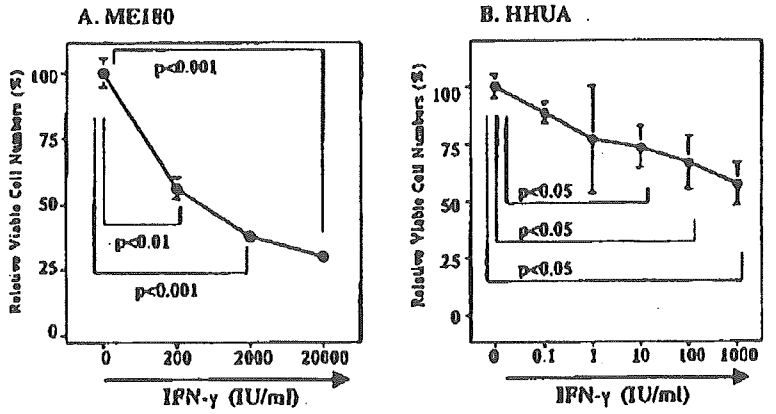

Figure 2. Effects of IFNγ on the proliferation of ME180 and HHUA cells. INFγ dose-dependently inhibits the proliferation of ME180 (A) and HHUA (B) cells.

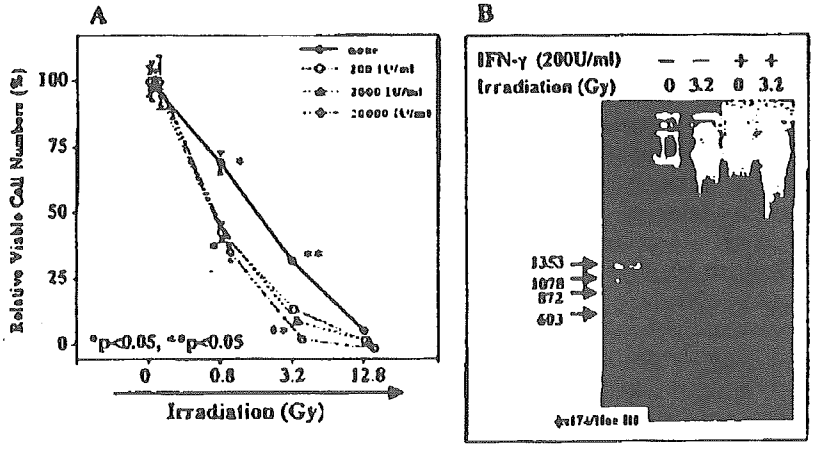

Figure 3. Effects of IFNγ on the radiosensitivity of ME180 cells. A, effects of IFNγ on the radiosensitivity curves of ME180 cells. IFNγ significantly enhances the irradiation-induced growth inhibition of ME180 cells. B, DNA degradation assay. DNA degradation in irradiated ME180 cells is slightly enhanced by IFNγ. No DNA fragmentation is detected.

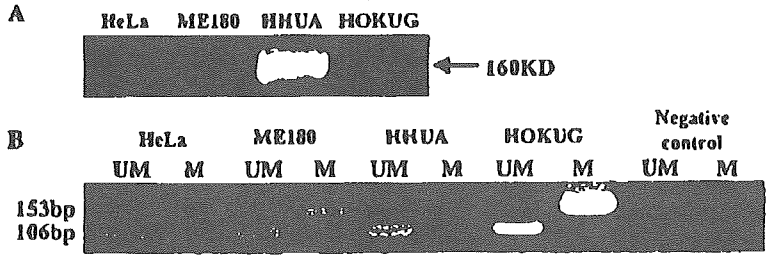

Figure 4. DAPK protein expression and DAPK-MS-PCR in 4 cancer cell lines. A, Western blot analysis of DAPK protein expression in the 4 human uterine carcinoma cell lines. B, DAPK-MS-PCR. The presence of a 153-bp product in lane M indicates the presence of DAPK gene promoter methylation, while a 106-bp product in lane UM indicates the presence of the unmethylated promoter sequence. The DAPK gene promoter is hypermethylated in ME180 and HOKUG cells. Distilled water was loaded in the lanes labeled negative control.

In order to examine whether DAPK expression is involved in the radiosensitivity of cancer cells, Western blot analyses were carried out to detect DAPK protein expression in ME180, HHUA and HOKUG cells. DAPK protein expression was undetectable in both ME180 cells, which were the most radiosensitive, and HOKUG cells, which were the least radiosensitive (Fig. 4A). HHUA cells, which were moderately radiosensitive, showed strong DAPK expression. To investigate the relationships between DAPK protein expression and CpG island hypermethylation of the DAPK gene promoter regions

Table I. Comparisons of the radiosensitivity and DAPK expression in 3 human uterine cancer cell lines.

	ME180	HHUA	HOKUG
Radiosensitivity	++	+	-
DAPK protein expression	-~±	+++	+
Hypermethylation of the DAPK gene promoter	++	-	++

in the 3 cell lines as well as in HeLa cells, MS-PCR for DAPK was performed. Methylation of the CpG islands was detected in ME180 and HOKUG cells, but not in HeLa or HHUA cells (Fig. 4B). These results indicate that the DAPK expression in these 4 cell lines was regulated by methylation of the DAPK CpG island. Taking all these results together, there was no relationship between radiosensitivity and DAPK protein expression or DAPK CpG island hypermethylation in the ME180, HHUA and HOKUG cell lines (Table I).

To investigate the relationships between radiation-induced cell death and DAPK-mediated apoptosis, changes in the DAPK expression in irradiated ME180 cells were examined. A Western blot analysis revealed that the DAPK protein expression in ME180 cells was slightly, but not significantly, increased after irradiation (Fig. 5A). Considering the result that radiation may induce DAPK expression and the report that DAPK stimulates IFNγ-induced apoptosis in HeLa cells (1), it was hypothesized that irradiation would not reduce the sensitivity of ME180 cells to IFNγ. In fact, however, irradiation significantly reduced the sensitivity of ME180 cells to IFNγ in a dose-dependent manner (Fig. 5B).

INFγ-induced apoptosis in HeLa cells is inhibited by suppression of DAPK expression (1), suggesting that the DAPK expression level plays an important role in IFNγ-induced cell death. In ME180 cells, however, IFNγ inhibited cell proliferation but did not induce cell death (Figs. 2A and 3B). The different effects of IFNγ on growth inhibition and cell death in HeLa and ME180 cells may arise from two independent signals induced by IFNγ, namely growth-inhibitory signals and apoptotic signals. Therefore, CDDP, which inhibits cancer cell growth with the induction of apoptosis, was used to investigate the relationships among CDDP-induced cell death, DAPK protein expression and radiation-induced cell death. We examined DAPK protein expression and radiosensitivity in 6 CDDP-resistant subclones established from ME180 cells in order to investigate the mechanisms of their drug-resistance. As shown in Fig. 6B, all 6 CDDP-resistant subclones were strongly radioresistant. Moreover, Western blot analysis revealed that DAPK protein expression was undetectable in all 6 CDDP-resistant subclones, similar to the case for the parent ME180 cells (Fig. 6C).

Discussion

The results of the present study indicate that the radiosensitivity of cancer cells is highly unlikely to be affected by the DAPK protein expression level. The radiosensitivity of ME180 cells, in which DAPK protein expression is strongly suppressed, is very high, as is usual for cervical squamous cell carcinoma cells. Furthermore, we established 6 CDDP-resistant subclones from ME180 cells that were all much more radioresistant than the parent cells and found that their DAPK expressions were undetectable. These results indicate that the acquisition of CDDP-resistance or radioresistance does not require the induction of DAPK protein expression. From the results of experiments on 3 of the cell lines (summarized in Table I), the radiosensitivity of cancer cells has no relationship with either DAPK protein expression or DAPK CpG island methylation. We strongly suggest that the DAPK protein expression level may have no regulatory function in the radiosensitivity of cancer cells.

The present study has proven that DAPK-mediated signals are not essential for the growth-inhibitory signals of IFNγ. In HeLa cells, IFNγ-induced apoptosis can be inhibited by suppression of DAPK protein expression (1), suggesting that DAPK-mediated signals are essential for the IFNγ-induced apoptosis in these cells. Growth inhibition by IFNγ was found in ME180 cells, in which DAPK protein expression

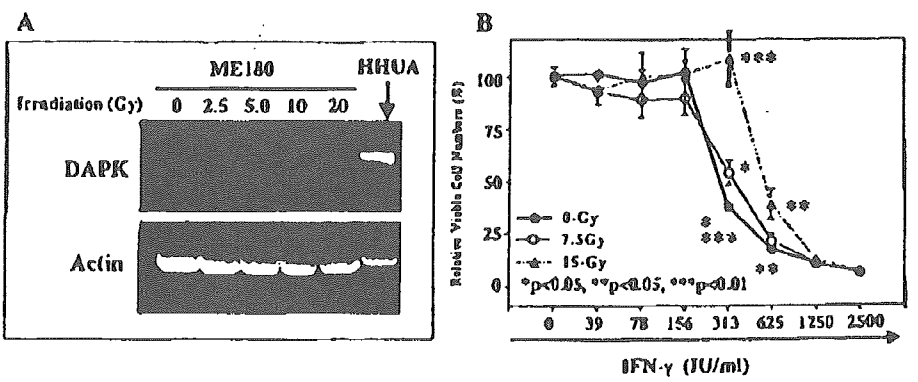

Figure 5. Effects of radiation on DAPK protein expression and sensitivity to IFNγ. A, effects of irradiation on DAPK protein expression in ME180 cells examined by Western blot analysis. DAPK protein expression (normalized to the actin protein expression) is slightly increased after irradiation. B, effects of irradiation on the sensitivity of ME180 cells to IFNγ. Irradiation significantly reduces the IFNγ-sensitivity in a dose-dependent manner.

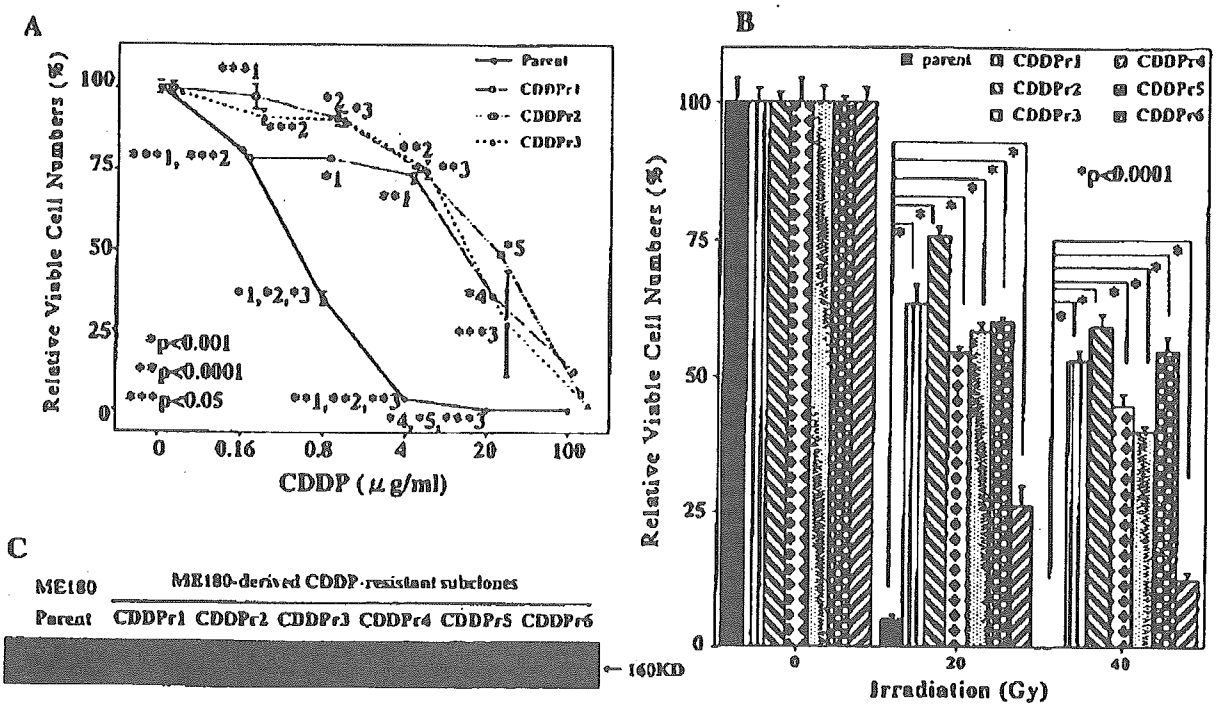

Figure 6. DAPK protein expression and radiosensitivity in ME180-derived CDDP-resistant subclones. A, CDDP-sensitivity of the CDDP-resistant subclones. The CDDP-sensitivity curves of 3 CDDP-resistant subclones, namely CDDPr1, CDDPr2 and CDDPr3, are shown. The other 3 CDDP-resistant subclones showed the same results (data not shown). All the established subclones are significantly more resistant to CDDP than the parent ME180 cells (closed circles with a solid line). Open circles with a dotted line, CDDPr1; open squares with a dotted line, CDDPr2; closed triangles with a dotted line, CDDPr3. B, radiosensitivities of the parent ME180 cells and 6 CDDP-resistant subclones. All 6 CDDP-resistant cells are sigoificantly more radioresistant than the parent cells. C, Western blot analysis of DAPK protein expression in the parent ME180 cells and 6 CDDP-resistant subclones. No DAPK protein expression is detected in any of the 6 CDDP-resistant subclones.

is strongly suppressed. However, although IFNγ strongly inhibited ME180 cell proliferation, it did not induce detectable cell death. In other words, the cell growth inhibition of ME180 cells by IFNγ was not associated with apoptotic changes. These results mean that DAPK-mediated signals are not essential for the growth-inhibitory signals induced by IFNγ-stimulation in ME180 cells. Further studies are required to determine whether or not DAPK-mediated signals are essential for the apoptosis induced by IFNγ, since IFNγ inhibited cell proliferation but did not induce apoptosis in HHUA cells, which show a very high level of DAPK protein expression.

IFNγ enhanced radiation-induced cell death in ME180 cells. On the other hand, radiation significantly reduced the sensitivity to IFNγ, while DAPK protein expression was slightly, but not significantly, enhanced after irradiation. As described above, DAPK-mediated signals are not essential for the growth-inhibitory signals induced by IFNγ in ME180 cells. Therefore, we cannot exclude the possibility that this slight increase in DAPK expression induced by irradiation may enhance radiation-induced cell death. However, since HHUA cells with a high level of DAPK protein expression were much less radioresistant than ME180 cells with an undetectable level of DAPK protein expression, it appears very unlikely that DAPK-mediated signals affect radiation-induced cell death.

In conclusion, the present study has almost proven that there is no relationship between DAPK protein expression level and radiosensitivity in ME180 cells, whose radiosensitivity can be enhanced by IFNγ. From our results, DAPK-mediated signals do not appear to have any effect on radiation-induced cell death although DAPK has been reported to be involved in IFNγ-induced cell death, apoptosis mediated by Fas or TNF-receptor and cell death induced by detachment of cells from the extracellular matrix (1-4). Since DAPK expression in tumor cells is thought to regulate metastasis and prognosis in cancer patients, DAPK has been proposed as a candidate molecular target for gene therapy. However, the current results indicate that DAPK is highly unlikely to be either a molecular target for combined radiotherapy with any gene therapy or a prognostic marker for radiotherapy in cancer patients. Radiation-induced cell death signals are considered to be independent of the DAPK-mediated apoptotic signals.

Acknowledgments

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Radiation enhances cisplatin-sensitivity in human cervical squamous cancer cells *in vitro*

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Summary

Purpose and methods of investigation: Cisplatin (CDDP) is regularly used in concurrent chemoradiotherapy in patients with advanced cervical cancer although an effective protocol of chemoradiotherapy with CDDP has not yet been established. In search of a better chemoradiotherapy protocol, we investigated both CDDP effects on radiosensitivity and irradiation effects on CDDP-sensitivity using the radiosensitive human cervical squamous cell carcinoma cell line ME180.

Results: We found that CDDP did not affect cellular radiosensitivity, and that irradiation significantly enhanced CDDP-sensitivity. Moreover, all the four post-irradiation surviving subclones obtained from repetitively irradiated ME180 cells showed significantly higher CDDP sensitivities than those of the non-irradiated parent cells.

Conclusion: These results suggest that an effective protocol would involve the concurrent administration of CDDP with radiotherapy and further administration following completion of radiotherapy in order to achieve higher CDDP-sensitivities.

Key words: Cisplatin; Chemoradiotherapy; Cervical cancer; Squamous cell carcinoma; Radiosensitivity.

Introduction

Since most cervical squamous cell carcinoma (SCC) cells are radiosensitive, patients with unresectable advanced cervical SCC are usually treated by radiotherapy as the first choice of therapy. However, standard radiotherapy of cervical cancer patients is often non-radical for locally advanced cervical cancers with either huge primary tumors, wide invasion to pelvic walls, many lymph node metastases, or possible distant micrometastases. Therefore, in order to i) eradicate cancer cells outside irradiated fields, ii) enhance the radiosensitivity of cancer cells during radiotherapy, iii) and kill surviving cancer cells after irradiation, chemoradiotherapy has sometimes been concurrently applied to patients with locally advanced cervical cancer.

Cisplatin (CDDP) is thought to be the most effective anticancer drug for cervical cancer. Therefore, CDDP has been the most frequently used worldwide in concurrent chemoradiotherapy in patients with advanced cervical SCC. Several research groups interested in concurrent chemoradiotherapy for cervical cancer patients reported significant increases in survival ratios of cancer patients treated with concurrent chemoradiotherapy using CDDP [1-5]. On the other hand, a few studies could not find any beneficial effects on survival times for patients receiving CDDP chemoradiotherapy [6]. In these studies, usual administration protocols of CDDP in concurrent chemoradiotherapy involved weekly injections of 40-75 mg/m^2 CDDP [1-6]. Recently, a few basic investigations reported the effective concurrent use of CDDP with radiotherapy. In addition, Tabata *et al.* [7] showed that cervical cancer patients pretreated with bleomycin, vincristine, mitomycin and cisplatin (BOMP) chemotherapy including CDDP before radiotherapy demonstrated a lower survival ratio compared with patients treated with radiotherapy alone. These results suggest that the use of BOMP chemotherapy before radiotherapy reduces radiosensitivity of cervical cancer cells. In the present study, we used radiosensitive human cervical squamous carcinoma cells to investigate several options of chemoradiotherapy with CDDP in order to achieve optimal treatment.

Materials and Methods

Cell line and cell culture

In this study, the radiosensitive human cervical SCC cell line ME180 with wild-type p53 genes [8] was used. The ME180 cells were obtained from Japan Resources of Cell Bank (JRCB, Tokyo, Japan). All cells used in this study were cultured in OPTI-MEM (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 5% fetal calf serum (FCS) (EQUITECH BIO Inc., Ingram, TX, USA) and a mixture of 100 U/ml penicillin/100 μg/ml streptomycin (GIBCO-BRL). CDDP used in this study was a gift from Nihon-Kayaku Co. (Tokyo, Japan).

Cell viability assay

Cell proliferation was assessed with the XTT non-RI colorimetric assay kit (Boerhinger Mannheim, Mannheim, Germany). Growth-inhibitory effects of radiation and CDDP on ME180 cells were investigated as follows. Cells in the log phase were initially dispersed with 0.25% trypsin/1 mM EDTA (GIBCO-BRL), and subsequently cultured overnight in 96-well culture plates (5,000 cells/well). On the second day, various doses of γ-rays were used to irradiate the cells using a MBR 1520A irradiator (Hitachi-Medico, Tokyo, Japan). On the fourth day, viable cells were counted with the XTT kit. In order to examine the modulatory effects of CDDP on cell death induced by irradiation, cells were treated with various concentrations of CDDP

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and immediately γ-irradiated at different doses, followed by a 2-day culture. Finally, relative viable cell numbers (expressed as a percentage) were calculated using the XTT kit. All experiments were repeated two or three times to verify the results. Data are reported as means followed by standard deviations (SD), and comparative data (n = 6) were statistically analyzed by ANOVA.

Establishment of surviving subclones following repetitive irradiations

Post-irradiation surviving subclones were established as follows. ME180 parent cells cultured in a 96-well culture plate (10,000 cells/well) were subjected to four consecutive doses of radiation (10 Gy each) once a week, and cultured for about four weeks. In a preliminary experiment, more than 90% of ME180 cells were killed after a single dose of 10 Gy γ-ray irradiation. Cells were collected from each of the four wells containing surviving cancer cell colonies and sub-cultured with a lower cell density (0.1-20 cells/well) using a limiting dilution protocol. Cloning efficiencies assessed from the limiting dilution cultures were below 10% (3.7%-9.1%). Finally, four months following the initial irradiation, four monoclonal post-irradiation surviving subclones were established.

Results

First of all, effects of CDDP on radiosensitivity of ME180 cells were examined. As illustrated in Figure 1, CDDP did not have any significant effect on ME180 radiosensitivity curves. Secondly, effects of irradiation on ME180 CDDP-sensitivity were investigated and we found that irradiation significantly enhanced CDDP-sensitivity (Figure 2). In order to determine whether post-irradiated cancer cells maintain the higher CDDP-sensitivity following irradiation, we established four post-irradiation surviving subclones according to methods described above and examined their sensitivity to CDDP. All four established subclones demonstrated significant higher CDDP-sensitivities than the parent ME180 cells as shown in Figure 3, suggesting that post-irradiation increases CDDP-sensitivity and this can be maintained for at least four months after initial irradiation.

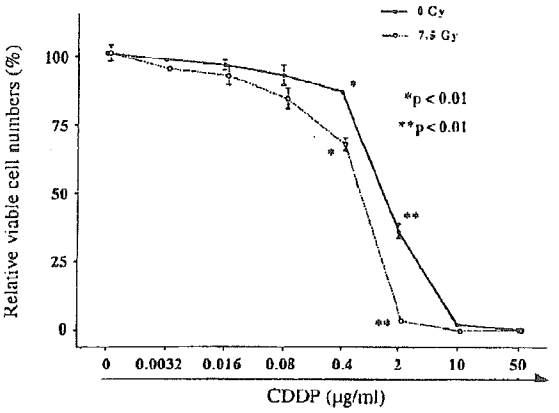

Figure 2. — *Effects of irradiation on CDDP-sensitivity of ME180 cells.*
Within 20 minutes after various concentrations of CDDP were added to ME180 cells, γ-rays were used for radiation. The solid line with closed circles shows the control CDDP-sensitivity curve of cells cultured without irradiation. γ-ray irradiation (7.5 Gy) significantly enhanced the CDDP-sensitivity (dotted line with open circles) (*p < 0.01, **p < 0.01).

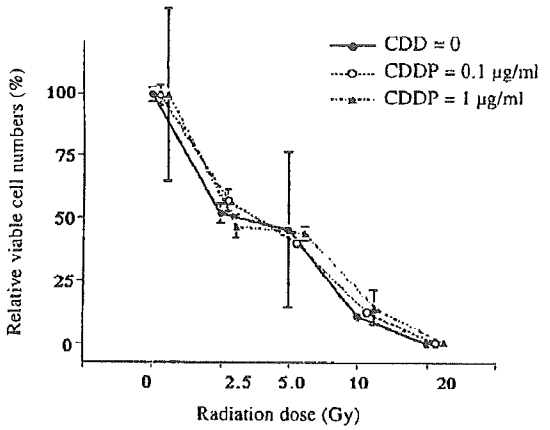

Figure 1. — *Effects of CDDP on radiosensitivity of ME180 cells.*
Within 20 minutes after initial addition of CDDP to ME180 cells, various doses of γ-rays were used for radiation. Final CDDP concentrations in culture media are 0, 0.1, and 1 μg/ml, respectively. The solid lines with closed circles show control radiosensitivity curves of cells cultured without CDDP. The dotted lines with open circles and closed triangles are those of cells cultured with CDDP. There was no significant difference between the three radiosensitivity curves.

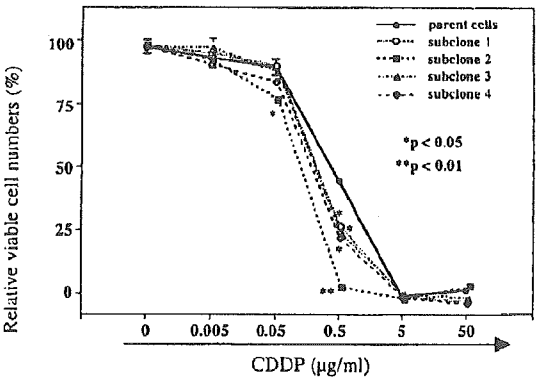

Figure 3. — *CDDP-sensitivity of post-irradiated surviving cells.*
CDDP-sensitivity of the four subclones established from surviving cells following irradiation was compared with the CDDP-sensitivity of non-irradiated ME180 parent cells. The solid line with closed circles shows the control CDDP-sensitivity curve of ME180 parent cells. All four post-irradiated surviving subclones (dotted lines) displayed significantly higher sensitivities to CDDP compared with non-irradiated parent cells (*p < 0.05, **p < 0.01).

Discussion

Radiotherapy is the most commonly used therapy for locally advanced cervical cancers and CDDP is also one of the most effective anticancer drugs used for advanced cervical cancers. Therefore, CDDP has been widely used in concurrent chemoradiotherapy for advanced cervical cancer patients [1-6]. Although many reports showed that concurrent chemoradiotherapy with CDDP resulted in significantly higher survival ratios than those of radiotherapy alone [1-5], there have been few reports on how to effectively administer CDDP during radiotherapy. Studies reporting concurrent chemoradiotherapy with CDDP used weekly injections of 40-75 mg/m^2 CDDP. When and how CDDP should be injected to cancer patients during radiotherapy has not been investigated yet. Tabata et al. reported that BOMP chemotherapy with the use of CDDP before radiotherapy in cervical cancer patients resulted in significantly lower survival ratios than those in patients treated with radiotherapy alone [7]. This report suggests that radiosensitivity of cancer cells may be reduced by BOMP chemotherapy. Additionally these results make us aware that chemoradiotherapy of cervical cancers may provide worse treatment than radiotherapy alone in cases of inadequate administration of chemotherapeutic drugs.

In our study, we used radiosensitive human cervical SCC cells to report optimal treatment conditions for combined chemoradiotherapy with CDDP. Although CDDP does not affect radiosensitivity of cancer cells, irradiation significantly enhances CDDP-sensitivity. Moreover, we found that surviving cancer cells following irradiation have a higher CDDP-sensitivity. These results suggest that CDDP should be administered to cervical cancer patients not before but after irradiation. Our results coincide with clinical results reported by Tabata *et al.* [7]. In conclusion, post-irradiation CDDP injection may be a better treatment than concurrent chemoradiotherapy alone because irradiated cancer cells may retain higher CDDP-sensitivity for several months after irradiation. Moreover, our proposed investigative procedures can be applied to other studies aimed to optimize other combinations of anticancer drugs with radiotherapy in cervical cancer patients.

Acknowledgments

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A novel mechanism for acquired cisplatin-resistance: Suppressed translation of death-associated protein kinase mRNA is insensitive to 5-aza-2'-deoxycitidine and trichostatin in cisplatin-resistant cervical squamous cancer cells

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Abstract. The molecular mechanism for cisplatin (CDDP)-resistance of cancer cells has not yet been clarified, despite extensive studies. Here, we investigated whether death-associated protein (DAP) kinase, an apoptosis modulator, was involved in CDDP-resistance by examining the ME180 human cervical squamous cancer cell line and 6 monoclonal ME180-derived CDDP-resistant subclones. Co-treatment with CDDP and 5-aza-2'-deoxycytidine (5-aza-CdR), a demethylating agent, significantly enhanced the CDDP-sensitivities of the parent cells and CDDP-resistant subclones. Subsequent removal of 5-aza-CdR rapidly reversed the CDDP-sensitivity of the CDDP-resistant subclones to their original levels, whereas the parent cells retained the enhanced CDDP-sensitivity for at least 24 h. Quantitative RT-PCR revealed that the CDDP-resistant subclones expressed higher DNA methyltransferase (DNMT) mRNA levels than the parent cells, suggesting that increased DNMT expressions easily restored the CDDP-resistance of the CDDP-resistant subclones following 5-aza-CdR removal. Although the parent cells showed hypermethylation in the DAP kinase promoter region, corresponding methylated bands were not detected in 2 of the 6 CDDP-resistant subclones by methylation-specific PCR. All 6 CDDP-resistant subclones expressed higher DAP kinase mRNA levels than the parent cells, as evaluated by quantitative RT-PCR. Although DAP kinase protein expression was strongly suppressed in the parent cells and CDDP-resistant subclones, 5-aza-CdR treatment of the parent cells dose-dependently stimulated the DAP kinase protein expression, and this was synergistically enhanced by inhibiting histone deacetylation via trichostatin treatment in addition to 5-aza-CdR. However, DAP kinase protein expression in the CDDP-resistant subclones was not stimulated by treatment with 5-aza-CdR and/or trichostatin. These results indicate that post-transcriptional translation of DAP kinase mRNA is strongly suppressed and insensitive to treatment with 5-aza-CdR and trichostatin in the CDDP-resistant subclones established from ME180 human cervical squamous cancer cells. This CDDP-resistance is accompanied by molecular changes that disturb the post-transcriptional translation of the DAP kinase mRNA, and these molecular changes are transiently restored by demethylation.

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Key words: death-associated protein kinase, cisplatin-resistance, methylation, squamous cell carcinoma, cervical cancer

Introduction

Combination chemotherapy remains the predominant treatment approach before and after surgery for advanced malignant tumors although both *de novo* and acquired anticancer drug-resistance limits further treatment clinically. Among the important chemotherapeutic agents, cisplatin (CDDP) is particularly effective and clinically useful against various types of malignant tumor (1,2). The ability of CDDP to become incorporated into DNA, where it forms intra- and inter-strand crosslinks, is generally considered to be one of its main anticancer effects (3,4). However, a variety of observations reported for certain tumor types and cell lines suggest that the mechanism of CDDP-resistance is multifactorial, and involves alterations in drug-uptake and -efflux, defective mismatch repair due to hypermethylation of the human mut-L homologue 1 (hMLH1) gene promoter (5,6), p53 mutations (7,8), elevated cellular glutathione (GSH) levels and a concomitant increase of multidrug resistance protein 2 (MRP2) expression (9). Based on these potential mechanisms, a variety of approaches have been adopted to reverse or overcome CDDP-resistance, including demethylation (6). 5-aza-2'-deoxycytidine (5-aza-CdR), a demethylating agent, has been shown to induce demethylation by inhibiting DNA methyltransferases (DNMTs) and synergistically potentiating the cytotoxicity of CDDP due to DNA topologic changes (10,11). It was reported that treatment of ovarian and colon carcinoma xenografts with 5-aza-CdR and CDDP *in vivo*

could reverse drug-resistance by restoring hMLH1 expression, which is silenced by promoter methylation (6). 5-aza-CdR inhibits DNA methylation by reducing DNMT enzymatic activity via stable complex formation between the enzyme and 5-aza-CdR-substituted DNA (12). In addition to DNA methylation, histone deacetylation is also involved in methylation-induced gene silencing (13). Furthermore, inhibition of histone deacetylation was reported to act synergistically with inhibition of DNA methylation to induce gene expression (14).

Death-associated protein (DAP) kinase is a cytoskeleton-localized Ca^{2+}/calmodulin (CaM)-regulated serine/threonine kinase which modulates the cell death induced by interferon-γ, tumor necrosis factor-ß, Fas, transforming growth factor-ß, the oncogenes c-myc and E2F, ceramide and detachment from the extracellular matrix (15-20). Conversely, several lines of evidence have indicated that DAP kinase functions as an anti-apoptotic factor in cells under normal growth conditions, depending on the apoptotic stimuli (21,22). Hypermethylation of normally unmethylated CpG islands in the promoter regions of many genes, including DAP kinase, during the development of various malignancies results in transcriptional inactivation and loss of protein expression, which serves as an alternative mechanism to genetic alterations (23-28). Our previous study revealed that DAP kinase expression was strongly reduced, possibly via aberrant methylation, in many ovarian and uterine carcinoma cell lines (29). Recently, we established 6 monoclonal CDDP-resistant subclones from a cervical squamous cell carcinoma cell line, ME180 (30), in order to investigate the molecular mechanisms of CDDP-resistance in cervical cancer and squamous cell carcinoma. In the present study, we examined the effects of 5-aza-CdR treatment on DAP kinase expression, DAP kinase promoter methylation status and the CDDP-sensitivity of the CDDP-resistant subclones in order to investigate the potential therapeutic applications of regulating the DAP kinase expression and CDDP-sensitivity of CDDP-resistant cancers.

Materials and methods

Cell lines and culture. The human cervical squamous cell carcinoma cell line, ME180, was purchased from the JCRB Cell Bank (Japan Collection of Research Bioresources Cell Bank, Tokyo, Japan). All cells were cultured in OPTI-MEM (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FCS; Equitech Bio Inc., Ingram, TX, USA), 100 U/ml penicillin (PC), 100 μg/ml streptomycin (SM) and 0.25 μg/ml fungizone (Invitrogen) in 5% CO_2/95% air at 37°C.

Establishment of CDDP-resistant subclones from ME180 cells. To establish CDDP-resistant subclones, ME180 cells were cultured with various concentrations of CDDP (courtesy of Nihon-Kayaku Co. Ltd., Tokyo, Japan) for 3-5 weeks, and the surviving cells were collected. This collection procedure after CDDP exposure was repeated 4 times. Finally, 6 single cell-derived CDDP-resistant subclones, designated CDDPrA, CDDPrB, CDDPrC, CDDPrD, CDDPrE and CDDPrF, were established by the limiting dilution method. The monoclonality of each CDDP-resistant subclone was confirmed by chromosome analysis (data not shown). The establishment of these CDDP-resistant subclones took 1 year.

CDDP-sensitivity assay. Cell viability was assayed using a non-RI colorimetric assay kit (XTT; Boehringer-Mannheim, Mannheim, Germany). The inhibitory effects of CDDP on cell growth were assayed as follows. Cells in the log-phase were detached using 0.25% trypsin/1 mM EDTA (Invitrogen), and cultured overnight in 96-well plates ($5x10^3$ cells/well). On day 2, various concentrations of CDDP were added to the cells. On day 4, the numbers of viable cells were evaluated using the XTT kit and expressed as the percentage of viable cells (%) relative to the mean number of viable unstimulated cells. All experiments were performed 3 times to verify the results. The data are shown as the mean ± SD, and comparative data (n=6) were analyzed by ANOVA.

DNA fragmentation assay. ME180 parent cells and CDDP-resistant subclones in the log phase were detached using 0.25% trypsin/1 mM EDTA, and then cultured overnight in culture dishes ($3x10^6$ cells/dish) containing OPTI-MEM/5% FCS/PC/SM. On day 2, CDDP (final concentrations: 4 and 20 μg/ml) was added to the cells. On day 4, genomic DNA was extracted from all cells, including the dead ones, using a SepaGene DNA extraction kit (Sankyo-Junyaku Co. Ltd., Tokyo, Japan) and treated with 100 μg/ml of RNase A (Sigma, St. Louis, MO, USA) in TE buffer (10 mM Tris, pH 8.0, 2 mM EDTA) for 90 min at 37°C to remove any contaminating RNA. Then, approximately 20 μg of the genomic DNA isolated from $5x10^5$ cells was electrophoresed in a 1.4% agarose gel at 50 V for approximately 2 h, stained with 5 μg/ml of ethidium bromide and visualized by UV fluorescence.

Effects of the methyltransferase inhibitor, 5-aza-CdR, on the CDDP-sensitivity of the parent cells and CDDP-resistant subclones. Approximately $5x10^3$ ME180 parent cells or ME180-derived CDDP-resistant subclones were seeded in 0.1 ml OPTI-MEM in 96-well culture plates and incubated for 24 h. The medium was then replaced with medium containing the designated concentrations of CDDP with or without 1 μM 5-aza-CdR (Sigma) and the cells were incubated for a further 48 h. Then, the viable cell numbers were determined using Cell Counting Kit (Dojindo Chemical Laboratory Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a 96-well plate reader (Dainihon-Seiyaku Co., Osaka Japan). The absorbance obtained from control cells without drug administration was set as 100% viability. All experiments were performed 3 times to verify the results. The data are shown as the mean ± SD, and comparative data (n=4) were analyzed by ANOVA.

Effects of 5-aza-CdR pretreatment on the CDDP-sensitivity of the parent cells and CDDP-resistant subclones. To examine the effects of sequential administration of 5-aza-CdR and CDDP, $5x10^3$ ME180 parent cells or ME180-derived CDDP-resistant subclones were plated in 0.1 ml OPTI-MEM in 96-well culture plates and incubated for 24 h. Then, the cells were exposed to 1 μM 5-aza-CdR or left untreated for a further 24 h, followed by treatment with the indicated

Table I. Primer sequences for DAPK-MS-PCR analysis.

PCR analysis	Primer sequence	Amplicon size (bp)	Refs.
Stage I PCR	Forward 5'-GGTTGTTTCGGAGTGTGAGGAG-3' Reverse 5'-GCTATCGAAAACCGACCATAAAC-3'	209	(33)
Stage II PCR			
Unmethylation	Forward 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3' Reverse 5'-CAAATCCCTCCCAAACACCAA-3'	106	(33)
Methylation	Forward 5'-ATAGTCGGATCGAGTTAACGTC-3' Reverse 5'-AAAACTAACCGAAACGACGACG-3'	153	(33)

concentrations of CDDP for 48 h. Finally, the viable cell numbers were determined as described above. All experiments were performed 3 times to verify the results. The data are shown as the mean ± SD, and comparative data (n=4) were analyzed by ANOVA.

Western blot analysis of DAP kinase protein expression. The ME180 parent cells and CDDP-resistant subclones were incubated with 1 μM 5-aza-CdR for 96 h and/or with 300 nM trichostatin (TSA), a histone deacetylase inhibitor (Wako-Junyaku Co. Ltd., Tokyo, Japan), for 48 h, after which they were harvested and lysed with 0.3 ml lysis buffer (Sigma). For dose-dependency experiments, ME180 parent cells were treated with various concentrations of 5-aza-CdR (0.5-10 μM) for 96 h and then lysed as described above. The protein contents of the cell lysates were quantified using a Coomassie Plus Protein assay (Pierce Biotechnology Inc., Rockford, IL, USA) and aliquots (25 μg total protein) were dissolved in Laemmli SDS-PAGE sample buffer prior to separation by 7.5% SDS-PAGE. The separated proteins were transferred to a polyvinylidene fluoride membrane (ATTO Corp., Tokyo, Japan) by using a wet transfer method. The membrane was blocked with 5% skim milk for 1 h at room temperature and subsequently incubated with a mouse monoclonal anti-human DAP kinase antibody (clone 55, 1:5000 dilution; Sigma) for 1 h at room temperature. After washing with TBS-T (20 mM Tris, pH 7.6, 0.3 M NaCl, 0.1% Tween-20), the membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma) for 1 h at room temperature. The bound antibodies were detected using an ECL plus kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and the membrane was scanned using a Luminocapture (type AE6955; ATTO Corp.).

Genomic DNA extraction and bisulfite modification for DAP kinase methylation-specific polymerase chain reaction (DAPK-MS-PCR). Genomic DNA was isolated from cultured cells using a SepaGene kit (Sanko-Junyaku Ltd.) according to the manufacturer's instructions. The DNA concentrations were calculated from the UV absorptions at 260 and 280 nm. Genomic DNA was modified by chemical treatment with sodium bisulfite (Sigma) as described previously (31,32). In this reaction, all unmethylated cytosines were converted to uracils, while methylated cytosines remained unaltered. Briefly, 2 μg/50 μl of DNA was denatured by adding freshly prepared sodium hydroxide (final concentration: 0.3 M) and incubating the mixture for 20 min at 37°C. Then, 30 μl of freshly prepared 10 mM hydroquinine (Sigma) and 520 μl of 3 M sodium bisulfite (pH 5.0) were added to the DNA solutions, mixed and incubated for 16 h at 55°C. The DNA samples were desalted using a Wizard DNA Clean-Up System (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol, redissolved in 50 μl of autoclaved distilled water, desulfonated with 0.3 M NaOH for 20 min at 37°C and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 20 μl of autoclaved distilled water and either used immediately or stored at -70°C.

DAP kinase-methylation-specific polymerase chain reaction (DAPK-MS-PCR). DAPK-MS-PCR was performed as described previously (33). Briefly, the bisulfite-modified DNA was used as a template for stage I PCR amplification to generate a 209-bp fragment of the DAP kinase gene that included a portion of its CpG-rich promoter region. The stage I PCR primers recognized the modified DNA but could not discriminate between methylated and unmethylated alleles. Stage I PCR amplification was carried out as follows: 95°C for 15 min; 35 cycles of 94°C for 1 min for denaturation, 58°C for 150 sec for annealing and 72°C for 150 sec for extension; followed by a final extension at 72°C for 10 min. The stage I PCR products were diluted 50-fold, and 5 μl was subjected to stage II PCR amplification using primers specifically designed for methylated or unmethylated DNA in the promoter region of the DAP kinase gene. The primers used for stage I and II PCR amplification are summarized in Table I. For stage II PCR, the annealing temperature was increased to 65°C and the annealing time was reduced to 90 sec for 40 cycles. Stage II PCR amplified 153-bp and 106-bp products from methylated and unmethylated DAP kinase genes, respectively. Finally, these PCR products were electrophoresed in a 2.0% agarose gel at 100 V for approximately 30-40 min, and visualized by staining with 5 μg/ml ethidium bromide.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR of DAP kinase and DNMT genes. Total RNA was isolated from cultured cells using

Table II. Primer sequences for RT-PCR and quantitative real-time RT-PCR analysis.

Gene	Primer sequence	Amplicon size (bp)	Refs.
DAP kinase	Forward 5'-TGGATCCACCAGCAAAGCAC-3' Reverse 5'-GTGTTGGTTAGTGAGGTTTC-3'	350	(34)
DNMT-1	Forward 5'-GTTCTTCCTCCTGGAGAATGTCA-3' Reverse 5'-GGGCCACGCCGTACTG-3'	138	(35)
DNMT-3A	Forward 5'-CCTGTGGGAGCCTCAATGTTA-3' Reverse 5'-TTCTTGCAGTTTTGGCACATTC-3'	72	(35)
DNMT-3B	Forward 5'-GACTCGAAGACGCACAGCTG-3' Reverse 5'-CTCGGTCTTTGCCGTTGTTATAG-3'	97	(35)
ß-actin	Forward 5'-ATTGCCGACAGGATGCAGAA-3' Reverse 5'-GCTGATCCACATCTGGTGGAA-3'	150	Original

TRIzol reagent (Invitrogen) for RT-PCR and an RNeasy mini kit (Qiagen Inc., Valencia, CA, USA) for real-time RT-PCR. Aliquots containing 1 μg of total RNA were pretreated with DNase I (Invitrogen) and then used for cDNA synthesis with a reverse transcriptase kit (Bio-Rad, Hercules, CA, USA) in a reaction volume of 20 μl. Each cDNA product was diluted to 100 μl. The PCR reaction mixture (25 μl) contained 5 μl of diluted cDNA, 0.125 μl Hotstart polymerase (Qiagen Inc.), 0.2 mM dNTP, 1xQ solution and 0.5 μM primers. The primers used for DAP kinase, DNMT 1, DNMT 3A and DNMT 3B are summarized in Table II. ß-actin was used as a positive control for the mRNA amount. For RT-PCR, an initial hot start at 95°C for 15 min was followed by 35 amplification cycles (30 sec at 94°C, 30 sec at the annealing temperature and 60 sec at 72°C). The annealing temperatures were 55°C for DAP kinase and 60°C for DNMT 1, DNMT 3A and DNMT 3B. The PCR products were electrophoresed in a 1.5-2.0% agarose gel at 100 V for approximately 30-40 min and visualized by staining with 5 μg/ml ethidium bromide. Quantitative real-time RT-PCR was performed using an iCycler (Bio-Rad). The PCR reaction mixture (25 μl) contained 2.5 μl of diluted cDNA, 12.5 μl of iQ™ SYBR-Green Supermix (Bio-Rad) and 0.5 μM of the above-described primers. An initial hot start at 95°C for 3 min was followed by 40 cycles of amplification (95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec). The relative values of DAP kinase mRNA in the ME180 parent cells and CDDP-resistant subclones were calculated based on the $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ CDDP\text{-}resistant\ subclones} - \Delta C_{T\ ME180\ parent\ cells} = (C_{T\ DAP\ kinase} - C_{T\ actin})_{CDDP\text{-}resistant\ subclones} - (C_{T\ DAP\ kinase} - C_{T\ actin})_{ME180\ parent\ cells}$.

Results

Establishment of 6 monoclonal CDDP-resistant subclones from the ME180 human cervical squamous cell carcinoma cell line. To investigate the mechanisms involved in anticancer drug-resistance, we established 6 independent monoclonal CDDP-resistant subclones from the ME180 cervical squamous [illegible] using the limiting dilution method. The CDDP-sensitivities of these subclones were determined using XTT assays. As shown in Fig. 1, all 6 CDDP-resistant subclones showed significantly lower CDDP-sensitivities than the parent cells. To confirm the resistance of the subclones against CDDP-induced apoptosis, we carried out DNA fragmentation assays. The parent cells and CDDP-resistant subclones were exposed to two different concentrations of CDDP (4 and 20 μg/ml), based on the XTT assays shown in Fig. 1. After incubation with CDDP for 48 h, DNA was extracted and chromatin fragmentation was examined by agarose gel electrophoresis. Typical DNA ladder patterns were detected in the parent cells treated with both 4 and 20 μg/ml CDDP, whereas DNA ladders were hardly seen in any CDDP-resistant subclones examined after culture with 4 μg/ml of CDDP (Fig. 2).

Effects of demethylation on the CDDP-sensitivity of the CDDP-resistant subclones. To address whether the acquired CDDP-resistance of the 6 subclones was caused by aberrant methylation of gene promoters, we analyzed the effects of demethylation on the CDDP-sensitivity of the CDDP-resistant subclones (Fig. 3). Preliminary culture experiments with 5-aza-CdR (0-20 μM) treatment showed no growth-inhibitory effects on the ME180 parent cells (data not shown). Next, we treated the parent cells and CDDP-resistant subclones with various concentrations of CDDP and 10 μM 5-aza-CdR for 48 h to examine the effects of concurrent treatment with 5-aza-CdR and CDDP on the CDDP-sensitivity. As shown in Fig. 3, parent cells co-treated with CDDP and 5-aza-CdR showed significantly increased CDDP-sensitivity compared to parent cells treated with CDDP alone. Among the 6 CDDP-resistant subclones, 3 subclones (B, D and F) showed a much higher CDDP-sensitivity, while the other 3 subclones (A, C and E) showed a slightly, but significantly, higher CDDP-sensitivity when co-treated with CDDP and 5-aza-CdR compared with the same subclones treated with CDDP alone. To further elucidate the reversibility of the CDDP-sensitivity after demethylation in CDDP-resistant malignancies, we investigated the persistence of the demethylation-mediated sensitization

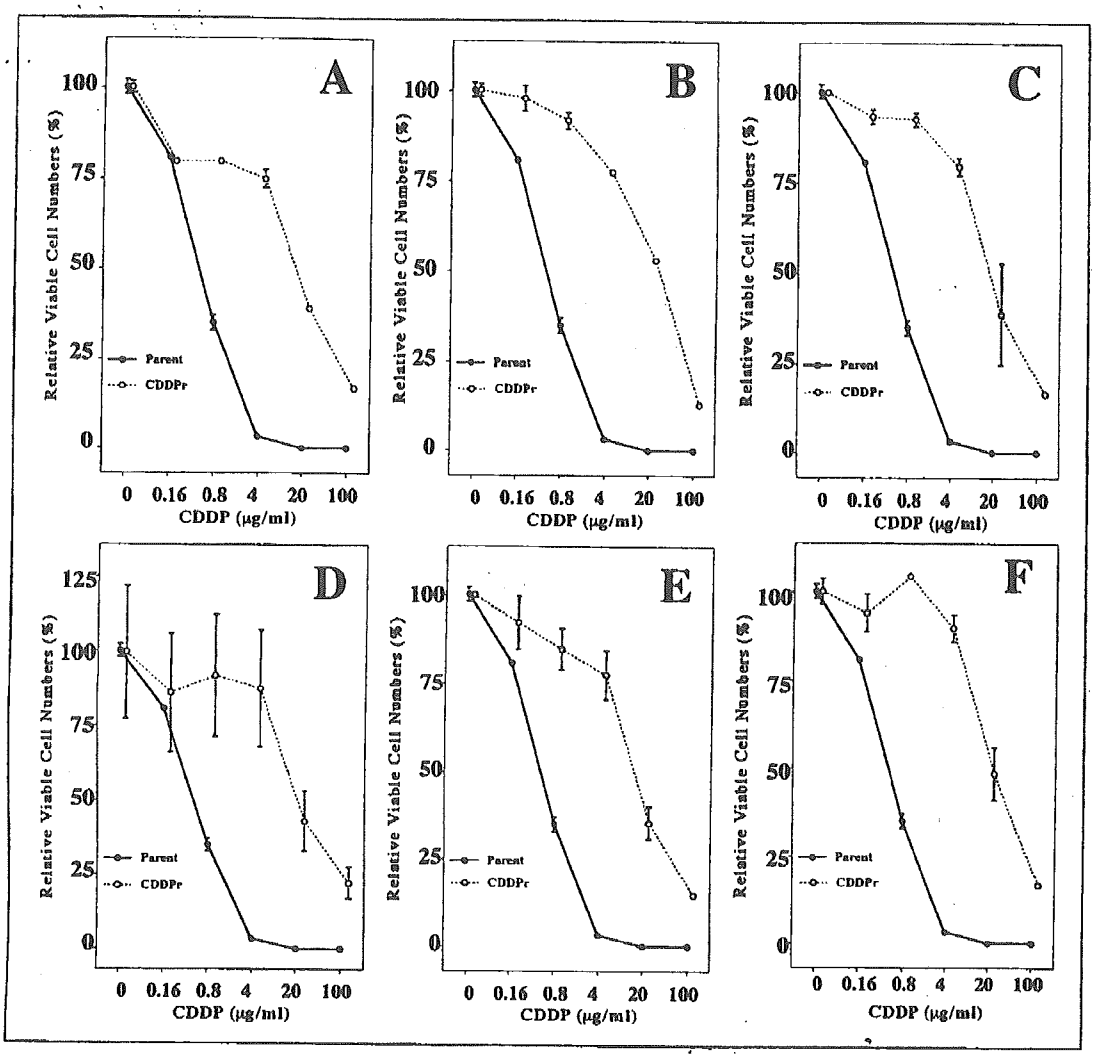

Figure 1. CDDP sensitivities of the CDDP-resistant subclones established from ME180 cells. Six monoclonal subclones (CDDPrA, B, C, D, E and F) were established. The solid lines with closed circles are CDDP-sensitivity curves of the parent cells and the dotted lines with open circles are CDDP-sensitivity curves of the CDDP-resistant subclones. All the subclones are clearly more resistant to CDDP-induced inhibition of cell growth than the parent cells.

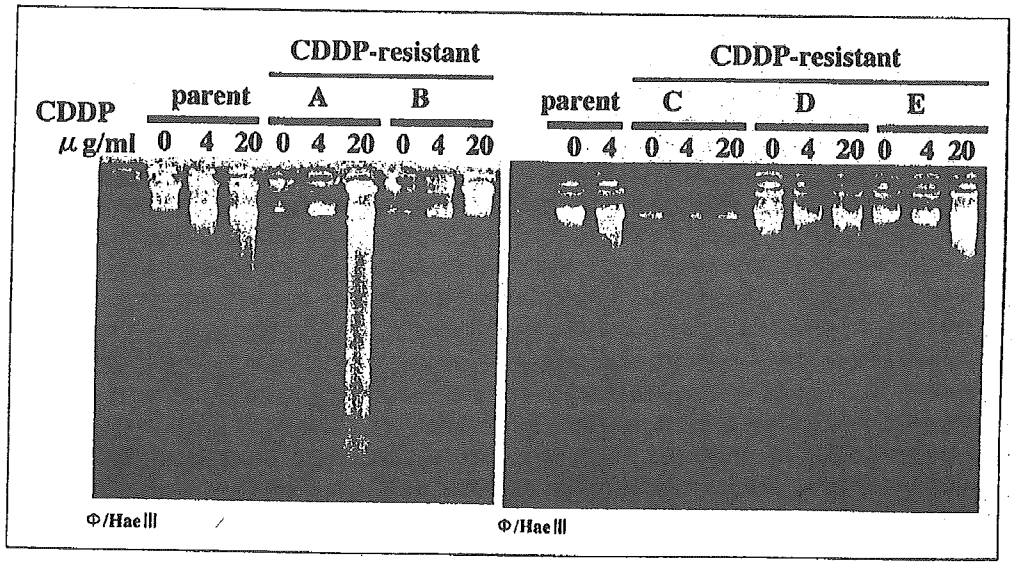

Figure 2. DNA fragmentation assay of the CDDP-resistant subclones. CDDP-induced DNA fragmentation is inhibited in the CDDP-resistant subclones (CDDPrA, B, C, D and E).

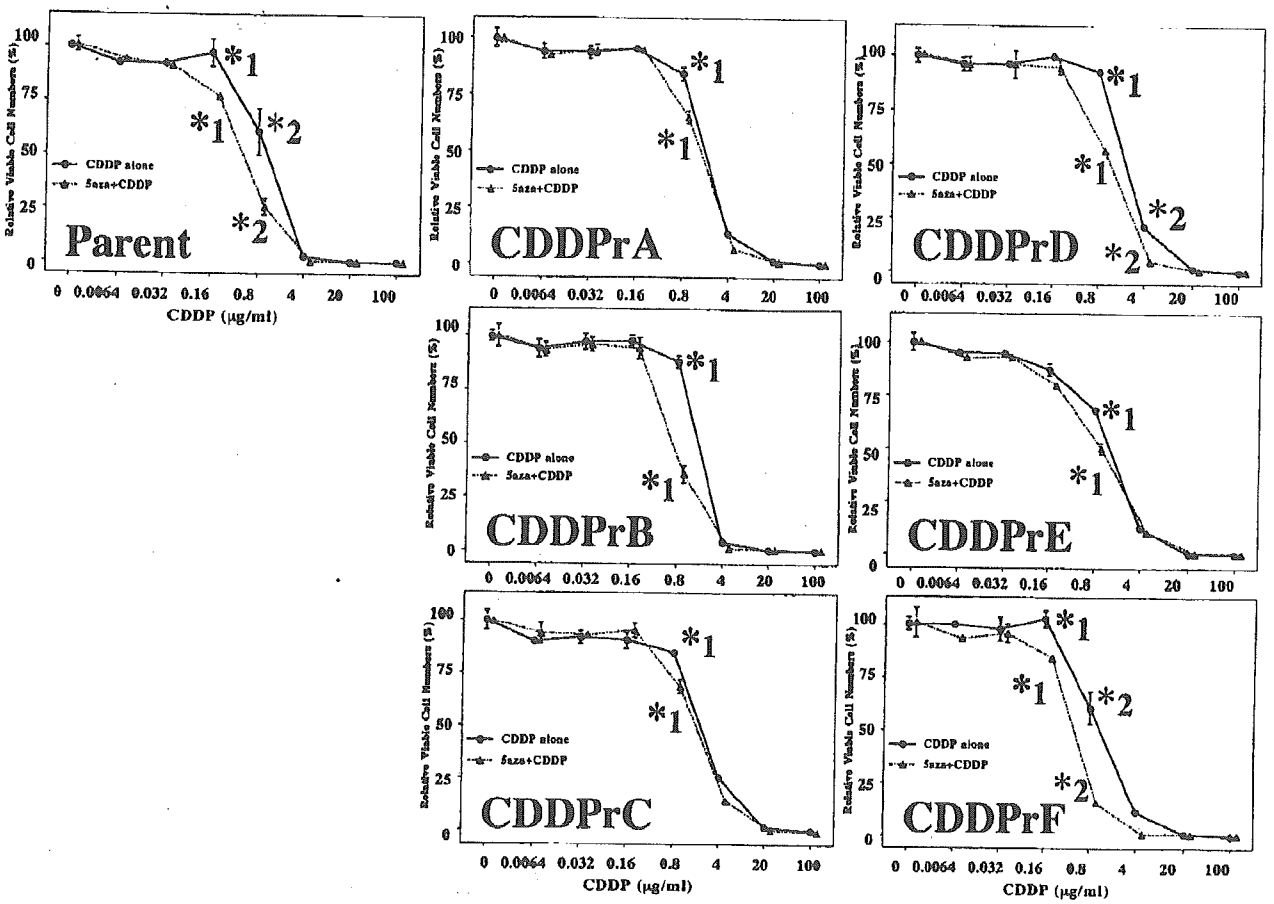

Figure 3. Effects of 5-aza-CdR treatment on the CDDP-sensitivities of the parent cells and CDDP-resistant subclones. The cells were concurrently treated with CDDP and 5-aza-CdR. The solid lines with closed circles are CDDP-sensitivity curves of cells without 5-aza-CdR treatment and the dotted lines with closed triangles are CDDP-sensitivity curves of cells treated with 5-aza-CdR. All the cells treated with 5-aza-CdR and CDDP show significantly higher CDDP-sensitivities than cells treated with CDDP alone. *p<0.05.

to CDDP in the parent cells and CDDP-resistant subclones for 24 h after 5-aza-CdR-pretreatment (Fig. 4). The results showed that the parent ME180 cells retained the CDDP-sensitivity restored by the 5-aza-CdR treatment after its removal, whereas all the CDDP-resistant subclones lost their restored CDDP-sensitivity.

Increased expression of DNMT genes in CDDP-resistant subclones. Since the CDDP-resistant subclones failed to maintain their restored CDDP-sensitivity, we speculated that the status of the DNMTs in the CDDP-resistant subclones may be altered during exposure to CDDP. To clarify whether DNMT expression was up-regulated in CDDP-resistant subclones compared with parent ME180 cells, we assessed the transcriptional expression of DNMTs by RT-PCR and quantitative real-time RT-PCR. As shown in Fig. 5A, RT-PCR readily detected the expression of DNMT 1, DNMT 3A and DNMT 3B in parent ME180 cells and all CDDP-resistant subclones. Moreover, quantitative real-time RT-PCR revealed that 3 CDDP-resistant subclones (A, C and E) showed significantly higher DNMT 3B mRNA expression and slightly higher DNMT 1 mRNA expression than the parent cells. As shown in Fig. 3, these 3 subclones showed smaller increases in their cellular CDDP-sensitivity than the other subclones upon co-treatment with 5-aza-CdR and CDDP, suggesting that the increased DNMT 3B and DNMT 1 levels could inhibit the effects of 5-aza-CdR in these 3 subclones.

The methylation status of the DAP kinase promoter in CDDP-resistant subclones. Several lines of evidence have shown that the hypermethylation of gene promoters is involved in drug-resistance (5,31,32). Previously, we reported that abnormal methylation of the DAP kinase gene promoter occurred and the level of DAP kinase protein was markedly diminished in the ME180 cell line (29). In view of the ability of DAP kinase to modulate apoptosis positively or negatively, depending on the cell type and apoptotic stimuli, we hypothesized that DAP kinase may be involved in anticancer drug-induced apoptosis and regulate the drug-sensitivity of malignant cells. Since the parent cells and CDDP-resistant subclones all showed increased CDDP-sensitivity upon co-treatment with CDDP and 5-aza-CdR, it is likely that the CpG island in the DAP kinase gene promoter is hypermethylated in CDDP-resistant subclones. To address this possibility, we examined the methylation status of the DAP kinase gene promoter using DAPK-MS-PCR (Fig. 6). In 2 of the 6 CDDP-resistant subclones (C and E), the band indicating hypermethylation of the DAP kinase gene promoter was not detected, while the others showed aberrant methylation.

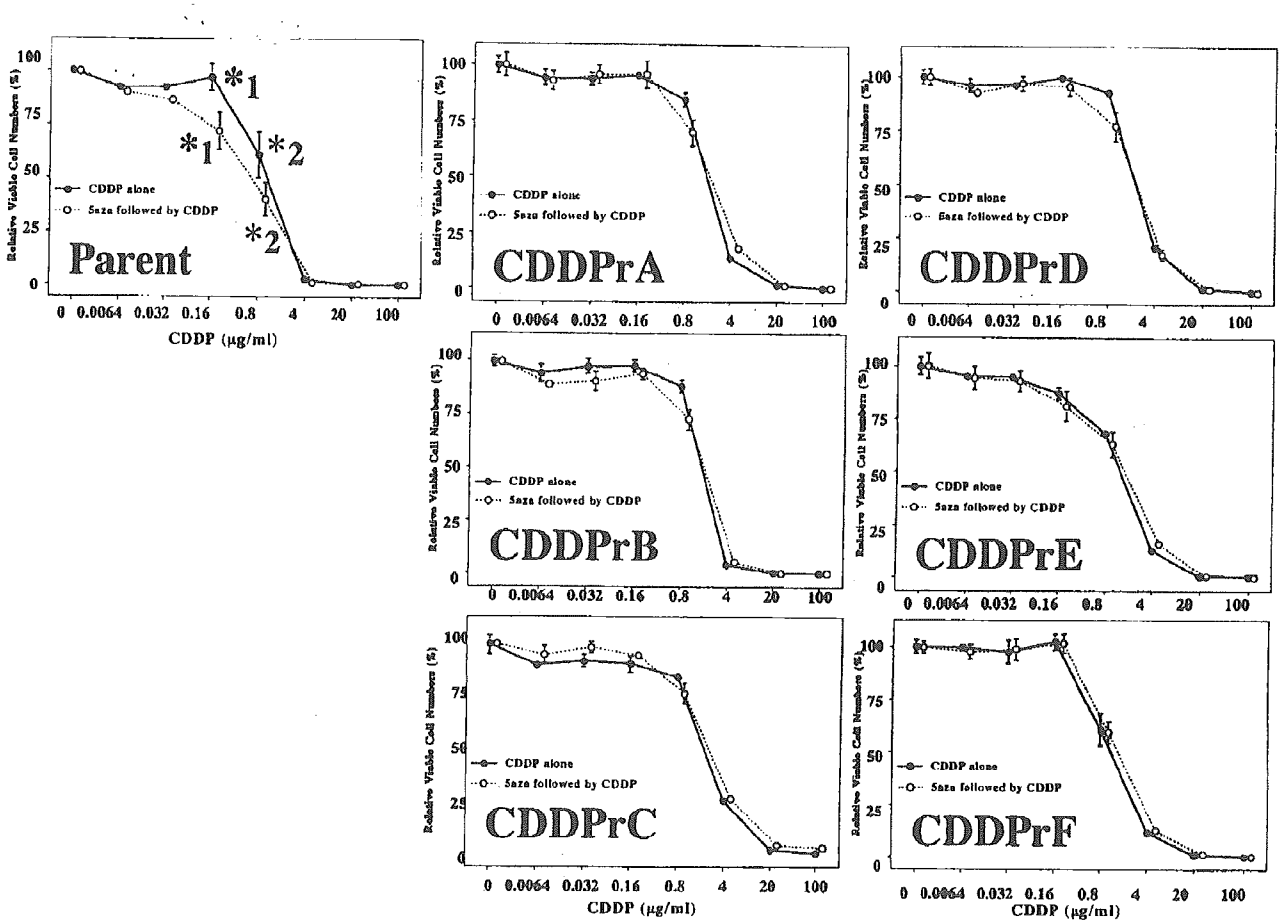

Figure 4. Effects of 5-aza-CdR removal on the CDDP-sensitivity of parent cells and CDDP-resistant subclones. The cells were stimulated with CDDP alone after pretreatment with 5-aza-CdR. The solid lines with closed circles are CDDP-sensitivity curves of cells treated with CDDP alone and the dotted lines with open circles are CDDP-sensitivity curves of cells treated with CDDP after 5-aza-CdR pretreatment. Although the parent cells exhibit a significantly higher CDDP-sensitivity after pretreatment with 5aza-CdR, all 6 CDDP-resistant subclones show no apparent changes in their CDDP-sensitivity with and without 5-aza-CdR pretreatment. *p<0.05.

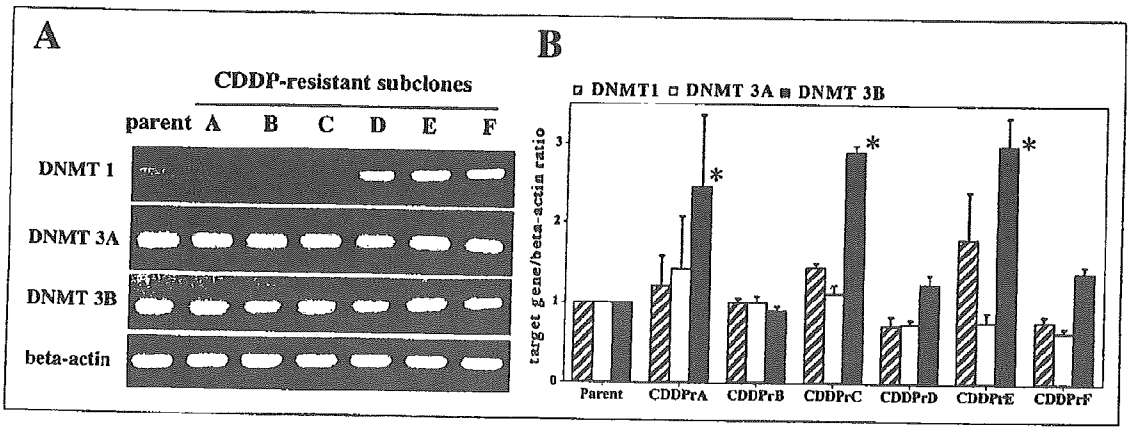

Figure 5. RT-PCR and quantitative real-time RT-PCR analyses of DNA methyltransferase mRNAs in CDDP-resistant subclones. A, RT-PCR analyses of DNA methyltransferase genes in CDDP-resistant subclones. Both the parent cells and CDDP-subclones express the 3 DNA methyltransferase mRNAs examined. B, Quantitative real-time RT-PCR analyses of 3 DNA methyltransferase genes: DNMT 1, DNMT 3A and DNMT 3B. *DNMT 3B mRNA expression is more than 2-fold higher in 3 CDDP-resistant subclones (CDDPrA, CDDPrC and CDDPrE) than in the parent cells. DNMT 1 mRNA expression is also slightly increased in these subclones, albeit to lesser extents.

The expression of DAP kinase protein in parent cells and CDDP-resistant subclones before and after treatment with 5-aza-CdR and/or TSA. Next, we examined the DAP kinase protein expression in parent cells and CDDP-resistant subclones by Western blot analysis. First, we investigated whether the DAP kinase protein expression in ME180 parent

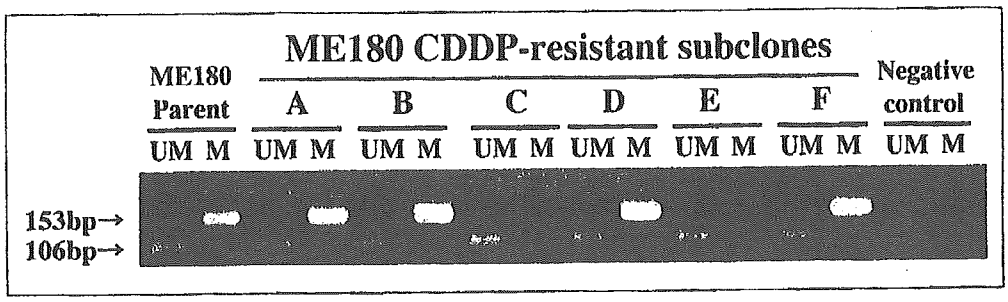

Figure 6. MS-DAPK-PCR analyses of CDDP-resistant subclones. Methylation-specific PCR of the DAP kinase gene was performed in ME180 parent cells and CDDP-resistant subclones. The parent cells have both the methylated and unmethylated bands. Two CDDP-resistant subclones (C and E) do not contain the methylated band. U, unmethylated; M, methylated.

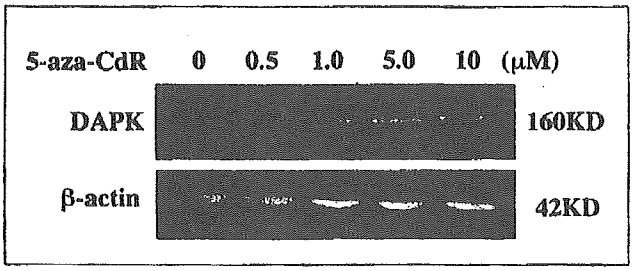

Figure 7. Effects of 5-aza-CdR treatment on DAP kinase protein expression in parent cells. The DAP kinase protein expression in parent ME180 cells is induced by 5-aza-CdR treatment in a dose-dependent manner.

cells was regulated by 5-aza-CdR treatment. In the parent cells, DAP kinase protein expression was remarkably suppressed. However, after treatment with various concentrations of 5-aza-CdR (0.5-10 μM) for 96 h, the parent cells showed increased DAP kinase protein expression in a dose-dependent manner (Fig. 7).

Secondly, we examined the DAP kinase protein expression in parent cells and CDDP-resistant subclones before and after treatment with 5-aza-CdR and/or TSA. Similar to the case for the parent ME180 cells, 5 of the 6 CDDP-resistant subclones (A, B, C, D and F) showed slightly detectable DAP kinase protein expression (Fig. 8). Next, we treated the parent cells and CDDP-resistant subclones with 1 μM 5-aza-CdR and/or 300 nM TSA, a histone deacetylase inhibitor (Fig. 8). In the parent cells, 5-aza-CdR treatment alone restored the expression of DAP kinase, and combined treatment of 5-aza-CdR and TSA caused a synergistic increase in DAP kinase protein expression. However, treatment of the parent cells with TSA alone induced little increase in the DAP kinase protein expression. In contrast to the parent cells, no distinguishable changes in DAP kinase protein expression were detected in any of the 6 CDDP-resistant subclones after treatment with 5-aza-CdR and/or TSA. These results demonstrated that the induction of DAP kinase protein expression was not responsible for the restoration of CDDP-sensitivity by 5-aza-CdR treatment shown in Figs. 3 and 4, since DAP kinase protein expression was not enhanced by 5-aza-CdR treatment in CDDP-resistant subclones whose CDDP-sensitivity was restored by 5-aza-CdR.

Transcription of the DAP kinase gene in parent cells and CDDP-resistant subclones. Although hypermethylation of the DAP kinase promoter was not detected in 2 of the 6 CDDP-resistant subclones by DAPK-MS-PCR, all 6 CDDP-resistant subclones failed to recover DAP kinase protein expression in response to demethylation after 5-aza-CdR treatment, suggesting that defective DAP kinase gene transcription may occur in CDDP-resistant subclones. Therefore, RT-PCR analysis was performed to estimate the DAP kinase gene transcription in parent cells and CDDP-resistant subclones. As shown in Fig. 9A, all of the cells tested showed readily detectable DAP kinase mRNA expression by RT-PCR. Next, quantitative real-time RT-PCR was carried out to investigate the mRNA expression levels in the parent cells and CDDP-resistant subclones. As shown in Fig. 9B, all 6 CDDP-resistant subclones expressed significantly higher levels of DAP kinase mRNA than the parent cells. These results indicated that impaired translation of the DAP kinase mRNA, rather than transcriptional repression of the DAP kinase gene, strongly reduced the DAP kinase protein expression in CDDP-resistant subclones.

Discussion

The epigenetic hypermethylation of gene promoters plays an important role in tumorigenesis as an alternative mechanism of genetic changes (36). The pattern of hypermethylation can be divided into age- or cancer-specific methylation (37,38). On the other hand, CDDP-resistance is a multifactorial condition, which involves decreased drug-uptake, increased drug-efflux and increased intracellular GSH (39) as well as genetic and epigenetic alterations, such as p53 mutations and hMLH1 hypermethylation (7). Furthermore, CDDP-resistance has also been reported to be associated with reciprocal EGF receptor and p21 expression in ME180 cells (40). Here, we focused on DAP kinase, an apoptosis modulator that has been reported to show abnormal methylation in many tumor types, including cervical cancer (25,41). We made several important observations regarding the relationship between DAP kinase expression and CDDP-resistance in the ME180 cervical squamous cell carcinoma cell line. To the best of our knowledge, this study represents the first report to investigate the relationships between CDDP-resistance and the regulatory system of DAP kinase expression. To evaluate the differing

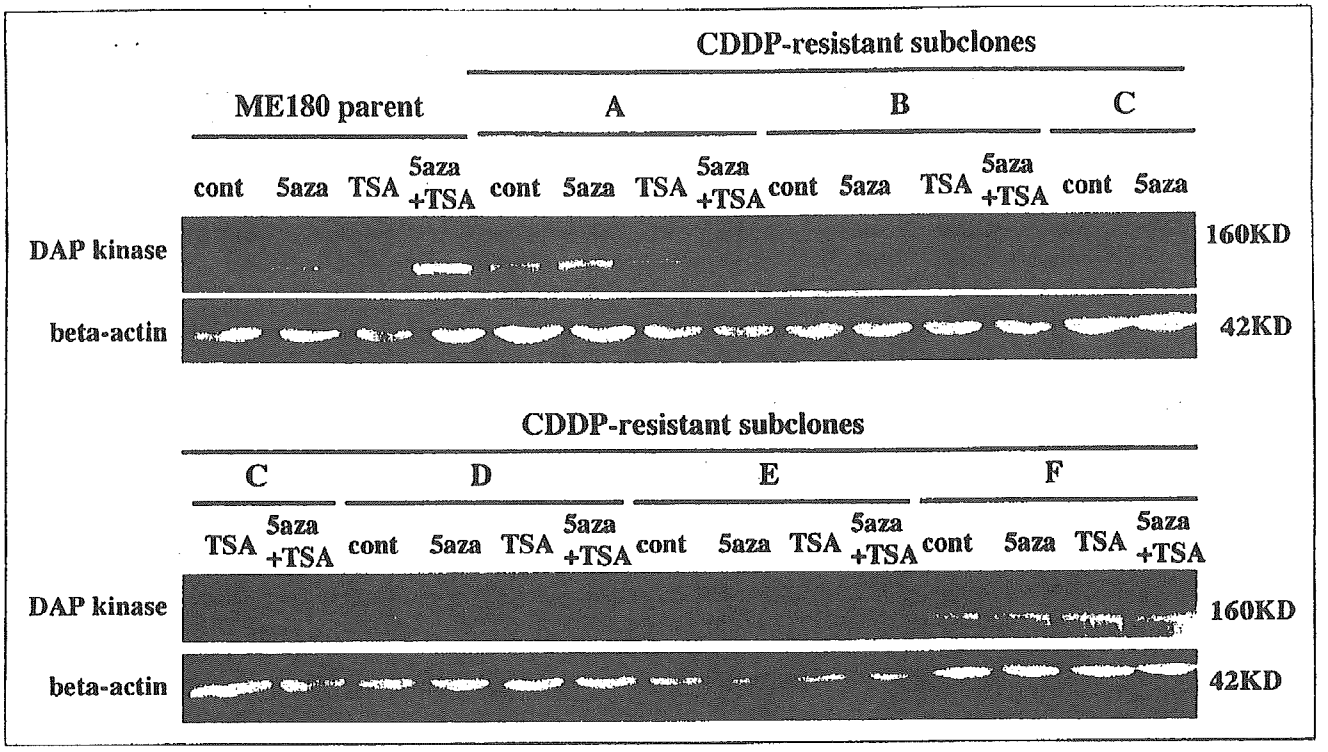

Figure 8. Effects of stimulation with 5-aza-CdR and/or TSA on DAP kinase protein expression in parent cells and CDDP-resistant subclones. Treatment with 5-aza-CdR clearly induces DAP kinase protein expression in parent cells. TSA synergistically enhances the DAP kinase protein expression induced by 5-aza-CdR in the parent cells. None of the CDDP-resistant subclones show induction of DAP kinase protein expression following treatment with 5-aza-CdR and/or TSA.

responsiveness to CDDP, we established 6 monoclonal CDDP-resistant subclones from ME180 cells, and then examined the effects of CDDP in combination with the demethylating agent, 5-aza-CdR, or the sequential treatment with 5-aza-CdR and CDDP of parent cells and CDDP-resistant subclones. The CDDP-sensitivity of the parent cells and CDDP-resistant subclones was significantly enhanced by 5-aza-CdR treatment, consistent with the previous finding that the cytotoxicity of a combination of CDDP and 5-aza-CdR against several tumor cell lines was synergistic (10). Based on the pattern of enhanced sensitivity to CDDP exposure after 5-aza-CdR treatment, the CDDP-resistant subclones were divided into two groups: one consisting of subclones B, D and F, which showed a relatively high responsiveness to 5-aza-CdR treatment; and the other consisting of subclones A, C and E, with a comparatively low responsiveness. We speculated that differences in the DNMT levels among these CDDP-resistant subclones may be responsible for the differing responsiveness to 5-aza-CdR between the 2 groups. DNMT 3A and DNMT 3B are thought to be the major *de novo* methylases that affect the methylation status of normally unmethylated CpG sites (42-44). Furthermore, *in vitro* methylation assays have shown that DNMT 3A and DNMT 3B cooperate with DNMT 1 to extend methylation within the Micrococcus luteus genome (45). Therefore, it seems likely that the lower CDDP-sensitivities of subclones A, C and E compared with the other subclones upon co-treatment with 5-aza-CdR and CDDP is partly attributable to the enhanced expression of DNMT 1 and DNMT 3B. Collectively, these observations suggest the

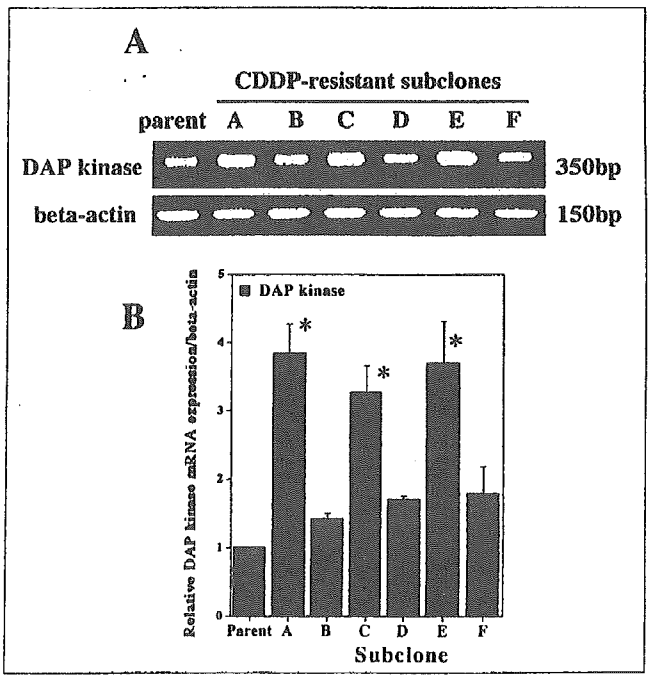

Figure 9. RT-PCR and quantitative real-time RT-PCR analyses of DAP kinase mRNA in CDDP-resistant subclones. A, RT-PCR analysis of CDDP-resistant subclones. B, Quantitative real-time RT-PCR analysis of DAP kinase mRNA. All of the CDDP-resistant subclones express higher DAP kinase mRNA levels than the parent cells. *DAP kinase mRNA expression is more than 2-fold higher in 3 CDDP-resistant subclones (CDDPrA, CDDPrC and CDDPrE) than in the parent cells.

possible application of a demethylating treatment, such as 5-aza-CdR, to cancer therapy, which could overcome acquired CDDP-resistance. In the present study, however, the CDDP-susceptibilities of the CDDP-resistant subclones rapidly returned to their original CDDP-sensitivity levels after the removal of 5-aza-CdR. Therefore, in order to overcome CDDP-resistance, long-acting demethylating drugs combined with CDDP may be essential for therapeutic use.

We also examined whether the CDDP-sensitivity of the parent cells and CDDP-resistant subclones correlated with the methylation status of the DAP kinase gene. Our previous study demonstrated that DAP kinase protein expression was strongly suppressed in ME180 cells, possibly due to aberrant methylation of the DAP kinase gene promoter (29). Thus, we examined the methylation status of the DAP kinase gene in CDDP-resistant subclones. We observed that the CDDP-resistant subclones showed little DAP kinase protein expression, similar to the ME180 parent cells. Interestingly, DAPK-MS-PCR was unable to detect a hypermethylated band in 2 of the 6 CDDP-resistant subclones (C and E), while hypermethylation of the DAP kinase promoter was easily detected in the other 4 CDDP-resistant subclones (A, B, D and F). A possible explanation is that our DAPK-MS-PCR is not sufficiently sensitive to identify the hypermethylation status of all of the targeted genes, and that undetected methylation sites are located in the DAP kinase promoter region in the 2 CDDP-resistant subclones (C and E). Further sequencing analyses of the full-length promoter region of the DAP kinase gene are necessary to verify this possibility. Alternatively, it is possible that these CDDP-resistant subclones isolated independently from the ME180 cell line may have diverged and acquired certain alterations during the selection cultures with CDDP. In addition, we noted that, even in the parent ME180 cells and 4 methylation-positive CDDP-resistant subclones, the methylated bands were always accompanied by unmethylated bands, suggesting that the 2 methylation-negative CDDP-resistant subclones expressing normal unmethylated DAP kinase promoters may originate from ME180 clones that possessed the unmethylated allele of the DAP kinase gene. Quantitative RT-PCR revealed that all of the CDDP-resistant subclones showed higher DAP kinase mRNA expression levels than the parent cells. In particular, much higher DAP kinase mRNA expression levels were found in the 3 subclones (A, C and E) whose CDDP-sensitivity was less affected by 5-aza-CdR than in the other 3 subclones (B, D and F). Since the 2 CDDP-resistant subclones without methylated bands in MS-DAPK-PCR (C and E) expressed much higher DAP kinase mRNA levels than the parent cells, DAP kinase mRNA transcription is partly regulated by methylation of the promoter region even in the CDDP-resistant subclones.

We then analyzed whether hypermethylation of the DAP kinase gene was sufficient to silence DAP kinase gene transcription and whether 5-aza-CdR-mediated demethylation affected the methylation status in the parent cells and CDDP-resistant subclones. Unexpectedly, we noted that the reduced DAP kinase protein expression in ME180 cells did not result from silenced transcription of the DAP kinase gene by hypermethylation of the DAP kinase gene promoter CpG island. DAP kinase mRNA expression was easily detected in the parent cells and all of the CDDP-resistant subclones. Thus, the post-transcriptional translation of DAP kinase mRNA was strongly suppressed in the ME180 parent cells and CDDP-resistant subclones. A similar discrepancy between the mRNA expression level and amount of protein expression was previously reported for the transcriptional regulatory factor, PAX6, in the SW837 human colorectal carcinoma cell line (40). However, the expression of DAP kinase protein was recovered to detectable levels in the ME180 parent cells upon 5-aza-CdR treatment in a dose-dependent manner, suggesting that the post-transcriptionally suppressed DAP kinase protein expression was at least partially attributable to aberrant methylation. Treatment with TSA alone had little effect on the DAP kinase protein re-expression in parent ME180 cells. Furthermore, combined treatment with 5-aza-CdR and TSA synergistically enhanced the DAP kinase protein expression in parent cells compared to treatment with 5-aza-CdR or TSA alone, consistent with a previous observation suggesting a more dominant role for methylation over histone deacetylase activity in mammals for the maintenance of gene silencing in association with CpG methylation (14). In contrast to the ME180 parent cells, 5 of the CDDP-resistant subclones (A, B, C, D and F) showed slightly detectable DAP kinase protein expression and all 6 retained DAP kinase mRNA expression. Moreover, quantitative RT-PCR analysis revealed that all 6 CDDP-resistant subclones had apparently higher DAP kinase mRNA expression levels than the parent cells. However, unlike the parent cells, all 6 CDDP-resistant subclones failed to show induction of DAP kinase protein expression upon treatment with 5-aza-CdR and/or TSA. Therefore, our results indicate that the acquired CDDP-resistance in the ME180-derived CDDP-resistant subclones is not caused directly by induction of DAP kinase protein expression. The impaired translation of DAP kinase mRNA after demethylation and/or inhibition of histone deacetylation was a common characteristic among all of the CDDP-resistant subclones. Thus, the acquisition of CDDP-resistance may be caused by gene mutations that strongly interrupt the post-transcriptional modulation of DAP kinase mRNA and simultaneously reduce the CDDP-sensitivity.

Our present results revealed that treatment with 5-aza-CdR could overcome CDDP-resistance in CDDP-resistant subclones established from the ME180 cervical carcinoma cell line, and that neither the methylation status of the DAP kinase promoter nor the DAP kinase protein expression level was directly involved in the acquisition of CDDP-resistance. CDDP-resistance may be caused by unknown molecular changes in the post-transcriptional mechanism that modulate the translation of DAP kinase mRNA. These currently unknown molecular changes in CDDP-resistant subclones are considered to be transiently restored by demethylation treatment. The present study is the first to demonstrate that the impaired translation mechanisms of DAP kinase mRNA are involved in the acquired CDDP-resistance of human cancer cells.

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Glutathione-S-transferase and p53 polymorphisms in cervical carcinogenesis

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Abstract

Objective. To investigate the clinical significance of glutathione-S-transferase GSTM1, GSTT1 and p53 codon 72 polymorphisms in cervical carcinogenesis.

Methods. GSTM1, GSTT1 and p53 codon 72 polymorphisms together with human papillomavirus (HPV) types were examined in a total of 198 cervical smear samples using multiplex polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (RFLP) techniques.

Results. Forty-two patients with high-grade squamous intraepithelial lesion (HSIL) had higher frequency of high-risk HPV and null GSTT1 genotype than 102 with low-grade SIL (LSIL) and 54 controls. Thirty-one patients with HSIL had also statistically higher frequency of null GSTT1 genotype than 28 with LSIL among 69 patients with high-risk HPV. There was no statistical difference in p53 Arg, Arg/Pro and Pro genotypes between SILs and controls with or without high-risk HPV.

Conclusion. GSTT1 null genotype in cervical cell samples may be associated with more severe precancerous lesions of the cervix in a Japanese population. The p53 codon 72 polymorphism is unlikely to be related to HPV status and the onset of cervical cancer.

Keywords: GST; p53; Polymorphism; SIL; Cervical carcinogenesis

Introduction

Cervical cancer is the second most common cancer in women worldwide, and is both a preventable and a curable disease especially if identified at an early stage. It is widely accepted that specific human papillomavirus (HPV) types are the central etiologic agent of cervical carcinogenesis. Recently, several candidate markers for cervical cancer risk, such as glutathione-S-transferase (GST) and p53, have been described [1–5]. Such markers could be used to direct high-risk women to more frequent cervical screening.

The genes of GST family encode enzymes that appear to be critical in cellular protection against the cytotoxic effects. GSTs play an important role in conjugating glutathione to the products of endogenous lipid peroxidation and inactivating organic hydroperoxides via selenium-independent glutathione peroxidase activity, thus protecting the cell from the deleterious effects of oxidative stress. GST classes mu (GSTM1) and theta (GSTT1) gene deletions may promote the development of cervical dysplasia by moderating the activation and detoxification of polycyclic hydrocarbons and other compounds that influence oxidative stress and DNA adduct formation [3]. A polymorphism at codon 72 of the p53 gene results in the substitution of arginine (Arg) for proline (Pro) in the gene product. It has been suggested that the homozygous Arg genotype increased the susceptibility

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