DDP-induced cytotoxicity is not influenced by p53 in nine human ovarian cancer cell lines with different p53 status

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Summary Nine human ovarian cancer cell lines that express wild-type (wt) or mutated (mut) p53 were used to evaluate the cytotoxicity induced by cisplatin (DDP). The concentrations inhibiting the growth by 50% (IC50) were calculated for each cell line, and no differences were found between cells expressing wt p53 and mut p53. Using, for each cell line, the DDP IC50, we found that these concentrations were able to induce an increase in p53 levels in all four wt-p53-expressing cell lines and in one out of five mut-p53-expressing cell lines. WAF1 and GADD45 mRNAs were also increased by DDP treatment, independently of the presence of a wt p53. Bax levels were only marginally affected by DDP, and this was observed in both wt-p53- and mut-p53-expressing cells. DDP-induced apoptosis was evident 72 h after treatment, and the percentage of cells undergoing apoptosis was slightly higher for wt-p53-expressing cells. However, at doses near the IC50, the percentage of apoptotic cells was less than 20% in all the cell lines investigated. We conclude that the presence of wt p53 is not a determinant for the cytotoxicity induced by DDP in human ovarian cancer cell lines.

Keywords: p53; ovarian cancer; cisplatin; apoptosis

Cisplatin (DDP) is one of the most effective agents in the treatment of ovarian cancer, in which it has shown activity either alone or in combination with other chemotherapeutic agents (Ozols et al., 1991; Cannistra, 1993; NIH, 1994). Its mechanism of action involves the interaction and alkylation of DNA, which results in the formation of monoadducts, interstrand and intranucleotide crosslinks (Eastman 1983; Sherman et al., 1987). These lesions, which are thought to be responsible for the cytotoxicity induced by DDP, are mostly repaired through a nucleotide excision repair mechanism (Hoeijmakers, 1993, 1994; Damia et al., 1996). In this regard, the tumour-suppressor protein p53 has been shown to be implicated in DNA repair by either increasing the levels of p21WAF1 or by interacting with nucleotide excision repair proteins (Kastan et al., 1991; Lane, 1992; Wang et al., 1994, 1995). Considering that in more than 50% of human tumours the p53 gene is mutated (Greenblatt et al., 1994), it is important to define its role in determining the sensitivity of cancer cells to anti-cancer agents. Different studies have reported the effects of the presence of mutated p53 on the response of cells to DDP treatment. There are data supporting that the presence of a wt p53 confers a greater sensitivity or resistance to DDP, depending on the cell types investigated (Brown et al., 1993; Fan et al., 1995; Gjerset et al., 1995; Perego et al., 1996).

Additional data have shown that in some cell types DDP-induced cytotoxicity is independent on the presence of wt or mutated p53 (Graniela Siré et al., 1995; Vikhanskaya et al., 1995; Wosikowski et al., 1995).

In this report, we analysed the DDP-induced cytotoxicity in human ovarian cancer cell lines expressing wt or mutated p53. The data were analysed together with the effects of DDP on the levels of p53 and of p53-downstream genes (WAF1, GADD45 and bax) as well as with the DDP-induced apoptosis in these cell lines.

MATERIALS AND METHODS

Cell lines and treatment

Four human ovarian cancer cell lines expressing wild-type p53, i.e. PA-1, IGROV-1, A2780 and A2774, and five expressing no p53 or mutated p53, i.e. OVCAR-3 (mutation at codon 248, R to Q), OVCAR-5 (insertion of 3 bp at 224), OVCAR-8 (deletion 126–132), SW626 (mutation at codon 262 G to V) and SKOV-3 (deletion), were used. They were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The p53 status was determined for A2774 and SW626 cell lines by polymerase chain reaction (PCR) amplifications and sequence of exons 5–8. In these experiments, A2780 and OVCAR-3 cells were used as controls and their status was confirmed.

Clones were obtained from the p53-null line SKOV-3 upon transfection with the temperature-sensitive murine p53 (SK23) (Vikhanskaya et al., 1994) or with the human wild-type p53 under the control of tetracycline (SKT4) after selection in G418-containing medium (500 μg ml−1).

Cytotoxicity was evaluated using the MTT test in 96-well plates (Nunc) at different times after treatment with different concentrations of DDP.
DDP-induced cytotoxicity and p53 status

Figure 1  IC_{50} values of DDP in the different ovarian cancer cell lines. The upper part shows a graphic representation of the IC_{50} values according to the status of p53

IC_{50} values were calculated at 72 h of recovery after DDP treatment (24 h).

Northern blot analysis

Total RNA was extracted from untreated or DDP-treated cells (after 1, 6 or 24 h of treatment) using the guanidine/caesium chloride gradient method (Sambrook et al., 1989). After fractionation through 1% agarose–formaldehyde gels, RNA was blotted on nylon membranes and hybridized with WAF1, GADD45 and bax. Each c-DNA was ^32P labelled using a Rediprime kit (Amersham). Hybridizations were performed at 42°C for 16 h, followed by two washes at room temperature with 2 x standard saline citrate (SSC) (150 mm sodium chloride–15 mm sodium citrate) and one wash at 65°C in 2 x SSC–1% sodium dodecyl sulphate (SDS). GADD45 and WAF1 probes were obtained by PCR using primers deduced from the published sequence as described (Vikhanskaya et al., 1994).

Western blot analysis

Total cell extracts were prepared from untreated or DDP-treated cells, after 6 or 24 h of treatment, according to standard procedures. Twenty micrograms of proteins for each sample were electrophoresed through 12% SDS–polyacrylamide gels and transferred to nitrocellulose. Filters were hybridized with monoclonal antibody against p53 (DO-1, Santa Cruz Biotechnology) and were detected with the electrochemiluminescence (ECL) system after addition of anti-mouse IgG (Santa Cruz Biotechnology).

Evaluation of apoptosis

The staining with DAPI was used to detect apoptotic cells. Cells were seeded on glass coverslips in 24-well plates (25 000 cells ml^{-1}) and treated with DDP at the respective IC_{50}. After 24, 48 and 72 h, cells were fixed in 70% ethanol, air-dried and stained with DAPI and sulphorhodamine (Darzynkiewicz et al, 1992). After washes in phosphate-buffered saline (PBS), coverslips were mounted in Mowiol 4-88 (Hoechst, Frankfurt/Main, Germany) and observed in a Zeiss Axiopt photomicroscope equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany). Fluorescent images were recorded on Kodak films.

Filter binding assay

The method previously described (Bertrand et al, 1995) was used. Briefly, 5 x 10^6 cells prelabelled with 0.02 μCi ml^{-1}[^{14}C]thymidine in 3 ml of a solution containing 0.2% sodium sarcosyl, 2 M sodium chloride, 0.04 M EDTA pH 10.0. After washing with 5 ml of 0.02 M EDTA pH 10.0, radioactivity was measured in filters, loading fraction, wash lysis fraction and EDTA wash. DNA fragmentation was determined as the fraction of [^{14}C]-labelled DNA in the lysis fraction + EDTA wash relative to total intracellular [^{14}C]-labelled DNA. Results are expressed as the percentage of DNA fragmented in treated cells compared with the DNA fragmented in control untreated cells (background) using the formula: [(F–F0) / (1–F0)] x 100, where F and F0 represent DNA fragmentation in treated and control cells respectively.

RESULTS

Figure 1 reports the IC_{50} values calculated 72 h after 24-h exposure to DDP. No clear correlation between the differential expression of p53 and DDP-induced cytotoxicity was found. The figure also shows a graphical representation of the same data where the lack of correlation between cytotoxicity and p53 status is more evident. We also assessed the IC_{50} values of DDP in SKOV3-derived clones obtained upon transfection with the temperature-sensitive mutant murine p53 (clone SK23a) and again no differences could be found.

We then used for each cell line, the calculated IC_{50} for DDP to investigate the changes induced by the drug in the levels of p53 and p53-downstream genes (WAF1, GADD45 and bax).
Figure 2 reports the western blot results, which indicate that DDP treatment was able to induce a rise in p53 levels (measured after 6 and 24 h of treatment) in all the wt-p53-expressing cells. In addition an increase in p53 levels was also observed in the mut-p53-expressing OVCAR-3 cell line, while they were unaffected in all the other mut-p53-expressing cell lines.

We then evaluated if the increase in p53 levels observed in the wt-p53-expressing cell lines after DDP treatment resulted in an increase in mRNA levels of WAF1, GADD45 and bax. Figure 3 reports the northern analysis in the four wt-p53-expressing cell lines (Figure 3B) and in the five mut-p53-expressing cell lines (Figure 3A). The relative increase over controls of the three

| A | SW626 | OVCAR-3 | OVCAR-5 | OVCAR-8 | SKOV-3 |
|---|-------|---------|---------|---------|--------|
| Hours after DDP | 0 | 6 | 24 | 0 | 1 | 6 | 24 | 0 | 1 | 6 | 24 | 0 | 1 | 6 | 24 |
| WAF-1 | | | | | | | | | | | | | | | |
| GADD 45 | | | | | | | | | | | | | | | |
| Bax | | | | | | | | | | | | | | | |
| α-Actin | | | | | | | | | | | | | | | |

| B | IGROV-1 | A2780 | PA-1 | A2774 |
|---|---------|-------|------|-------|
| Hours after DDP | 0 | 1 | 6 | 24 | 0 | 1 | 6 | 24 | 0 | 1 | 6 | 24 |
| WAF-1 | | | | | | | | | | | | | |
| GADD 45 | | | | | | | | | | | | | |
| Bax | | | | | | | | | | | | | |
| α-Actin | | | | | | | | | | | | | |
mRNAs measured by densitometric scanning of the autoradiographs are reported in Figure 4.

As can be seen, in all the cell lines examined, DDP treatment was able to increase the levels of WAF1 mRNAs independently of the status of p53. GADD45 mRNA levels were also increased by DDP, essentially with similar profiles as those observed for WAF1; this was with the exception of PA-1 and SW626 cells, in which GADD45 levels were not increased. When tested by Western blotting, the levels of p21WAF1 were also found to be increased after DDP, in accordance with the mRNA levels (data not shown).

Bax mRNA levels were not increased to a similar extent and only minor changes could be observed. It is interesting to note that the most striking increase (relative to untreated cells) in bax mRNA levels after DDP has been found in the mut-p53-expressing cell line OVCAR-8.

We then analysed the WAF1, GADD45 and bax expression in clones derived from SKOv3 cells after transfection with a temperature-sensitive mutant murine p53. Figure 5 shows that when SK23a cells were incubated at 32°C there was an increase in the basal levels of WAF1 and GADD45 mRNAs as a result of the shift from the mutant to the wild-type form of p53 protein, while bax levels were almost unmodified. In these clones, DDP was able to induce an increase in WAF1 and GADD45 and only slightly in bax mRNAs. At 32°C, DDP was able to increase the levels of WAF1 and GADD45 in the mock-transfected SKN cells, while in SK23a cells, in which the basal levels of WAF1 and GADD45 were already increased by the presence of wt p53, DDP was unable to further increase these mRNA levels.

DDP-induced apoptosis was investigated with two different techniques. The DAPI staining was used to evaluate apoptosis in cells treated with the IC50 calculated for DDP in each cell line, while the filter-binding assay was used to evaluate DDP-induced apoptosis at three different concentrations (1, 10 and 100 μM) for all the cell lines examined.

Figure 6 shows representative pictures obtained after DAPI staining in the different cell lines after 72 h of treatment with DDP. The number of apoptotic cells was very low in the cells examined.
Similar results were obtained at earlier (24 and 48 h) times (data not shown). A more quantitative assay (Table 1) was used to evaluate the percentage of DNA fragmentation induced by DDP. As can be seen, DDP-induced apoptosis was again generally greater in wt-p53-expressing cells than in mut-p53-expressing cells but, comparing the data with the IC_{50} values reported in Figure 1, it appears evident that at doses close to the DDP IC_{50} the percentage of fragmentation was less than 20%, perhaps with the exception of the A2774 cell line in which, at 10 μM DDP (the calculated IC_{50} was 7.5 μM), the percentage of DNA fragmentation was 69% at 72 h.

**DISCUSSION**

We report here evidence that DDP-induced cytotoxicity in ovarian cancer cells in vitro is independent of the presence of a wt p53.

| Cell line | 24 h | 48 h | 72 h |
|-----------|------|------|------|
| OVCAR-3   | 1.5  | 0.5  | 0    |
| OVCAR-5   | 0    | 1.0  | 2.5  |
| OVCAR-8   | 0    | 0    | 2.0  |
| SW626     | 0    | 0    | 0    |
| SKOV-3    | 0    | 1.5  | 0    |
| A2774     | 0    | 1.0  | 2.0  |
| A2780     | 0.4  | 0    | 0.3  |
| PA-1      | 2.0  | 11.0 | 21.0 |
| IGROV-1   | 1.5  | 0    | 3.0  |
| SK23a (37°C) | 0.4 | 1.2  | 4.4  |
| SK23a (32°C) | 0.5 | 1.6  | 0    |
| SKN (37°C) | 0.7  | 0    | 0.7  |
| SKN (32°C) | 1.2  | 0    | 0.1  |

DDP concentration (μM)

| Cell line | 1   | 24 h | 48 h | 72 h |
|-----------|-----|------|------|------|
| OVCAR-3   | 2.0 | 9.5  | 24.0 | 7.0  |
| OVCAR-5   | 0   | 7.5  | 12.0 | 0    |
| OVCAR-8   | 0   | 4.5  | 4.5  | 27.0 |
| SW626     | 0   | 0    | 0.5  | 0    |
| SKOV-3    | 1.0 | 25.0 | 41.5 | 22.0 |
| A2774     | 0   | 22.0 | 69.0 | 39.4 |
| A2780     | 1.5 | 1.0  | 1.0  | 38.0 |
| PA-1      | 19.0| 76.0 | 35.0 | 75.0 |
| IGROV-1   | 0   | 0    | 7.0  | 1.5  |
| SK23a (37°C) | 5.2 | 26.3 | 38.0 | 33.4 |
| SK23a (32°C) | 0   | 3.5  | 8.1  | 2.5  |
| SKN (37°C) | 0.3 | 10.3 | 7.5  | 17.4 |
| SKN (32°C) | 0.8 | 0    | 1.1  | 1.2  |

DNA fragmentation calculated as described in Materials and methods after 24, 48 or 72 h of treatment.
independently of the presence of p53, ectopic overexpression of bax increases the sensitivity to anti-cancer drug treatment in vitro, and this was associated with an increased fraction of apoptotic cells after treatment (Sakakura et al, 1996; Wagener et al, 1996).

We have found an unexpected induction of bax, although at very low level, after DDP treatment in mut-p53-expressing cell lines. This is a new finding, which deserves further, specifically addressed experiments and which could support the existence of new DNA damage and p53-independent activation of bax.

In conclusion, our data show the independence of DDP-induced cytotoxicity from p53 status in ovarian cancer cell lines growing in vitro.

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