p53 Status Does Not Affect Sensitivity of Human Ovarian Cancer Cell Lines to Paclitaxel

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ABSTRACT

Nine human ovarian cancer cell lines that express wild-type (wt) or mutated p53 were used to evaluate the cytotoxicity induced by paclitaxel. The IC50 calculated in the five mutated p53-expressing cell lines was not different from the four wt p53-expressing cell lines. The introduction of wt p53, by using a temperature-sensitive mutant murine p53 or the human p53 under the control of a tetracycline-dependent promoter, did not change the cytotoxicity of paclitaxel as compared to mock-transfected cells. By using for each cell line the paclitaxel IC50, we found that these concentrations were sufficient to induce an increase in p53 levels in all of the four wt p53-expressing cells, whereas in the mutated p53-expressing cells, the levels were unaffected. This increase in p53 led to an increase in the mRNA and protein levels of p53 downstream genes (WAF1, GADD45, and bax). In none of the cell lines examined was paclitaxel able to induce apoptosis, evaluated by terminal deoxyribonucleotidyl transferase-mediated nick end labeling staining and filter binding assay at concentrations closed to the IC50. By increasing the concentration of paclitaxel in the filter binding assay, we could see fragmentation of DNA in the different cell lines.

We conclude that the presence of p53 is not a determinant for the cytotoxicity induced by paclitaxel in human ovarian cancer cell lines. Differences in the activation of p53 downstream genes could be observed in wt versus mutated p53-expressing cells, but this does not account either for a differential induction of apoptosis or for a change in cytotoxicity induced by paclitaxel.

INTRODUCTION

Paclitaxel is one of the most promising agents for the treatment of ovarian and breast cancer (1). In ovarian cancer, in particular, paclitaxel has been reported to be effective in patients refractory to standard chemotherapy (1, 2). The drug does not exert its antitumor activity by interacting with DNA but rather by binding tubulin and stabilizing microtubule formation; this results in a cell block of the cell cycle at the G2-M phase transition, thus preventing completion of mitosis (3). In some cell types, the paclitaxel-induced G2-M block resulted in activation of apoptosis (4—6).

A possible determinant of the activity of anticancer agents is the tumor suppressor protein p53. This protein has been reported to act as a guardian of the genome, and its levels rapidly increase after treatment of cells with DNA-damaging agents (7—9). The rise in p53 protein levels, which is primarily because of a stabilization of the protein itself, activates a cascade of genes that in turn bring about cell cycle arrest or apoptosis, depending on the cell type. For many anticancer agents, the presence of a wild-type p53 gene in tumors has been associated with an increase in drug activity compared with tumors harboring mutations in the p53 gene (7, 9). There are data, however, showing that expression of wild-type p53 protein allows more complete repair of DNA damage to occur by blocking cells either in the G1 phase or in the G2 phase of the cell cycle (10, 11).

It has been reported recently that in different cell lines in culture (HeLa and fibroblast-derived cell lines), the disruption of the wild-type p53 function resulted in an increased sensitivity of cells to paclitaxel treatment compared to cells expressing wild-type p53 (12). In an ovarian cancer cell line expressing wild-type p53, the disruption of p53 by transfection with the E6 protein of human papillomavirus type 16 led to a decrease in sensitivity to paclitaxel with a corresponding decrease in apoptosis induced by DNA-damaging agents (13). In another ovarian cancer cell line not expressing p53, the introduction of a wild-type p53 did not change the sensitivity of these cells to paclitaxel (14). In addition, paclitaxel has been reported to increase the levels of p53 in some cell types (15) but not in others (14).

In the present study, we report the cytotoxicity induced by paclitaxel in different ovarian cancer cell lines with a different p53 status. The cytotoxicity was studied in parallel with the changes in the protein levels of p53 as well as in the changes in mRNA and protein levels of the genes p21/WAF1, GADD45, and bax, which are directly activated by p53 and represent the downstream effector of the p53 gene. In addition, we evaluated the induction of apoptosis after treatment with paclitaxel.

MATERIALS AND METHODS

Cell Lines and Treatment. Nine human ovarian cancer cell lines (four expressing wild-type p53: PA-1, IGROV-1, A2780, and A2774, and five expressing no or mutated p53: OVCAR-3, OVCAR-5, OVCAR-8, SW626, and SKOV-3) were used. Cell lines were obtained from American Type Culture Collection (Rockville, MD) except for A2774, kindly obtained from Dr. Pommier (Instituto Nazionale per la Ricerca sul Cancro, Genova, Italy), and OVCAR-5, OVCAR-8, and IGROV-1, kindly obtained from Dr. Pommier (National Cancer Institute, Bethesda, MD). The cell lines were maintained in RPMI 1640 supplemented with 10% FCS.

Clones were obtained from the p53 null line SKOV3 upon transfection with the temperature-sensitive murine p53 (SK23a; Ref. 16) or with the human wild-type p53 under the control of a tetracycline-dependent promoter (SK74T). In both cases, cotransfection with plasmids containing the neomycin resistance gene was used to allow selection in G418 containing medium (500 μg/ml).

Cytotoxicity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay in 96-well plates (Nunc) at different times after treatment with different concentrations of paclitaxel (Taxol, obtained from Bristol-Meyers, dissolved in DMSO at a concentration of 500 μM and stored at −20°C). Concentrations inhibiting the growth by 50% (IC50) were calculated at 72 h of recovery in drug-free medium after paclitaxel treatment (24 h).

Northern Blot Analysis. Total RNA was extracted from untreated or paclitaxel-treated cells (after 6 or 24 h treatment) with the guanidine/cesium chloride gradient method (17). After fractionation through 1% agarose-formaldehyde gels, RNA was blotted on nylon membranes (GeneScreen Plus; DuPont) and hybridized with cDNAs encoding bax (kindly supplied by Dr. Kornmayer, St. Louis, MI), WAF1, and GADD45. Each cDNA was 32P-labeled using a Rediprime kit (Amersham, United Kingdom). Hybridizations were
performed in 50% formamide, 10% dextran sulfate, 1% SDS, and 1 M NaCl at 42°C for 16 h, followed by two 10-min washes at room temperature with 2× SSC (150 mM sodium chloride, 15 mM sodium citrate) and one wash for 30 min at 65°C in 2× SSC-1% SDS. GADD45 and WAF1 cDNAs were obtained by PCR, as described previously (16). Each filter was hybridized with α-actin cDNA to normalize for RNA loading.

Table 1 Paclitaxel IC_{50} in the different human ovarian cancer cell lines

<table>
<thead>
<tr>
<th>p53 status</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR-3 mut (248 R to Q)</td>
<td>4.0</td>
</tr>
<tr>
<td>OVCAR-5 mut (insertion 3 bp at 224)</td>
<td>6.0</td>
</tr>
<tr>
<td>OVCAR-8 mut (deletion 126—132)</td>
<td>7.0</td>
</tr>
<tr>
<td>SW626 mut (262 G to V)</td>
<td>3.5</td>
</tr>
<tr>
<td>SKOV-3 del</td>
<td>4.6</td>
</tr>
<tr>
<td>A2774 wt</td>
<td>6.3</td>
</tr>
<tr>
<td>A2780 wt</td>
<td>387</td>
</tr>
<tr>
<td>PAI wt</td>
<td>3.5</td>
</tr>
<tr>
<td>IGROV-1</td>
<td>8.2</td>
</tr>
<tr>
<td>SK4T + tetra</td>
<td>62</td>
</tr>
<tr>
<td>SK4 tetra wt</td>
<td>45</td>
</tr>
<tr>
<td>SK23a 37°C mut (135 C to V)</td>
<td>7.2</td>
</tr>
<tr>
<td>SK23a 32°C wt</td>
<td>9.2</td>
</tr>
<tr>
<td>SKN 37°C del</td>
<td>7.2</td>
</tr>
<tr>
<td>SKN 32°C del</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* The p53 status was determined for each cell line by PCR amplifications and sequencing of exons 5—8. In parentheses is reported the codon and the sequence changed for the mutated (mut) p53-expressing cell line. wt, wild type.

* Concentrations inhibiting by 50% the growth of the cells were calculated 72 h after cells were treated with paclitaxel for 24 h.

* Cells cultured in the presence (+) or absence (−) of 1 μg/ml of tetracycline.

* Calculated at 24 h.

Western blot analysis. Total cell extracts were prepared from untreated or paclitaxel-treated cells, after 6 or 24 h treatment, according to standard procedures (18). Twenty μg of proteins for each sample were electrophoresed through 12% polyacrylamide-SDS gels and electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in transfer buffer (50 mM Tris, 100 mM glycine, 0.01% SDS, and 20% methanol) for 2 h at 50 V. Filters were stained with Ponceau Red, hybridized with monoclonal antibody against p53 (clone DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), and detected with the enhanced chemiluminescence (ECL) system after the addition of antirabbit or antimouse IgG (Santa Cruz Biotechnology). The experiments were repeated in all of the cell lines at last twice.

Evaluation of Apoptosis. The filter binding assay method described previously (19) was used. Briefly, 5 × 10^5 cells prelabeled with 0.02 μCi/ml [14C]thymidine were loaded onto polyvinyl chloride filters, washed with PBS, and lysed with 5 ml of a solution containing 0.2% sodium sarcosyl, 2 M NaCl, and 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 fractions, washes, lysis fractions, and EDTA washes. DNA fragmentation was determined as the fraction of 14C-labeled DNA in the lysis fraction and in the EDTA wash relatively to total intracellular 14C-labeled DNA. Results are expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in control untreated cells (background). The formula [(F — F0)/(1 — F0)] times 100, where F and F0 represent the fraction of DNA fragmented in treated and control cells, respectively. Results obtained with the filter binding assay were confirmed using the TUNEL<sup>3</sup> method (20).

RESULTS

We first examined paclitaxel-induced cytotoxicity in the different human ovarian cancer cell lines. The calculated IC_{50} were reported in Table 1, together with the status of the p53 gene for the different ovarian cell lines.

Paclitaxel was active in all the cell lines except for one (A2780) at very low concentrations (IC_{50} of approximately 10 nm or below). The presence of mutations and/or deletions in the p53 gene did not affect the sensitivity to paclitaxel, which had comparable IC_{50} in all the cell lines.
lines examined. The only exception was represented by the wild-type p53 expressing cell line A2780 in which paclitaxel IC50 was 387 nM, much higher than that found in the other wild-type p53-expressing cell lines or mutated p53-expressing cell lines.

In addition, the introduction of wild-type p53, either by using a temperature-sensitive mutant p53 or a wild-type p53 gene under the control of tetracycline in SKOV3 cells (which do not express p53) did not result in a significant change in the IC50s (Fig. 1). In fact, in the clone SK23a which expresses mutated p53 at 37°C and wild-type p53 at 32°C, IC50s were 7.1 and 9.4, respectively, at the two temperatures. As shown in Table 1 and in data reported previously (14), the change in temperature from 37°C to 32°C of the mock-transfected clone SKN does not induce any change in the cytotoxicity induced by paclitaxel. The sensitivity to paclitaxel of the clone SK4T was similar in the presence of tetracycline (i.e., without expression of wild-type p53) or in the absence of tetracycline (i.e., with expression of wild-type p53), again confirming that paclitaxel-induced cytotoxicity is not related to p53 expression (note that these IC50s were calculated at 24 h instead of 72 h because the removal of tetracycline for a longer period resulted in complete arrest of the growth of control cells due to the continuous presence of a wild-type p53.

We analyzed the levels of p53 after 6 and 24 h treatment with paclitaxel by using the respective IC50s reported in Table 1 for each cell line. A typical experiment is reported in Fig. 2. Paclitaxel treatment caused an increase in the levels of p53 in cells expressing wild-type p53, although in IGROV-1 only a slight induction was observable. In mutated p53-expressing cell lines, p53 was not detectable in SKOV3 and OVCAR-5, whereas p53 was even reduced in OVCAR-8 and SW626.

The levels of mRNAs encoding WAF1, GADD45, and bax (three genes which have been previously shown to be inducible by wild-type p53) were analyzed by Northern blotting 1, 6, and 24 h after paclitaxel treatment using the same experimental conditions used for the Western blotting.

Fig. 3A shows the result obtained in the mutated (or null) p53-expressing cell lines SW626, OVCAR-3, OVCAR-5, OVCAR-8, and SKOV-3. In none of the cell lines was paclitaxel able to induce the mRNA levels of WAF-1 and bax genes, whereas we observed a slight increase in GADD45 expression 24 h after paclitaxel treatment in SW626. We also observed in the five cell lines a different basal expression of the three genes (compare, for example, GADD45 and bax expression in OVCAR-3 and SKOV-3 cell lines).

A different picture was obtained when p53-expressing cell lines were analyzed (Fig. 3B). WAF-1 mRNA expression was increased
over controls in all the cell lines examined, although some differences could be found (for example, in IGROV-1 WAF-1 mRNA, expression was increased as early as 1 h after treatment, without further increase, whereas in A2780, A2774, and PA-1, the maximum increase was found at 24 h). GADD45 mRNA expression was activated by paclitaxel in IGROV-1, A2774, and PA-1 and to a much lower extent in A2780. bax was only clearly increased over controls in PA-1; it was not induced in IGROV-1, where the basal mRNA level of this gene was almost undetectable, whereas in the A2780 and A2774 cell lines, only minor changes could be detected.

In the SK23a clone, the Northern analysis was performed at either 37°C (mutated p53) or at 32°C (wild-type p53). The results of the experiments, performed in parallel with a mock-transfected cell clone (SKN), are reported in Fig. 4. In these clonal cell lines, paclitaxel was unable to increase the levels of WAF-1, GADD45, and bax in all of the conditions tested. When SK23a cells were incubated at 32°C, there was an increase in the basal levels of WAF-1 and GADD45 mRNAs due to the shift from the mutant to the wild-type form of p53 protein, but also in these conditions, paclitaxel treatment did not further increase these levels.

We also analyzed paclitaxel-induced apoptosis after the same treatment conditions used for Northern and Western analysis. We used the the filter binding assay and the TUNEL staining method to evaluate the induction of apoptosis. Using the different paclitaxel doses for each of the cell lines, we could see evidence of DNA fragmentation using the filter binding assay (Table 2) only at 100 nM, whereas at 10 nM (i.e., concentrations much closer to the IC50), no significant apoptosis could be determined. The absence of significant apoptosis in the different cell lines was confirmed by TUNEL staining using the paclitaxel IC50,8 reported in Table 1 for each cell line (data not shown).

**DISCUSSION**

Paclitaxel is one of the most promising anticancer agents for the therapy of ovarian cancer, where it has shown activity also in tumors refractory to cisplatin treatment (1, 2). It is, therefore, important to understand if there are cellular factors that can play a role as determinant of the response of ovarian cancer cells to paclitaxel treatment. p53 is one of the proteins that plays a central role in the response to anticancer agent treatment (8, 21). It has, in fact, been shown that in different cell types, the presence of a wild-type p53 induces a sensitization to DNA-damaging agents (7, 9), although more recent evidence of a wild-type p53-induced chemoresistance has been described (10, 11). Paclitaxel, which does not interact directly with DNA, was found to be able to activate p53 in some cell types, and this increase has been associated mainly with its ability to activate the raf-1 cascade (15, 22, 23). In other cell types, including one human ovarian cancer cell line, p53 did not increase after paclitaxel treatment (14), and the presence of a wild-type p53 did not result in change in sensitivity to paclitaxel in respect to cells expressing mutated p53. Recently, the presence of wild-type p53 has been reported to decrease the cytotoxicity of paclitaxel (compared to the same cell lines not expressing wild-type p53), and this was explained with a p53-dependent block in G1 after treatment that would prevent the cells from getting to G2-M, where paclitaxel is known to exert its activity (12). In that work, however, no ovarian tumors were used. Another report, however, showed that in a human ovarian cancer cell line, the disruption of wild-type p53 did reduce the cytotoxicity induced by paclitaxel (13).

We here report that the presence of wild-type p53 does not change the sensitivity to paclitaxel treatment by examining five mutant p53 and four wild-type p53-expressing human ovarian cancer cell lines. In addition, in clones obtained by introduction of a wild-type p53 (from cells not expressing p53), no differences could be observed in paclitaxel-induced cytotoxicity.

An increase in the levels of p53 after paclitaxel treatment and consequently an activation of the transcription of downstream genes such as WAF1, GADD45, and bax could be observed in wild-type conditions.
p53-expressing cells. This increase, however, did not lead to a change in cytotoxicity in respect to cells (expressing mutated p53) in which paclitaxel treatment did not increase the levels of p53 downstream genes. It is also interesting to note that the rise in p53 levels observed in wild-type p53-expressing cells after paclitaxel treatment led to a different kinetic of induction of the three examined genes, transcriptionally inducible by p53, in the different cell lines. In fact, for example, in A2780 a clear induction of WAF-1 was found, with GADD45 and bax only slightly affected, whereas in PA-1, all of the three genes were clearly induced.

In SKOV-3 cells transfected with wild-type p53 (SK23a), we could not see an increase in p53 downstream genes after paclitaxel treatment, in agreement with data published previously (14). It could be that the basal, high level of WAF-1 and GADD45 determined by the presence of wild-type p53 is already high enough that paclitaxel treatment cannot induce a further increase. In the same system, however, other drugs such as cisplatin and doxorubicin were able to further increase WAF-1 and GADD45 mRNA (14, 24). We also did not observe apoptosis in all of the cell lines examined independently of the presence of wild-type p53 at different times after paclitaxel treatment, even if in other cell types paclitaxel was reported to activate apoptosis (5, 23, 25–27).

In conclusion, our data show that in human ovarian cancer cell lines, paclitaxel-induced cytotoxicity is independent of the presence of a wild-type p53 and that this is not due to the inability of paclitaxel to induce a functional p53 in wild-type p53-expressing cells since a clear induction of p53 downstream genes after paclitaxel treatment could be observed. The lack of induction of apoptosis in all the cell lines used (independently on the presence of p53) could explain the similar cytotoxicity observed in the different ovarian cancer cell lines. These would imply that, at least in ovarian cancer cell lines, p53 can be a determinant of the cellular response to anticancer agents only when programmed cell death can be activated.

REFERENCES

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