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Cisplatin-resistant HeLa Cells Are Resistant to Apoptosis via p53-dependent and -independent Pathways

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Since HeLa cells possess very little functional p53 activity, they could be originally resistant to genotoxic stress-induced apoptosis. Therefore, it is likely that the drug-resistant cells derived from HeLa cells are more resistant to apoptosis. The aim of this study was to determine whether cisplatin-resistant cells derived from HeLa cells have an apoptosis-resistant phenotype. A cisplatin-resistant cell subline, HeLa/CDDP cells, showed a 19-fold resistance to cisplatin compared with the parent cells. The subline showed a collateral sensitivity to paclitaxel. An equitoxic dose (IC_{50}) of cisplatin produced DNA fragmentation in HeLa cells but not in HeLa/CDDP cells. Transfection of wild-type *p53* gene enhanced the cytotoxicity of cisplatin and cisplatin-induced apoptosis in HeLa cells but not in HeLa/CDDP cells, although it caused p53 overexpression in both cell lines. The expression of *caspase 1* (interleukin-1β-converting enzyme, ICE) mRNA and the overexpression of bax protein were observed only in HeLa cells. Paclitaxel-induced DNA fragmentation appeared less in HeLa/CDDP cells than in HeLa cells. *p53* gene transfection did not affect the extent of DNA fragmentation in either cell line, suggesting that paclitaxel may induce p53-independent apoptosis. These findings suggest that HeLa/CDDP cells may have an acquired phenotype that is resistant to p53-dependent and -independent apoptosis.

Key words: HeLa cells — Cisplatin — Drug-resistance — Apoptosis — p53

Cisplatin is one of the most active anticancer agents now used. Despite its broad clinical applications, however, the resistance of cancer cells to cisplatin often culminates in chemotherapeutic failure. Recently, the disruption of apoptotic response to cisplatin has been suggested as a possible basis of the resistance. Most anticancer agents may exert their toxicity, at least in part, by induction of apoptosis.[1, 2] Cisplatin, as well as a variety of cytotoxic drugs, has the potential to cause cell death by apoptosis.[3, 4] p53 is regarded as a key regulator of cellular response to anticancer agents.[5–7] Many studies, whose results have been conflicting, have examined the relationship between the status of p53 and cellular sensitivity to cisplatin.[8–16]

We recently developed a recombinant adenovirus carrying a wild-type *p53* gene (AxCAp53), and showed that p53 transduction increased sensitivity to cisplatin via the induction of apoptosis in *p53* gene-deficient ovarian cancer cells.[17]

Because HeLa cells possess very little functional p53 activity,[18] owing to the constitutive expression of human papilloma virus (HPV) E6 protein,[19] they could be originally resistant to genotoxic stress-induced apoptosis mediated by a wild-type *p53* gene product. Therefore, it is likely that the cisplatin-resistant cells derived from HeLa cells are more resistant to cisplatin-induced apoptosis.

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Paclitaxel as a promoter of tubulin polymerization, changes the dynamic equilibrium of assembling and disassembling microtubules, disrupts the formation of the normal spindle at metaphase, and causes the blockade of cell division.[20] Paclitaxel exhibited no cross-resistance or collateral sensitivity in cisplatin-resistant cell lines.[21] Although several studies have discussed the role of p53 in determining cellular responses to paclitaxel treatment, the results have been controversial.[22–26] On the other hand, paclitaxel-induced apoptosis, at least in part, has been shown to occur via a p53-independent pathway.[27–29]

To determine whether cisplatin-resistant cells derived from HeLa cells have an apoptosis-resistant phenotype in p53-dependent and -independent pathways, we examined the apoptotic response to both cisplatin and paclitaxel under conditions of transient p53 overexpression in HeLa and cisplatin-resistant HeLa cells.

MATERIALS AND METHODS

Cell lines and culture HeLa cells (ATCC CCL-2) and a cisplatin-resistant subline, HeLa/CDDP cells[30] were maintained in Eagle's minimum essential medium (Biowhittaker, Walkersville, MD) containing 2 m*M* glutamine, 100 IU/ml of penicillin, and 10% fetal bovine serum (FBS) (Cansera, Ontario, Canada) at 37°C in a humidified incubator with 95% air and 5% CO_2.

Reagents Cisplatin and paclitaxel were obtained from

Bristol Myers-Squibb, Tokyo. A multiple primers kit to detect apoptosis-related gene expression was purchased from Maximum Biotech, Inc. (San Francisco, CA). Anti-p53 and anti-bax monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish-peroxidase-conjugated antimouse antibody and enhanced chemiluminescence western blotting kits were obtained from Amersham Corp. (Lexington Heights, IL).

Sensitivity to cisplatin or paclitaxel The sensitivity of HeLa and HeLa/CDDP cells to cisplatin and paclitaxel was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[31] Cells were diluted with culture medium to the seeding density, suspended in 96-well tissue culture plates (120 μl, containing 10^4 cells, per well) (Sumitomo Bakelite, Tokyo), and preincubated at 37°C for 4 h. Cells were incubated for 72 h with cisplatin (0.1–100 μM) or paclitaxel (0.01 nM–10 μM) to obtain a dose-response curve for each drug. After incubation for 72 h, the absorbance of the MTT-formazan product at 570 nm was measured with a microplate reader Model 450 (BIO-RAD, Richmond, CA).

To determine the efficacy of p53 transduction to the cells, the cells were infected with AxCALacZ, encoding for the bacterial *LacZ* gene, and the transduced cells were detected by β-galactosidase staining. HeLa and HeLa/CDDP cells were seeded in an 8-well chamber slide (Nunc, Roskilide, Denmark). After 4 h preincubation, the cells were infected with AxCALacZ at 12.5–100 multiplicities of infection (MOI). After 48 h, the cells were rinsed with phosphate-buffered saline (PBS), and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS. The magnitude of staining was quantitated by counting the percentage of blue cells. The efficacy of the recombinant adenovirus to transduce adenovirus for HeLa or HeLa/CDDP cells was 100% over 25 MOI.

The effect of wild-type *p53* gene transfer mediated by AxCAp53 on the cell viability was examined by using MTT assay. After 4 h preincubation, HeLa and HeLa/CDDP cells (10^4 cells/well) were infected with 25 MOI of AxCAp53.[17] The cell growth suppression rate was calculated as: suppression rate (%) = (the absorbance of non-infected control cells − the absorbance of infected cells)/the absorbance of non-infected control cells.

Next, the combined effect of wild-type *p53* gene transfer and cisplatin or paclitaxel was examined. One hour after 25 MOI of AxCAp53 infection, various concentrations of cisplatin or paclitaxel were added and the cells were incubated for 72 h. The change in IC_{50} was determined from the dose-response curve.

Analysis of cisplatin- or paclitaxel-induced apoptosis The cells were treated with cisplatin or paclitaxel at a dose of IC_{50}, with or without AxCAp53 infection. For the analysis of the DNA laddering characteristic of apoptotic cell death, DNA was isolated by the salting-out procedure described by Miller *et al.*,[32] from cells floating in the medium and cells attaching on the dish. The DNA samples were subjected to electrophoresis on a 2% agarose gel in 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0 buffer at 50 V for 2 h. A 123-bp DNA ladder was used as the standard. DNA was visualized by ethidium bromide staining and photographed under ultraviolet illumination.

To assess apoptosis morphologically, Hoechst 33258 (Calbiochem-Novabiochem, San Diego, CA) staining was performed using cells fixed with Clarke fixative (ethanol: acetic acid, 3:1) under the indicated conditions. Over 3000 cells were counted in each experiment. The apoptotic index (AI) was defined as: AI (%) = 100×apoptotic cells/total cells.

For flow cytometric analysis, cells (2×10^6) were trypsinized, collected by centrifugation, fixed in 70% ethanol at 4°C for 1 h and then resuspended in PBS containing 40 μg/ml propidium iodide and 0.1 mg/ml RNase. After 30 min at 37°C, the cells were analyzed with a FACSCaliber cytofluorometer (Becton Dickinson, San Jose, CA).

p53 and bax protein expressions were analyzed by western blot analysis. Cells were solubilized on ice in a lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% NP40, 5 mM ethylenediaminetetraacetic acid, 50 mM NaF, 0.1% phenylmethylsulfonyl fluoride, and protease inhibitor) and centrifuged at 25,000g for 5 min. The total protein concentration in the supernatant was measured, and samples of 60 μg protein were separated by electrophoresis on 4–20% gradient polyacrylamide gels. The separated proteins were transferred onto a polyvinylnylidene difluoride membrane (Millipore Co., Bedford, MA). p53 and bax were probed with monoclonal antip53 (DO-7) and antibax antibodies, then visualized with antimouse or antirabbit IgG coupled to horseradish peroxidase using enhanced chemiluminescence according to the manufacturer's recommendation.

Caspase 1 (ICE) and *p53* gene expressions were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted, using RNAzol according to the manufacturer's protocol (Biotec Laboratories Inc., Houston, TX). cDNA was synthesized from the isolated RNA by RT as described by Wei *et al.*[33] *Caspase 1* and *p53* genes were simultaneously amplified by PCR according to the manufacturer's protocol (Maximum Biotech, Inc.), in which the primers were designed to generate products of 658 bp from *caspase 1*, and 204 bp from *p53*. Thermal cycling was performed in a DNA amplifier by using the following profile: (a) 2 cycles of initial denaturation at 96°C and annealing at 57°C for 4 min; (b) 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 2.5 min; and (c) synthesis at 70°C for 10 min. The primers of G3PDH designed for amplification of the gene, as a control, are 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-

Table I. Sensitivity to Cisplatin or Paclitaxel and *p53* Gene Transfer

	IC_{50} (μM)		Resistance factor
	HeLa cells	HeLa/CDDP cells	
cisplatin	2.2	42.0	19.1
cisplatin+AxCAp53	1.5	38.8	25.9
paclitaxel	20.0×10^{-3}	6.8×10^{-3}	0.3
paclitaxel+AxCAp53	18.6×10^{-3}	6.2×10^{-3}	0.3

The resistance factor is the IC_{50} of HeLa/CDDP cells divided by IC_{50} of HeLa cells.

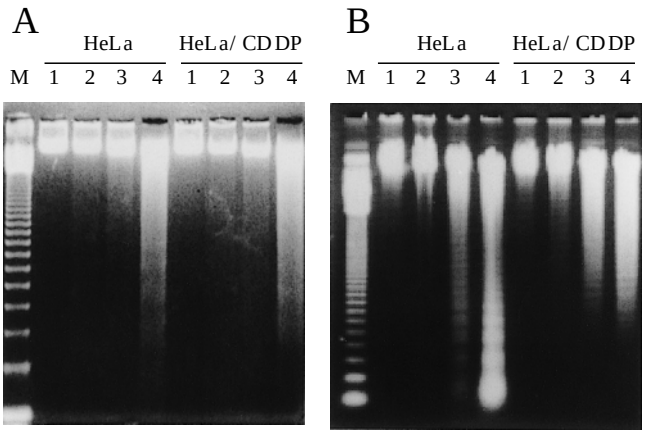

Fig. 1. Cisplatin (A) or paclitaxel (B)-induced DNA cleavage assessed by agarose gel electrophoresis. Cells were exposed to IC_{50} of cisplatin or paclitaxel for 72 h. M, 123-bp DNA ladder as standard; lane 1, 0 h; lane 2, 24 h; lane 3, 48 h; lane 4, 72 h.

3′ (antisense). The primers were designed to generate a product of 450 bp. The PCR products were electrophoresed in 2% agarose gel and stained with 0.5 μg of ethidium bromide.

RESULTS

Sensitivity to cisplatin or paclitaxel Compared with HeLa cells, HeLa/CDDP cells showed a 19-fold resistance to cisplatin. In contrast, the IC_{50} value to paclitaxel was greater for HeLa cells than for HeLa/CDDP cells, suggesting that HeLa/CDDP cells acquired a collateral sensitivity to paclitaxel (Table I).

The suppression rate at 25 MOI of AxCAp53 was significantly greater in HeLa cells than in HeLa/CDDP cells (12.8% vs. 4.2%). With AxCAp53 infection, the IC_{50} value to cisplatin for HeLa cells was reduced to 68.2% of the value for non-infected cells. In contrast, the *p53* gene transfer did not change the value in HeLa/CDDP cells (92.4% of the value for non-infected cells). On the other hand, AxCAp53 infection did not affect their sensitivity to paclitaxel under this experimental condition (Table I).

Table II. Percentage of Cells Morphologically Showing Apoptotic Change (AI) 48 h after Each Treatment

Treatment	HeLa cells	HeLa/CDDP cells
AxCAp53	19.3±7.8 *a)*	7.8±2.5 *f)*
cisplatin	25.6±3.9 *b)*	13.4±3.2 *g)*
cisplatin+AxCAp53	43.2±11.6 *c)*	17.5±6.4 *h)*
paclitaxel	34.6±8.2 *d)*	22.2±5.1 *i)*
paclitaxel+AxCAp53	39.5±13.7 *e)*	25.9±7.7 *j)*

a) vs. *f*), *b*) vs. *g*), *d*) vs. *i*), $P<0.01$; *b*) vs. *c*), $P<0.001$; *g*) vs. *h*), NS; *d*) vs. *e*), *i*) vs. *j*), NS (Student's *t* test).

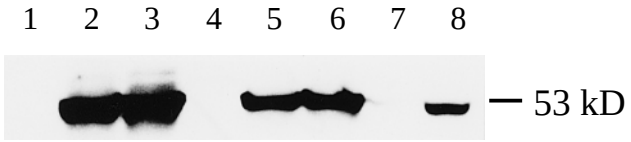

Fig. 2. Western blot analysis of p53 protein in HeLa and HeLa/CDDP cells after infection with 25 MOI of AxCAp53. Lane 1 and lane 4, no-infection control for HeLa cells and HeLa/CDDP cells, respectively; lane 2 and lane 5, 24 h after transfection for HeLa cells and HeLa/CDDP cells, respectively; lane 3 and lane 6, 48 h after transfection for HeLa cells and HeLa/CDDP cells, respectively; lane 7, blank; lane 8, positive control of p53 protein. As a positive control of p53 protein expression, we used KF cells, an ovarian cancer cell line with a wild-type *p53* gene, exposed to etoposide to induce p53 protein.

Cisplatin- or paclitaxel-induced apoptosis DNA fragmentation was observed in HeLa cells 72 h after cisplatin exposure (Fig. 1A). In contrast, in HeLa/CDDP cells, some smearing, rather than DNA fragmentation, appeared on the gel, indicating random continuous degradation of cellular DNA, a process that is characteristic of necrosis. On the other hand, paclitaxel induced clear DNA fragmentation in both cell lines after exposure for 48 h (Fig. 1B). Moreover, the degree of the fragmentation was obviously less for HeLa/CDDP cells than for HeLa cells. The

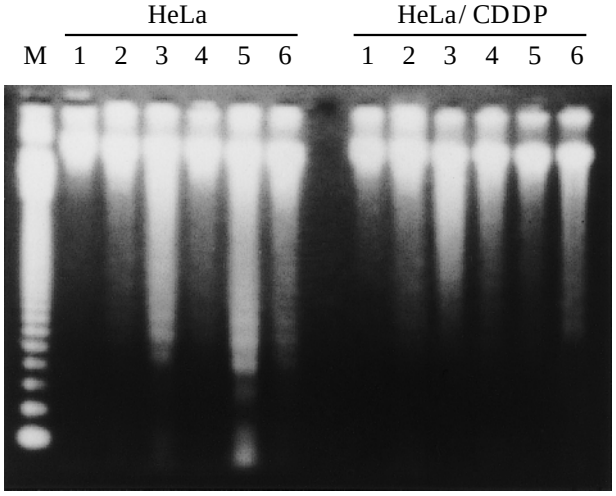

Fig. 3. The effect of AxCAp53 infection on cisplatin- or paclitaxel-induced DNA fragmentation 48 h after treatment. M, 123-bp DNA ladder as standard; lane 1, no treatment; lane 2, cisplatin; lane 3, paclitaxel; lane 4, AxCAp53; lane 5, cisplatin with AxCAp53; lane 6, paclitaxel with AxCAp53.

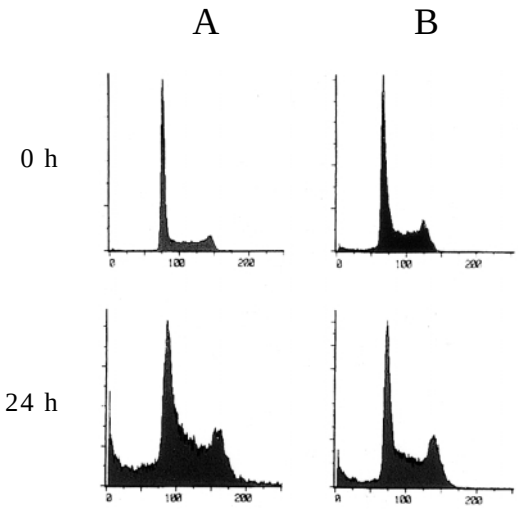

Fig. 4. Combination effect of cisplatin and AxCAp53 on cell cycle progression. A, HeLa cells, 48 h after exposure to the IC_{50} of cisplatin combined with AxCAp53 infection. B, HeLa/CDDP cells similarly treated.

Hoechst 33258 staining also showed that AI 48 h after cisplatin exposure was significantly greater in HeLa cells than in HeLa/CDDP cells. Also, AI 48 h after paclitaxel exposure was significantly greater in HeLa cells than in HeLa/CDDP cells (Table II). These findings suggest that HeLa/CDDP cells are resistant to apoptosis induced by cisplatin or paclitaxel.

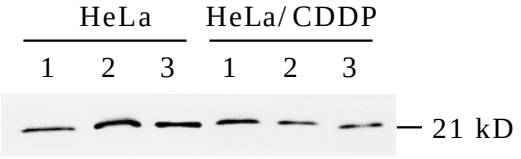

Fig. 5. Western blot analysis of bax protein expression 48 h after cisplatin exposure with or without AxCAp53 infection. Lane 1, no-exposure control; lane 2, cisplatin exposure at IC_{50}; lane 3, cisplatin exposure combined with AxCAp53 infection,

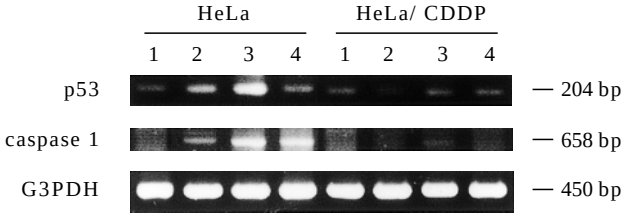

Fig. 6. RT-PCR for *p53* and *caspase 1* mRNA expression 48 h after cisplatin exposure with or without AxCAp53 infection. Lane 1, no-exposure control; lane 2, cisplatin exposure at IC_{50}; lane 3, AxCAp53 infection; lane 4, cisplatin exposure combined with AxCAp53 infection.

Twenty-five MOI of AxCAp53 infection caused p53 overexpression in both HeLa and HeLa/CDDP cells, indicating that p53 transduction was successful (Fig. 2).

AxCAp53 infection apparently increased cisplatin-induced DNA fragmentation in HeLa cells, but not in HeLa/CDDP cells (Fig. 3). After exposure to cisplatin and AxCAp53 infection, AI significantly increased in HeLa cells, but not in HeLa/CDDP cells (Table II). Although flow cytometric analysis showed accumulations of cells in the pre-G_1 and G_2-M phases after exposure to cisplatin and AxCAp53 in both cell lines, the proportion of pre-G_1 cells, presumably apoptotic cells, was greater in HeLa cells than in HeLa/CDDP cells (Fig. 4).

Bax protein expression was increased only in HeLa cells by cisplatin exposure and the combination of cisplatin and AxCAp53 infection (Fig. 5). In contrast, for HeLa/CDDP cells, the protein expression did not change with either treatment. After cisplatin exposure, AxCAp53 infection, or the combination of cisplatin and AxCAp53 infection, *caspase 1* mRNA was detected in HeLa cells, but not in HeLa/CDDP cells (Fig. 6).

In contrast to the collateral sensitivity to paclitaxel observed in HeLa/CDDP cells, paclitaxel-induced DNA fragmentation was less in HeLa/CDDP cells than in HeLa cells. Therefore, we also investigated the cell cycle changes after paclitaxel exposure. An accumulation of pre-G_1 cells and a reduction of G_0/G_1 cells was apparent

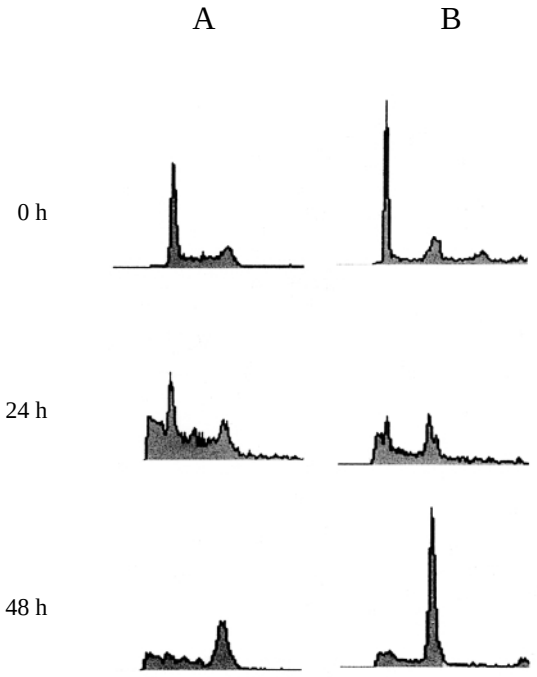

Fig. 7. Effect of paclitaxel on cell cycle progression. Cells were incubated for 48 h with paclitaxel at the IC_{50}. Cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. A, HeLa cells; B, HeLa/CDDP cells.

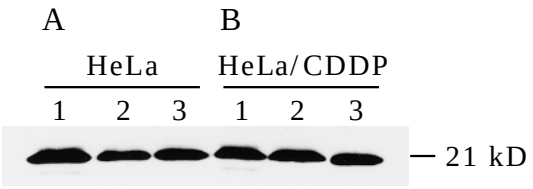

Fig. 8. Western blot analysis of bax protein expression 48 h after paclitaxel exposure with or without AxCAp53 infection. Lane 1, no-exposure control; lane 2, paclitaxel exposure at the IC_{50}; lane 3, paclitaxel exposure combined with AxCAp53 infection; A, HeLa cells, 48 h after cisplatin exposure at the IC_{50} combined with AxCAp53 infection; B, HeLa/CDDP cells similarly treated.

among HeLa cells (Fig. 7). For HeLa/CDDP cells, a prominent accumulation of G_2-M cells and a reduction of G_0/G_1 cells appeared, suggesting that paclitaxel induced M block more effectively in the resistant cells.

In both the parent and cisplatin-resistant cells, AxCAp53 infection did not affect DNA fragmentation and AI compared with paclitaxel exposure alone (Fig. 3, Table II). Furthermore, the bax protein expression did not change after paclitaxel exposure or the combination of AxCA p53 infection (Fig. 8), suggesting that paclitaxel-induced apoptosis may occur in a p53-independent manner in both cell lines.

DISCUSSION

In the present study, equitoxic and relatively low-dose (IC_{50}) cisplatin induced apoptosis in HeLa cells, but not in HeLa/CDDP cells. In our preliminary study, polymerase chain reaction single-strand confirmation polymorphism analysis showed that the *p53* gene in both cell lines was the wild-type. However, p53 protein expression before and after exposure to cisplatin with a dose of IC_{50} could not be detected in either of the cell lines by western blot analysis (data not shown). Therefore, the above-mentioned result might have two different explanations: first, endogenous p53-mediated apoptosis might occur only in HeLa cells, but the p53 protein level might be under the detection level by the usual western blotting; second, cisplatin might induce apoptosis via a p53-independent pathway in HeLa cells.

p53 overexpression enhanced cisplatin-induced apoptosis and increased the sensitivity to cisplatin in HeLa cells. This indicates that cisplatin may induce apoptosis via a p53-dependent pathway in HeLa cells. In the recent literature, resistance to cisplatin parallels a reduced cell susceptibility to cisplatin-induced apoptosis.[9, 34–38] Several authors have indicated that loss of p53 function due to p53 gene mutation may be responsible for the resistance to the apoptotic process,[9, 34, 38] and transient p53 overexpression could mediate cisplatin-induced apoptosis and increase sensitivity to cisplatin.[9, 38] In the HeLa/CDDP cells, p53 overexpression did not enhance the sensitivity and could not induce apparent apoptosis. This indicates that the cisplatin-resistant cell line may be resistant to p53-mediated cisplatin-induced apoptosis.

In the HeLa/CDDP cells, bax protein expression did not change after cisplatin exposure with or without *p53* gene transfection. Furthermore, *caspase 1* mRNA was not detected after cisplatin exposure with or without *p53* gene transfection. Even when the thermal cycling number was elevated to 33 cycles in the latter case, the result was the same (data not shown). Other authors have reported reduced or deficient apoptotic response to cisplatin in the cisplatin-resistant cells, irrespective of p53 function.[36, 37] However, they did not describe the effect of p53 overexpression on the cisplatin-induced apoptotic process in their cell systems. In these cisplatin-resistant cells, including ours, changes in an apoptosis-regulating gene located transcriptionally downstream in the apoptotic pathway, such as caspase 1-related protease,[37] seemed to be responsible for the resistance to apoptosis. A recent study showed that paclitaxel-induced apoptosis paralleled cytotoxicity in cisplatin-resistant cells.[39] In this study, paclitaxel induced DNA fragmentation in the parent and cisplatin-resistant

cells, but it appeared less in HeLa/CDDP cells than in HeLa cells, despite the collateral sensitivity to paclitaxel observed in HeLa/CDDP cells. This phenomenon might imply that paclitaxel-induced apoptosis does not always parallel the cytotoxicity. Furthermore, following paclitaxel exposure, an accumulation in the pre-G_1 phase was dominant in HeLa cells and in the G_2-M phase in HeLa/CDDP cells. This result suggests that the mode of paclitaxel action may be different between the parent and cisplatin-resistant cells. Recent studies have shown that paclitaxel-induced cell death may result from two different mechanisms which are dependent on drug concentration, time after drug exposure, or cell type.[28–30] The first may be the result of a mitotic arrest followed by rapid apoptotic response, while the second may occur after an aberrant mitosis (abnormal mitotic exit) followed by G_1 block, which may lead to relatively slow apoptosis. Therefore, it is likely that the former mechanism might predominantly act in HeLa/CDDP cells and the latter might act predominantly in HeLa cells. Apoptosis may occur more rapidly in the former situation. Nevertheless, HeLa/CDDP cells showed fewer apoptotic changes. Therefore, the HeLa/CDDP cells may have a phenotype that is resistant to paclitaxel-induced apoptosis, irrespective of the sensitivity.

The correlation between p53 status and sensitivity to paclitaxel has not been confirmed.[29–33] Depending on cell type, p53 has been shown to have no effect or to increase or decrease paclitaxel-induced cytotoxicity.[23–27] Also, comparisons of the sensitivity to paclitaxel in clones of the same cell line in which p53 has been disrupted (in the case of wild-type p53-expressing cells) or inserted (in cells not expressing p53) showed contrasting results.[23, 25, 26] We found that p53 overexpression did not affect the apoptotic response, when compared with paclitaxel exposure alone, in both HeLa and HeLa/CDDP cells. Therefore, paclitaxel-induced apoptosis may be p53-independent in these cells, although the mechanisms involved remain unclear.

In conclusion, cisplatin-resistant HeLa cells may have an apotosis-resistant phenotype in p53-dependent and -independent pathways.

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