Taxol-induced growth arrest and apoptosis is associated with the upregulation of the Cdk inhibitor, p21$^{WAF1/CIP1}$, in human breast cancer cells

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Abstract. The anticancer agent, taxol, stabilizes tubulin polymerization, resulting in arrest at the G2/M phase of the cell cycle and apoptotic cell death. However, the molecular mechanism of this growth inhibition and apoptosis is poorly understood. In this study, we used MCF-7 and MDA-MB-231 human breast carcinoma cells which have different estrogen receptor (ER) and tumor suppressor p53 statuses to examine the mechanisms of taxol-induced growth inhibition and apoptosis. Treatment of the cells with taxol resulted in a time-dependent inhibition of cell viability, which was accompanied by an accumulation of cells at G2/M and the sub-G1 apoptotic region, determined by flow cytometric analysis. Furthermore, chromatin condensation, DNA ladder formation and proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) in both cell lines were observed following treatment with taxol, indicating the occurrence of apoptotic cell death. Western blot analysis using whole cell lysates from MCF-7 and MDA-MB-231 cells treated with taxol demonstrated that taxol treatment inhibited expression of cyclin A and cyclin B1 proteins in a time-dependent manner. The inhibitory effects of taxol on cell growth and apoptosis induced by taxol were also associated with the downregulation of Wee1 kinase expression and a marked induction in the activity of the cyclin-dependent kinase inhibitor, p21$^{WAF/CIP1}$. Furthermore, taxol elevated p21 promoter activity in both cell lines. These findings suggest that taxol-induced G2/M arrest and apoptosis in human breast carcinoma cells is mediated through the ER- and p53-independent upregulation of p21.

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Introduction

Cell cycle progression is a highly regulated process involving the sequential activation of a series of cell cycle control proteins, the cyclins, and their catalytic subunits, the cyclin-dependent kinases (Cdks). Different cyclin/Cdk complexes are required at different phases of the cell cycle. In mammalian cells, cyclin A is produced in the late G1 phase, and its expression accumulates during the S and G2 phases (1,2). The onset of the M phase is regulated by the maturation-promoting factor (MPF), which consists of at least two subunits, a regulatory subunit, cyclin B, and its catalytic subunit, Cdc2 (also known as Cdk1). The kinase activity of cyclin B/Cdc2 is activated specifically at the G2/M transition and thereafter inactivated at the onset of anaphase. At the end of mitosis, the activity of the Cdc2 kinase is abolished suddenly by proteolysis of cyclin B (3,4). The progression from the G2 phase into mitosis is negatively regulated by Cdc2 phosphorylation on threonine-14 (Thr^{14}) and tyrosine-15 (Tyr^{15}) residues (4). Wee1 is one of the inhibitory protein kinases and is capable of phosphorylating Cdc2 on Tyr^{15}, but not Thr^{14} (5).

The cyclin/Cdk complexes may be further regulated by certain types of proteins, the Cdk inhibitors, which bind to the complexes and inhibit their activity (2,6). The first mammalian Cdk inhibitor identified, p21$^{WAF1/CIP1}$, is an important mediator of cell cycle arrest imposed by the tumor suppressor, p53, in response to DNA damage (7,8). Accumulating data have implicated growth arrest accompanied by the upregulation of p21 not only in inhibiting proliferation, but also in promoting differentiation (2,7,9). p21 is also known to be an inhibitor of DNA replication by inhibiting the proliferating cell nuclear antigen, which is required for cell cycle progression (2,9,10).

Cell death occurs either by necrosis or apoptosis. Necrosis is usually considered to result from physical injury and not genetically controlled, whereas apoptosis, programmed cell death, is a deliberate and genetically controlled cellular response to specific development or environmental stimuli, such as DNA damage, viral infection, cellular damage, and loss of cell-cell or

cell-substrate contact (11-13). For cells that have sustained irreversible levels of DNA damage, apoptosis is an important means for elimination of these cells, whose presence may ultimately be damaging to the organism. Apoptosis occurs either in a cell cycle-dependent or -independent manner. Deregulation of cell cycle progression and inappropriate Cdk activity may be signals that trigger apoptotic cell death (14,15). The molecular mechanism implicated in apoptosis has been partially elucidated, and the induction of apoptosis is partly mediated intracellularly by a number of genes, such as p53, Bcl-2 and Bax. The p53 protein functions in part by responding to DNA damage and inducing apoptosis, which is likely to be a crucial aspect of the function of p53 as a tumor suppressor. Wild-type p53 protein arrests DNA-damaged cells in the late G1 phase by inducing p21 activity, and non-repaired cells may be eliminated by apoptosis, by inducing Bax activity and repressing Bcl-2 activity (16,17). However, the mechanism of apoptosis occurring in the G2/M phase is not well understood.

Paclitaxel (taxol), a diterpene with a natural chemotherapeutic activity first described in 1971, has been isolated from the bark of the Pacific Western yew tree, *Taxus brevifolia* (18). This antineoplastic drug is one of the most effective antitumor agents currently used for therapy against ovarian and breast cancer, as well as non-small cell lung cancer, melanoma and lymphoma (19,21). Unlike other antimicrotubulin agents, taxol is characterized by a strong affinity for tubulin protein. Thus, taxol achieves its antitumor effect by promoting tubulin dimerization and inhibiting the depolymerization of microtubules, resulting in the formation of abnormally stable and non-functional microtubules (19,22,23). As a result of this unique mechanism of action, the continuous exposure to taxol prevents the completion of mitosis, resulting in mitotic metaphase arrest and cellular toxicity (24,25). The cell death induced by taxol appears to result from apoptosis, which is the mechanism of most antineoplastic agents (25,26). However, despite these accumulating data, the precise mechanism of taxol-induced apoptosis remains poorly understood.

In the present study, we investigated the mechanism of taxol-induced growth arrest and apoptosis in the MCF-7 and MDA-MB-231 human breast carcinoma cells, which have different estrogen receptor (ER) and tumor suppresser p53 statuses. We demonstrate that taxol treatment induces a similar inhibition of cell growth, due to cell cycle arrest in the G2/M phase and apoptosis in both cell lines, which are associated with a decrease in Wee1 expression and the induction of p21 activity. Our data suggest that taxol induces apoptosis in cells arrested in the G2/M phase, which may be explained at least in part by the induction of Cdk inhibitor p21 activity in an ER- and p53-independent manner in human breast carcinoma cells.

Materials and methods

Cell culture and taxol treatments. The MCF-7 and MDA-MB-231 human breast carcinoma cell lines were purchased from the American Type Culture Collection (Rockville, MD). They were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Taxol was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution in dimethyl sulfoxide (DMSO) was prepared and stored at 4˚C. The final concentration of DMSO did not exceed 0.05% in the culture medium, which did not affect growth.

Growth study and morphology. For growth inhibition analysis, the cells were plated at $0.5x10^4$ cells per 100-mm plate and incubated for 24 h. The cells were cultured in the presence or absence of taxol. After every 12 h of culture, the cells were trypsinized and washed with phosphate-buffered saline (PBS), and the viable cells were scored using a Neubauer hemocytometer with trypan blue exclusion. For the morphological study, cells were grown on coverslips, treated with taxol for 48 h, and Wright-stained (Fisher Scientific, Pittsburgh, PA), as recommended by the manufacturer.

DAPI staining. The cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were washed two more times with PBS and analyzed via a fluorescence microscope at x400 magnification.

Assessment of DNA degradation. The cells were incubated with taxol for 24 and 48 h and then trypsinized. Cells were washed with PBS and resuspended in lysis buffer [1 mM EDTA, 10 mM Tris (pH 8.0), 1% SDS, 1 μg/ml proteinase K]. After 1 h of incubation at 37˚C, RNase A was added, and incubation was continued for another hour. Crude DNA preparations were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). Cell lysate samples were subsequently run at 120 V on a 0.8% agarose gel containing ethidium bromide (EtBr, Sigma). The gel was examined on an ultraviolet light source and photographed (27).

DNA flow cytometric analysis. The cells were incubated for various periods of time in the presence of taxol and detached with trypsin. After centrifugation, cell pellets were resuspended gently in cold PBS and fixed with ice-cold 70% ethanol for 30 min. The cells were resuspended in citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6), and frozen at -80˚C. Before staining, the cells were thawed rapidly and treated with RNase A at room temperature for 30 min. The nuclei were stained with propidium iodide (PI, Sigma). The fluorescence intensity of the PI-stained DNA in each cell nucleus was examined with a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Immunoprecipitation and western blot analysis. Total cell lysates were lysed in extraction buffer. The supernatant was collected, and protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at -80˚C or immediately used for immunoblotting and immunoprecipitations. For immunoprecipitation, cell extracts were incubated with immunoprecipitating antibodies in extraction buffer for 1 h at 4˚C. The immunocomplexes were precipitated with protein A-Sepharose beads (Sigma) for 1 h and washed five times with extraction buffer prior to boiling in an SDS sample buffer. The immunoprecipitated proteins or aliquots containing 40 μg protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Western blot analysis was performed as previously described (28). Monoclonal antibodies to Cdc2, cyclin A, cyclin B1 and pRB,

and polyclonal antibodies to Cdk2 and Wee1 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal anti-poly(ADP-) polymerase (PARP) and anti-p53 antibodies were purchased from Calbiochem (Cambridge, MA). Monoclonal anti-p21 antibody was obtained from Transduction Laboratories (Lexington, KY). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp. (Arlington Heights, IL).

p21 promoter-luciferase constructs and transfection assay. The cells were transiently transfected with p21 promoter-luciferase reporter constructs using Lipofectamine (Gibco BRL, Gaithersburg, MD), as recommended by the manufacturer. Following transfection, the cells were incubated for 12 h, the medium was exchanged, and the cells were incubated for various periods of time in the presence of 20 nM taxol. The cells were then lysed, and luciferase activity in the lysates was assayed using a Dynatech ML1000 luminometer (Dynatech Laboratories, Chantilly, VA). Luciferase activity was normalized to β-galactosidase activity, which was assayed using the β-galactosidase Enzyme Assay System (Promega, Madison, WI). All the luciferase assays were carried out at least in triplicate, and the experiments were repeated at least three times.

Statistical analysis. Data are presented as the means ± SD of at least three separate experiments. Comparisons between groups were analyzed using Student's t-test. P-values <0.05 were considered to indicate statistically significant differences.

Results

Growth inhibition by taxol. To evaluate the effect of taxol on the proliferation of human breast carcinoma cell lines, we initially determined the effect of taxol on the growth of MCF-7 and MDA-MB-231 cells. The cells were cultured in the absence or presence of 20 nM taxol for various time-periods, and viable cells were scored by a hemocytometer (Fig. 1A). The results showed that the untreated control cells displayed exponential growth during the 48-h incubation, whereas taxol treatment resulted in a dramatic decrease in the number of viable cells. This growth inhibition was accompanied by membrane shrinking and cells rounding up (Fig. 1B). These distinct morphological changes were even more pronounced with higher concentrations and prolonged exposure to taxol (data not shown).

Apoptosis induction by taxol. We then investigated whether taxol can induce apoptosis in human breast cancer cells, using DAPI staining, DNA fragmentation, PARP cleavage and flow cytometry. Fig. 2A shows the morphological changes in the MCF-7 and MDA-MB-231 cells incubated with or without taxol. The control cells displayed an intact nuclear structure, while the cells treated with taxol displayed chromosomal condensation and the formation of apoptotic bodies, indicating that the prolonged exposure to taxol induced programmed cell death. As reported, during the apoptotic process, endonucleases are activated, and they degrade chromosomal DNA to small nucleosomal fragments; the biochemical hallmark for apoptosis is the cleavage of DNA at an internucleosomal ladder. Thus, we then determined whether taxol can induce this type of chromatin destruction, by

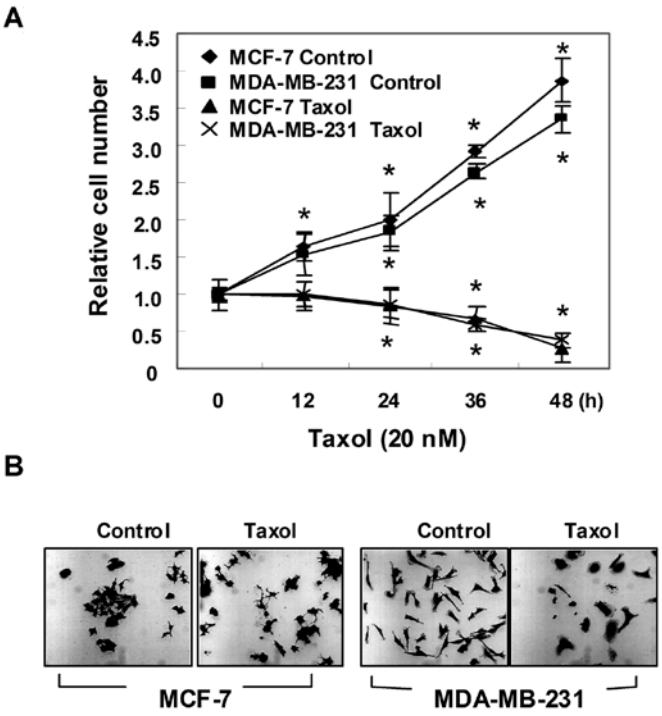

Figure 1. Taxol-induced growth inhibition and morphological changes of MCF-7 and MDA-MB-231 cells. (A) After 24 h of inoculation, the cells were cultured in the absence or presence of 20 nM taxol for the times indicated, and the total numbers of viable cells were counted using a hemocytometer with exclusion trypan blue. The data are reported as the means ± SD of three independent experiments. The significance was determined by a Student's t-test (*p<0.05, compared with the control). (B) Exponentially growing cells were incubated with taxol for 48 h. Following incubation, the cells were sampled, subjected to Wright's staining, and examined under a light microscope. Magnification, x200.

using agarose gel electrophoresis. After a 24-h and 48-h treatment of the cells with taxol, typical DNA laddering was clearly visible in the EtBr-stained gels in a time-dependent manner, which indicated the occurrence of apoptosis (Fig. 2B). Western blot analysis of PARP cleavage also showed that the PARP protein was degraded, with the concomitant disappearance of full-size (116 kd) molecules and accumulation of 85-kd fragments in the MCF-7 cells after 48 h of taxol treatment (Fig. 2C). The 85-kd PARP fragments in the MDA-MB-231 cells treated with taxol were not detected. However, the steady-state levels of PARP protein were markedly decreased between 24 and 48 h, suggesting that the PARP protein was degraded by taxol (Fig. 2C). The levels of apoptosis were also quantified by means of flow cytometry following PI staining. As shown in Fig. 2D, the percentage of apoptotic cells increased in a time-dependent manner in both cell lines following taxol treatment.

Cell cycle arrest by taxol. We subseqently examined the effect of taxol on cell cycle progression. At the indicated time-points, cell aliquots were collected and analyzed for DNA content by a fluorescence-activated cell sorter. As demonstrated in Table I, the percentage of G2/M cells in the untreated controls was approximately 23% for MCF-7 cell line and approximately 27% for the MDA-MB-231 cell line. However, >60% of MCF-7 cells and 50% of MDA-MB-231 cells were in the G2/M phase following 24 h of taxol treat-

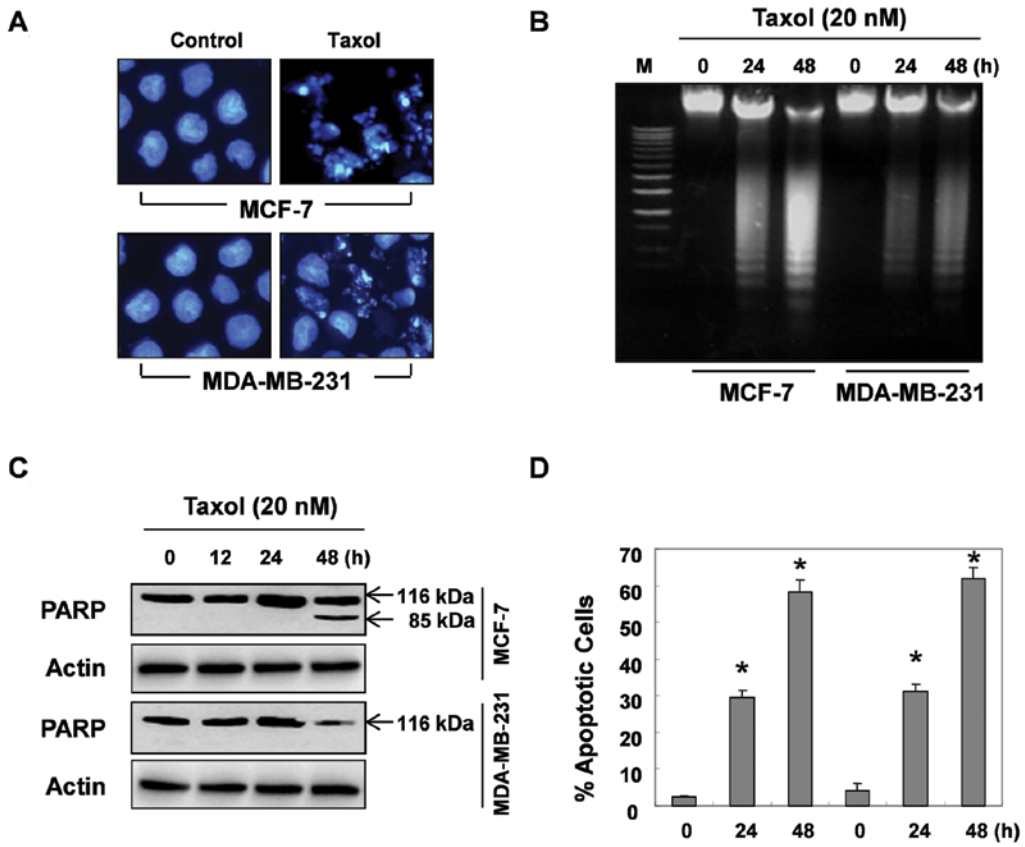

Figure 2. Induction of apoptotic cell death by taxol in MCF-7 and MDA-MB-231 cells. (A) Cells were cultured in the presence or absence of 20 nM taxol for 48 h before they were fixed and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope using a blue filter. Magnification, x400. (B) Induction of internucleosomal DNA fragmentation by taxol. Cells were incubated without (lanes 2 and 5) or with 20 nM taxol for 24 h (lanes 3 and 6) or 48 h (lanes 5 and 7). DNA was extracted and analyzed by 0.8% agarose gel electrophoresis in the presence of EtBr. Molecular weight standards of 1 kp DNA ladder are shown in lane 1. (C) Western blot analysis of PARP cleavage. MCF-7 and MDA-MB-231 cells were treated with taxol. At the indicated time-points, cells were lysed, and equal proteins were resolved on 10% SDS-polyacrylamide gels. Proteins were visualized by western blot analysis, using anti-PARP antibody and ECL detection. Actin was used as the internal control. (D) Time dependence of the apoptotic effect induced in both cell lines by taxol. Cells were incubated for various periods of time in the presence of 20 nM taxol. The cells were then fixed and stained with PI. The number of apoptotic sub-G1 cells was determined by flow cytometry. Data are the means ± SD of three separate experiments. The significance was determined by a Student's t-test (*$p<0.05$, compared with the control).

Table I. Taxol inhibits cell cycle progession at the G2/M phase in MCF-7 and MDA-MB-231 cells.[a]

		No. of cells (%) in each phase of the cell cycle		
Cell line	Time (h)	G1	S	G2/M
MCF-7	0	57.55	19.19	23.26
	12	25.69	18.12	56.19
	24	20.20	15.00	64.80
	48	34.89	27.85	37.25
MDA-MB-231	0	49.68	23.09	27.23
	12	27.19	29.80	43.01
	24	23.77	25.47	50.76
	48	30.91	36.00	33.09

[a]Exponentially growing cells at 60-70% confluency were treated at time 0 with 20 nM taxol. At the indicated time-points, the cells were trypsinized, and the pellets were collected. The cells were fixed and digested with RNase A, and then cellular DNA was stained with PI and analyzed by flow cytometry.

ment, which suggested the existence of a block at the G2/M phase of the cell cycle. Taken together, these results indicate that the growth inhibition in response to taxol in both cell lines is due to growth arrest and cell death.

Effect of taxol on G2/M cell cycle regulatory proteins. Since taxol blocked cell cycle progression in the G2/M phase, we determined the expression of cell cycle regulatory components at the G2/M boundary, such as cyclin A, cyclin B1, Cdk2, Cdc2 and Wee1. Western blot analysis of the samples obtained from taxol treatment for various time-periods showed no significant change in the intracellular protein levels of Cdk2 and Cdc2 (Fig. 3A), whereas taxol treatment resulted in a marked decrease in cyclin A expression between 12 and 24 h (Fig. 3). The expression levels of cyclin B1 first increased at 12 h and then decreased at 24 h following treatment with 20 nM taxol (Fig. 3). Under the same conditions, there was a dramatic reduction in the expression of Wee1 kinase protein (Fig. 3). These results indicate that the G2/M arrest induced by taxol is associated with a marked alteration in the expression of G2/M cell cycle regulatory proteins, such as cyclin A, cyclin B1 and Wee1.

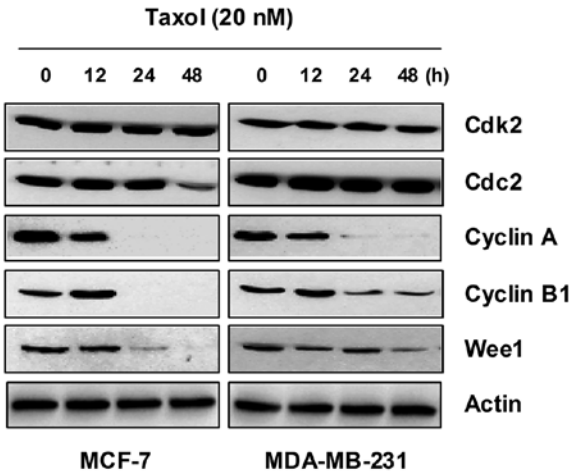

Figure 3. Effect of taxol on the expression of Cdks, cyclins and Wee1 proteins in MCF-7 and MDA-MB-231 cells. Cells were treated with 20 nM taxol for the times indicated. At the indicated time-points, the cells were lysed, and equal proteins were resolved on 8-10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Proteins were detected by western blot analysis using antibodies against cyclin A, cyclin B1, Cdk2, Cdc2 and Wee1, and an ECL detection system. Actin was used as the internal control.

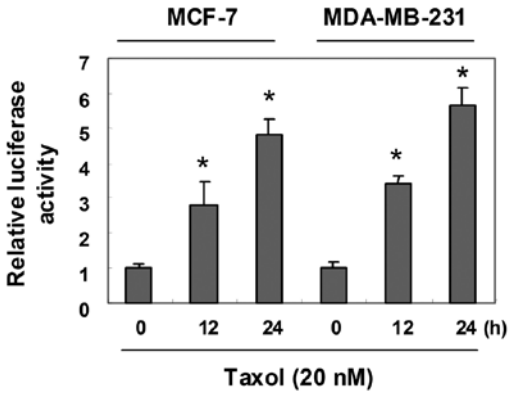

Figure 5. Time-dependent change in p21 full-length promoter-luciferase activity in response to taxol in MCF-7 and MDA-MB-231 cells. The p21 promoter fused to the luciferase reporter gene was transiently transfected into the cells. After 6 h of incubation, the medium was replaced with fresh medium, and the cells were incubated for the indicated time-points in the presence of 20 nM taxol. The activities of the luciferase reporter genes in each sample were normalized with respect to β-galactosidase activity in the same cells. The results are expressed as the means ± SD of three separate experiments. The significance was determined by a Student's t-test (*p<0.05, compared with the control).

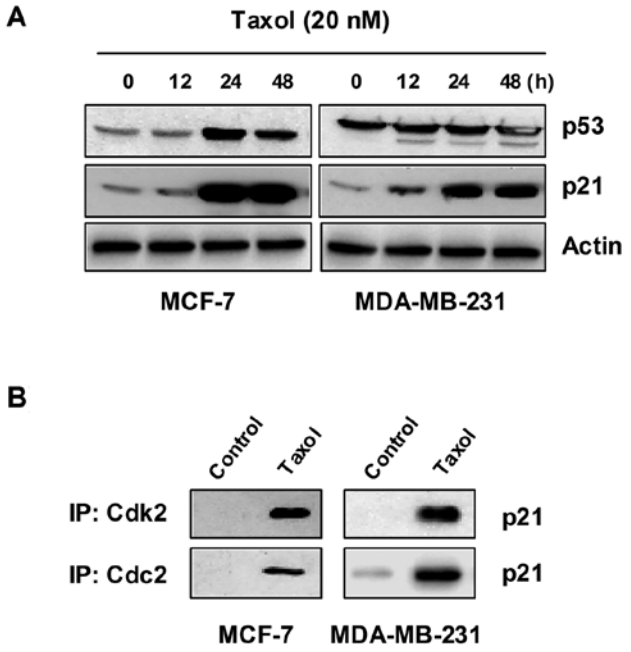

Figure 4. Induction of Cdk inhibitor p21 expression and its association with Cdks in response to taxol treatment in MCF-7 and MDA-MB-231 cells. (A) The cells were incubated with 20 nM taxol for the time indicated. Equal amounts of total cell lysates were subjected to 10% or 12% SDS-polyacrylamide gels, transferred, and probed with antibodies against p53 and p21. (B) After treatment with taxol for 36 h, total cell lysates were immunoprecipitated with anti-Cdk2 or anti-Cdc2 antibody, separated on 12% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. p21 protein levels were detected with anti-p21 antibody and ECL detection system.

Induction of Cdk inhibitor p21 by taxol. We then investigated whether Cdk inhibitors are involved in taxol-induced growth arrest and apoptosis. As shown in Fig. 4A, the incubation of cells with taxol caused a striking time-dependent induction of p21 protein activity in the MCF-7 and MDA-MB-231 cells. However, other Cdk inhibitors, such as p27 and p16, were not detectable in either cell line, without or with taxol treatment (data not shown). MCF-7 cells harbor wild-type p53, whereas MDA-MB-231 cells express abundant mutant p53. In this experiment, we used anti-p53 antibody, which reacts with both human wild-type and mutant p53. In the MCF-7 cells, treatment with taxol induced a consistent increase in the level of p53 with respect to the untreated control cells; however, the expression of p53 in the MDA-MB-231 cells was not affected by taxol (Fig. 4A). As the p53 gene is mutated in MDA-MB-231 cells, it is highly likely that the induction of p21 activity by taxol is mediated in a p53-independent manner.

Since it was well known that the Cdk inhibitor, p21, inhibits the activity of Cdks by direct association with various cyclin/Cdk complexes, the complex formation of cyclins/Cdks/p21 is increased in cells arrested by DNA damaging agents. As shown in Fig. 4B, the association of p21 with Cdks was almost undetectable by co-immunoprecipitation analysis of the untreated log phase cells. However, the treatment of cells with taxol resulted in a significant increase in the binding of Cdk2 and Cdc2 with p21 in both cell lines.

Taxol stimulates p21 promoter activity. Since p21 expression was markedly induced by taxol in the MCF-7 and MDA-MB-231 cells, we subsequently investigated whether the upregulation of p21 expression by taxol involves the transcriptional regulation of the p21 gene promoter. The cells were transiently transfected with wild-type p21 promoter-luciferase fusion plasmids, and luciferase activity was measured in the untreated control cells and in the cells treated with taxol (Fig. 5). Following exposure to taxol, the activity of the p21 promoter was time-dependently activated in both cell lines.

Discussion

Taxol is one of the most effective antitumor agents currently used in the treatment of drug refractory tumors (19-21). This

drug is a potent inhibitor of microtubule depolymerization, which results in the formation of abnormally stable and non-functional microtubules, mitotic arrest at the metaphase of the cell cycle and apoptotic cell death (22,23). However, the precise molecular mechanism of taxol-induced apoptosis remains to be fully elucidated. The purpose of the present study was to investigate the mechanisms involved in the inhibition of proliferation and the induction of apoptosis by taxol in the ER-positive MCF-7 and ER-negative MDA-MB-231 human breast carcinoma cell lines. Taxol similarly inhibited the growth of the ER-positive and -negative cells (Fig. 1). Flow cytometry confirmed that this growth inhibition was associated with G2/M cell cycle arrest up to 24 h after taxol exposure in both cell lines (Table I).

Apoptosis is an active cellular death process induced by normal physiological or pathological factors for the elimination of unwanted or damaged cells. It has been proposed that DNA fragmentation results from the loss of the compartmentalization of DNase I, which would reach the nucleus due to the breakdown of the endoplasmic reticulum and the nuclear membrane (29). Fragmentation of DNA at the internucleosomal linker regions has been observed in cells undergoing apoptosis induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180-200 bp) (30,31). Since their characteristic patterns are shown by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. The cleavage of PARP has also been used as a marker of chemotherapy-induced apoptosis (32). In addition, flow cytometry analysis has been conducted to detect and quantify cells undergoing apoptosis (12). An increase in the number of apoptotic cells was recognized following exposure to taxol by flow cytometry analysis, chromatin condensation, cleavage of PARP and DNA ladder formation (Fig. 2). At 48 h, flow cytometry demonstrated the highest peak of sub-G1 fraction, implying that the apoptotic cells outnumbered the cells arrested in G2/M. Thus, taxol arrested the examined cells in G2/M, and then induced apoptosis.

In eukaryotic cells, two cyclins, cyclin A and cyclin B, play a critical role in regulating cell cycle progression from the G2 through the M phase, including exit from the M phase. Cyclin A is associated with and primarily activates Cdk2 in the regulation of S and G2/M phase transition (1,2). It has been shown that entry into and exit from the M phase of the cell cycle are controlled by fluctuations in the activity of MPF, of which Cdc2 is the catalytic subunit and cyclin B is a regulatory element. Cyclin B accumulates during the S and G2 phases, and its induction and activation of MPF are necessary to initiate the transition from the G2 to the M phase. The cyclin B/Cdc2 complex allows for the phosphorylation of Cdc2 at several sites, maintaining it in an inactive state and ensuring that mitosis does not occur prematurely (3,4). This phosphorylation is regulated by protein kinases, such as Wee1 (5). Based on these data, we investigated the effects of taxol on the expression of Cdk2, Cdc2, cyclin A, cyclin B and Wee1 proteins using western blot analysis. Taxol did not affect the levels of Cdk2 and Cdc2 expression; however, the expression levels of cyclin A and cyclin B1 decreased after 24 h (Fig. 3), suggesting that the synthesis of cyclin A and cyclin B1 was suppressed following the onset of taxol-induced apoptosis. Wee1 kinase protein expression also decreased in a time-dependent manner in both cell lines (Fig. 3). These results demonstrate that the G2/M arrest of the cell cycle and apoptosis are associated with the inhibition of cyclin A and cyclin B1 expression, as well as a decrease in Wee1 expression.

Wild-type p53 protein arrests DNA damaged cells in the late G1 phase by inducing the Cdk inhibitor, p21 (7,8), and non-repaired cells may be eliminated by apoptosis by inducing Bax and repressing Bcl-2 activity (33). It has been suggested that the ratio between the levels of pro-apoptotic Bax protein and the anti-apoptotic factor, Bcl-2, determines whether a cell responds to an apoptotic signal (34). However, Tang *et al* (35) found that Bcl-2 did not affect taxol-induced microtubular binding and G2/M arrest in human pre-B leukemia cells, although it did delay the induction of apoptosis. Thus, it is possible that apoptosis provoked by DNA damage is mediated, in other cases, by mechanisms independent of p53 (36). On the contrary, an increased level of p21 in cyclin-containing complexes is associated with decreasing cyclin-dependent activity in damaged cells destined for apoptosis.

In addition to being induced by p53, p21 is also be induced by other factors in p53-independent pathways (2,9,10). However, the mechanism of p21-involved apoptosis occurring in the G2/M phase is not yet well understood. We therefore investigated whether taxol can induce the levels of p53 and p21. As shown in Fig. 5A, the levels of both p21 and p53 protein expression significantly increased after 24 h in the MCF-7 cells, which harbor wild-type p53. However, MDA-MB-231 cells harbor mutant p53 and express abundant mutant p53 protein (37). The treatment of MDA-MB-231 cells with taxol did not change the level of mutant p53, whereas p21 activity was markedly induced by taxol (Fig. 4A). The p21 proteins levels that increased due to 36-h treatment with taxol were significantly associated with Cdk2 and Cdc2 (Fig. 4B). Since p21 expression was markedly induced by taxol in MCF-7 and MDA-MB-231 cells, we subsequently investigated whether the upregulation of p21 expression by taxol involves the transcriptional regulation of the p21 gene promoter. Both cell lines were transiently transfected with wild-type p21 promoter-luciferase fusion plasmids, and luciferase activity was measured in both the untreated control cells and the cells treated with taxol (Fig. 5). Following 24 h of exposure to taxol, promoter activity increased by approximately 5.5-fold compared to the control in both cell lines. Since p21 is usually associated with G1 arrest, the G2/M arrest and apoptosis noted in this study may be specifically related to a unique taxol pathway.

In conclusion, the findings from our present study demonstrate that taxol has a signficant and similar inhibitory effect on cell proliferation in both ER-positive and -negative human breast carcinoma cell lines. This inhibitory effect on cell proliferation is the result of G2/M arrest and is accompanied by the downregulation in the expression of G2/M regulatory proteins, such as cyclin A, cyclin B1 and Wee1. The anti-proliferative effect of taxol also resulted in the onset of apoptosis, which followed the induction of p21 activity and G2/M arrest. It is possible that the induction of p21 activity occurs through a p53-independent pathway, and it may be one of the molecular mechanisms through which taxol induces apoptosis. Thus, taxol-induced cell death may provide a model of the apoptosis that occurs in the G2/M phase in an ER-independent manner,

which is associated with upregulation of p21 in a p53-independent manner.

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