Overexpression of p21\textsuperscript{waf1} Decreases G\textsubscript{2}-M Arrest and Apoptosis Induced by Paclitaxel in Human Sarcoma Cells Lacking Both p53 and Functional Rb Protein

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ABSTRACT

We examined the effect of overexpression of p21\textsuperscript{waf1} on cytotoxicity of paclitaxel, a microtubule stabilizer, using a tetracycline-inducible expression system in human sarcoma cells (SaOs-2) that lack both functional retinoblastoma protein and p53. Under normal growth conditions, p21\textsuperscript{waf1} is not detectable in SaOs-2 cells. Upon p21\textsuperscript{waf1} induction by tetracycline withdrawal, we observed a reduced apoptotic response to paclitaxel with a 3- to 6-fold increase in IC\textsubscript{50} values compared with that of cells not induced by p21\textsuperscript{waf1}. We also observed a 5-fold increase in the IC\textsubscript{50} value when cytotoxicity to vincristine, another microtubule-disrupting agent, was assessed, whereas we observed a marked decrease in the IC\textsubscript{50} value after p21\textsuperscript{waf1} induction in response to etoposide, a topoisomerase II inhibitor. After treatment with paclitaxel, less accumulation of G\textsubscript{2}-M was observed in p21\textsuperscript{waf1}-induced cells compared with non-p21\textsuperscript{waf1}-induced cells (57% versus 74%). p21 \textsuperscript{waf1} induction also inhibited the increased cyclin B1-associated kinase activity induced by paclitaxel. Overexpression of p21\textsuperscript{waf1} in SaOs-2 cells lacking both p53 and functional retinoblastoma protein may decrease the G\textsubscript{2}-M arrest induced by paclitaxel due to suppression of the S-G\textsubscript{2} checkpoint, resulting in a decreased apoptotic response of cells to paclitaxel.

Cell cycle checkpoints are considered to be essential in controlling the ordered progression of cell cycle and DNA repair (Sherr, 1996; Paulovich et al., 1997). Alterations in expression of genes that control progression through the cell cycle have been demonstrated to affect chemosensitivity (Kohn et al., 1994). The most well studied alterations (and the most likely to be important) in cancer cells occur in genes encoding p53 and retinoblastoma protein (pRb). In particular, the absence of or presence of mutated forms of p53 and/or pRb have been shown to be associated with increased resistance of tumor cells to various anticancer drugs and irradiation, mainly because of disruption of cell cycle checkpoints (Lowe et al., 1993; Li et al., 1995). Alterations of other cell cycle regulators, such as E2F-1, cyclin/Cdkks (cyclin D1/Cdk4, cyclin A/Cdk2), and cyclin-dependent kinases (Cdk) inhibitors (p16, p21\textsuperscript{waf1}, and p27) may also play an important role in regulation of drug sensitivity (Loan et al., 1995; Hochhauser et al., 1996; St. Croix et al., 1996; Stone et al., 1996; Li et al., 1997).

p53-dependent and -independent expression of p21\textsuperscript{waf1} may result in a universal inhibition of cyclin-dependent kinase activities (Xiong et al., 1993). In the presence of functional pRb, p21\textsuperscript{waf1} may inhibit cyclin D/Cdk4 and cyclin E/Cdk2, thereby increasing hypophosphorylated pRb, decreasing free E2F, and predominantly arresting cells in the G\textsubscript{1}-S-phase transition. By regulating the G\textsubscript{1} checkpoint, p21\textsuperscript{waf1} may decrease the sensitivity of cells to S-phase-specific drugs, such as doxorubicin (Waldman et al., 1996), whereas deficiency of p21\textsuperscript{waf1} may result in a defective G\textsubscript{1} checkpoint control (Deng et al., 1995) and an increased apoptotic response of cells to such DNA-damaging agents as cisplatin (CDDP) and nitrogen mustard (Fan et al., 1997). In contrast, in the absence of pRb, p21\textsuperscript{waf1} overexpression leads to S-phase retardation (Ogryzko et al., 1997) and increased sensitivity to such S-phase-specific drugs as methotrexate and doxorubicin in sarcoma cells, which may be a result of decreased phosphorylation of E2F-1 and consequent S-G\textsubscript{2} phase arrest (Li et al., 1997).

The p53 status seems not to affect the sensitivity of tumor cells to M-phase-specific drugs such as paclitaxel, a microtubule stabilizer (O’Connor et al., 1997); in fact, tumor cells lacking normal p53 function may be more sensitive to paclitaxel (Wahl et al., 1996). However, it is still to be established whether and how p21\textsuperscript{waf1} affects sensitivity of M-phase-specific drugs with regard to the status of p53 and pRb. Two recent studies show that in colon cancer cells containing both


**Materials and Methods**

**Chemicals**

Plasmid TC-p21 \(^{\text{waf1}}\) contains the full-length cDNA of p21waf1 (a gift from Dr. David Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) cloned into the pUHD10-3-Tc site of the expression vector pUHD10-3. The tetracycline (TC)-responsive system, which is composed of two plasmids (pUHD10-3-TC-p21 \(^{\text{waf1}}\) and pUHD 15-1 neo), was used to construct the inducible vectors for the controlled expression of p21 \(^{\text{waf1}}\). SaOs-2 cells were transfected with the TC-responsive system containing the above two plasmids (pUHD10-3-TC-p21 \(^{\text{waf1}}\), Bel-2, Bel-X \(_L\), Bax, cyclin B, and Cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Histone H1 was purchased from Boehringer Mannheim (Indianapolis, IN).

**Cell Culture**

SaOs-2, an osteosarcoma cell line that lacks both p53 and functional Rb protein (Masuda et al., 1987; Shew et al., 1990), was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained as monolayer cultures in RPMI 1640 medium containing 10% fetal bovine serum.

**Plasmids and Expression of p21 \(^{\text{waf1}}\)**

Plasmids pUHD 10-3 and pUHD 15-1 were gifts from Dr. Herman Bujard (Zentrum für Molekulare Biologie, Universität Heidelberg, Germany) (Gossen and Bujard, 1992). Plasmid TC-p21 \(^{\text{waf1}}\) contains full-length cDNA of p21waf1 (a gift from Dr. David Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) cloned into the XhoI site of the expression vector pUHD10-3. The tetracycline (TC)-responsive system, which is composed of two plasmids (pUHD10-3-TC-p21 \(^{\text{waf1}}\) and pUHD 15-1 neo), was used to construct the inducible vectors for the controlled expression of p21 \(^{\text{waf1}}\). SaOs-2 cells were transfected with the TC-responsive system containing the above two plasmids by lipofection (Gossen and Bujard, 1992). Cells were then transfected with the TC-responsive system containing the above two plasmids by lipofection (Gossen and Bujard, 1992). Cells were then transfected with the TC-responsive system containing the above two plasmids by lipofection (Gossen and Bujard, 1992).

**Cytotoxicity Assay**

Cytotoxicity of drugs was determined by the sulforhodamine B (SRB) assay in 96-well microtiter plates as described previously (Skehan et al., 1990). Cells were plated in duplicate wells (2000 cells/well) and exposed to paclitaxel, CDDP, and VP-16 at concentrations of 1.0 nM to 10 \(\mu\)M in the absence or presence of TC for 24 h. After washing and incubating in drug-free medium for an additional 96 h, cells were then fixed with 50% trichloroacetic acid solution for 1 h, 0.4% SRB (Sigma) was added to each well. The mixture was then incubated for 30 min. After washing with 1% acetic acid, plates were read at 570 nm on a BioWhittaker microplate reader 2001 (BioWhittaker, Walkersville, MD). The wells with cells containing no drugs and with medium plus drug but no cells were used as controls.

**Western Blot Analysis**

Cell extract (100 \(\mu\)g), prepared as described previously (Li et al., 1997), was electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were probed with various primary antibodies using standard techniques (Sambrook et al., 1989) and protein expression was detected using enhanced chemiluminescence detection reagents (Amersham, Buckinghamshire, UK).

**Results**

**Overexpression of p21 \(^{\text{waf1}}\) Increases Resistance to Paclitaxel.** As shown in our previous study, p21 \(^{\text{waf1}}\) expression was barely detectable in SaOs-2/p6 cells incubated in the presence of TC (1 \(\mu\)g/ml), whereas high levels of p21 \(^{\text{waf1}}\) were expressed in these cells 24 h after TC withdrawal (Li et al., 1997). SaOs-2/p6 cells, both p21 \(^{\text{waf1}}\)-induced and non-p21 \(^{\text{waf1}}\)-induced, were treated with paclitaxel for 24 h. Cells were more resistant to paclitaxel when p21waf1 was induced than when p21waf1 was not induced (Fig. 1).
more resistant to VCR, an M-phase-specific drug, but more sensitive to VP-16, an S-phase-specific agent (Table 1). No difference in sensitivity to CDDP was observed in p21waf1-induced and non-p21waf1-induced cells (Table 1).

Increased Resistance to Paclitaxel Is Linked to Reduced Apoptosis Induced by Paclitaxel. Cells were exposed to paclitaxel for either 24 h or 24 h followed by an additional 48-h incubation in drug-free medium in the presence or absence of TC; apoptosis was then measured. As shown in Fig. 2A, DNA fragmentation was not observed in either p21waf1-induced or non-p21waf1-induced cells without drug treatment. DNA fragmentation was also not significant in cells exposed to paclitaxel after 24 h. However, after the 24-h exposure to paclitaxel followed by the 48-h washout, p21waf1-induced cells showed decreased DNA laddering compared with non-p21waf1-induced cells. The FACS assay also showed that percentage of apoptotic cells (sub-G1) after treatment with paclitaxel (48-h washout) was reduced in p21waf1-induced cells (24.5%) compared with non-p21waf1-induced cells (41.5%) (Fig. 2B).

Induction of p21waf1 Decreases G2-M Accumulation Induced by Paclitaxel. Paclitaxel has been demonstrated to be able to block cells in the G2-M-phase, and apoptosis induced by paclitaxel is most probably caused by a G2-M arrest-triggered disruption of mitotic spindles (Bhalla et al., 1993). Therefore, we examined cell cycle progression in p21waf1-induced and non-p21waf1-induced cells in the presence of paclitaxel. After incubation with paclitaxel for 24 h, non-p21waf1-induced cells exhibited an accumulation of G2-M-phase cells (59.1 ± 1.2%). In contrast, a reduction of G2-M-phase cells was observed in p21-induced cells (44.0 ± 1.3%, p < .01). After a 24-h incubation with paclitaxel followed by a 48-h washout, more cells were arrested at G2-M-phase under both p21waf1-induced and non-p21waf1-induced conditions. However, there were still significantly fewer G2-M-phase cells in p21waf1-induced cells than in non-p21waf1-induced cells (57.1 ± 5.5% versus 73.9 ± 6.0%). Without paclitaxel treatment, p21waf1 induction did not seem to increase significantly the accumulation of G2-M cells, even when cells were cultured for 72 h. A representative result is shown in Fig. 3.

p21waf1 Overexpression Reduces Cyclin B1/Cdc2 Kinase Activity after Treatment with Paclitaxel. Because cyclin B1-Cdc2 is a major cyclin kinase in controlling the G2-M transition and reduction of M-phase entrance by DNA damage is associated with the relative inactivation of cyclin B1/Cdc2 complexes (Solomon, 1993; Ling et al., 1996), we examined the expression of cyclin B1 and Cdc2 and their kinase activity after treatment with paclitaxel under p21waf1-induced and non-p21waf1-induced conditions. As shown in Fig. 4, expression of cyclin B1 and Cdc2 was not significantly changed when p21waf1 was induced. However, after p21waf1-induced cells were treated with paclitaxel, expression of cyclin B1 was reduced, whereas the level of Cdc2 was still not significantly changed (Fig. 4A). Cyclin B1-associated histone H1 kinase activity measured was also not affected by p21waf1 induction. Paclitaxel treatment seemed to increase the cyclin kinase activity, however, p21waf1 induction significantly inhibited the increased cyclin kinase activity induced by paclitaxel. Even more significant inhibition of this cyclin kinase activity by p21waf1 was observed in cells treated with paclitaxel for 24 h followed by an additional 48-h incubation in drug-free medium and then tested for this enzyme activity. Total Cdc2 kinase activity was also reduced in p21waf1-induced cells after paclitaxel treatment, but not to the same degree as cyclin B1-associated kinase activity (Fig. 4B).

Discussion

In a previous study, we showed that overexpression of p21waf1 in SaOs-2 cells lacking both p53 and functional pRb leads to increased inhibition of E2F-1 phosphorylation by inhibiting cyclin A/Cdk2, thereby resulting in S-G2 arrest and increased sensitivity to S-phase-specific drugs (Li et al., 1997). In this study, we- show that sensitivity to G2-M phase-specific drugs (i.e., paclitaxel and vincristine) is reduced by p21waf1-mediated cell-cycle change in these sarcoma cells. These results are consistent with those reported by O’Connor et al. (1997), who observed that high p21waf1 expression

![Figure 1](image-url) Effect of p21waf1 overexpression on resistance of SaOe-2/p6 cells to paclitaxel. Cells were exposed to different concentrations of paclitaxel for 24 h in the presence or absence of 1 μg/ml TC, washed, and then incubated in drug-free medium for an additional 96 h. Cell growth inhibition was then determined by using the SRB assay. Shown are means ± S.E. of three different experiments. ○, TC +; ■, TC –.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Paclitaxel</th>
<th>VCR</th>
<th>VP-16</th>
<th>CDDP</th>
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<tr>
<td>TC+</td>
<td>30.2 ± 4.4</td>
<td>17.9 ± 3.8</td>
<td>384.4 ± 18.5</td>
<td>1707.8 ± 206</td>
</tr>
<tr>
<td>TC−</td>
<td>205.6 ± 19.5</td>
<td>95.0 ± 12.2</td>
<td>15.7 ± 2.8</td>
<td>2375.0 ± 306</td>
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TABLE 1 Effect of p21waf1 expression on the inhibitory effect of paclitaxel, VCR, VP-16, and CDDP in SaOs-2/p6 cells

Cells were exposed to different concentrations of drugs for 24 h, washed, and incubated in drug-free medium for an additional 96 h. Cell growth inhibition was then determined by using the SRB assay. See Materials and Methods for details. Mean ± S.E. of three different experiments.
tended to increase sensitivity to S-phase-specific drugs but not to M-phase-specific drugs in 60 human tumor cell lines.

Increased paclitaxel resistance in p21<sup>waf1</sup>-induced sarcoma cells as measured by SRB assay was associated with a decreased apoptotic response of cells to paclitaxel, demonstrated by DNA fragmentation assay and accumulation of sub-G<sub>1</sub> cells by FACS measurement. The relationship of expression of tumor suppression genes and oncogenes to paclitaxel-induced apoptosis has been studied extensively in recent years (Haldar et al., 1996; Strobel et al., 1996; Frankel et al., 1997; Ibrado et al., 1997; Lanni et al., 1997). Paclitaxel-triggered apoptosis seems to be p53-independent (O’Connor et al., 1997). An increased apoptotic response to paclitaxel was also observed in cells containing a high level of Bcl-2, which was attributed to paclitaxel-induced Bcl-2 phosphorylation (Haldar et al., 1996). Bax expression also enhances paclitaxel-induced apoptosis (Strobel et al., 1996). In contrast, Bcl-X<sub>L</sub> overexpression leads to decreased paclitaxel-induced apoptosis (Ibrado et al., 1997). We did not observe a signif-

Fig. 3. Effect of p21<sup>waf1</sup> expression on cell cycle arrest in SaOs-2/p6 cells treated with paclitaxel (0.5 μM). Cells were incubated in complete medium with or without TC for 24 h and then treated with paclitaxel and subjected to FACS analysis. A, control; untreated cells. B, 24-h exposure to paclitaxel. C, cells were exposed to paclitaxel for 24 h, washed, and reincubated in drug-free medium for additional 48 h.

Fig. 2. Induction of apoptosis by paclitaxel in p21<sup>waf1</sup>-induced and non-p21<sup>waf1</sup>-induced cells. A, DNA fragmentation in 0.8% agarose gel. M, marker; C, control, untreated cells; T1, 24-h exposure to paclitaxel (0.5 μM); T2, cells were exposed to paclitaxel (0.5 μM) for 24 h, washed, and reincubated in drug-free medium for an additional 48 h. B, representation of FACS display of cells with a DNA content less than that of G1 cells. □, TC +; ▪, TC −.
Fig. 4. Expression of cyclin B1 and cdc2 and cyclin B1/cdc2-associated histone H1 activity in p21<sup>waf1</sup>-induced and non-p21<sup>waf1</sup>-induced cells. Cells were incubated in media with or without TC for 24 h. Whole-cell lysates were extracted as described in Materials and Methods. A, expression of cyclin B1 and cdc2. Cell extracts were separated on SDS/PAGE and levels of the proteins indicated were detected with anticyclin B1 and cdc2 antibodies. B, cyclin B1 and cdc2-associated histone H1 activity. Cell lysates were immunoprecipitated with anticyclin B1 or cdc2 antibody. Precipitates were subjected to 10% SDS/PAGE and autoradiography. C, control; T1, 24-h exposure to paclitaxel (0.5 μM); C, control; T2, cells were exposed to paclitaxel for 24 h, washed, and reincubated in drug-free medium for an additional 48 h.

Marked difference of Bcl-X<sub>L</sub> and bax expression between p21<sup>waf1</sup>-induced and non-p21<sup>waf1</sup>-induced cells even after treatment with paclitaxel (data not shown). Bcl-2 expression was barely detectable in these cells before or after p21<sup>waf1</sup> induction. Therefore, increased resistance to paclitaxel-induced apoptosis in p21<sup>waf1</sup>-induced cells did not seem to be attributable to changes in the levels of these proteins.

Paclitaxel-induced apoptosis has been directly linked to G<sub>2</sub>-M arrest. Tumor cells arrested in G<sub>2</sub>-M underwent apoptosis in the presence of paclitaxel (Bhalla et al., 1993). In G<sub>2</sub>-M, paclitaxel results in microtubule disorganization, which may trigger apoptotic cell death by as yet unclear mechanisms. Therefore, it is not surprising that a decrease in the percentage of cells arrested at the G<sub>2</sub>-M checkpoint may also reduce paclitaxel-induced apoptosis. As shown in this study, paclitaxel-induced G<sub>2</sub>-M accumulation in these sarcoma cells was reduced by overexpression of p21<sup>waf1</sup>, whereas a larger fraction of p21<sup>waf1</sup>-induced cells remained in S-phase after treatment with paclitaxel. Recently, Niculescu et al. (1998) reported that p21<sup>waf1</sup> induction may cause DNA endoreduplication in pRB-negative cells. It is worthwhile exploring whether paclitaxel treatment in these cells further increases DNA endoreduplication under both p21<sup>waf1</sup>-induced and non-p21<sup>waf1</sup>-induced conditions.

Activation of cyclin B1/Cdc2 kinase has been demonstrated to trigger transition of cells from S-phase to G<sub>2</sub>-M-phase and is capable of increasing paclitaxel-induced apoptosis (Donaldson et al., 1994). Activation of this complex is mainly caused by binding to Cdc25 phosphatase, which dephosphorylates Cdc2 phosphotyrosine 15 and phosphothreonine 14 residues in Cdc2 (Solomon et al., 1993). During S-phase, Cdc2 kinase is inactive because of phosphorylation at these two amino acids, and cyclin B1 protein expression is also suppressed (Shimizu et al., 1995; Maity et al., 1996). Therefore, a decreased cyclin B1/Cdc2 kinase activity would contribute to a decrease in G<sub>2</sub>-M transition and paclitaxel-induced apoptosis. An S-phase block or delay by p21<sup>waf1</sup> would reduce both cyclin B1 expression and cyclin B1/Cdc2 kinase activity. The reduced cyclin B1 expression and activation of cyclin B1/Cdc2 complex in p21<sup>waf1</sup>-induced cells observed after paclitaxel treatment may result in reduced G<sub>2</sub>-M transition. Other studies also demonstrated that drug-induced decrease in G<sub>2</sub>-M accumulation was correlated with delayed cyclin B1 expression or decreased cyclin B1/cdc2 kinase activity (Shimizu et al., 1995; Maity et al., 1996).

Because mutations of p53 and inactivation of pRB are commonly found together in human tumors and because paclitaxel-induced apoptosis is mainly p53-independent, the level of p21<sup>waf1</sup> expression may play an important role in determining sensitivity of tumor cells to paclitaxel. Determination of p21<sup>waf1</sup> expression may help to determine anticancer therapeutic strategy in tumors lacking both p53 and pRB. For example, paclitaxel may be more effective in the tumors that lack p21<sup>waf1</sup> expression. In contrast, other drugs, such as VP-16, may be more selective in the tumors containing high levels of p21<sup>waf1</sup> (Li et al., 1997).

As this article was being revised, Yu et al. (1998) reported that up-regulation of p21 contributes to resistance to paclitaxel-induced apoptosis by inhibiting p34<sup>cdc2</sup> kinase in p18<sup>erbB2</sup>-overexpressing breast cancer cells (Yu et al., 1998), which supports our results obtained in sarcoma cells.

**References**


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