease inhibitor cocktail tablets (Roche Diagnostics K.K., Tokyo) according to the manufacturer's instructions. Cells were centrifuged at 14,000g for 10 min at 4°C. Supernatants were quantified using the Bradford assay (Bio-Rad Laboratories, Richmond, CA), and stored at−80°C. Aliquots of protein (40 µg) from each cell group were electrophoresed on 8% SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon-P, Millipore Co.). Mouse monoclonal antibodies for MRP-1 and actin were purchased from Santa Cruz Biotechnology, CA. After incubation with 5% non-fat skim milk in TBS for 1 h, membranes were probed with mouse polyclonal antibodies at 4°C overnight, and reactivity was detected using the BM chemiluminescence Western Blotting Kit (Roche Diagnostics K.K.) according to the manufacturer's instructions.

Data analysis. Statistical analysis was performed using Fisher's direct probability test, the χ^2 test, or Student's t test. A

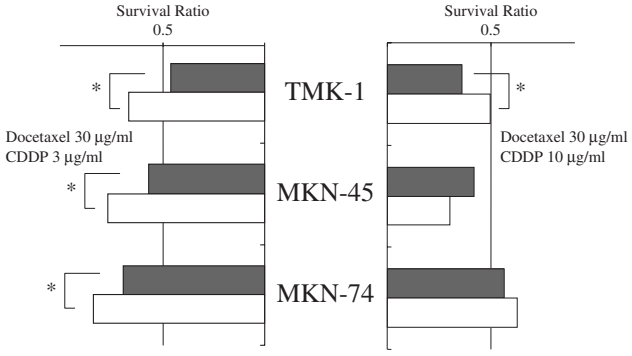

Fig. 1. Cells (3000 MKN-45 or TMK-1 cells/well or 5000 MKN-74 cells/well) were pre-incubated in 96-well microplates in RPMI 1640 medium including 10% fetal bovine serum (FBS) for 24 h, and then exposed to 30 µg/ml of DOC and/or 3 or 10 µg/ml of CDDP. Viability and survival of the cancer cells *in vitro* were evaluated by MTT assay. Solid bars show DOC followed by CDDP (DC treatment), and open bars show CDDP followed by DOC (CD treatment). * Statistically significant difference (P<0.05).

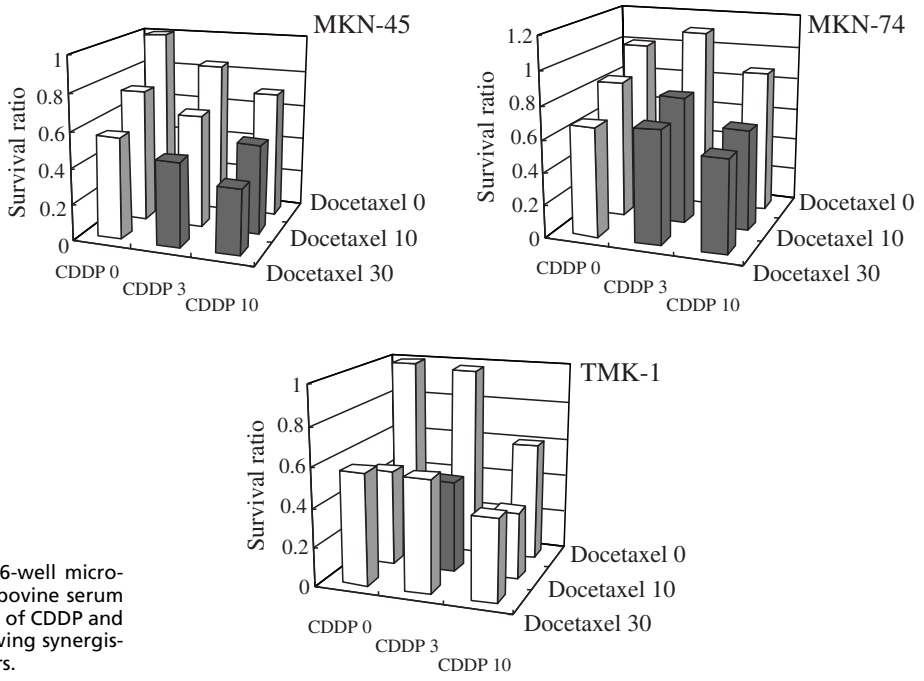

Fig. 2. Each cell line was pre-incubated in 96-well microplates, cultured in RPMI 1640 plus 10% fetal bovine serum (FBS) for 24 h, and exposed to 0, 3, or 10 μg/ml of CDDP and 0, 10, or 30 μg/ml of DOC. Combinations showing synergistic antitumor activity are indicated as solid bars.

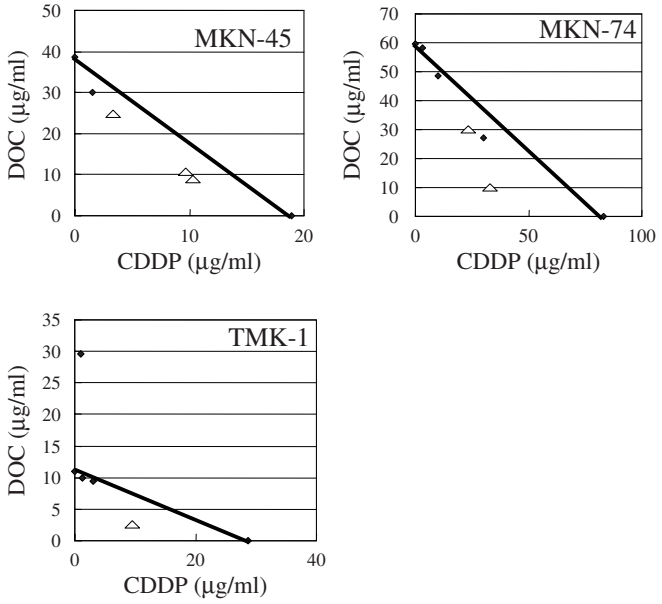

Fig. 3. Isobolograms demonstrating synergistic/antagonistic antitumor activity of DOC and CDDP combinations. The DC combination showed synergistic antitumor activity in MKN-45 and MKN-74 cells *in vitro*, while antitumor activity of DC against TMK-1 was only additive or antagonistic. Open triangles indicate a significant difference between the survival rate with the combination, and the expected rate calculated from the survival rates with the single agents ($P<0.05$).

significance level of $P<0.05$ was regarded as statistically significant.

Results

DOC followed by CDDP is the optimal sequential regimen in the combination. The sequence dependency of the *in vitro* antitumor activity of the DOC/CDDP combination was evaluated. Three

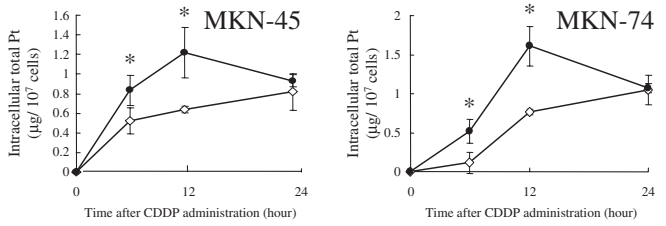

Fig. 4. Total platinum (Pt) levels in 10^7 cancer cells (μg/10^7 cells) were quantified by atomic absorption spectrophotometry (AAS) using a Z-8000R polarized Zeeman atomic absorption spectrophotometer (Hitachi). After incubation with DOC and/or CDDP for 0, 6, 12 and 24 h, cancer cells were collected and counted, and total Pt in each sample was measured by AAS. Solid symbols indicate DOC followed by CDDP and open symbols indicate CDDP alone. * Statistically significant difference ($P<0.05$).

gastric cancer cell lines were treated with 30 μg/ml of DOC and then 3 or 10 μg/ml of CDDP and the cell survival ratios were calculated (Fig. 1). The survival ratios of cells treated with 30 μg/ml of DOC followed by 10 μg/ml of CDDP (DC treatment) vs. that of cells treated with 10 μg/ml of CDDP followed by 30 μg/ml of DOC (CD treatment) were 0.462 vs. 0.666 for MKN-45 cells, 0.691 vs. 0.838 for MKN-74 cells, and 0.570 vs. 0.766 for TMK-1 cells, respectively. These results indicate a significantly higher antitumor activity for DC treatment compared to CD treatment.

Synergistic antitumor activity of the DC regimen in the cell lines. As shown in Fig. 2, the DC regimen exhibited synergistic activity against MKN-45 and MKN-74 cells using 10 or 30 μg/ml of DOC followed by 3 or 10 μg/ml of CDDP, while an additive antagonistic effect was observed in TMK-1 cells. Isobolograms to evaluate synergistic/antagonistic effects of the two agents (Fig. 3) confirmed that the DOC/CDDP combination displayed synergistic antitumor activity against MKN-45 and MKN-74 cells *in vitro*.

DOC-treatment induced accumulation of intracellular Pt after CDDP exposure. Total intracellular Pt levels were measured at 0, 6, 12, and 24 h in MKN-45 and -74 cells incubated with 3 μg/

ml of CDDP, and the results compared with those for untreated control cells and cells pretreated for 24 h with 10 μg/ml of DOC (DC) (Fig. 4). At 12 h after CDDP exposure, CDDP-treated cells without DOC pretreatment and untreated control cells displayed similar levels of intracellular Pt accumulation. In contrast, DC-treated cells exhibited almost two-fold higher Pt accumulation than control cells (1.22±0.26 vs. 0.64±0.03 μg/10^7 for MKN-45 cells, 1.61±0.34 vs. 0.77±0.06 μg/10^7 for MKN-74 cells, respectively), which was statistically significant (P<0.05). By 24 h after CDDP exposure, the amount of intracellular Pt in DC-treated cells had returned to control levels.

DOC treatment increased intracellular total GSH after CDDP exposure. DOC pretreatment for 24 h did not increase total intracellular GSH levels, as the GSH levels in control and DC-treated cells at the 0 h time point were not significantly different (145±30 and 135±56 μmol/10^6 for MKN-45 cells, and 308±69 and 260±30 μmol/10^6 for MKN-74 cells, respectively) (Fig. 5). DC-treated cells showed increased GSH levels for both cell lines after 6, 12, and 24 h incubation using 3 μg/ml CDDP. Statistically significant differences were observed at 6, 12, and 24 h after CDDP-only treatment using MKN-45 cells and at 12 h after CDDP-only treatment using MKN-74 compared to both control and DC-treated cells. The greatest differences between treatments were observed at 6 h after CDDP treatment for MKN-45 cells (165±31 μmol/10^6 cells for DC vs. 421±38 μmol/10^6 cells for CDDP-only), and at 12 h after CDDP treatment for MKN-74 cells (197±11 μmol/10^6 cells for DC vs. 548±87 μmol/10^6 cells for CDDP-only).

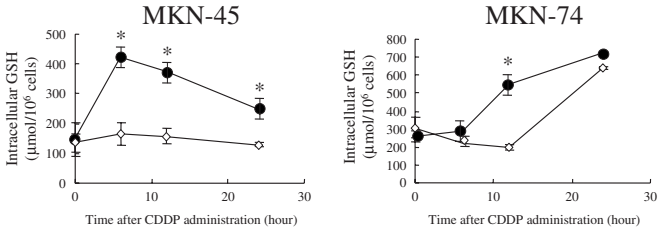

Fig. 5. Total GSH levels in 10^7 cells were quantified using the Total Glutathione Quantification Kit. After incubation with/without DOC/CDDP for 6, 12, and 24 h, 10^5 cells were collected and homogenized in 10 m*M* HCl, and placed into a 96-well microplate. Intracellular GSH was reacted with 5,5′-dithiobis(2-nitrobenzoic) acid (DNTB) and the absorbance was measured at 412 nm. Solid symbols indicate DOC followed by CDDP and open symbols indicate CDDP alone. * Statistically significant difference (P<0.05).

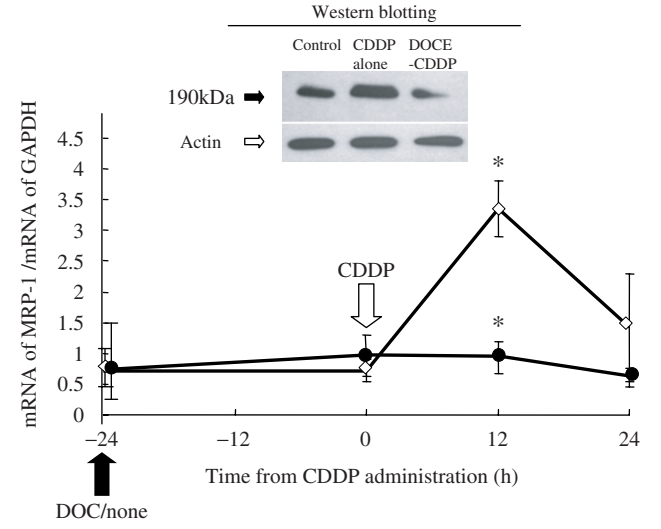

Fig. 6. Total RNA was isolated using an RNeasy mini kit (Qiagen, Inc.) and DNase-treated with an RNase-Free DNase Set (Qiagen, Inc.), and first-strand cDNA was synthesized according to the manufacturer's instructions. Quantification of mRNA for MRP-1 and an internal reference gene (*GAPDH*) was performed by fluorescence-based real-time PCR (*Taq*Man PCR with an ABI PRISM 7700 Detection System, PE Biosystems) using the relative standard curve method. Relative target gene expression values were obtained after division by the control GAPDH value. Open circles indicate CDDP alone and closed circles indicate DOC followed by CDDP. * Statistically significant difference (P<0.05). The protein expression of MRP-1 was also checked by western blotting (upper photograph). The results indicate suppression of CDDP-induced MRP-1 up-regulation by DOC pretreatment.

CDDP-induced MRP-1 up-regulation was suppressed by pretreatment with DOC. Expression levels of MRP-1 mRNA in MKN-45 cells were calculated as: (ratio of amount MRP-1 mRNA/amount GAPDH mRNA in CDDP-treated cells)/(ratio amount MRP-1 mRNA/amount GAPDH mRNA in cells at 0 h after CDDP treatment). As shown in Fig. 6, MRP-1 mRNA expression in MKN-45 cells was enhanced by CDDP treatment. Levels peaked at 12 h after treatment (4.67 times the control), but had decreased by 24 h (1.97 times the control). In DOC-pretreated cells, a statistically significant (P<0.05) suppression of enhanced MRP-1 expression by CDDP exposure was observed at the 12 h time point. This suggested that DOC pretreatment enhanced the CDDP toxicity against MKN-45 cells through suppression of the MRP-1 expression, which would normally be enhanced by CDDP exposure in DOC non-treated cells. Fig. 6 also shows suppressed CDDP-induced MRP-1 up-regulation by DOC pretreatment at the protein level, as well as the mRNA level.

Discussion

While the efficacy rate of conventional anticancer agents, such as mitomycin C, doxorubicin, or 5-fluorouracil, against gastric cancer is reported to be only 10–20%,[13–16] newly developed anticancer agents such as oxaliplatin, CPT-11, and taxanes (paclitaxel and DOC) show comparatively high efficacy rates, especially when used in combination chemotherapies alongside conventional agents.[6–9] DOC has been used clinically for a variety of cancers, including cancer of the lung, ovary, and stomach, and when used as a single-use agent gave efficacy rates of approximately 20%.[1] While the combination of CDDP and DOC has demonstrated good efficacy against lung and breast cancer,[6–9] the optimal DOC/CDDP regimen has not yet been determined, experimentally or clinically. DOC has a unique mechanism as a chemotherapeutic agent; it exhibits antimicrotubule properties, inhibiting microtubule depolymerization, causing disruption of the microtubule system at cell division and inducing cell death.[17, 18] As the mechanism of DOC-mediated cell death is different from that of conventional antitumor agents, and DOC appears not to show cross resistance with other agents,[2, 3] it was thought that the potent antitumor properties of DOC would improve the overall efficacy of chemotherapies based on conventional agents such as CDDP.

The results of our study showed that treatment of MKN-45 and MKN-74 gastric cancer cells *in vitro* with DOC followed by CDDP (DC) showed a higher efficacy than treatment with CDDP followed by DOC (CD). While the synergistic effects of DC were also clear in an isobologram, synergy was not observed using the TMK-1 cell line. GSH metabolism after CDDP exposure was not altered by DOC treatment in TMK-1 cells (data not shown), which supports the hypothesis that the synergistic antitumor activity of DC is related to cellular Pt and GSH metabolism.

Several studies using *in vitro* cell lines have suggested that acquired cisplatin resistance is associated with defects in the apoptotic program, decreased cisplatin accumulation, and in-

creased drug inactivation via several mechanisms.[19–21] Other studies have reported that altered expression of molecules such as c-myc and the adenosine triphosphate (ATP) binding cassette (ABC) superfamily of transport proteins, and modifications in signal transduction pathways such as that of phosphatidylinositol 3′-kinase are associated with cisplatin resistance.[22, 23] Multidrug resistance has been shown to be conferred by various integral membrane proteins, including the 170-kDa P-glycoprotein (multidrug resistance gene-1; MDR-1) and the 190-kDa multidrug resistance associated proteins (MRPs), which belong to the adenosine triphosphate (ATP) binding cassette (ABC) family of transport proteins.[24–26] In the present study, we evaluated MRP-1 expression in connection with the alteration of platinum metabolism by DOC, since several reports have concluded that MDR-1 and MRP-1 are molecules related to CDDP-resistance,[27–32] and overexpression of MRP-1 is known to be associated with a reduction in intracellular cisplatin accumulation.[2] In addition, MRP-1 is reported to be related with CDDP drug resistance in a variety of tumor types including lung carcinoma,[27–29] gastric carcinoma,[30] retinoblastoma,[31] and acute myeloid leukemia,[32] and there is much indirect evidence that MRP-1 is involved in CDDP-resistance.[33–35]

Increases in both total Pt accumulation and total GSH in MKN-45 and MKN-74 cells were observed in our study, with the largest differences in Pt and GSH levels in cells treated with DC or CDDP-only being observed relatively early (6 to 12 h) after CDDP administration. These results demonstrated that CDDP exposure may stimulate cancer cells during early phases and maintain cell viability, with increased Pt efflux combined with GSH outside the cells leading to decreased Pt accumulation inside the cells. We also observed up-regulation of MRP-1 mRNA expression after 12-h CDDP exposure in MKN-45 cells without DOC pretreatment, in agreement with a previous study[19] that investigated the cellular response to CDDP stimulation. While the levels of other molecules may also be altered by CDDP stimulation,[36] it is highly likely that it is the increased MRP-1 expression in response to CDDP exposure that underlies the synergism of the DOC/CDDP combination. Nonpretreated MKN-45 or -74 cells increased GSH levels 6 h after CDDP treatment, which would effectively bind the Pt within the cell. This, coupled with enhanced MRP-1 mRNA expression, and the resultant increase in MRP-1 protein levels, would increase Pt efflux and maintain cell viability. In contrast, DOC-pretreated cells failed to increase MRP-1 expression, for some as yet unknown reason. This would decrease the efflux of the Pt-GSH complexes compared to that in non-DOC-treated cells, thereby increasing the intracellular Pt-GSH levels and enhancing CDDP toxicity against the gastric cancer cell lines.

Conclusion

The results of our study have uncovered a mechanism that may underlie the synergistic relationship between the components of the DC regimen in terms of alterations in the levels of particular proteins expressed by cancer cells. To allow the further development of effective combination regimens involving DOC, it will be useful to investigate the regulatory networks modulated by DOC in cancer cells, with regard to gene regulation and transcription factors, in addition to the direct functional roles of the DOC molecule.

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