Journal of Cellular Biochemistry 98:1645–1652 (2006)

$p27^{kip1}$ Overexpression Promotes Paclitaxel-Induced Apoptosis in pRb-Defective SaOs-2 Cells

Chiara Gabellini,[1]* Bruna Pucci,[2] Paola Valdivieso,[3] Giuseppina D'Andrilli,[4] Marco Tafani,[5] Antonio De Luca,[6] and Valeria Masciullo[4]**

[1]Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Rome, Italy
[2]Department of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, Rome, Italy
[3]Centro di Ricerca e Formazione ad Alta Tecnologia delle Scienze Biomediche, Catholic University of Campobasso, Italy
[4]Sbarro Institute for Cancer Research, Center for Biotechnology, Temple University, Philadelphia, Pennsylvania
[5]Department of Experimental Medicine and Pathology, La Sapienza University, Rome, Italy
[6]Department of Medicine and Public Health, Section of Clinical Anatomy, Second University of Naples, Naples, Italy

Abstract $p27^{kip1}$ is a cyclin-dependent kinase (CDK) inhibitor, which controls several cellular processes in strict collaboration with pRb. We evaluated the role of $p27^{kip1}$ in paclitaxel-induced apoptosis in the pRb-defective SaOs-2 cells. Following 48 h of exposure of SaOs-2 cells to 100 nM paclitaxel, we observed an increase in $p27^{kip1}$ expression caused by the decrease of the ubiquitin-proteasome activity. Such increase was not observed in SaOs-2 cells treated with the caspase inhibitors Z-VAD-FMK, suggesting that $p27^{kip1}$ enhancement at 48 h is strictly related to apoptosis. Finally, we demonstrated that SaOs-2 cells transiently overexpressing the $p27^{kip1}$ protein are more susceptible to paclitaxel-induced apoptosis than SaOs-2 cells transiently transfected with the empty vector. Indeed, after 48 h of paclitaxel treatment, 41.8% of SaOs-2 cells transiently transfected with a pcDNA3-$p27^{kip1}$ construct were Annexin V-positive compared to 30.6% of SaOs-2 cells transfected with the empty vector ($P < 0.05$). In conclusion, we demonstrated that transfection of the pRb-defective SaOs-2 cells with the $p27^{kip1}$ gene via plasmid increases their susceptibility to paclitaxel-induced apoptosis. The promoting effect of $p27^{kip1}$ overexpression on apoptosis makes $p27^{kip1}$ and proteasomal inhibitors interesting tools for therapy in patients with pRb-defective cancers. J. Cell. Biochem. 98: 1645–1652, 2006.

Key words: $p27^{kip1}$; apoptosis; paclitaxel; Osteosarcoma

Apoptosis is an active process of programmed cell death that is preserved throughout evolution [Vaux and Korsmeyer, 1999]. The cell cycle is the highly conserved innate mechanism by which eukaryotic cells replicate themselves. In metazoans the processes of cell loss and cell gain are homeostatically balanced in order to maintain the normal development and architecture of tissues. Accumulating evidence shows that the manipulation of the cell cycle may either prevent or induce an apoptotic response [Pucci et al., 2000]. This ability has been recognized for several cell cycle proteins such as p53 and the retinoblastoma family members, pRb/p105, p107, and pRb2/p130 [Morgenbesser et al., 1994; Paggi et al., 1996; Pucci et al., 2002; Tonini et al., 2004]. Data concerning the role of cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors (CKIs) also support a direct coupling between the proliferating machinery and the apoptotic process [Kasten and Giordano, 1998].

Abbreviations used: PTX, paclitaxel.

Grant sponsor: Ministero della Sanità; Grant sponsor: FIRB-MIUR n.RBNE01PZER.

*Correspondence to: Chiara Gabellini, Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro, 156 00158 Rome, Italy.
E-mail: gabellini@ifo.it.

**Correspondence to: Valeria Masciullo, Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, BioLife Science Bldg., Suite 333, 1900 N 12th Street Philadelphia, PA 19122. E-mail: masciull@temple.edu.

Received 14 December 2005; Accepted 18 January 2006
DOI 10.1002/jcb.20900

Growing evidence suggests that apoptosis frequently occurs in cells in the G_1 phase of the cell cycle [Meikrantz and Schlegel, 1995], thus CKIs expressed during late G_1 like $p27^{kip1}$ are logical targets for the apoptotic process. The cyclin-dependent kinase inhibitor $p27^{kip1}$ is an important negative regulator in cell cycle identified as a 27 kDa protein that binds to and inhibits the activity of the cyclin E-CDK2 complexes in cells treated with transforming growth factor TGF-β and is regulated by growth inhibitory cytokines and by contact inhibition [Koff et al., 1993; Hengst et al., 1994; Slingerl et al., 1994; Polyak et al., 1994a,b].

In several cellular systems $p27^{kip1}$ is regulated primarily post-transcriptionally by the ubiquitin-proteasome pathway and phosphorylation of $p27^{kip1}$ at Thr187 by cyclin E-CDK2 is required to initiate the ubiquitination-proteasomal degradation [Sheaff et al., 1997; Montagnoli et al., 1999].

Although $p27^{kip1}$ has been shown to prevent drug-induced apoptosis in carcinoma cells [St Croix et al., 1996], other studies have pointed toward its pro-apoptotic role. Recombinant adenovirus overexpressing $p27^{kip1}$ triggers apoptosis in several cancer cell lines [Craig et al., 1997; Katayose et al., 1997] and cell death induction by $p27^{kip1}$ overexpression is mediated by the presence of pRb in lung cancer cell lines [Naruse et al., 2000]. Moreover, it has been recently shown that the adenoviral transduction of a proteolysis-resistant form of $p27^{kip1}$ is able to increase apoptosis in breast cancer cells [Zhang et al., 2005].

Paclitaxel is an antimitotic agent [Kumar, 1981] that inhibits the formation of mitotic spindle [Schiff and Horwitz, 1980], thus causing G_2/M arrest as well as cellular toxicity via apoptosis [Roberts et al., 1990; Bhalla et al., 1993]. Its cytotoxic effects have been shown in several tumor cell lines [Wani et al., 1971; Douros and Suffness, 1978; Pucci et al., 1999], in a variety of murine tumors, and human xenografts [Riondel et al., 1986, 1988], as well as in advanced human carcinomas resistant to traditional chemotherapy [Rowinsky and Donehower, 1995]. However, the underlying mechanism by which paclitaxel induces cell death remains poorly understood. Among apoptotic factors, few of the Bcl-2 family proteins have been shown to be involved in paclitaxel-induced apoptosis [Ferlini et al., 2003]. For example, Bcl-2 is phosphorylated and consequently inactivated by Raf-1 during paclitaxel apoptosis [Ling et al., 1998]. Overexpression of another anti-apoptotic protein, Bcl-x_L, confers resistance to paclitaxel-induced apoptosis in HL-60 cells. On the other hand, the overexpression of the pro-apoptotic proteins Bax and Bcl-x_S respectively sensitize SW626 and MCF7 cells to paclitaxel treatment [Sumantran et al., 1995; Strobel et al., 1996; Wang et al., 2000]. Several cell cycle regulators also appear to be involved in paclitaxel-induced cell death. Among those, p34cdc2 is activated during paclitaxel-induced apoptosis and is responsible for G_2/M arrest and cell death [Yu et al., 1998]. Overexpression of the CKIs $p21^{cip1}$ or $p27^{kip1}$ produces a differential resistance to paclitaxel in RKO colon carcinoma cells, causing a G_1 but mostly G_2 arrest, which prevents cells from entering M phase and becoming paclitaxel-susceptible [Schmidt et al., 2000]. When transferred from monolayer to three-dimensional culture, A2780 and CAOV3 ovarian cancer cell lines show an increase of resistance to paclitaxel treatment that correlates with the upregulation of $p27^{kip1}$ and multidrug resistance P-glycoprotein expression [Xing et al., 2005].

However, induction of $p27^{kip1}$ conferred resistance to paclitaxel in Rb-positive but not Rb nonfunctional adenocarcinoma cell lines [Schmidt and Fan, 2001].

In the present study, we showed that $p27^{kip1}$ overexpression increases paclitaxel-induced apoptotic rate in the pRb defective SaOs-2 osteosarcoma cell line.

MATERIALS AND METHODS

Cell Culture

SaOs-2 (p53 and pRb-null) human osteosarcoma cell line was purchased from ATCC (Rockville, MD), and grown in monolayer cultures in Dulbecco's modified Eagle's medium D-MEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone), 2 mM L-glutamin (GIBCO, Invitrogen) and gentamicin 50 μg/ml (GIBCO, Invitrogen), at 37°C in a humidified atmosphere 5% CO_2 and 95% air.

Treatments

Paclitaxel stock solutions (10 mM) were dissolved in dimethyl sulfoxide (DMSO) and protected from light. Drug dilutions were obtained at the appropriate concentration with

medium and were freshly prepared before each experiment. Broad-range caspase inhibitor Z-VAD-FMK (Bachem Bioscience, Inc., Philadelphia, PA) was prepared from 100 mM in DMSO stock solution. Twenty-four hours after plating, cells were treated with 100 nM paclitaxel from 16 to 48 h in the presence 100 μM of the caspase inhibitor. Z-VAD-FMK was added fresh daily to prevent its degradation.

Degradation Assay

SaOs-2 cells untreated or treated with 100 nM paclitaxel for 16 and 48 h were collected and protein extracts were prepared by preserving ubiquitinating enzymes. Purified histidine-tagged p27^{kip1} (0.06 μl) was incubated at 37°C for 72 h in 30 μl of degradation mix containing 50 μg of extracts, 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate, and 5 μM ubiquitin [Pagano et al., 1995]. The degradation of p27^{kip1} was analyzed by immunoblotting with an anti-human p27^{kip1} polyclonal antibody (clone C-19, Santa Cruz, CA).

Transfections

SaOs-2 cells were seeded on six-well plates (1×10^5 cells/well) and after 24 h pcDNA3 empty vector and pcDNA3-p27^{kip1} [Sgambato et al., 1998] were transiently transfected with Lipofectamine^{TM} 2000 and Plus^{TM} Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's guidelines. Briefly, SaOs-2 cell line was transfected with a ratio1:2 (0.5 μg DNA and 1 μl Lipofectamine^{TM} 2000) in OptiMEM® I (GIBCO) for 16 h. 24 h after transfection SaOs-2 treated with 100 nM paclitaxel (Calbiochem, La Jolla, CA) for 16, 24 and 48 h. SaOs-2 cell line was also transfected with a plasmid vector expressing GFP and the transfection efficiency was assessed to be around 50% by flow cytometry analysis.

Western Blot Analysis

Cells were harvested, washed and lysed in lysis buffer (50 mmol/L Tris-Cl, pH 7.4; 5 mmol/L EDTA; 250 mmol/L NaCl; 50 mmol/L NaF; 0.1% Triton X-100; 0.1 mmol/L Na_3VO_4; 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin). Protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL) and 50 μg of protein extracts from each sample were separated on denaturing SDS–PAGE gels and blotted on nitrocellulose membrane. Immunodetections were performed using anti-p27^{kip1} (clone c-19) antibody, obtained from Santa Cruz Biotecnology (Santa Cruz, CA), and anti-pRb antibody (clone G3-245; BD PharMingen). To check the amount of proteins transferred to nitrocellulose membrane, β-actin was used as control and it was detected by anti-β-actin antibody (clone AC-15; Sigma, Saint Louis, MI). Protein detection was performed using the enhanced chemiluminescence detection reagents (Amersham Corp, Buckinghamshire, UK). Densitometric evaluation of Western blot analysis of p27^{kip1} expression was performed using Molecular Analyst Software (Bio-Rad) and normalized with relative β-actin expression.

Flow Cytometry Analysis

Cells were trypsinized, counted with Coulter Counter (model ZM, Kontron Instruments, Watford, Herts, UK) and washed with PBS. For cell cycle analysis, cells were fixed 1×10^6 cells/ml in ice-cold 70% EtOH while vortexing and stored at 4°C. After a wash in PBS, cells were stained in 10 μg/mL propidium iodide (Sigma, Saint Louis, MI), 250 μg/mL RNAase (Sigma) PBS solution and incubated for 30 min at 37°C in the dark. Apoptosis was assessed using Vybrant^{TM} Apoptosis Assay Kit (Molecular Probes) according to the manufacturer's instructions. All flow cytometry acquisitions were performed using a FACSCalibur instrument (Becton Dickinson Biosciences). Cell cycle distribution was evaluated using ModFit software and Annexin V-positive/PI negative cell percentage was quantified using CellQuest software.

RESULTS

Increase of p27^{kip1} Levels in Paclitaxel-Induced Apoptosis of SaOs-2 Cell Line

The role of p27^{kip1} was evaluated in SaOs-2 cells undergoing paclitaxel-induced apoptosis [Pucci et al., 1999]. Cells were grown in medium containing 100 nM paclitaxel, harvested after 16, 24, and 48 h of treatment and analyzed by Western blot analysis for p27^{kip1} levels. As shown in Figure 1, p27^{kip1} expression decreased at 16 h; however, starting from 24 to 48 h following paclitaxel treatment, p27^{kip1} was expressed again. β-actin was used as a loading control (Fig. 1).

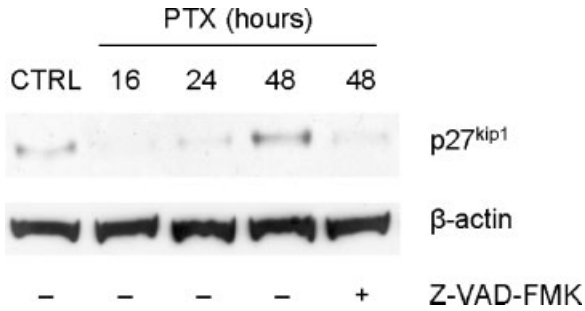

Fig. 1. $p27^{kip1}$ expression during paclitaxel treatment of osteosarcoma Rb-null SaOs-2 cell line. Western blot analysis of $p27^{kip1}$ expression in SaOs-2 cell line, treated with 100 nM paclitaxel at indicated times. Untreated control cells were collected following 16 h of treatment. SaOs-2 cells were treated for 48 h with 100 nM paclitaxel together with 100 μM of the broad-range caspase inhibitor Z-VAD-FMK.

Next step was to test if $p27^{kip1}$ increase at 48 h of paclitaxel treatment was specifically related to apoptosis. In a previous study [Pucci et al., 1999], we demonstrated that the broad-range caspase inhibitor Z-VAD-FMK is able to block the apoptotic process induced by paclitaxel. Thus, we treated SaOs-2 cells with 100 nM paclitaxel in the presence of Z-VAD-FMK for 48 h and the levels of $p27^{kip1}$ were determined by Western blot analysis. As Figure 1 shows, $p27^{kip1}$ levels did not increase following 48 h of paclitaxel treatment in presence of Z-VAD-FMK.

Inhibition of $p27^{kip1}$ Protein Degradation During Paclitaxel-Induced Apoptosis

To verify if the proteasomal activity was involved in modulation of $p27^{kip1}$ protein during paclitaxel treatment, SaOs-2 cells untreated or treated with 100 nM paclitaxel for 16 and 48 h were collected and a degradation assay was performed by adding purified recombinant $p27^{kip1}$ to the protein extracts [Esposito et al., 1997]. This assay was designed for testing a specific proteasome-dependent degradation activity, indeed protein extracts were prepared by preserving ubiquitinating enzymes and the assay was conducted in presence of ubiquitin. Figure 2 shows that recombinant $p27^{kip1}$ protein was degraded only in the extracts obtained following 16 h of treatment with paclitaxel. No degradation was observed in the extracts obtained following 48 h of treatment. As expected, $p27^{kip1}$ protein was not degraded in either of the extracts from untreated cells nor in the reaction mix, which consisted of the buffer plus the recombinant protein.

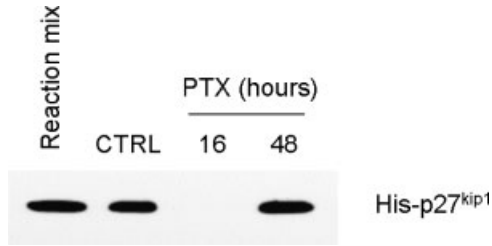

Fig. 2. Degradation activity of SaOs-2 cells, untreated or treated with 100 nM paclitaxel at the indicated times. Purified histidine-tagged $p27^{kip1}$ was used as substrate of reaction.

$p27^{kip1}$ Overexpression Enhances Paclitaxel-Induced Apoptosis in SaOs-2 Cell Line

Finally, to study the role of $p27^{kip1}$ increase during paclitaxel-induced apoptosis, we examined the effect of a transient overexpression of $p27^{kip1}$ on the cytotoxicity of paclitaxel. SaOs-2 cells were transiently transfected with the constructs pcDNA3 and pcDNA3-$p27^{kip1}$ [Sgambato et al., 1998]. The day after transfection, the cells were treated with 100 nM paclitaxel for 16, 24, and 48 h. At the end of each treatment, the cells were collected for Western blot and flow cytometry analyses. SaOs-2 cells transfected with the empty vector or pcDNA3-$p27^{kip1}$ were collected at 16 h and used as an internal control. Figure 3A shows that $p27^{kip1}$ was overexpressed at each time point studied and β-actin expression was used as the loading control. SaOs-2 trasfection with pcDNA3-$p27^{kip1}$ vector determined an increase of $p27^{kip1}$ protein level of about 100 times compared to pcDNA3-trasfected cell line. Figure 3B shows the densitometric evaluation of Western blot analysis showed in panel A. $p27^{kip1}$ levels following paclitaxel treatment of SaOs-2 cells transfected with empty vector (Fig. 3B, left panel) are comparable with those observed in SaOs-2 parental cell line in Figure 1. Also in $p27^{kip1}$-overexpressing SaOs-2 cell line (Fig. 3B, right panel), paclitaxel treatment induced a decrease of exogenous $p27^{kip1}$ protein level at 16 h of treatment followed by an increase at 24 and 48 h of paclitaxel treatment. Flow cytometry analysis of Annexin V positivity was performed to assess the percentage of cells committed to apoptosis in the presence or absence of $p27^{kip1}$ overexpression (Fig. 3C–D). Cell cycle distribution in transfected cells treated by paclitaxel was also evaluated: induction of $p27^{kip1}$ expression did not cause

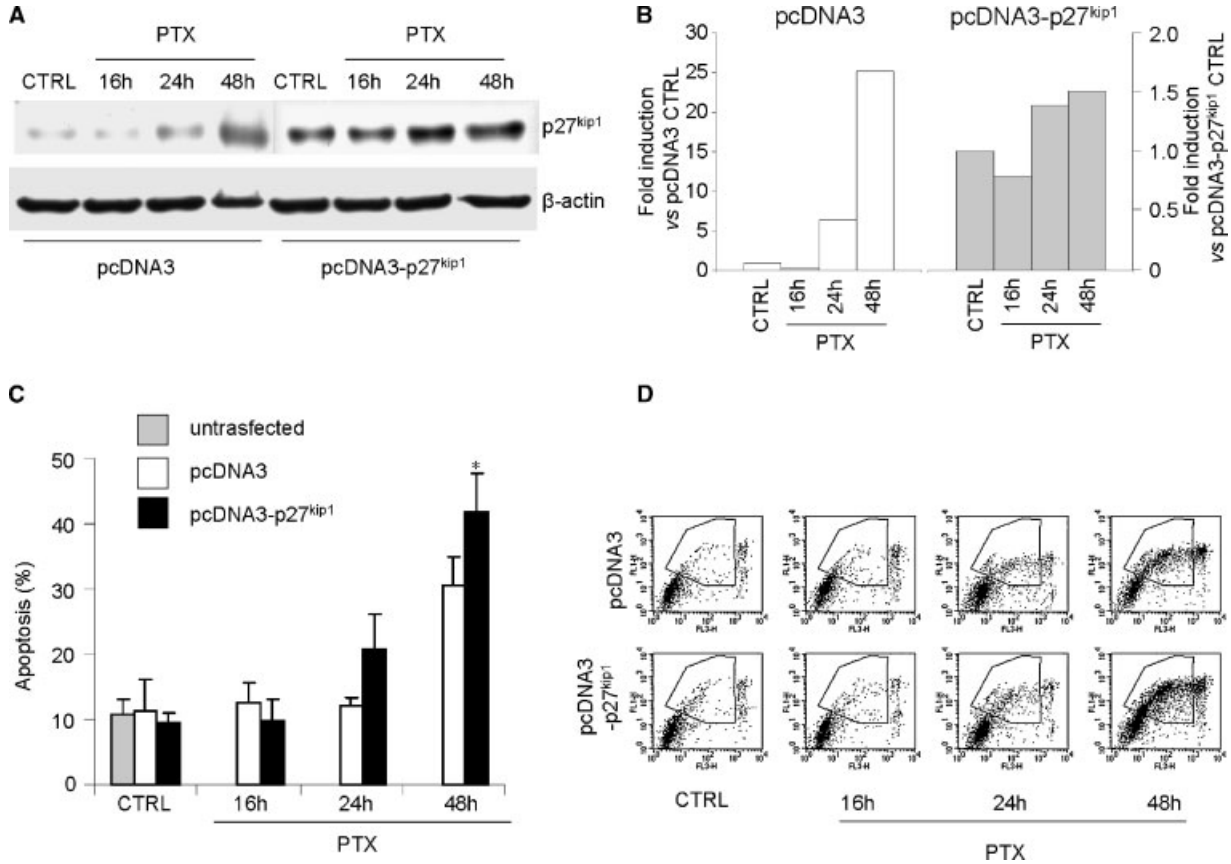

Fig. 3. **A**: Western blot analysis of $p27^{kip1}$ expression in SaOs-2 cells transfected with pcDNA3-$p27^{kip1}$ expression vector or control vector and treated with 100 nM paclitaxel at the indicated times. All the samples were run on the same gel but protein expression in $p27^{kip1}$-overexpressing samples was obtained from a less exposed film, to avoid the issue of a saturated signal of $p27^{kip1}$ protein. β-actin expression was used as the loading control. **B**: Densitometric evaluation of $p27^{kip1}$ protein level obtained by the Western blot analysis showed in **panel A**. The two groups of histograms represent the effect of paclitaxel treatment on $p27^{kip1}$ level, normalized to β-actin expression, on SaOs-2 cell line trasfected with pcDNA3 empty vector (**left panel**, white bars) or with pcDNA3-$p27^{kip1}$ vector (**right panel**, gray bars) compared to their untreated control samples. **C**: Annexin V-positive/PI negative apoptotic cell percentages in SaOs-2 cells transfected with pcDNA3-$p27^{kip1}$ or pcDNA3 control vector and treated with 100 nM paclitaxel. The data are presented as the mean ± standard deviation of three different experiments. $^*P < 0.05$ (Student's *t*-test of $p27^{kip1}$-overexpressing cells vs. pcDNA3-transfected ones). **D**: A representative result of flow cytometric evaluation of apoptosis showed in **panel C**.

G_1 arrest or apoptosis compared to the parental cell line or to SaOs-2 cells transfected with empty vector only (Table I). As Figure 3C shows, after 48 h of paclitaxel treatment, 41.8% of SaOs-2 cells transiently transfected with the pcDNA3-$p27^{kip1}$ construct are committed to apoptosis compared to 30.6% of SaOs-2 cells transfected with the empty vector ($P < 0.05$).

TABLE I. Effect of 100nM Paclitaxel on Cell Cycle Phases of SaOs-2 Cell Line Overexpressing $p27^{kip1}$

			G_0/G_1	S	G_2	Apoptosis
Control	16 h	Parental	56.1 ± 5.7	28.7 ± 3.3	15.1 ± 2.4	10.7 ± 2.3
		pcDNA3	51.0 ± 0.5	20.2 ± 1.5	28.8 ± 2.0	11.3 ± 4.7
		$p27^{kip1}$	49.4 ± 1.0	26.5 ± 6.7	24.1 ± 7.7	9.6 ± 1.4
Paclitaxel	16 h	pcDNA3	21.3 ± 1.0	32.7 ± 2.5	46.0 ± 1.5	12.1 ± 1.1
		$p27^{kip1}$	21.4 ± 0.7	31.1 ± 4.3	47.5 ± 5.1	9.7 ± 3.4
	24 h	pcDNA3	4.6 ± 0.1	25.4 ± 7.1	70.0 ± 7.1	12.1 ± 1.1
		$p27^{kip1}$	5.6 ± 2.3	29.4 ± 1.7	65.0 ± 4.0	20.8 ± 5.3
	48 h	pcDNA3	4.2 ± 0.8	3.8 ± 4.0	92.3 ± 4.5	30.6 ± 4.2
		$p27^{kip1}$	3.7 ± 0.6	6.5 ± 1.8	89.8 ± 1.3	41.8 ± 5.8

Data are presented as the mean ± standard deviation of three different experiments.

DISCUSSION

In a previous study, we demonstrated that 100 nM paclitaxel treatment induces apoptosis in the SaOs-2 osteosarcoma cell line [Pucci et al., 1999]. In this study, at 16 h of continuous paclitaxel treatment, we observed a decrease of $p27^{kip1}$ protein levels. Following 24 to 48 h of treatment, times of paclitaxel exposure that induced apoptosis in SaOs-2 cell line [Pucci et al., 1999], we observed that $p27^{kip1}$ is expressed again, suggesting that $p27^{kip1}$ increase in paclitaxel-treated SaOs-2 cells is in conjunction with the activation of the apoptotic program. The broad range caspase inhibitor Z-VAD-FMK treatment is able to inhibit paclitaxel-induced apoptosis [Pucci et al., 1999] and $p27^{kip1}$ increase following 48 h of paclitaxel treatment, suggesting that, in SaOs-2 cells, the increase of $p27^{kip1}$ observed following drug exposure is strictly related to the apoptotic process.

It has been demonstrated that $p27^{kip1}$ is regulated primarily at the post-transcriptional level by the ubiquitin-dependent proteasome pathway [Pagano et al., 1995]. Paclitaxel treatment induces an activation of the ubiquitin-proteasome complex, determining a degradation of $p27^{kip1}$ during the first 16 h of treatment. In conjunction with the activation of apoptotic program, the proteasome activity is inhibited determining an increase of $p27^{kip1}$ protein. These results suggest that the inhibition of the proteasome activity observed in SaOs-2 cells following 48 h of paclitaxel treatment may account for the abnormal accumulation of $p27^{kip1}$, which may in turn play an important role in sustaining the apoptosis of transformed cells. Although the mechanism responsible for the decrease of proteasome activity in SaOs-2 cells at 48 h of treatment is unclear, it is conceivable that several subunits of the proteasome undergo caspase-dependent proteolysis and that this results in diminished protein turnover, as recently reported [Adrain et al., 2004]. Moreover, the pro-apoptotic role of lack of proteasome activity in paclitaxel-induced cell death has been observed in a human lung adenocarcinoma cell line (LC-2-AD): inhibition of proteasome by the proteasome inhibitor 1 (PS1) in these cells treated with paclitaxel leads to an increase in the number of apoptotic cells [Oyaizu et al., 2001]. The involvement of $p27^{kip1}$ accumulation in proteasome inhibitor-induced apoptosis has already been reported in several human carcinoma cells [An et al., 1998; Kudo et al., 2000] and the adenoviral expression of a degradation-resistant form of $p27^{kip1}$ can induce growth arrest and apoptosis in a breast cancer cell line model [Zhang et al., 2005].

To understand the significance of $p27^{kip1}$ protein modulation during paclitaxel-induced apoptosis, we examined the effect of a transient overexpression of $p27^{kip1}$ on SaOs-2 cell line sensibility to paclitaxel. Unexpectedly, $p27^{kip1}$ did not cause any G_1 arrest of SaOs-2 cells in our study. The explanation may be found in the genotype of SaOs-2 cells. Indeed, this osteosarcoma cell line expresses a truncated form of pRb that is defective for E2F binding and cell cycle regulation [Shew et al., 1990]; therefore, even if cyclin D/cdk4 and cyclin E/cdk2 are inhibited by $p27^{kip1}$, the cell cycle is not arrested.

In our study, transient overexpression of $p27^{kip1}$ protein in SaOs-2 cell line determines an increase of paclitaxel-induced apoptosis. Several studies, mostly in normal cells and in vivo, have pointed toward a protective role in apoptosis for $p27^{kip1}$ [St Croix et al., 1996; Eymin et al., 1999]. However, additional research using adenoviral vectors to overexpress $p27^{kip1}$ in several cancer cells cell lines [Craig et al., 1997; Katayose et al., 1997; Wang et al., 1997] has shown that $p27^{kip1}$ may play a role in promoting apoptosis by inducing G_1 arrest in the presence of a functional pRb [Schreiber et al., 1999; Naruse et al., 2000].

Regarding the pro-apoptotic role of $p27^{kip1}$ following paclitaxel treatment, our data are in contrast with a previous study in RKO human colon adenocarcinoma cells, where overexpression of $p27^{kip1}$ slightly inhibited taxol-induced cell death [Schmidt et al., 2000]. The use of two different cell lines may account for this difference. Again, SaOs-2 cells are pRb-defective, thus $p27^{kip1}$ could be ineffective in inducing G_1 arrest, whereas in RKO cells, $p27^{kip1}$ can induce G_1 arrest by increasing the levels of pRb, thus preventing cells from arresting in the G_2/M phase.

In this study, we have shown that an increase in $p27^{kip1}$ levels occurs in vivo during paclitaxel treatment of SaOs-2 cells due to the blockade of proteasomal activity. Moreover, $p27^{kip1}$ overexpression is able to inhibit cell proliferation leading to apoptosis in a G_1-independent

manner. The mechanism underlying a pro-apoptotic role of p27^{kip1} is still unclear. It is conceivable that p27^{kip1} overexpression could act by sending an aberrant signal to those cells that are able to escape paclitaxel-induced apoptosis in G_2/M phase, or by downregulating anti-apoptotic molecules such as mcl-1 [Woltman et al., 2003].

In conclusion, we have demonstrated that transfection of pRb-null SaOs-2 cells with the p27^{kip1} gene via plasmid leads to an increase of paclitaxel-induced apoptosis. The combined effect of p27^{kip1} on proliferation and apoptosis in tumor cells makes p27^{kip1} and proteasomal inhibitors interesting tools for therapy in patients with pRb-defective cancers.

ACKNOWLEDGMENTS

FIRB-MIUR n.RBNE01PZER (Tafani M). Gabellini C is a recipient of a fellowship from the Italian Foundation for Cancer Research.

REFERENCES

Adrain C, Creagh EM, Cullen SP, Martin SJ. 2004. Caspase-dependent inactivation of proteasome function during programmed cell death in *Drosophila* and man. J Biol Chem 279:36923–36930.

An B, Goldfarb RH, Siman R, Dou QP. 1998. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ 5:1062–1075.

Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME, Huang Y. 1993. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia 7:563–568.

Craig C, Wersto R, Kim M, Ohri E, Li Z, Katayose D, Lee SJ, Trepel J, Cowan K, Seth P. 1997. A recombinant adenovirus p27^{kip1} induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells. Oncogene 14:2283–2289.

Douros J, Suffness M. 1978. New natural products of interest under development at the National Cancer Institute. Cancer Chemother Pharmacol 1:91–100.

Esposito V, Baldi A, De Luca A, Groger AM, Loda M, Giordano GG, Caputi M, Baldi F, Pagano M, Giordano A. 1997. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res 57:3381–3385.

Eymin B, Sordet O, Droin N, Munsch B, Haugg M, Van de Craen M, Vandenabeele P, Solary E. 1999. Caspase-induced proteolysis of the cyclin-dependent kinase inhibitor p27Kip1 mediates its anti-apoptotic activity. Oncogene 18:4839–4847.

Ferlini C, Raspaglio G, Mozzetti S, Distefano M, Filippetti F, Martinelli E, Ferrandina G, Gallo D, Ranelletti FO, Scambia G. 2003. Bcl-2 down-regulation is a novel mechanism of paclitaxel resistance. Mol Pharmacol 64: 51–58.

Hengst L, Dulie V, Slingerl J, et al. 1994. A cell cycle regulated inhibitor of cyclin-dependent kinases. Proc Natl Acad Sci 91:5291–5294.

Kasten MM, Giordano A. 1998. pRb and the cdks in apoptosis and the cell cycle. Cell Death Differ 5:132–140.

Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P. 1997. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res 57:5441–5445.

Koff A, Ohtsuki M, Polyak K, et al. 1993. Negative regulation of G1 in mammalian cells: Inhibition of cyclinE-dependent kinase by TGF-β. Science 260:536–539.

Kudo Y, Takata T, Ogawa I, Kaneda T, Sato S, Takekoshi T, Zhao M, Miyauchi M, Nikai H. 2000. p27Kip1 accumulation by inhibition of proteasome function induces apoptosis in oral squamous cell carcinoma cells. Clin Cancer Res 6:916–923.

Kumar N. 1981. Taxol-induced polymerization of purified tubulin. Mechanism of action. J Biol Chem 256:10435–10441.

Ling YH, Tornos C, Perez-Soler R. 1998. Phosphorylation of Bcl-2 is a marker of M phase events and not a determinant of apoptosis. J Biol Chem 273:18984–18991.

Meikrantz W, Schlegel R. 1995. Apoptosis and the cell cycle. J Cell Biochem 58:160–174.

Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev 13:1181–1189.

Morgenbesser SD, Williams BO, Jacks T, DePinho RA. 1994. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 371:72–74.

Naruse I, Hoshino H, Dobashi K, Minato K, Saito R, Mori M. 2000. Over-expression of p27kip1 induces growth lung cancer cell lines. Int J Cancer 88:377–383.

Oyaizu H, Adachi Y, Okumura T, Okigaki M, Oyaizu N, Taketani S, Ikebukuro K, Fukuhara S, Ikehara S. 2001. Proteasome inhibitor 1 enhances paclitaxel-induced apoptosis in human lung adenocarcinoma cell line. Oncol Rep 8:825–829.

Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269:682–685.

Paggi MG, Baldi A, Bonetto F, Giordano A. 1996. Retinoblastoma protein family in cell cycle and cancer: a review. J Cell Biochem 62:418–430.

Polyak K, Kato JY, Solomon MJ, et al. 1994a. p27^{kip1}, a cyclin-CDK inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev 8: 9–22.

Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massague J. 1994b. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78: 59–66.

Pucci B, Bellincampi L, Tafani M, Masciullo V, Melino G, Giordano A. 1999. Paclitaxel induces apoptosis in Saos-2

cells with CD95L upregulation and Bcl-2 phosphorylation. Exp Cell Res 252:134–143.
Pucci B, Kasten M, Giordano A. 2000. Cell cycle and apoptosis. Neoplasia 2:291–299.
Pucci B, Claudio PP, Masciullo V, Bellincampi L, Terrinoni A, Khalili K, Melino G, Giordano A. 2002. pRb2/p130 promotes radiation-induced cell death in the glioblastoma cell line HJC12 by p73 upregulation and Bcl-2 downregulation. Oncogene 21:5897–5905.
Riondel J, Jacrot M, Picot F, Beriel H, Mouriquand C, Potier P. 1986. Therapeutic response to taxol of six human tumors xenografted into nude mice. Cancer Chemother Pharmacol 17:137–142.
Riondel J, Jacrot M, Nissou MF, Picot F, Beriel H, Mouriquand C, Potier P. 1988. Antineoplastic activity of two taxol derivatives on an ovarian tumor xenografted into nude mice. Anticancer Res 8:387–390.
Roberts JR, Allison DC, Donehower RC, Rowinsky EK. 1990. Development of polyploidization in taxol-resistant human leukemia cells in vitro. Cancer Res 50:710–716.
Rowinsky EK, Donehower RC. 1995. Paclitaxel (taxol). N Engl J Med 332:1004–1014.
Schiff PB, Horwitz SB. 1980. Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 77: 1561–1565.
Schmidt M, Fan Z. 2001. Protection against chemotherapy-induced cytotoxicity by cyclin-dependent kinase inhibitors (CKI) in CKI-responsive cells compared with CKI-unresponsive cells. Oncogene 20:6164–6171.
Schmidt M, Lu Y, Liu B, Fang M, Mendelsohn J, Fan Z. 2000. Differential modulation of paclitaxel-mediated apoptosis by p21Waf1 and p27Kip1. Oncogene 19:2423–2429.
Schreiber M, Muller WJ, Singh G, Graham FL. 1999. Comparison of the effectiveness of adenovirus vectors expressing cyclin kinase inhibitors p16INK4A, p18INK4C, p19INK4D, p21(WAF1/CIP1) and p27KIP1 in inducing cell cycle arrest, apoptosis and inhibition of tumorigenicity. Oncogene 18:1663–1676.
Sgambato A, Zhang YJ, Ciaparrone M, Soh JW, Cittadini A, Santella RM, Weinstein IB. 1998. Overexpression of p27Kip1 inhibits the growth of both normal and transformed human mammary epithelial cells. Cancer Res 58:3448–3454.
Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. 1997. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev 11:1464–1478.
Shew JY, Lin BT, Chen PL, Tseng BY, Yang-Feng TL, Lee WH. 1990. C-terminal truncation of the retinoblastoma gene product leads to functional inactivation. Proc Natl Acad Sci USA 87:6–10.
Slingerl JM, Hengst L, Pan C-H, et al. 1994. A novel inhibitor of cyclin-CDK activity detected in transforming growth factor β-arrested epithelial cells. Mol Cell Biol 14: 3683–3694.
St Croix B, Florenes VA, Rak JW, Flanagan M, Bhattacharya N, Slingerland JM, Kerbel RS. 1996. Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. Nat Med 2:1204–1210.
Strobel T, Swanson L, Korsmeyer S, Cannistra SA. 1996. BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway. Proc Natl Acad Sci USA 93: 14094–14099.
Sumantran VN, Ealovega MW, Nunez G, Clarke MF, Wicha MS. 1995. Overexpression of Bcl-XS sensitizes MCF-7 cells to chemotherapy-induced apoptosis. Cancer Res 55:2507–2510.
Tonini T, Gabellini C, Bagella L, D'Andrilli G, Masciullo V, Romano G, Scambia G, Zupi G, Giordano A. 2004. pRb2/p130 decreases sensitivity to apoptosis induced by camptothecin and doxorubicin but not by taxol. Clin Cancer Res 10:8085–8093.
Vaux DL, Korsmeyer SJ. 1999. Cell death in development. Cell 96:245–254.
Wang QM, Luo X, Studzinski GP. 1997. Cyclin-dependent kinase 6 is the principal target of p27/Kip1 regulation of the G1-phase traverse in 1,25-dihydroxyvitamin D3-treated HL60 cells. Cancer Res 57:2851–2855.
Wang TH, Wang HS, Soong YK. 2000. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. Cancer 88:2619–2628.
Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc 93:2325–2327.
Woltman AM, van der Kooij SW, Coffer PJ, Offringa R, Daha MR, van Kooten C. 2003. Rapamycin specifically interferes with GM-CSF signaling in human dendritic cells, leading to apoptosis via increased p27KIP1 expression. Blood 101:1439–1445.
Xing H, Wang S, Hu K, Tao W, Li J, Gao Q, Yang X, Weng D, Lu Y, Ma D. 2005. Effect of the cyclin-dependent kinases inhibitor p27 on resistance of ovarian cancer multicellular spheroids to anticancer chemotherapy. J Cancer Res Clin Oncol 131:511–519.
Yu D, Jing T, Liu B, Yao J, Tan M, McDonnell TJ, Hung MC. 1998. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol Cell 2:581–591.
Zhang Q, Tian L, Mansouri A, Korapati AL, Johnson TJ, Claret FX. 2005. Inducible expression of a degradation-resistant form of p27Kip1 causes growth arrest and apoptosis in breast cancer cells. FEBS 579:3932–3940.