Cell Cycle–Dependent Antagonistic Interactions between Paclitaxel and γ-Radiation in Combination Therapy

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ABSTRACT

Purpose: The promising clinical activity of paclitaxel, a naturally occurring antimitotubule agent, has promoted considerable interest in combining this drug with radiation therapy, but it remains unclear whether such a combination would increase the therapeutic efficacy. This study is to assess the potential interactions between paclitaxel and γ-radiation against human tumor cells in vitro.

Experimental Design: Paclitaxel and γ-radiation were administered in three different sequences designated as pre-irradiated, co-irradiated, and post-irradiated to BCap37 (human breast cancer cell line) and KB (human epidermoid carcinoma cell line) cells. The cytotoxic interactions between and mutual influences of these two agents on their antitumor activities were analyzed by a series of assays including cytotoxic, morphological, and biochemical examinations.

Results: The combination of paclitaxel and γ-radiation did not produce a synergistic or additive effect. Instead, the overall in vitro cytotoxicity of these combinations was much lower than that of paclitaxel treatment alone. DNA fragmentation and flow cytometric assays showed that the addition of γ-radiation interfered with paclitaxel-induced apoptosis. Further analyses indicated that the addition of γ-radiation resulted in a transient or prolonged cell cycle arrest at G2 phase, which likely prevented the cytotoxic effects of paclitaxel on both mitotic arrest and apoptosis. In addition, biochemical examinations revealed that γ-radiation inhibited paclitaxel-induced IκBα degradation and bcl-2 phosphorylation and increased the protein levels of cyclin B1 and inhibitory phosphorylation of p34<sup>cdc2</sup>.

Conclusions: Our results suggest that γ-radiation might specifically block the cell cycle at G2 phase, which in turn prevents the cytotoxic effects of paclitaxel on both mitotic arrest and apoptosis. Therefore, it eventually results in a cell cycle-dependent antagonistic effect on the antitumor activity of paclitaxel. This finding may be relevant to the clinical application of combination therapy with paclitaxel and radiation.

INTRODUCTION

Paclitaxel (Taxol), a novel naturally occurring antineoplastic agent, has shown great promise in the treatment of a variety of tumor types including breast, head and neck, ovarian, and lung cancers (1–3). Although the exact mechanism of the cytotoxicity of paclitaxel against tumor cells is not entirely clear, previous studies demonstrated that paclitaxel is a unique antimitotubule agent that acts by inhibiting microtubule depolymerization and thereby disrupting the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation (4, 5). Thus, it was generally believed that the antitumor effects of paclitaxel result mainly from interference with the normal function of microtubules and the blockage of cell cycle progression in late G2-M phase via prevention of mitotic spindle formation. On the other hand, recent studies have shown that paclitaxel is able to induce internucleosomal DNA fragmentation and the typical morphological features of apoptosis in a number of tumor cell lines (6), indicating that paclitaxel also possesses cell-killing activity by induction of apoptosis.

Recently, the combination of paclitaxel and radiation therapy has been of greater interest in both research and clinical settings. Ideally, combination therapy with these two agents or modalities would increase the therapeutic efficacy via synergistic or additive effects. Because cells at the G2-M phase were considered to be excessively radiosensitive (7–9), it was theorized that the combination of paclitaxel with radiation would behave synergistically due to the ability of paclitaxel to arrest cells at the G2-M phase. However, studies in a variety of tumor cell lines (10–18), animal models (13, 19), and some clinical investigations (3, 20–22) have produced contradictory results. Some investigators reported that paclitaxel enhanced the efficacy of radiation therapy (10–15, 19), whereas others suggested that the combination of paclitaxel and radiation might not result in any synergistic or additive effects (11, 14, 16–18), although the potential mechanisms involved in these responses have seldom been explored and still remain unclear.

In this study, we assessed the in vitro interaction between paclitaxel and γ-radiation in BCap37 (human breast cancer cell line) and KB (human epidermoid carcinoma cell line) cells. These two modalities were administered in three different sequences, so that radiation treatment was either before, concurrent with, or after paclitaxel treatment. Meanwhile, the interactions between γ-radiation and two other promising anticancer drugs, cisplatin and doxorubicin, were also evaluated and compared with paclitaxel. The interactions between these drugs and radiation were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, clonogenic assay,
DNA fragmentation, flow cytometric analysis, and morphological examinations. We found that the combination of paclitaxel and \( /H9253\)-radiation did not show any synergistic or additive effect. Instead, such a combination resulted in a cell cycle-dependent antagonistic interaction between paclitaxel and \( /H9253\)-radiation.

**MATERIALS AND METHODS**

**Cell Culture and Drugs.** The human breast cancer BCap37 cell line and the human epidermoid carcinoma KB cell line (American Type Culture Collection, Manassas, VA) were cultured in 96-well microculture plates. Cells were treated with 2 or 10 Gy of radiation, 100 nM paclitaxel, 40 \( \mu \)M cisplatin, 2.5 \( \mu \)M doxorubicin alone or their combinations at various schedules. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed 48 h after exposure to anticancer drugs as described in “Materials and Methods.” Data presented in the bar graphs are means ± SD, based on three independent experiments. CTL, control; PTX, paclitaxel; R2, 2 Gy of radiation; R10, 10 Gy of radiation; Pre-R, cells exposed to radiation 12 h before drug treatment; Co-R, cells treated simultaneously with radiation and drug; Post-R, cells exposed to radiation 12 h after drug treatment.

**Fig. 1** The combination of \( \gamma \)-radiation and paclitaxel decreases the overall cytotoxicity of paclitaxel. Approximately \( 5 \times 10^4 \) BCap37 and KB cells were cultured in 96-well microculture plates. Cells were treated with 2 or 10 Gy of radiation, 100 nM paclitaxel, 40 \( \mu \)M cisplatin, 2.5 \( \mu \)M doxorubicin alone or their combinations at various schedules. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed 48 h after exposure to anticancer drugs as described in “Materials and Methods.” Data presented in the bar graphs are means ± SD, based on three independent experiments. CTL, control; PTX, paclitaxel; R2, 2 Gy of radiation; R10, 10 Gy of radiation; Pre-R, cells exposed to radiation 12 h before drug treatment; Co-R, cells treated simultaneously with radiation and drug; Post-R, cells exposed to radiation 12 h after drug treatment.

**MTT Assay.** Cell cytotoxicity due to drug and \( \gamma \)-radiation was determined by the MTT assay. Briefly, cells were harvested by trypsinization and resuspended to a final concentration of \( 5 \times 10^4 \) cells/ml in fresh medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Aliquots of the cell suspension (150 \( \mu \)l/well) were evenly distributed into 96-well tissue culture plates (Falcon, Oxnard, CA) with lids. After

**Drugs and \( \gamma \)-Radiation Treatment.** Both BCap37 and KB cells were treated with a single drug, with or without radiation, using different sequences and time points. We designated the time point at which the tumor cells were exposed to paclitaxel (or other drugs) as 0 h. The radiated groups were subjected to a single dose of 2 or 10 Gy of \( \gamma \)-radiation at room temperature, using the model 143 irradiator (JL Shepherd & Associates, San Fernando, CA). Three different sequences or schedules were assessed, in which the tumor cells were exposed to either 2 or 10 Gy \( \gamma \)-radiation 12 h before drug treatment (pre-radiated), simultaneously with drug treatment (co-radiated), or 12 h after drug treatment (post-radiated).

**Fig. 1** The combination of \( \gamma \)-radiation and paclitaxel decreases the overall cytotoxicity of paclitaxel. Approximately \( 5 \times 10^4 \) BCap37 and KB cells were cultured in 96-well microculture plates. Cells were treated with 2 or 10 Gy of radiation, 100 nM paclitaxel, 40 \( \mu \)M cisplatin, 2.5 \( \mu \)M doxorubicin alone or their combinations at various schedules. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed 48 h after exposure to anticancer drugs as described in “Materials and Methods.” Data presented in the bar graphs are means ± SD, based on three independent experiments. CTL, control; PTX, paclitaxel; R2, 2 Gy of radiation; R10, 10 Gy of radiation; Pre-R, cells exposed to radiation 12 h before drug treatment; Co-R, cells treated simultaneously with radiation and drug; Post-R, cells exposed to radiation 12 h after drug treatment.

**MATERIALS AND METHODS**

**Cell Culture and Drugs.** The human breast cancer BCap37 cell line and the human epidermoid carcinoma KB cell line (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco Life Technologies Inc., Rockville, MD). Paclitaxel was purchased from Mead Johnson Co. (Princeton, NJ) and stored at 4°C in the dark. Cisplatin and doxorubicin were purchased from Sigma (St. Louis, MO) and dissolved in 100% DMSO (Sigma) and distilled water, respectively. Cisplatin and doxorubicin were stored at −20°C and protected from light. All drugs were diluted in culture medium to obtain the desired concentrations.
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Materials and Methods.  

Clonogenic Assay. As described in “Materials and Methods,” BCap37 cells were exposed to the indicated doses of γ-radiation, paclitaxel, or both agents simultaneously. After 48 h of treatment, all of the plates were centrifuged to save all of the cells, followed by two washes with fresh medium. The cells were then incubated in paclitaxel-free medium for 2 weeks, and colonies were counted for calculation of surviving fractions.  

Survival curves of the cells treated with a range of paclitaxel concentrations (2–100 nM) and radiation doses (2–10 Gy).  

Surviving fraction of the cells treated with the indicated doses of paclitaxel and in combination with the indicated doses of γ-radiation. * P < 0.001; ** P < 0.05 (compared with the group treated with paclitaxel alone).

Fig. 2 Clonogenic assays. As described in “Materials and Methods,” BCap37 cells were exposed to the indicated doses of γ-radiation, paclitaxel, or both agents simultaneously. After 48 h of treatment, all of the plates were centrifuged to save all of the cells, followed by two washes with fresh medium. The cells were then incubated in paclitaxel-free medium for 2 weeks, and colonies were counted for calculation of surviving fractions.  

Surviving fraction of the cells treated with the indicated doses of paclitaxel and in combination with the indicated doses of γ-radiation. * P < 0.001; ** P < 0.05 (compared with the group treated with paclitaxel alone).

one night of incubation, the designated columns were treated with drug regimes, γ-radiation, or a combination of both at different sequences. At the end of each time point, the 96-well plates were centrifuged to collect all of the detached cells, and the medium was removed carefully. Then, 100 μl of 0.5 mg/ml MTT solution, diluted in culture medium, were added to each well. The plates were incubated at 37°C in a 5% CO2 atmosphere for 4 h, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. At the end of 4 h of incubation, the MTT solution was removed, and 100 μl of DMSO were added to each well to dissolve the formazan crystals. To ensure complete crystal dissolution, the plates were mixed gently at low speed for 20 min. The absorbance in individual wells was determined at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Clonogenic Assay. The cytotoxicity of paclitaxel, γ-radiation, and their combinations was also evaluated with clonogenic assays according to the method described by Dunne et al. (23). Briefly, cells were plated on 6-well plates (35 mm) with 200–10,000 cells/well based on the dose of radiation and the concentration of paclitaxel to achieve a concentration of 20–200 colonies/well. After plating, tumor cells were incubated for 8 h before exposure to γ-radiation, paclitaxel, and their combinations. After 48 h of treatment, all of the plates were centrifuged to save all of the cells, followed by two washes with fresh medium. The cells were then incubated in paclitaxel-free medium for 2 weeks to allow colony formation. Colonies consisting of ≥50 cells were counted, and the surviving fractions were calculated after correction for plating efficiency of control cells.

Detection of Internucleosomal DNA Fragmentation. After exposure to various drugs and radiation, cells were harvested and suspended in lysis solution [5 mM Tris-HCl, 20 mM EDTA, and 0.5% (v/v) Triton X-100] for 30 min on ice. Crude DNA samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1). The remaining steps for detection of DNA fragmentation were performed as described previously (24). DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.2 μg/ml ethidium bromide and visualized under UV illumination.

Light Microscopy Examination and Cytospin Preparation. Cells were cultured in 6-cm dishes and treated with drugs and radiation as described above. At the end of each time point, duplicate dishes in each group were examined and photographed using a regular phase-contrast microscope. For cytospin preparation, both detached and attached cells were collected or harvested by trypsinization and washed twice with PBS. Cell numbers were determined with a hemocytometer, and approximately 0.5–1 × 105 cells from each group were plated onto microscope slides using a Cytospin 3 cell preparation system (Shandon, Pittsburgh, PA). As described previously (25), slides were air dried and fixed in absolute methanol before Giemsa staining. Slides for various groups were examined and photographed using bright-field microscopy. The number of mitotic cells was also counted under the microscope for comparisons among different groups.

Flow Cytometric Analysis. Cell cycle progression and apoptosis were determined by flow cytometric analysis. Cell sample preparation, including propidium iodide staining, was performed according to the method described by Nicoletti et al. (26). Briefly, at the end of each time point, both detached and attached cells were harvested and washed twice with PBS, followed by fixation in 70% ethanol diluted in PBS. Cells were then incubated in PBS containing 100 μg/ml RNase and 40 μg/ml propidium iodide at room temperature for 0.5–1.0 h before flow cytometry analysis. Cell cycle distribution and DNA content were determined using a Coulter Epics V instrument (Beckman Coulter, Inc., Fullerton, CA) with an argon laser set to excite at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, CA).

Western Blotting. After various treatments, BCap37 cells were harvested and washed twice with PBS. Cellular protein was isolated with a protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Protein concentrations were determined with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Equal amounts (40 μg/lane) of proteins were fractionated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated
with anti-IκBα (Santa Cruz Biotechnology, CA), bcl-2 (DAKO Corp.), p34<sup>cdc2</sup> (Sigma), and cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies, respectively. After washing with PBS containing 0.1% (v/v) Tween 20, the membranes were incubated with peroxidase-conjugated goat antirabbit or antimouse secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) followed by enhanced chemiluminescent staining using the ECL system (Amersham Biosciences). β-Actin (Sigma) was used for normalization of protein loading.

**Statistical Analysis.** Student’s t test was used to determine the statistical difference between various experimental and control groups. P < 0.05 was considered significant.

**RESULTS**

**γ-Radiation Interferes with the Overall Cytotoxicity of Paclitaxel.** To evaluate potential synergistic or antagonistic interactions, we first performed MTT assays to examine the overall cytotoxicity of these anticancer drugs, with or without radiation. The results depicted in Fig. 1 show that the cell viability rates ranged from around 38% to 45%, respectively, in BCap37 and KB cells treated with 100 nM paclitaxel for 48 h. The in vitro cytotoxicity of 2 Gy of radiation alone on these tumor cells was relatively low. When these cells were exposed to 10 Gy of γ-radiation at various schedules, the cell viability rates were around 80–85% at 48 h (Fig. 1). However, the overall cytotoxicity of paclitaxel was dramatically decreased in both BCap37 and KB cells when paclitaxel was combined with radiation, particularly when these cells were exposed to 10 Gy of γ-radiation (P < 0.001). This result indicates that the addition of γ-radiation significantly interferes with the cytotoxic effect of paclitaxel in vitro. In addition, as a comparison, similar MTT assays were also conducted to assess the possible influence of γ-radiation on the cytotoxicity of cisplatin and doxorubicin. The results, presented in Fig. 1, show that radiation has very little effect on the overall cytotoxicity of these two drugs (P > 0.05).

To further determine the inhibitory effect of γ-radiation on the cytotoxicity of paclitaxel, we performed clonogenic assays in both BCap37 and KB cells treated with a range of paclitaxel concentrations (2–100 nm) and radiation doses (2–10 Gy). As shown in Fig. 2A, the survival fraction of BCap37 cells after exposure to these two agents is essentially dose dependent. Although treatment with 4 Gy of radiation and 10 nm paclitaxel seemed to slightly increase the overall cytotoxicity, the antagonistic interaction was clearly observed in all groups treated with higher concentrations of paclitaxel (50 and 100 nm) in combination with 2–10 Gy of radiation (Fig. 2, A and B). Similar results were also observed in KB cells (data not shown).

**Combination of Radiation Inhibits Paclitaxel-Induced Apoptotic Cell Death.** Next, we investigated the possible influence of γ-radiation on paclitaxel-induced apoptosis. An important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180-bp units, producing a characteristic ladder on agarose gel. Thus, we first performed a DNA fragmentation assay to determine whether paclitaxel-induced apoptosis would be affected by the addition of radiation. As reported previously (24, 27–29), the characteristic DNA fragmentation ladders were observed in both BCap37 and KB cells after treatment with 100 nm paclitaxel for 48 h (Fig. 3, Lanes 3). When these cells were exposed to radiation alone (either 2 or 10 Gy), essentially no clear DNA fragmentation was observed. However, on combination treatment with paclitaxel and radiation, particularly when these cells were pre-exposed or co-exposed to 10 Gy of γ-radiation, paclitaxel-induced DNA fragmentation was dramatically inhibited (Fig. 3, Lanes 7 and 11), suggesting that the exposure to radiation could repress paclitaxel-induced apoptotic cell death in both tumor cell lines. Furthermore, a flow cytometric assay was performed to analyze the kinetic changes of cell cycle distribution and apoptotic cell death in BCap37 cells treated with paclitaxel, radiation, and a combination of the two. The results depicted in Fig. 4 indicate that paclitaxel first caused cell cycle arrest at the G<sub>2</sub>-M phase. This was clearly seen at 24 h, whereas the apoptotic peak (Ap) was not detected at this time point. The apoptotic peak, which represents apoptotic cell population, was clearly observed after BCap37 cells were treated with 100 nm paclitaxel for 48 h. However, this apoptotic peak was dramatically decreased when the cells were exposed to paclitaxel combined with 10 Gy of γ-radiation in all three schedules (Fig. 4). From the flow cytometric assay, we also see that paclitaxel-induced apoptosis was slightly reduced by combination with 2
Gy of radiation (Fig. 4). Similar flow cytometric results were observed with the KB cell line (data not shown).

γ-Radiation Induces G2 Arrest That Prevents Paclitaxel-Induced Mitotic Arrest. In the above-mentioned flow cytometric assay, we noted that radiation alone also caused an elevated G2-M peak (Fig. 4). It seems that γ-radiation, like paclitaxel, may also induce cell cycle arrest at the G2-M phase. Interestingly, such a phenomenon was not observed when we conducted morphological examinations. As shown in Fig. 5, when BCap37 cells were treated with 100 nM paclitaxel, a significant number of apparent mitotic cells (rounded and detached from the dish) were observed beginning 12 h after drug treatment, and the number of these cells increased continuously until 48 h. However, such rounded and detached mitotic cells were not observed in the cells treated with radiation alone (Fig. 5), although an elevated G2-M peak was clearly detected by flow cytometric assay. Moreover, when these cells were treated with a combination of paclitaxel and 10 Gy of radiation, the number of mitotically arrested cells was significantly decreased in comparison with that seen after treatment with paclitaxel alone (Fig. 5). This phenomenon suggests that γ-radiation may arrest the cell cycle at the G2 phase, but not the mitotic phase. Instead, this blockage of cells at the G2 phase may then prevent cells from entering M phase. To confirm this hypothesis, we prepared cytospin slides in which the mitotically arrested cells were easily identified by their morphological features, e.g., condensed chromosomes. Through bright-field microscopy, we...
found that, compared with BCap37 cells treated with paclitaxel alone, the number of cells arrested at mitotic phase was significantly decreased when paclitaxel was combined with 10 Gy of radiation (Fig. 6). Furthermore, we counted those cells that appeared to contain condensed chromosomes, i.e., mitotic figures, and summarized these results in Table 1. The statistical analysis of the percentage of mitotic cells indicated that there were significant differences \((P < 0.001)\) between treatment with paclitaxel alone and combination treatment of paclitaxel with 2 or 10 Gy of \(\gamma\)-radiation at various schedules, with the exception of the combination of paclitaxel and 2 Gy of pre-radiation. These results suggested that \(\gamma\)-radiation might specifically arrest cells at \(G_2\) phase, which in turn prevented the effect of paclitaxel on mitotic arrest.

\(\gamma\)-Radiation Up-Regulates Phosphorylated p34\(^{cdk2}\) and Inhibits Paclitaxel-Induced I\(\kappa\)B\(\alpha\) Degradation and bcl-2 Phosphorylation. The above-mentioned data reveal that \(\gamma\)-radiation antagonizes the cytotoxic effect of paclitaxel on both mitotic arrest and apoptotic cell death. To investigate the potential mechanisms and genes that may be involved in these processes, we examined several regulatory proteins associated with the \(G_2\)-M phase of the cell cycle and paclitaxel-induced apoptosis. We first analyzed the possible alterations of bcl-2 and I\(\kappa\)B\(\alpha\) that were previously reported to play important roles in paclitaxel-induced apoptosis (30–34). The results depicted in Fig. 7 show that radiation alone has no effect on either I\(\kappa\)B\(\alpha\) or bcl-2 (Fig. 7A). As reported previously (31, 33, 34), paclitaxel caused bcl-2 phosphorylation and degradation of I\(\kappa\)B\(\alpha\), but the combination of paclitaxel and \(\gamma\)-radiation (except for the group treated with paclitaxel and 2 Gy of pre-radiation) could significantly block paclitaxel-induced I\(\kappa\)B\(\alpha\) degradation and bcl-2 phosphorylation (Fig. 7B). On the other hand, we also examined two important \(G_2\)-M checkpoint proteins, p34\(^{cdk2}\) and cyclin B1 (35–38). The results indicated that the protein levels of phosphorylated p34\(^{cdk2}\) increased in BCap37 cells after exposure to...
radiation for 12, 24, and 36 h (Fig. 7A), but this was not seen in cells treated with paclitaxel alone for 24 h (Fig. 7B). However, the increased phosphorylation of p34<sup>cdc2</sup> occurred when paclitaxel was combined with radiation at various schedules (Fig. 7B). In addition, compared with the control groups, increased protein levels of cyclin B1 were also observed in BCap37 cells treated with radiation or paclitaxel alone or a combination of the two (Fig. 7).

**DISCUSSION**

Combination therapy with multiple drugs or multiple modalities is a common practice in the treatment of cancer. The purpose of using different drugs or modalities in combination is to achieve therapeutic effects greater than those provided by a single drug or modality alone. Paclitaxel is a naturally occurring antimicrotubule agent that has shown great promise in the treatment of a variety of human cancers. Its favorable clinical profile has also promoted considerable interest in combining paclitaxel with other antineoplastic agents/modalities, including radiation therapy. The combination therapy of paclitaxel plus radiation is under extensive investigation on the rationale that paclitaxel is able to arrest cells at the G<sub>2</sub>-M phase, which is the most radiosensitive phase of the cell cycle (7–9). Previously, it was generally believed that paclitaxel might act cooperatively with radiation, serving as a radiosensitizer and resulting in supra-additive or even synergistic effects (11–15, 19). However, the accumulating literature did not always support this hypothesis. Many investigators reported that paclitaxel combined with radiation might not result in any synergistic or additive effects (11, 14, 16–18), although the mechanisms involved in these interactions are not entirely clear.

Fig. 6 The combination of radiation and paclitaxel prevents paclitaxel-induced mitotic arrest. BCap37 cells were treated with 100 nM paclitaxel, with or without radiation, for 24 h. The cells were then harvested for preparation of cytosin slides as described in “Materials and Methods.” The slides were examined and photographed with a light microscope. The mitotic cells exhibit the typical morphological feature of condensed chromosomes. Magnification, ×40.
presented as the mean

radiation can also cause cell cycle perturbations such as G1 or delayed G2-M phase delay (39). Indeed, from the flow cytometric analysis, we observed that both radiation, especially 10 Gy of radiation, interfered with the effects of paclitaxel on both mitotic arrest and apoptotic cell death (see the elevated G2-M peak in Fig. 4). Interestingly, far fewer cells underwent apoptosis in the groups exposed to the combination of paclitaxel and radiation, even though the high G2-M peak was clearly observed. This result indicates that the G2-M arrest is not sufficient to produce cooperative interaction between these two agents. Moreover, by morphological examination, we also noted that there were far fewer mitotic cells (cells that were rounded and detached from the bottom of culture dishes) in the group treated with both paclitaxel and radiation. This phenomenon implied that radiation might actually interfere with the ability of paclitaxel to induce mitotic arrest. Because flow cytometric assay is unable to separate the G2 phase from the mitotic phase, we prepared cytospin slides, by which we demonstrated that the combination of radiation and paclitaxel significantly reduced the number of mitotic cells. These findings indicate that γ-radiation caused only G2 arrest but not mitotic arrest, although a similar G2-M peak was also observed in flow cytometry. Obviously, radiation caused cell cycle arrest at G2 phase, which subsequently prevented paclitaxel-induced mitotic arrest and apoptotic cell death. These results may explain, in part, the phenomenon that the combination of radiation and paclitaxel results in a cell cycle-dependent interaction between these two agents.

In the current study, we have carefully evaluated the therapeutic effect of paclitaxel with or without the addition of γ-radiation in two paclitaxel-sensitive human tumor cell lines (28, 29). Our results showed that the overall cytotoxicity generated by the combination of paclitaxel and γ-radiation was significantly less than that produced by paclitaxel alone, particularly at higher concentrations (50–100 nM) of paclitaxel (Figs. 1 and 2). Further analyses revealed that the addition of radiation interfered with the effects of paclitaxel on both mitotic arrest and apoptotic cell death (see Figs. 3–5). These findings implied that the combination of γ-radiation and paclitaxel might not show an additive or synergistic effect in vitro. Instead, the interaction between paclitaxel and γ-radiation might result in an antagonistic effect in all three different sequences between the two modalities. Thus, our findings have also raised a clinically relevant question as to whether combination therapy with paclitaxel can really increase therapeutic efficacy.

Previously, studies on the combination of paclitaxel and radiation therapy focused on whether paclitaxel can potentiate radiation therapy, but little attention was given to the possible influence of radiation on the cytotoxic effects of paclitaxel. Because cells at G2-M phase are considered to be exquisitely sensitive to radiation (7–9), many investigators believe that the combination of paclitaxel and radiation may produce an additive or synergistic effect due to the ability of paclitaxel to induce cell cycle arrest at the G2-M phase. In fact, it is well known that radiation can also cause cell cycle perturbations such as G2 or G2-M phase delay (39). Indeed, from the flow cytometric analyses, we observed that both radiation, especially 10 Gy of γ-radiation, and paclitaxel increased the cell population at G2-M phase (see the elevated G2-M peak in Fig. 4). Interestingly, far fewer cells underwent apoptosis in the groups exposed to the combination of paclitaxel and radiation, even though the high G2-M peak was clearly observed. This result indicates that the G2-M arrest is not sufficient to produce cooperative interaction between these two agents. Moreover, by morphological examination, we also noted that there were far fewer mitotic cells (cells that were rounded and detached from the bottom of culture dishes) in the group treated with both paclitaxel and radiation. This phenomenon implied that radiation might actually interfere with the ability of paclitaxel to induce mitotic arrest. Because flow cytometric assay is unable to separate the G2 phase from the mitotic phase, we prepared cytospin slides, by which we demonstrated that the combination of radiation and paclitaxel significantly reduced the number of mitotic cells. These findings indicate that γ-radiation caused only G2 arrest but not mitotic arrest, although a similar G2-M peak was also observed in flow cytometry. Obviously, radiation caused cell cycle arrest at G2 phase, which subsequently prevented paclitaxel-induced mitotic arrest and apoptotic cell death. These results may explain, in part, the phenomenon that the combination of radiation and paclitaxel results in a cell cycle-dependent interaction between these two agents.
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antagonistic interaction between these two agents. As a comparison, this study also examined the possible interaction of γ-radiation with cisplatin or doxorubicin. The results show that γ-radiation has little effect on either cisplatin or doxorubicin (Fig. 1). Because the antitumor effects of cisplatin and doxorubicin are caused mainly by interfering with the integrity of DNA, either by physical binding to DNA bases or by initiation of DNA damage via inhibition of topoisomerase II (40–43), they are much less cell cycle dependent and may induce cell death during all phases of the cell cycle. Therefore, in contrast to the interaction of paclitaxel and radiation, the addition of γ-radiation exhibited little influence on the antitumor activity of cisplatin or doxorubicin.

To investigate the possible mechanisms by which radiation inhibits paclitaxel-induced mitotic arrest and apoptosis, we have examined a number of genes or regulatory proteins that may contribute to paclitaxel activity in inducing cell cycle arrest and apoptosis. Fig. 6 presents four of these proteins (bcl-2, IkBα, p34<sup>cdc2</sup>, and cyclin B1) whose expression or phosphorylation was obviously regulated by radiation or paclitaxel. In previous studies, paclitaxel was found to cause bcl-2 phosphorylation, and this modification was believed to cause attenuation of bcl-2 antiapoptotic activity (30, 31). IkBα is the specific cytoplasmic inhibitory protein of transcription factor nuclear factor κB, and degradation of IkBα is generally believed to be the critical step for the activation of nuclear factor κB (44). Recent studies have suggested that activation of the nuclear factor κB/IκBα signal pathway may play an active role in the mediation of paclitaxel-induced apoptosis (33, 34). By Western blotting, we observed that paclitaxel caused bcl-2 phosphorylation and IkBα degradation, whereas γ-radiation alone was found to affect neither IkBα nor bcl-2 protein. However, the addition of γ-radiation (except for the combination of paclitaxel and 2 Gy of pre-radiation) could significantly block paclitaxel-induced IkBα degradation and bcl-2 phosphorylation. These results may explain in part the interference of γ-radiation with paclitaxel-induced apoptosis. In addition, it is well known that p34<sup>cdc2</sup> and cyclins play key roles in cell cycle control (35). Although the G2-M block after exposure to radiation appears to result from multiple mechanisms, it is widely believed that the p34<sup>cdc2</sup>/cyclin B1 complex plays a critical role (36). During the G2 phase, p34<sup>cdc2</sup> forms a heterodimeric complex with cyclin B1 but remains inactive by phosphorylation of p34<sup>cdc2</sup> on tyrosine 15 and threonine 14 residues (35, 36). The p34<sup>cdc2</sup>/cyclin B1 complex could be activated by dephosphorylation of p34<sup>cdc2</sup> on these inhibitory residues, and this step is indispensable for the entry to mitosis (37). Cyclin B1 is periodically expressed in the cell cycle and accumulates in both G2 phase and mitotic phase (35, 36). Our results indicate that radiation causes increased levels of both cyclin B1 and inhibitory phosphorylated p34<sup>cdc2</sup>, which coincides with radiation-induced G2 arrest after radiation. After 24 h of paclitaxel treatment, most of the cells were already arrested in mitotic phase, thus the inhibitory phosphorylation state of p34<sup>cdc2</sup> was absent, whereas the elevated levels of cyclin B1 can still be detected. However, when paclitaxel was combined with radiation at various schedules, the increased inhibitory phosphorylation of p34<sup>cdc2</sup> arose, which is consistent with the blockage of paclitaxel-induced mitotic arrest by radiation-induced G2 arrest. Moreover, activation of the p34<sup>cdc2</sup>/cyclin B1 complex has been suggested to play an important role in paclitaxel-induced apoptosis (38). These findings may provide additional evidence on the molecular mechanism for the cell cycle-dependent antagonistic effect between paclitaxel and radiation therapy.

In summary, this study has evaluated the interaction between paclitaxel and radiation therapy in combination against human cancer cells in vitro. Our results showed that the combination of paclitaxel and γ-radiation resulted in a significant decrease in overall cytotoxicity in comparison with treatment with paclitaxel alone. Further analyses demonstrated that the addition of γ-radiation interfered with the cytotoxic effects of paclitaxel on both mitotic arrest and apoptosis. Through morphological examination, cytospin preparation, and flow cytometric analyses, our results indicate that γ-radiation caused a transient or prolonged cell cycle arrest at the G2 phase, which may in turn prevent the cytotoxic effects of paclitaxel on both mitotic arrest and apoptosis. In addition, this study also found that γ-radiation was able to inhibit paclitaxel-induced IkBα degradation and bcl-2 phosphorylation and increase the protein levels of cyclin B1 and inhibitory phosphorylation of p34<sup>cdc2</sup>, which may provide further proof for the cell cycle-dependent interaction between the two modalities. Taken together, these findings suggest that γ-radiation might specifically block the cell cycle at the G2 phase and thereby result in a cell cycle-dependent antagonistic effect on the antitumor activity of paclitaxel, which may be relevant to the clinical application of combination therapy of paclitaxel and radiation.

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