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Cisplatin Restores p53 Function and Enhances the Radiosensitivity in HPV16 E6 Containing SiHa Cells

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Abstract Most HPV-positive cervical cancer cells possess wild type *p53* gene, but its normal p53 functions are disrupted by expression of HPVs E6. Treatment with 0–20 μM cisplatin for 24 h in HPV16 E6 containing SiHa cells suppressed E6 mRNA, reduced E6 protein, and restored p53 expression in dose-dependent manners. Dual-parameter flow cytometric analysis indicated that sub-G_1 apoptotic cells, but not necrotic cells were the major species for cisplatin-induced cytotoxicity in SiHa cells. After 0–10 μM cisplatin treatment, slightly more apoptotic cells appeared from SiHa cells than those from dominant negative p53-transfected SiHa cells. There was no different ionizing radiation (IR)-induced apoptosis in these two different cells. On the other hand, cisplatin enhanced more IR-induced sub-G_1 apoptosis in SiHa than mp53-SiHa cells. These accompanied with prolonged p53 restoration in irradiated-SiHa cells after 24 h cisplatin treatment and thereafter. In contrast, it was not found in cells after irradiation alone. Similar results were also shown in Mdm2 expression in SiHa cells after combined treatment. Therefore, cisplatin restored p53 expression and prolonged IR-induced p53 restoration would be possible candidates to response more sub-G_1 apoptosis in irradiated SiHa cells. These results provided another new explanation on cisplatin sensitizing radiotherapy for HPV16 E6 containing cancer cells. J. Cell. Biochem. 91: 756–765, 2004.

Key words: cisplatin; human papillomavirus; E6; p53; ionizing radiation; apoptosis

Cisplatin or cis-diamminedichloroplatinum (DDP) is a DNA damage agent that is widely used for the treatment of a variety of tumors [Cohen and Lippard, 2001].

Since the 1970s, cisplatin has been used in the treatment of testicular tumors; malignant melanoma; osteogenic sarcoma; carcinomas of the urinary bladder, lung (other than small cells), uterine cervix, and ovary; and squamous carcinoma of the head and neck region [Bradford et al., 2003].

Cisplatin is also known to act synergistically when administered in combination with various chemotherapeutic drugs [Kern et al., 1988; Boike et al., 1990; Peters et al., 1991]. Over the past few years the concurrent chemotherapy and radiation (chemoradiotherapy) for advanced cervical cancer has significantly improved the local control and overall survival compared with the traditional therapy with radiation. Cisplatin has become a standard part of the treatment, but it was not really known whether the dose and schedule are optimal or whether it is possible to achieve even better efficacy with some other drugs or drug combinations for specific cancer cells such as HPV-positive cervical cancer cells [Koivusalo et al., 2002].

In contrast to many other human tumor forms, most HPV-positive cervical cancer cells possess wild type *p53* gene [Crook et al., 1991; Scheffner et al., 1991]. But its normal functions are disrupted by expression of HPVs E6. Previous studies indicated that cells expressing HPV16 E6 were 6-fold more sensitive to cisplatin than were vector alone-transfected human foreskin fibroblasts. The restoration of p53 was not found in those S-phase synchronized E6-transfected cells after cisplatin treatment at

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various experimental designs [Hawkins et al., 1996]. Recently, it was reported that cisplatin could repress the HPV18 E6 protein and restore the p53 expression in HPV18 E6 containing Hep2 cells [Wesierska-Gadek et al., 2002]. Koivusalo et al. [2002] reported that stably transfected SiHa reporter cells treated with 70 μM cisplatin for 24 h increased the p53 reporter activation. However, they did not find any p53 restoration in above samples by Western analysis. On the other hand, we had found that cisplatin could restore p53 expression in HPV16 E6 containing SiHa cells after 10 μM cisplatin treatment for more than 4 h. In present study, we examined p53 restoration, HPV16 E6 mRNA, E6 protein, cell survival, and sub-G_1 apoptosis in SiHa cells after cisplatin treatment.

It had been reported that ionizing radiation (IR) did not restore p53 expression and downstream p53 tumor suppressor pathway in HPV16 E6 containing SiHa cells and TK6 cells after 2–4 h post-incubation [Huang et al., 1996; Chou and Huang, 2002a,b]. In this study, we continued to examine above mentioned parameters on p53 expression and sub-G_1 apoptosis in SiHa cells after IR exposure and more than 4 h post-incubation. Same parameters were also investigated in SiHa cells and dominant negative p53 transfected SiHa cells after IR alone, cisplatin alone, and concurrent treatment with IR and cisplatin.

MATERIALS AND METHODS

Materials

Cisplatin (Cis-diamminedichloroplatinum (II)) was provided by Strem Chemicals, Inc. (Newburyport, MA). Cisplatin was freshly prepared in distilled water and diluted in culture medium. Monoclonal anti-p53 (Do-1), anti-p21 (F-5), and anti-Mdm2 (SMP14) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal β-actin antibody was from Chemicon International (Temecula, CA). Several antibodies, chemicals, and media used in this study unless otherwise stated had been mentioned previously [Chou and Huang, 2002a].

Cells and Culture Conditions

The SiHa cells (ATCC number: HTB-35), SiHa-pcDNA3, and SiHa-pcDNA3-p53 mutant cells (stably transfected cells with vector alone or mutant *p53* gene, refer to [Chou and Huang, 2002a] were grown in DMEM at the same condition previously described [Chou and Huang, 2002a]. In general, stably transfected cells were grown in medium containing 0.4 mg/ml G418 before used in experiments. The TK6 cells (ATCC number: CRL-8015) and HPV16 E6 containing TK6 cells (T15 clone was used in this study) were grown in RPMI-1640 medium as previously described [Chou and Huang, 2002b].

Genomic DNA Extraction and Polymerase Chain Reaction (PCR)

In order to determine whether the *HPV16 E6* gene presented in SiHa cells, genomic DNAs were extracted. Approximately $1-5 \times 10^6$ cells were used to extract genomic DNA. Amplification of E6 sequences integrated into the genome was carried out as described previously [Huang et al., 1996].

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted by TriPure™ Kit (Genesis Biotech. Inc. Taipei, Taiwan), according to the manufacture's instruction. RT-PCRs from different samples were conducted as previously [Chou and Huang, 2002a]. For the synthesis of cDNA, 10 μg of total RNA from control or treated cells was used in the beginning of the reverse transcription. Then suitable amount of the synthesized cDNA was applied in PCR amplification [Chou and Huang, 2002a,b]. RNA concentrations and PCR cycle were titered to establish standard curves, to document linearity, and to permit semiquantitative analysis of single strength as previously described [Germolec et al., 1996]. The HPV16 E6 primer sequences and sizes of amplified products were described previously [Chou and Huang, 2002a]. The human β-actin primer sequences and sizes of amplified products are as followed: sense 5′-TGACTGACTACCTCATGAAG-3′, and antisense 5′-AAGGCTGGAAGAGTGCCTCA-3′, amplified PCR fragment, 239 bp. The PCR product of human β-actin served as an internal control.

Preparation of Polyclonal Antibody Against the HPV16 E6 Protein

Expression vector pQE30-HPV16 E6 was constructed according to manufacture's instruction (Qiagen, Germany). HPV16 E6 protein was

expressed after isopropyl-thio-β-D-galactoside (IPTG) induction at 25°C for 9 h from *E. coli* host SG13009 cells. His-tagged E6 protein was purified in using Ni-NTA Super flow (Qiagen, Germany) and used as antigen for immunizing procedures. E6 protein first was mixed with complete (for first injection only) or incomplete Freund's adjuvant to form emulsified suspension, then, the suspension was applied in subcutaneous injections into 2–3 female rabbits four times at 2-week interval. Blood samples from rabbits were tittered on 14 days or more after each injection. Blood samples containing more than 1:3,000 titer to detect 5 ng E6 antigen were collected for further usage in this study [Chao et al., 1994].

Western Blotting and Immunoprecipitation (IP)

Samples from control and treated cells were lysed on ice in lysis buffer containing 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 50 mM Tris-base at pH 8.0, 150 mM NaCl, 0.02% NaN_3, 1% NP-40, and 1 μg/ml aprotinine. After centrifugation, the protein concentration of the supernatant was determined by Bio-Rad D_C Protein Assay kit (Bio-Rad laboratories, Richmond, CA). Equal amounts of proteins from different samples were used in Western blotting. Samples after electrophoresis were electrotransferred onto PVDF membranes. Membranes were probed with appropriate antibody and the signals were detected as previously mentioned [Chou and Huang, 2002a].

For IP procedures, total cell extracts were prepared in lysis buffer as previously mentioned for Western blotting. Approximately 1.5–2.0 mg cell extract was mixed with 100 μl 10% protein A-Shepharose CL-4B in PBS containing 0.02% NaN_3 (for removal of non-specific binding to protein A in cell extract). The reaction mixtures were incubated at 4°C for 30 min. After centrifugation, the supernatant were collected and 0.5% NP-40 (in PBS solution) was added to adjust to 1 ml final volume. About $60 \sim 80$ μl anti-HPV16 E6 antibody was added to the above supernatant. Reaction mixture was incubated at 4°C overnight. After another addition of 100 μl 10% protein A-Shepharose CL-4B, the mixtures were further incubated at 4°C overnight. After centrifugation, the pellet was washed with 0.5% NP-40/PBS solution. After final centrifugation, pellets were suspended in 0.5% NP-40/PBS. One-tenth to one-fifth volume of the final suspension was used for Western blotting in this study.

Flow Cytometric Analysis

For sub-G_1 apoptosis analysis, samples were harvested and stained with propidium iodide (PI), followed by FACScan flow cytometric analysis [Chou and Huang, 2002a]. The percentage of cells in every cell cycle stage was analyzed by ModFit program (Becton Dickinson, UK). For HPV16 E6 protein detection, cells were incubated for 30 min with anti-HPV16 E6 antibodies (1:500 dilution in PBS with 1% FCS) after removal of the fixative. The cells were washed with PBS containing 1% FCS, then incubated in a solution containing anti-rabbit FITC conjugated IgG antibodies. FACScan flow cytometric analysis was conducted to detect E6 oncoprotein for various samples thereafter.

The method for dual-parameter flow cytometric analysis of normal, apoptotic, and necrotic cells was performed as previously described [Chou and Huang, 2002a]. In brief, after treatment, cells were washed with PBS and incubated with 2 μg/ml Hoechst 33258 at 37°C for 15 min. After centrifugation, cells were resuspended in 1 μg/ml PI at 4°C for 30 min and analyzed with FACScan flow cytometry and CellQuest program (Becton Dickinson).

Sulforhodamine B (SRB) Cell Survival Assay

Cells were grown in 6-well plates or 35 mm two dishes, 2×10^4 per each well. After various treatments, cells were refreshed with new medium and incubated for few days until the untreated control cells reached to 80% confluence. Then, SRB cell survival assay was conducted as previously mentioned [Huang et al., 1998; Chou and Huang, 2002a].

RESULTS

Expression of p53 and HPV16 E6 in SiHa Cells After Cisplatin Treatment

Because high-risk HPVs E6 binds to normal p53 protein, promotes its degradation through the ubiquitin pathway and abrogates its function, the expression of p53 and E6 in SiHa cells were examined after cisplatin treatment. After treated with 10 μM cisplatin for various time, p53 was dramatically restored in a time-dependent manner and persisted for at least 24 h (left panel in Fig. 1A). Similarly, after treated with 0–20 μM cisplatin for 24 h, p53 was restored in a

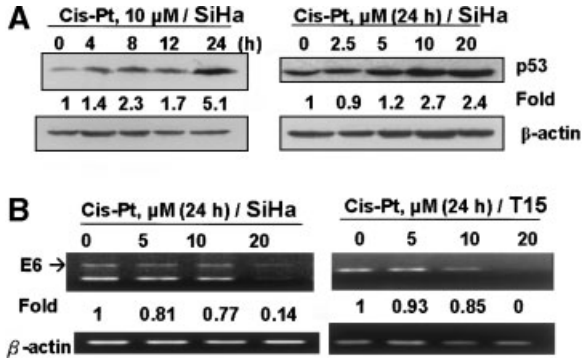


Fig. 1. Expressions of p53 protein and HPV16 E6 mRNA in cells after cisplatin treatment. **A**: SiHa cells at log phase were treated with 10 μM cisplatin for 0–24 h or 0–20 μM cisplatin for 24 h. Whole cellular proteins were isolated, and Western analysis of p53 was performed as described in ''Materials and Methods.'' The relative different p53 protein levels from various samples at same loading amount were also normalized by β-actin in each sample. Fold of p53 protein levels compared with untreated cells were shown between plots for p53 and β-actin. **B**: Cells were treated with 0–20 μM cisplatin for 24 h. Total cellular RNA was isolated and RT-PCR was performed as described in ''Materials and Methods'' to determine relative difference of E6 mRNA levels normalized by β-actin mRNA. Fold of control on E6 mRNA level for each sample was shown between plots for E6 and β-actin.

dose-dependent manner (right panel in Fig. 1A). In contrast, HPV16 E6 mRNA expressions were suppressed in SiHa cells after more than 5 μM cisplatin treatment for 24 h. Only 14% of control E6 mRNA expression was found in cells after 20 μM cisplatin treatment for 24 h (left panel in Fig. 1B). This was accompanied with more than 2-fold of p53 restoration in treated cells (right panel in Fig. 1A). Similarly, the E6 mRNA decreased to a minimum in T15 cells (TK6 cells containing stably transfected *HPV16 E6* gene) after 20 μM cisplatin treatment for 24 h (right panel in Fig. 1B).

Suppression of HPV16 E6 Protein in Cisplatin Treated Cells

The amount of E6 protein within untreated control and cisplatin-treated SiHa cells was assayed by means of anti-E6 polyclonal antibody. The results from flow cytometric analysis (described in "Materials and Methods") indicated that 40% less mean fluorescence intensity (or E6 amount) was found in cells treated with 10–20 μM cisplatin for 24 h in comparison with that in control cells (Fig. 2A). Results from IP plus Western blotting indicated that about 40% of control E6 protein remained in T15 cells after 10 μM cisplatin treatment for 24 h in comparison with untreated cells. On the other hand, T15 cells treated with cisplatin had more than 5-fold p53 restoration as compared to untreated control cells (Fig. 2B).

The Effect of Cell Cycle on Cisplatin Treatment in SiHa

SiHa cells at log phase were first treated with 0–20 μM cisplatin for 24 h, and post-incubated for another 72 h. There was a significant increase in cell population at G_2/M phase in cisplatin-treated cells, compared to untreated control. Additionally, while the G_2/M peak decline (data not shown), sub-G_1 apoptotic cells increased in a dose dependent manner. It indicated that cisplatin could block the cell cycle progression at G_2/M phase, which coincided with the appearance of sub-G_1 apoptosis ($1.7 \sim 23.8\%$) in SiHa cells. Dual-parameter flow cytometric analysis in these treated cells indicated that sub-G_1 apoptotic cells were the major species of cell death after 72 h of post-incubation (Fig. 3).

The Cytotoxic Effects of Cisplatin on Irradiated SiHa Cells

To examine whether cisplatin has synergistic effects on irradiated cells, we treated cells with IR alone, cisplatin alone, or IR plus low dose cisplatin and conducted SRB assay. The SRB survival fractions were 95, 90, and 50% in cells after treated with 0.5, 1.0, and 2.0 μM cisplatin for 24 h and incubated in drug-free medium for 5 days, respectively (Fig. 4A). After 1–4 Gy exposure and post-incubation for 5 days, the percentage of survival in cells decreased to 85% (1 Gy) and 72% (4 Gy). IR and post-treatment with 0.25 μM cisplatin for 24 h significantly decreased cell survival fractions to 60, 55, and 47%, which corresponded to exposure doses of 1, 2, and 4 Gy, respectively (Fig. 4B).

Since no difference in sensitivities to IR or cisplatin was found between SiHa and pcDNA3 vector transfected SiHa cells after SRB assay or sub-G_1 apoptosis, SiHa cells, instead of SiHa containing pcDNA3 cells, were used to conduct all cytotoxicity experiments in this study. Sub-G_1 apoptosis was first examined in SiHa and mp53-SiHa cells after all kinds of treatment and 72 h incubation. After 0–4 Gy exposure, no difference was found in sub-G_1 apoptosis between SiHa and mp53-SiHa cells. Slightly more sub-G_1 cells were produced in samples

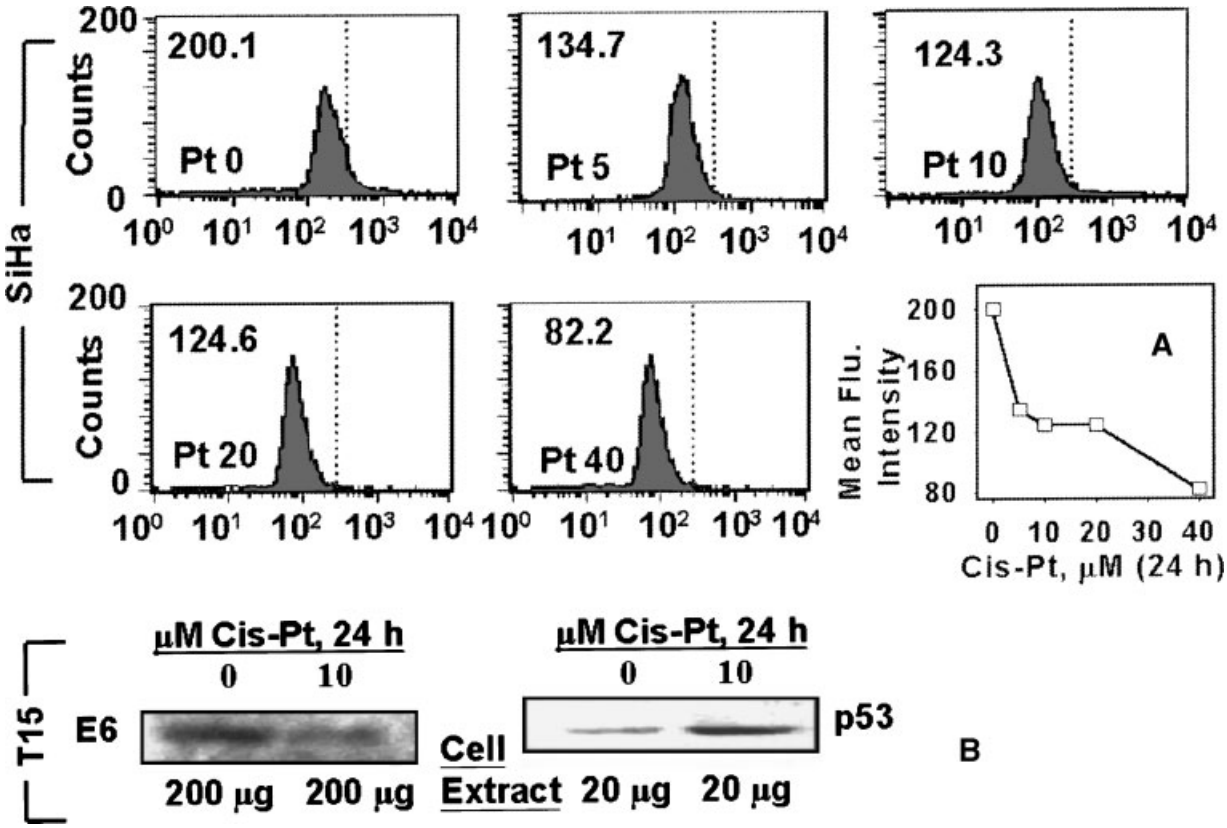


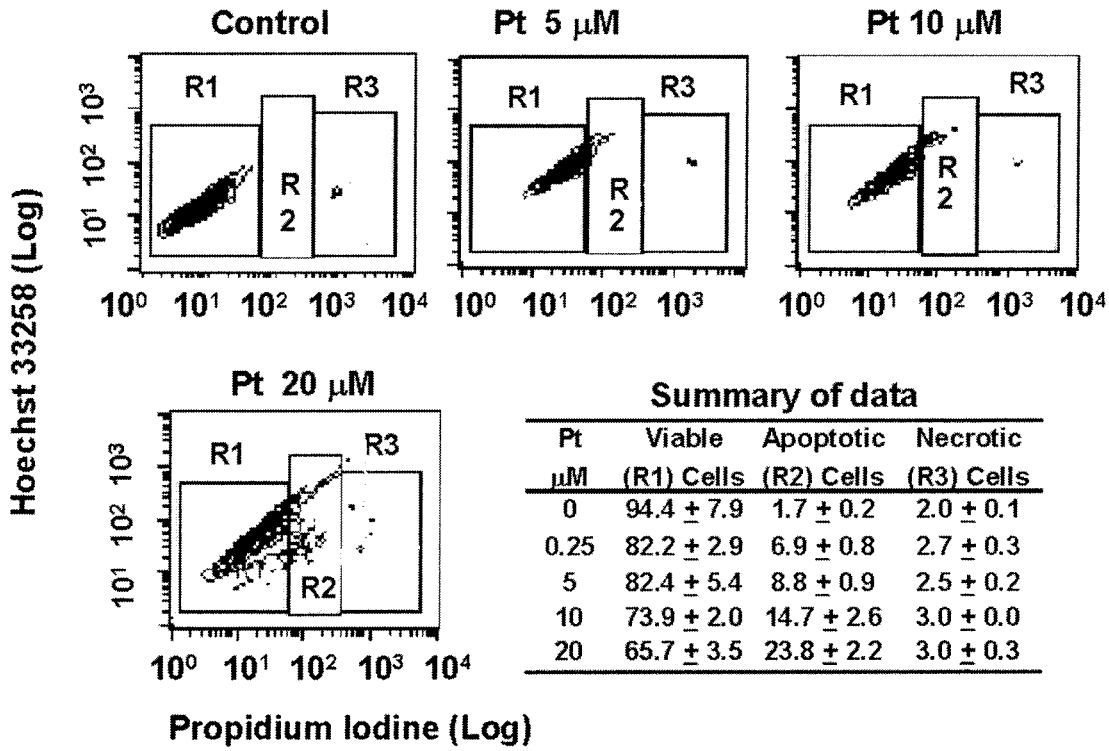
Fig. 2. Alterations of HPV16 E6 protein levels in cisplatin treated cells. **A**: SiHa cells at log phase were treated with 0–40 μM cisplatin for 24 h. Cells were washed with PBS and fixed in 70% ethanol at 4°C overnight. After removing ethanol by centrifugation, cells were then washed twice with ice-cold PBS and incubated for 30 min on ice with the indicated polyclonal antibody anti-6× His-E6 diluted 1:500 in PBS with 1% FCS. The cells were then washed twice in ice-cold PBS containing 1% FCS before flow cytometric analysis (Refer to ''Materials and Methods''). **B**: HPV16 E6 containing T15 cells at log phase were treated with 0–10 μM cisplatin for 24 h, then harvested cells to process immuno-precipitation (IP) and Western analysis for HPV16 E6 and p53 protein detection (Refer to ''Materials and Methods'').

from SiHa cells than those from mp53-SiHa cells after treatment with 0–10 μM cisplatin (Fig. 5A). Cisplatin enhanced IR-induced sub-G_1 cells in both SiHa and mp53-SiHa cells (Fig. 5B). SiHa cells had more sub-G_1 cells after combined treatment than mp53-SiHa cells. In contrast, less synergistic effects on enhancing sub-G_1 population were found in mp53-SiHa cells than in SiHa cells after similar combined treatments (Fig. 5B).

Concurrent Radiotherapy and Cisplatin Treatment Stabilize and Accumulate More p53 Protein in SiHa Cells

The p53 restoration was further examined in SiHa cells after IR alone or concurrent IR and cisplatin treatment. After irradiation at 6 Gy and post-incubation for 24 h, 2-fold p53 expression was restored in SiHa cells, as compared to untreated control cells (lane 2 in Fig. 6A–C). IR-induced p53 restoration then declined at 48 h (1.4-fold) and 72 h (0.9-fold) after irradiation (Fig. 6A). On the other hand, more p53 was restored in cells exposed to 6 Gy and post-treated with 10 μM cisplatin for 24 h (5.1-fold) than those exposed to 6 Gy and 24 h post-incubation (1.5-fold), or cisplatin (4.3-fold) for 24 h (lane 3, 2, and 4 in order in Fig. 6B).

Restoration of p53 expression was also examined after the above mentioned treatment and post-incubation of 24–72 h. After treatment with 5 μM cisplatin for 24 h, followed by irradiation at 6 Gy, about 2.1-fold p53 restoration were shown (lane 3 in Fig. 6C). Then, p53 restoration increased to 4-fold and declined to 1.5-fold in those cells after incubation for another 24 h (data point at 48 h in the protocol shown in Fig. 6, lane 5 in Fig. 6C) and 48 h (data point at 72 h, lane 7 in Fig. 6C), respectively. In contrast, p53 restoration (1.4-fold of control) in



Summary of data

Pt μM	Viable (R1) Cells	Apoptotic (R2) Cells	Necrotic (R3) Cells
0	94.4 ± 7.9	1.7 ± 0.2	2.0 ± 0.1
0.25	82.2 ± 2.9	6.9 ± 0.8	2.7 ± 0.3
5	82.4 ± 5.4	8.8 ± 0.9	2.5 ± 0.2
10	73.9 ± 2.0	14.7 ± 2.6	3.0 ± 0.0
20	65.7 ± 3.5	23.8 ± 2.2	3.0 ± 0.3

Fig. 3. Dual-parameter flow cytometric analysis of viable, apoptotic, and necrotic cells after cisplatin treatment. SiHa cells at log phase were treated with 0–20 μM cisplatin for 0–24 h. After 3 days in drug-free medium, cells were collected, incubated with Hoechst 33258 and propidium iodide (PI), and applied to flow cytometric analysis. The regions, R1, R2, and R3, indicate viable, apoptotic, and necrotic cells, respectively [Referred to Chou and Huang, 2002a]. The percentage of cells in each region was analyzed by CellQuest program. The summary results from 2–3 independent experiments were shown in table on the right corner of this panel. Sub-G_1 apoptotic cells from all treated samples were significantly different from untreated control after statistics analysis with *t*-test.

samples after 6 Gy irradiation alone lasted until 48 h post-irradiation (lane 4 in Fig. 6C). But it did not last for samples after 72 h post-irradiation (lane 4 in Fig. 6A and lane 6 in Fig. 6C). These indicated that concurrent cisplatin treatment and radiotherapy could prolong the existence of p53 in HPV16 E6 containing SiHa cells (Fig. 6).

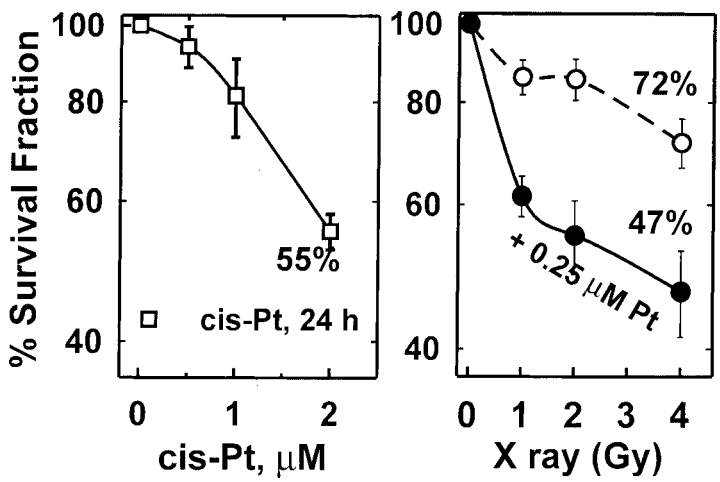


Fig. 4. Cytotoxicity of SiHa cells after X-irradiation and cisplatin treatment. SiHa cells at log phase were treated with 0–2 μM cisplatin for 24 h (**A**), or 0.25 μM for 24 h after 1–4 Gy exposure (**B**). Cells were incubated in drug-free medium for another 5 days before harvested for further SRB assay (Refer to ''Materials and Methods'').

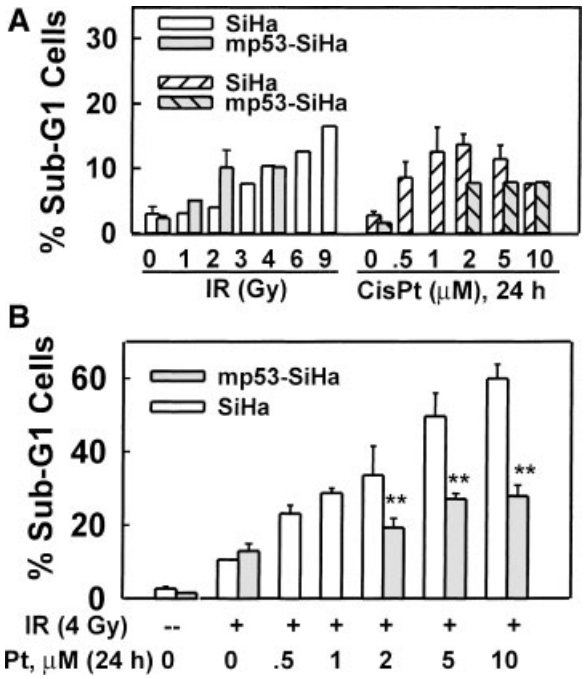


Fig. 5. Apoptotic responses of SiHa cells and mp53-SiHa cells after ionizing radiation (IR), cisplatin, and IR combined cisplatin treatment. **A**: Log-phase cells were either irradiated with 0–9 Gy or treated with 0–10 μM cisplatin for 24 h and incubated in drug-free medium for another 72 h. **B**: Log-phase cells were treated with cisplatin with and without 4 Gy X-irradiation. The IR was given immediately after the addition of cisplatin-containing medium. After treatment with 0–10 μM cisplatin for 24 h, cells were incubated in drug-free medium for another 72 h. Harvested cells were then applied to flow cytometric analysis for quantifying apoptotic cells with sub-G_1 DNA contents by ModFit program. Cisplatin enhanced IR-induced sub-G_1 cells in either SiHa or mp53-SiHa cells. SiHa cells had more sub-G_1 cells after IR combined cisplatin treatment than mp53-SiHa cells. Data shown here were from $2 \sim 3$ independent experiments and statistical analysis between two cells were performed with *t*-test. The symbols, ** indicates $P < 0.01$ in comparison with data from both cells.

Expression of p21 and Mdm2 in SiHa Cells After IR, Cisplatin, or IR plus Cisplatin Treatment

Similar to those previously reported by others [Abdulkarim et al., 2002], our preliminary data indicated that 4 Gy irradiation followed by incubation for 24 h restored p21 in SiHa cells to 1.4-fold. However, no p21 expression accompanied p53 protein restoration in the same cells after 24 h cisplatin treatment (data not shown). These correlated well with our finding mentioned above (this section on 3rd paragraph)—a significant increase in cell population at G_2/M phase (not G_1 arrest) in cisplatin treated cells in comparison with that in control cells. On the other hand, dose-dependent increase in Mdm2 was accompanied by p53 restoration in cells after cisplatin treatment for 24 h (Fig. 7A). Also, Mdm2 increased slightly in irradiated cells at 6 Gy plus 24 h incubation (lane 2 in Fig. 7B). In addition, IR combined with 5 μM cisplatin treatment for 24 h increased more Mdm2 than those from single treatment alone (lane 4 vs. lane 2–3 in Fig. 7B). The increase of Mdm2 in cells at 24 h after treatment confirmed the restoration of p53 and its down-stream pathway. Since most p53 restoration occurred at 48 h post-irradiation in cells treated with 5 μM cisplatin (lane 5 in Fig. 6C), it is expected to find more Mdm2 levels at 48 h data point.

DISCUSSION

In this study, treatment with 10 μM cisplatin for 24 h could significantly restore p53 expression in SiHa cells (Fig. 1A). In addition, cisplatin suppressed HPV16 E6 mRNA formation in E6 containing SiHa and T15 cells as indicated by RT-PCR (Fig. 1B). Suppression on E6 protein was also found in samples after flow cytometric analysis or IP procedures (Fig. 2). These results of p53 restoration and HPV16 E6 mRNA reduction partially supported recently published data by others [Koivusalo et al., 2002]. Report from Koivusalo et al. [2002] indicated that an increase in p53-responsive reporter activation was found in cisplatin-treated transfected SiHa cells. However, p53 protein restoration was not shown in their samples after 70 μM cisplatin treatment for 24 h by means of Western analysis. In contrast, we had demonstrated p53 restoration, together with E6 mRNA suppression and E6 protein reduction, in cisplatin treated SiHa cells (Figs. 1 and 2). These discrepancies might come from different source of cisplatin as well as SiHa cell strains that we had used in this study. Nevertheless, similar to our results in using human cervical cancer cell line HeLa, Wesierska-Gadek et al. [2002] reported that cisplatin could restore p53 protein and decrease HPV18 E6 proteins.

On the other hand, our results showed that dose-dependent induced sub-G_1 apoptosis was found in SiHa cells after cisplatin treatment (Figs. 3 and 5). The increase in sub-G_1 apoptosis seemed to correlate well with cisplatin-restored p53 expression (Fig. 1A). This suggested that p53 restoration at least partially activated apoptosis pathway in SiHa cells. In mp53-SiHa cells without normal function of p53, slightly

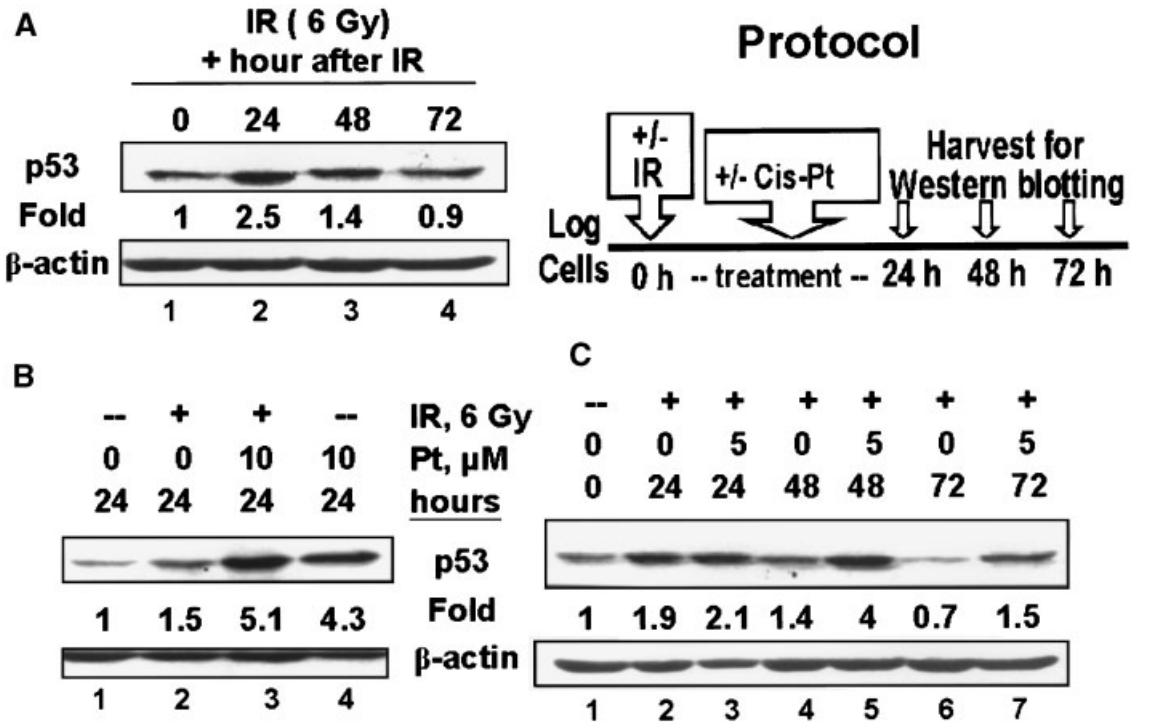


Fig. 6. Restoration of p53 protein in SiHa cells after IR, cisplatin or IR combined cisplatin treatment. Protocol for all treatments was shown on upper left corner of this picture. Cells at log phase were irradiated with 6 Gy and then harvested samples at 24, 48, or 72 h post-irradiation for further Western analysis (**A**). Cells irradiated with 6 Gy and treated with 10 μM cisplatin for 24 h were then harvested for Western analysis in (**B**). Cells irradiated with 6 Gy and treated with 5 μM cisplatin for 24 h. Samples were collected at 24, 48, and 72 h after irradiation for Western analysis (**C**).

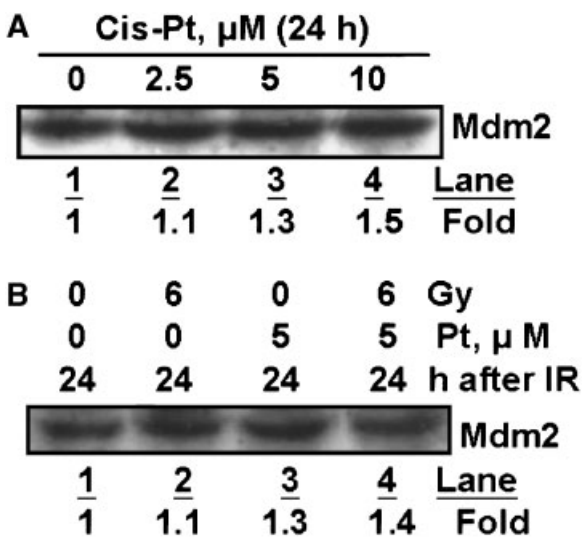


Fig. 7. Expressions of Mdm2 protein in SiHa cells after IR, cisplatin, or IR combined cisplatin treatment. **A**: SiHa cells at log phase were treated with 0–10 μM cisplatin for 24 h were then harvested for Western analysis. **B**: Cells irradiated with 6 Gy and treated with 5 μM cisplatin for 24 h. Samples were harvested and proteins were extracted for Western analysis.

less sub-G_1 cells after cisplatin treatment were found (Fig. 5A).

In the present study, we had obtained E6 DNA fragments of the same size from PCR reaction using genomic DNA of SiHa cells and HPV16 E6 primers (data not shown). However, results from RT-PCR in this study indicated that another uniform smaller mRNA presented besides the regular size of E6 mRNA in our SiHa cells (upper left panel in Fig. 1B). However, only one size of E6 protein from SiHa cells by means of IP and Western analysis was recognized (data not shown). Only one species of E6 mRNA or protein from HPV16 E6 transfected T15 cells was recognized from gel electrophoresis results (upper right panel on Figs. 1B and 2B).

Under normal growth conditions, p53 is turned over by the ubiquitin-proteasome system. In addition, p53 is regulated mainly through Mdm2-mediated degradation [Finlay, 1993]. But in cervical cancer cells infected with HPVs, the degradation of p53 is completely switched to HPVs E6-mediated ubiquitination [Hengstermann et al., 2001]. Therefore, the increase of p53 expression might partially be

due to the down-regulation of HPV16 E6 oncogene in cisplatin-treated SiHa cells (Figs. 1B and 2). It would reduce intracellular E6 levels to abrogate p53. On the other hand, cisplatin might directly or indirectly bind the thiol groups of E6 protein to affect its function or activity. Since E6 protein contains two zinc finger sequences that bind to DNA, RNA, and protein [Ullman et al., 1996]. It is possible that cisplatin may also bind to E6 protein in cells. However, E6 has been reported to bind to the core structure of p53 to mediate its degradation. Therefore, cisplatin might interfere with the binding of E6 to p53 and avoid the degradation of p53 by E6.

It has been known that E6 protein binds to p53 and promotes its ubiquitin-dependent degradation. Thus, it perturbs the control of cell cycle progression and apoptosis. In this study, the cell cycle progression in SiHa cells was directed toward sub-G_1 apoptosis after cisplatin treatment (Fig. 3). The E6 oncoprotein has been shown to recruit the cellular ubiquitin–protein ligase E6-AP to target the tumor-suppressor protein p53 for ubiquitin-proteasome-mediated degradation. E6 has been reported to interact with several cellular proteins, including E6BP, hDLG, IRF-3, Bak, and E6TP1, and it seems likely that at least some of these interactions contribute to HPV-induced cellular transformation [Hengstermann et al., 2001; Cooper et al., 2003].

IR is the most commonly used cytotoxic agent for cervical carcinoma and HNSCC cells. But, there was not much studies focusing on the effects of IR on the expression of E6/E7 oncoproteins or p53 protein restoration in cervical cancer cells [Abdulkarim et al., 2002]. Recently, the HPV16 E6 mRNA and p53 expression in several cervical cell lines were examined by several research groups [Abdulkarim et al., 2002; Koivusalo et al., 2002]. A close correlation between p53 activity and HPV E6 mRNA was suggested in all of the tested cell lines. However, treated the same SiHa cells with various agents or harvested them at various time points may have very different results on p53 down-stream pathway. For instance, previous studies from our laboratory indicated that there was no change in either HPV16 E6 mRNA or p53 levels in SiHa cells after 4, 8, and 12 Gy X-irradiation plus 2 h post-incubation (Fig. 1A in [Chou and Huang, 2002a]). Further examination in this study showed that p53 expression was restored (1.5–2.5-fold of control level) in cells after 6 Gy irradiation, followed by 24 h incubation (Fig. 6A–C). The restoration was suppressed again in cells after 48–72 h post-incubation (at 24 h data point: 2.5-fold; at 48 h: 1.4-fold; at 72 h: 0.9-fold, Fig. 6A). These results were not the same as those from other reports in which 5 Gy irradiation did not induce much p53 reporter activation (1.1 and 1.4-fold of control level) in p53 reporter-transfected SiHa cells after 24 and 48 h post-incubation [Koivusalo et al., 2002].

To investigate HPV E6 mRNA expression in irradiated cells, Abdulkarim et al. [2002] reported an increasing on HPV18 E6 mRNA to 1.3–2.1-fold of control level in HEP2 cell line after 2–9 Gy irradiation and 24 h post-incubation. In contrast to their data, our preliminary results indicated that about 70% E6 mRNA of control level was observed in SiHa cells after 4 Gy exposure and 24 h post-incubation (data not shown). Since we had prepared useful anti-E6 polyclonal antibody to detect E6 reduction in cells after treatment with cisplatin (Fig. 2), E6 oncoprotein in SiHa cells after IR and post-incubation for various times would be easily monitored.

Cisplatin enhanced IR-induced SRB-cytotoxicity and sub-G_1 apoptosis was found in SiHa cells (Figs. 4 and 5). These enhancements of radio-sensitivities (cytotoxicity and sub-G_1apoptosis) in SiHa cells seemed to be accompanied by the restoration of p53 expression, the increase of Mdm2 expression (Figs. 6 and 7) and the enhancement of IR-restored p53 expression hours in SiHa cells (Figs. 5 and 6).

In contrast, less synergistic effects have been found in irradiated mp53-SiHa cells after cisplatin treatment (Fig. 5B). Since p53 in mp53-SiHa cells would not be restored to have similar function as their parental SiHa cells, our data in this study coincided with the expected results (Figs. 6 and 5B).

In conclusion, we have provided evidence to show that the cisplatin treatment activated the p53 tumor suppressor pathway (p53 and Mdm2), increased cytotoxic effects, and induced apoptosis in E6-positive SiHa and TK6 cells. And the restoration of p53 function has been correlated with the decrease of E6 mRNA and E6 protein levels in E6-transfected cells after cisplatin treatment. In addition, we also demonstrated these above mentioned phenomena on p53 restoration and Mdm2 expression presented in SiHa cells after IR and

post-incubation for 24 h and thereafter. Cisplatin would prolong IR-restored p53 expression in SiHa cells. These seemed to correlate well with the fact that more sub-G_1 apoptosis occurred in cells after combined treatment. We therefore speculated that cisplatin may interact with E6 protein. Additional studies are required to know more about the interaction among cisplatin, E6, p53, and other associated proteins in E6/p53 degradation pathway.

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REFERENCES

Abdulkarim B, Sabri S, Deutsch E, Chagraoui H, Maggiorella L, Thierry J, Eschwege F, Vainchenker W, Chouaib S, Bourhis J. 2002. Antiviral agent Cidofovir restores p53 function and enhances the radiosensitivity in HPV-associated cancers. Oncogene 21:2334–2346.

Boike GM, Petru E, Sevin BU, Averette HE, Chou TC, Penalver M, Donato D, Schiano M, Hilsenbeck SG, Perras J. 1990. Chemical enhancement of cisplatin cytotoxicity in a human ovarian and cervical cancer cell line. Gynecol Oncol 38:315–322.

Bradford CR, Zhu S, Ogawa H, Ogawa T, Ubell M, Narayan A, Johnson G, Wolf GT, Fisher SG, Carey TE. 2003. P53 mutation correlates with cisplatin sensitivity in head and neck squamous cell carcinoma lines. Head Neck 25:654–661.

Chao CC, Shieh TC, Huang H. 1994. Use of a monoclonal antibody to detect DNA damage caused by the anticancer drug cis-diamminedichloroplatinum (II) in vivo and in vitro. FEBS Lett 354:103–109.

Chou RH, Huang H. 2002a. Restoration of p53 tumor suppressor pathway in human cervical carcinoma cells by sodium arsenite. Biochem Biophys Res Commun 293: 298–306.

Chou RH, Huang H. 2002b. Sodium arsenite suppresses human papillomavirus-16 *E6* gene and enhances apoptosis in E6-transfected human lymphoblastoid cells. J Cell Biochem 84:615–624.

Cohen SM, Lippard SJ. 2001. Cisplatin: From DNA damage to cancer chemotherapy. Prog Nucleic Acid Res Mol Biol 67:93–130.

Cooper B, Schneider S, Bohl J, Jiang Y, Beaudet A, Vande Pol S. 2003. Requirement of E6AP and the features of human papillomavirus E6 necessary to support degradation of p53. Virology 306:87–99.

Crook T, Tidy JA, Vousden KH. 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. Cell 67: 547–556.

Finlay CA. 1993. The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. Mol Cell Biol 13:301–306.

Germolec DR, Yoshida T, Gaido K, Wilmer JL, Simeonova PP, Kayama F, Burleson F, Dong W, Lange RW, Luster MI. 1996. Arsenic induces overexpression of growth factors in human keratinocytes. Toxicol Appl Pharmacol 141:308–318.

Hawkins DS, Demers GW, Galloway DA. 1996. Inactivation of p53 enhances sensitivity to multiple chemotherapeutic agents. Cancer Res 56:892–898.

Hengstermann A, Linares LK, Ciechanover A, Whitaker NJ, Scheffner M. 2001. Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells. Proc Natl Acad Sci USA 98:1218–1223.

Huang H, Li CY, Little JB. 1996. Abrogation of P53 function by transfection of *HPV16 E6* gene does not enhance resistance of human tumour cells to ionizing radiation. Int J Radiat Biol 70:151–160.

Huang H, Shu SC, Shih JH, Kuo CJ, Chiu ID. 1998. Antimony trichloride induces DNA damage and apoptosis in mammalian cells. Toxicology 129:113–123.

Kern DH, Morgan CR, Hildebrand-Zanki SU. 1988. In vitro pharmacodynamics of 1-beta-D-arabinofuranosylcytosine: Synergy of antitumor activity with cis-diamminedichloroplatinum(II). Cancer Res 48:117–121.

Koivusalo R, Krausz E, Ruotsalainen P, Helenius H, Hietanen S. 2002. Chemoradiation of cervical cancer cells: Targeting human papillomavirus E6 and p53 leads to either augmented or attenuated apoptosis depending on the platinum carrier ligand. Cancer Res 62:7364–7371.

Peters RH, Jollow DJ, Stuart RK. 1991. Role of glutathione in the in vitro synergism between 4-hydroperoxy-cyclophosphamide and cisplatin in leukemia cell lines. Cancer Res 51:2536–2541.

Scheffner M, Munger K, Byrne JC, Howley PM. 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci USA 88: 5523–5527.

Ullman CG, Haris PI, Galloway DA, Emery VC, Perkins SJ. 1996. Predicted alpha-helix/beta-sheet secondary structures for the zinc-binding motifs of human papillomavirus E7 and E6 proteins by consensus prediction averaging and spectroscopic studies of E7. Biochem J 319(Pt 1):229–239.

Wesierska-Gadek J, Schloffer D, Kotala V, Horky M. 2002. Escape of p53 protein from E6-mediated degradation in HeLa cells after cisplatin therapy. Int J Cancer 101: 128–136.