Paclitaxel-induced Apoptosis May Occur without a Prior G\textsubscript{2}/M-Phase Arrest

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Abstract. Background: Paclitaxel, a naturally occurring antineoplastic agent, can cause both mitotic arrest and apoptotic cell death, but the relationship between these two events is not entirely clear. The focus of this study was to determine whether apoptosis induced by paclitaxel may occur independent of mitotic arrest and to examine the underlying molecular mechanisms of paclitaxel-induced apoptosis. Materials and Methods: Two paclitaxel-sensitive tumor cell lines, human breast cancer BCap37 and human epidermoid carcinoma KB cells, were pulsed exposed to various concentrations of paclitaxel. Multiple methods were used to analyze the possible correlation between paclitaxel-induced apoptosis and paclitaxel-induced mitotic arrest. A series of assays were also performed in which we utilized parthenolide, a specific inhibitor of NF-\textit{kappa} B, to analyze the possible role that the NF-\textit{kappa} B/I\textit{kappa} B signal pathway has in mediating paclitaxel-induced apoptosis. Results: Both tumor cell lines treated with pulsed paclitaxel exposures exhibited a significant number of cells undergoing apoptosis, however many fewer cells were arrested at the \textit{G\textsubscript{2}/M}-phase of the cell cycle when compared to the continuous paclitaxel exposures. Short exposures to paclitaxel also induced the phosphorylation and degradation of I\textit{kappa} B-\alpha, which in turn caused the activation of NF-\textit{kappa} B/I\textit{kappa} B signaling pathway as well as apoptotic cell death. Conclusion: These findings suggest that paclitaxel-induced apoptosis might occur independent of a prior \textit{G\textsubscript{2}/M}-phase arrest and be mediated or regulated by the NF-\textit{kappa} B/I\textit{kappa} B signal pathway.

Abbreviations: NF-\textit{kappa} B, nuclear factor-kappa B; I\textit{kappa} B-\alpha, inhibitor-kappa B alpha; TX, paclitaxel; EMSAs, electrophoretic mobility shift assays; PBS, phosphate-buffered saline; PI, propidium iodide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

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Paclitaxel (Taxol\textsuperscript{[\textregistered]}) is a naturally occurring antineoplastic agent that is widely used in the treatment of a variety of solid tumors, including metastatic breast cancer and drug-refractory ovarian cancer (1-3). Previous studies indicate that paclitaxel is a unique antimicrotubule agent that acts by disrupting the normal microtubular network of the cell by inhibiting microtubular depolymerization and thereby forming unusually stable microtubules (4-6). This microtubular stabilization leads to a mitotic block in the late \textit{G\textsubscript{2}/M}-phase of the cell cycle and is generally believed to be the mechanism by which paclitaxel exerts its cytotoxic effects (7-9). However, the molecular events that are downstream from paclitaxel binding to microtubules are not clearly understood. In recent years, several laboratories have demonstrated that paclitaxel, at clinically relevant concentrations, is able to induce internucleosomal DNA cleavage, along with other morphological features typical of apoptosis in both solid and leukemic tumor cells (10-13). This clearly indicates that paclitaxel, along with the classic effects on microtubules and cell cycle arrest, also possesses cell-killing activity by the induction of apoptosis. However, it is currently unclear whether this finding represents a novel mechanism of action for paclitaxel, or just represents an end product of the well-known action of paclitaxel against microtubules and the cell cycle.

Recently, several lines of evidence have suggested that paclitaxel may induce apoptosis through a novel pathway independent of mitotic arrest (14-16). For example, previous studies have shown that paclitaxel binding to cytoplasmic microtubules is reversible (8). Briefly, when tumor cells were treated with \textsuperscript{[\textit{H}]}-paclitaxel for one hour and then washed and incubated in drug-free media, the binding of \textsuperscript{[\textit{H}]}-paclitaxel to microtubules was reversible after 90 minutes (8). This indicates that it is critical to maintain an intracellular concentration of paclitaxel to induce cytotoxicity. Studies from several other laboratories have also shown that low concentrations of paclitaxel induce apoptosis without a prior mitotic arrest (17-20). In addition, results in this laboratory have shown that baccatin III, the synthetic precursor of paclitaxel, could cause tumor cell apoptosis but had only minor effects on microtubule...
bundling and cell cycle arrest (21). All these results suggest that paclitaxel may induce apoptosis through a pathway independent of cell cycle arrest.

In this study, two paclitaxel-sensitive tumor cell lines, human breast cancer BCap37 and the human epidermoid carcinoma KB, were treated with pulsed exposures of paclitaxel and analyzed for the correlation between paclitaxel-induced apoptosis and paclitaxel-induced mitotic arrest. Further, we also investigated the possible involvement of the NF-κB/IκB signal pathway in the mediation of paclitaxel-induced apoptosis. Our results demonstrated that paclitaxel-induced apoptosis may occur independent of G2/M-phase arrest, and that the NF-κB/IκB signal pathway may mediate paclitaxel-induced apoptosis.

Materials and Methods

**Drugs and cell culture.** Paclitaxel and parthenolide were purchased from Sigma (St. Louis, MO, USA). Paclitaxel was dissolved in 100% DMSO to make a stock concentration of 100 μM and parthenolide was dissolved in 100% ethanol to make a stock concentration of 20 mM. The drugs were then diluted in culture media to obtain the desired concentrations when the cells reached approximately 60-70% confluence. The human breast cancer BCap37 cell line and the human epidermoid carcinoma KB cell line were propagated in RPMI 1640 supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Culture materials were obtained from Gibco/BRL Laboratories (Grand Island, NY, USA).

**Determination of internucleosomal DNA fragmentation.** Following incubation with the various drug regimes, approximately 1 x 10⁶ cells were harvested and suspended in lysis buffer containing 5 mM Tris-Cl pH 8.0, 20 mM EDTA and 0.5% (v/v) Triton X-100 for 20 minutes on ice. The remaining steps for DNA fragmentation were performed as previously described (31). DNA samples were analyzed by electrophoresis in a 1.4% agarose slab gel containing 0.2 μg/ml ethidium bromide and visualized under UV illumination.

**Flow cytometric analysis.** Cell sample preparation and PI staining were performed according to the methods described by Nicoletti *et al.* (32). At approximately 60-70% confluence, the cells were treated with the various drug regimes for 12 and 24 hours. The cells were collected and cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corp., USA) with an argon laser set to excite at 488 nm (24).

**Cytospin preparation and cell staining.** Cells were cultured to approximately 60-70% confluence in 60cm² dishes and treated with various drug regimes for the times indicated. Cells were harvested by trypsinization and lysed in hypotonic lysis buffer containing 10 mM HEPEs pH 7.8, 1.5 mM (MgCl₂)₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin and 2 μg/ml Pepstatin A (all Sigma). After a 10 minute incubation on ice, 25 μl of 10% Nonidet P-40 was added and vigorously mixed and centrifuged to collect the nuclei. A 50-μl volume of extraction buffer (10mM HEPEs, 0.5 mM (MgCl₂)₂, 10 mM KCl, 0.5 mM DTT, 0.5mM PMSF, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin and 2 μg/ml Pepstatin A) was added to the nuclear pellet, incubated on ice for 20 minutes, and centrifuged to produce supernatant containing nuclear proteins. Protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). EMSAs were performed using γ-32P-labeled double-stranded oligonucleotide probes containing the NF-κB binding sequence 5’-AGGGTTGAGGGAGTTTCCC-3’ (Santa Cruz). Probes were labeled with T4 polynucleotide kinase (Promega Co.) and [γ-32P]ATP (ICN) and purified using G-25 spin columns (Amersham Biosciences). EMSA binding reaction mixture contained 2 μg nuclear protein and 32P-labeled probe (3000 cpm) in binding buffer (10 mM HEPEs (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.2 μg/ml albumin). The binding reaction was incubated at room temperature for 30 minutes. Bound and free probes were then separated by electrophoresis on a 4% non-denaturing polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat AR film.

Western blot analysis. Cells were cultured to approximately 60-70% confluence in 60cm² dishes and treated with various drug regimes for the times indicated. Cells were harvested by trypsinization and lysed in 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 using Bio-Rad DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (~100 μg) were loaded into each lane and fractionated on a 10 or 12.5% SDS-PAGE gel. Following transfer to a PVDF membrane, the whole-cell fractionated proteins were immunoblotted with 3 μg/ml IκB rabbit polyclonal antibody or 1.5 μg/ml β-actin goat polyclonal antibody (Santa Cruz Bio.) diluted in 5% milk-PBS (5% dry milk powder-PBS-0.1% Tween-20). Immunoreactive bands were visualized using a chemiluminescence substrate for horseradish peroxidase (Amersham Biosciences) and exposed to Kodak X-OMAT AR film.

**Electrophoretic mobility shift assay (EMSA).** Cells were cultured to approximately 60-70% confluence in 60cm² dishes and treated with various drug regimes for the times indicated. Cells were harvested by trypsinization and lysed in hypotonic lysis buffer containing 10 mM HEPEs pH 7.8, 1.5 mM (MgCl₂)₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin and 2 μg/ml Pepstatin A (all Sigma). After a 10 minute incubation on ice, 25 μl of 10% Nonidet P-40 was added and vigorously mixed and centrifuged to collect the nuclei. A 50-μl volume of extraction buffer (10mM HEPEs, 0.5 mM (MgCl₂)₂, 10 mM KCl, 0.5 mM DTT, 0.5mM PMSF, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin and 2 μg/ml Pepstatin A) was added to the nuclear pellet, incubated on ice for 20 minutes, and centrifuged to produce supernatant containing nuclear proteins. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). EMSAs were performed using γ-32P-labeled double-stranded oligonucleotide probes containing the NF-κB binding sequence 5’-AGGGTTGAGGGAGTTTCCC-3’ (Santa Cruz). Probes were labeled with T4 polynucleotide kinase (Promega Co.) and γ-32P[ATP (ICN) and purified using G-25 spin columns (Amersham Biosciences). EMSA binding reaction mixture contained 2 μg nuclear protein and γ-32P-labeled probe (3000 cpm) in binding buffer (10 mM HEPEs (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol and 0.2 μg/ml albumin). The binding reaction was incubated at room temperature for 30 minutes. Bound and free probes were then separated by electrophoresis on a 4% non-denaturing polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat AR film.

**Immunofluorescence assays.** Cells were subcultured into Lab-Tek II Chamber Slide System (Nalge Nunc International). Upon reaching 60-70% confluence cells were treated with the various drug regimes. At the end of treatment the cells were fixed in 3.7% formaldehyde in PBS for 15 minutes at room temperature. Permeabilization was performed in 0.5% Triton X-100 in PBS for 15 minutes at room temperature. The slides were incubated with polyclonal antibody against human NF-κB subunit 65 (Santa Cruz Biotechnology, Inc.) at a dilution of 1:50 in 5% goat serum/0.1% Triton/PBS for 2 hours at room temperature with shaking. Immunoreactive NF-κB was revealed by one-hour incubation with streptavidin-FITC (1:100), diluted in 5% goat serum/0.1% Triton/PBS. Nuclei were visualized by incubating with the DNA...
The intercalating fluorescent marker Hoechst 33258 (0.4 ìg/ml) for 5 minutes. The cells were viewed and photographed with a Zeiss Axioplan epifluorescence microscope.

**Statistical analysis.** The Student’s *t*-test was used to determine the statistical differences between various experimental and control groups. A *p* value of <0.001 was considered significant.

**Results**

Short exposures to low doses of paclitaxel induce apoptosis in both BCap37 and KB cells. The fragmentation of genomic DNA is an important and unique feature of apoptotic cell death, producing a characteristic ladder on agarose gel electrophoresis. We first performed DNA fragmentation assays to determine if short exposures to paclitaxel induce apoptosis. As depicted in Figure 1A, tumor cells were cultured and split into four experimental groups. One group served as the control. The second group, labeled a one-hour pulse, was treated with various concentrations of paclitaxel for one hour; cells were then washed and replaced with drug-free media. After two hours the media was washed again and replaced with drug-free media. This allows time for any microtubule binding by paclitaxel to be reversed. The third group was treated with a three-hour pulsed exposure, which complements the one-hour exposure; only the cells were initially exposed to paclitaxel for three hours instead of one. The fourth group was treated with a continuous paclitaxel exposure. After the BCap37 and KB cells were treated with either pulsed or continuous paclitaxel exposures with the indicated concentrations of paclitaxel for 48 hours, DNA was extracted and analyzed for apoptosis by DNA fragmentation assay.

The results presented in Figure 1B and 1C show that the characteristic DNA fragmentation ladders were clearly observed following treatment of BCap37 and KB cells with 5 nM or higher concentrations of paclitaxel in all groups, although slightly less fragmented DNA was detected in the groups with the pulsed exposures to paclitaxel. Further, paclitaxel-induced apoptosis was analyzed by flow...
cytometric assay. Figure 2A demonstrates that pulsed exposures to paclitaxel induced a distinct sub-G₁ peak (AP peak), representing an apoptotic cell population, which was clearly observed in cells exposed to as low as 5 nM paclitaxel in the BCap37 cell line. The AP peaks clearly increased from the one-hour treatment to the three-hour treatment for all paclitaxel concentrations in both the BCap37 and KB cell lines. These results indicate that short exposures to low doses of paclitaxel are enough to induce apoptosis.

Short exposures to paclitaxel cause fewer cells to undergo mitotic arrest. Although pulsed exposures to paclitaxel clearly induce apoptosis, from flow cytometry analysis we noted that both the BCap37 (Figure 2A) and KB (Figure 2B) cell lines had significantly fewer cells undergoing mitotic arrest in the pulsed paclitaxel exposures when compared to the continuous paclitaxel exposures. To clarify this phenomenon, cytospin slides were prepared by which we counted the exact cell numbers arrested at G₂/M-phase that exhibited condensed DNA.

Figure 2. Flow cytometric analysis of BCap37 cells. Cells were treated with a one-hour pulse, three-hour pulse, or continuous exposure to various concentrations of paclitaxel for 24 hours total. DNA was extracted and stained with propidium iodide as described in "Materials and Methods". The distribution of cells in the G0/G1 and G2/M-phases of the cell cycle and apoptotic cells (Ap) are indicated above the corresponding peak. TX, paclitaxel.
Figure 3. Cytospin analysis of BCap37 and KB cells. (A) BCap37 and (B) KB cells were treated with a one-hour pulse, three-hour pulse, or continuous exposure to various concentrations of paclitaxel for 24 hours total. Cells were fixed and stained by Giemsa as described in "Materials and Methods". Three hundred cells were counted from each slide; only those cells with typical morphological features of condensed chromosomes were identified as mitotically-arrested cells. The results in the bar graph are means±SD of three separate experiments using non-linear regression.

Figure 4. Altered protein level of IκB-α and nuclear translocation of NF-κB following exposure to paclitaxel in BCap37 and KB cells. (A) Western blot: Cells were treated with a one-hour pulse, three-hour pulse, or continuous exposure to various concentrations of paclitaxel for 24 hours total. Protein levels were determined by Western blot analysis. One hundred μg of protein were analyzed in each lane. Membranes were probed with 3 μg/ml IκB-α rabbit polyclonal antibody or 1.5 μg/ml β-Actin goat polyclonal antibody (both Santa Cruz), as discussed in "Materials and Methods". (B) Immunofluorescence assay: BCap37 cells were treated as above. Cells were fixed and the nuclei were stained red using 0.4 μg/ml Hoechst 33258 and NF-κB was stained green using streptavidin-FITC as discussed in "Materials and Methods". (1) Control, (2) 20 nM paclitaxel for one-hour pulse, (3) 20 nM paclitaxel for three-hour pulse, (4) 20 nM paclitaxel for a continuous 48 hours.
chromosomes. The results summarized in Figure 3 indicate that BCap37 cells treated with 5 nM paclitaxel for a continuous 24 hours exhibited over 60% of cells arresting at the G2/M-phase. However, one- or three-hour pulsed exposures to 5 nM paclitaxel caused less than 10% of the cells to arrest at the G2/M-phase. All other paclitaxel concentrations for both BCap37 and KB cells followed this trend. These results clearly indicate that significantly fewer \( (p < 0.001) \) cells were arrested at the G2/M-phase when the cells were exposed to pulsed exposures of paclitaxel compared to the continuous paclitaxel exposures. This suggests that some apoptotic events were uncoupled from mitotic arrest.

The NF-\( \kappa B \)/\( \kappa B \) signal pathway is activated by short exposures to paclitaxel. Our previous results have shown that the NF-\( \kappa B \)/\( \kappa B \) signal pathway may mediate paclitaxel-induced apoptosis (14, 20, 24). To further examine the possible role of this pathway in the mediation of paclitaxel-induced apoptosis caused by short exposures to paclitaxel, BCap37 and KB cells treated with paclitaxel for a one-hour pulse, three-hour pulse or continuous exposures were harvested for protein extraction followed by Western blotting. The results show that a one-hour pulsed exposure to 20 nM paclitaxel also induced the nuclear translocation of NF-\( \kappa B \) (Figure 4B) in the BCap37 cell line. Further, we used EMSAs to determine that the NF-\( \kappa B \) DNA binding activity in the cells exposed to a one-hour pulsed paclitaxel treatment increased in both cell lines (Figure 5). Put together, these findings indicate that the activated NF-\( \kappa B \)/\( \kappa B \) signal pathway may mediate short-exposure paclitaxel-induced apoptosis.

Parthenolide blocks paclitaxel-induced apoptosis by inhibiting the NF-\( \kappa B \)/\( \kappa B \) signal pathway. Parthenolide, a novel NF-\( \kappa B \) inhibitor, was utilized to determine the actual role that the NF-\( \kappa B \)/\( \kappa B \) signal pathway has in the mediation or regulation of paclitaxel-induced apoptosis. Parthenolide is a plant-derived predominant sesquiterpene lactone that is used in Mexican-Indian folk remedies as an anti-inflammatory agent (33). Parthenolide has previously been reported to inhibit NF-\( \kappa B \) activation by inhibiting the core \( \kappa B \) Kinase complex, which mediates \( \kappa B \)-\( \alpha \) degradation and subsequent NF-\( \kappa B \) release (34).

In our experiments, parthenolide was given to cell cultures four hours before paclitaxel treatments. Parthenolide was not removed when the cells were treated with a one-hour pulse, three-hour pulse or continuous paclitaxel exposure. This experiment was performed simultaneously with pulsed paclitaxel exposures only. We first performed DNA
fragmentation and flow cytometric assays to evaluate the influence of parthenolide on paclitaxel-induced apoptosis. The results depicted in Figure 6 indicate that a one-hour pulsed exposure with paclitaxel alone was able to induce the characteristic DNA fragmentation in both cell lines. However, when pretreated for four hours with parthenolide (20 ÌM), paclitaxel-induced apoptosis was inhibited regardless of paclitaxel concentration or exposure time. Furthermore, flow cytometry assays revealed that 20 ÌM parthenolide significantly blocked apoptosis induced by short and continuous exposures to paclitaxel in both the BCap37 and KB cell lines (Figure 7). By Western blot, we then determined that a four-hour pretreatment with 20 ÌM parthenolide significantly blocked the degradation of ÎB-Îα induced by short and continuous paclitaxel exposures in both the BCap37 and KB cell lines (Figure 8). Subsequently, EMSAs were performed to examine the effect that parthenolide has on NF- ÎB DNA-binding activity. Activation of NF- ÎB DNA-binding

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Figure 6. Parthenolide attenuated paclitaxel-induced DNA fragmentation. BCap37 and KB cells were pretreated for four hours with 20 ÌM parthenolide and then treated with a one-hour pulse, three-hour pulse, or continuous exposure to various concentrations of paclitaxel for 48 hours for DNA fragmentation as described in "Materials and Methods".
activity is dramatically inhibited by 20 μM parthenolide in both cell lines (data not shown). These results suggest that IκB-α phosphorylation and degradation, as well as activation of NF-κB DNA-binding activity, might be a critical step for the induction of apoptosis by paclitaxel.

### Discussion

This study investigated the possibility that paclitaxel-induced apoptosis can occur independently of cell cycle arrest. It also examines the molecular mechanisms that underlie

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Figure 7. Parthenolide significantly inhibits apoptosis induced by short exposures to paclitaxel. BCap37 cells were pretreated for four hours with 20 μM parthenolide and then treated with a one-hour pulse, three-hour pulse, or continuous exposure to various concentrations of paclitaxel for 24 hours for flow cytometry analysis as described in “Materials and Methods.”

Figure 8. Parthenolide completely inhibits paclitaxel-induced IκB-α degradation in BCap37 and KB cell line. BCap37 and KB cells were pretreated for four hours with 20 μM parthenolide and then treated with a one-hour pulse, three-hour pulse, or continuous exposure to various concentrations of paclitaxel for 24 hours. Total cellular protein was extracted and subjected to Western blotting as described in “Materials and Methods.”
paclitaxel-induced apoptosis that may occur independently of mitotic arrest. Previous experiments from this laboratory and several others have suggested that paclitaxel might induce apoptosis independently of a prior G2/M-phase arrest (14-20). Utilizing baccatin III, a synthetic precursor of paclitaxel, our laboratory had previously determined that paclitaxel-induced apoptosis could be a separate event from microtubular stabilization and mitotic arrest at the G2/M-phase (21). More recently, our experiments have shown that there might be a correlation between paclitaxel-induced apoptosis and the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway (14, 20, 24). These findings implied that paclitaxel itself might be able to trigger a genetic pathway, subsequently leading to apoptotic cell death while bypassing any microtubule interaction.

Both the human breast cancer BCap37 and the human epidermoid carcinoma KB cell lines were chosen for this study because they are sensitive to paclitaxel-induced apoptosis (12 and 14) and express both the NF-\(\overline{I}\)B and \(\overline{I}\)B-\(\alpha\) proteins (14). Through DNA fragmentation and flow cytometric assays we determined that a one-hour pulsed exposure to low doses of paclitaxel (5 nM) can induce apoptosis in both cell lines (Figure 1B, 1C and 2). Flow cytometry and cytospin analysis revealed that there is a significantly (\(p<0.001\)) smaller number of cells treated with pulsed paclitaxel exposures that arrest at the G2/M-phase when compared to the continuous paclitaxel exposures (Figure 3). It has been shown that the process of microtubular bundling and stabilization by microtubules takes considerably longer than one hour. It has also been shown that the classic microtubule bundling effect of paclitaxel is reversible once the drug is removed from the cellular environment (8). Based on this feature, it would be necessary to maintain a certain intracellular concentration of drug to induce paclitaxel cytotoxicity. However, our DNA fragmentation and flow cytometry results indicate that a continuous paclitaxel exposure is not required to induce apoptosis in these two solid tumor cell lines. Some apoptotic events are triggered as long as the cells are exposed to paclitaxel for as short as one hour (Figure 1, 2). This finding suggests that paclitaxel-induced apoptosis may occur by a pathway independent of paclitaxel-induced mitotic arrest.

We have previously shown that the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway may contribute to the mediation or regulation of paclitaxel-induced apoptosis. Our current experiments have demonstrated that pulsed exposures to paclitaxel can cause tumor cell apoptosis, however many fewer cells were arrested at the G2/M-phase of the cell cycle, indicating that some apoptotic events occurred independent of mitotic arrest. Therefore, we examined whether the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway is involved in, or mediates, apoptosis induced by short exposures to paclitaxel. NF-\(\overline{I}\)B, and its specific intracellular inhibitor \(\overline{I}\)B-\(\alpha\), normally reside in the cytoplasm of the unstimulated cell. To become activated, a stimulus must target the core \(\overline{I}\)B Kinase complex, which then tags the \(\overline{I}\)B-\(\alpha\) protein for phosphorylation at two specific serines (S32 and S36). The phosphorylation is a marker that tags \(\overline{I}\)B-\(\alpha\) for degradation by the 26S proteasome, allowing NF-\(\overline{I}\)B to translocate to the nucleus and activate the transcription of genes involved in both cell proliferation and apoptotic cell death. By Western blots, we determined that the \(\overline{I}\)B-\(\alpha\) protein is degraded after only a one-hour pulsed exposure to paclitaxel (Figure 4A). Further, we tested the nuclear translocation and DNA-binding activity of NF-\(\overline{I}\)B by immunofluorescence assays and EMSAs. Both revealed that one-hour pulsed exposures to paclitaxel induced the activation of NF-\(\overline{I}\)B, indicating that this pathway is involved in the mediation of paclitaxel-induced apoptosis (Figures 4B, 5).

Next, we utilized an NF-\(\overline{I}\)B inhibitor, parthenolide, which has been shown to specifically inhibit \(\overline{I}\)B-\(\alpha\) phosphorylation, to examine whether the inhibition of this pathway could affect apoptosis induced by short exposures to paclitaxel. Not only did parthenolide inhibit paclitaxel-induced activation of the NF-\(\overline{I}\)B/\(\overline{I}\)B-\(\alpha\) signal pathway (Figure 8), it also inhibited paclitaxel-induced apoptosis (Figure 6 and 7). Therefore, when BCap37 and KB cells are pretreated with a compound that blocks the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway, paclitaxel-induced apoptosis is blocked. This apoptotic blockade is induced independently of paclitaxel concentration and exposure. This finding indicates that activation of the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway may be required to execute paclitaxel-induced apoptosis.

In summary, this study investigated the possible relationship between paclitaxel-induced apoptosis and paclitaxel-induced mitotic arrest, as well as the molecular mechanisms that underlie paclitaxel-induced apoptosis. Through characterization of two human solid tumor cell lines that are paclitaxel-sensitive, we demonstrated that paclitaxel-induced apoptosis may be a separate event from paclitaxel-induced mitotic arrest. Further analyses revealed that short exposures to paclitaxel could activate or regulate multiple components of the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway. Inactivation of this pathway by an NF-\(\overline{I}\)B inhibitor, parthenolide, caused a block of paclitaxel-induced apoptosis. These findings suggest that paclitaxel-induced apoptosis may occur independently of mitotic arrest and be mediated by the NF-\(\overline{I}\)B/\(\overline{I}\)B signal pathway.

Acknowledgements

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