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Disruption of Cell Adhesion and Caspase-Mediated Proteolysis of β- and γ-Catenins and APC Protein in Paclitaxel-Induced Apoptosis

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ABSTRACT

Cell adhesion is important in the regulation of cell proliferation, migration, survival, and apoptosis. The major components of cell adhesion are the cadherin family of proteins, α-, β- and γ-catenins, and cytoskeletons. In addition, β-catenin, when associated with adenomatous polyposis coli (APC) protein, an oncosuppressor, is implicated in the regulation of β-catenin/APC-related signaling pathways. To examine the correlation between impairment of cell adhesion events and apoptosis, we used human non–small-cell lung cancer H460 and H520 cell lines as models to determine whether paclitaxel-induced apoptosis is associated with disruption of the components of cell adhesion and their functions. Paclitaxel treatment resulted in cells rounding up and losing contact with their neighboring cells, suggesting that the drug does indeed affect cell adhesion and related events. Western blot analysis revealed that paclitaxel caused a time- and concentration-dependent cleavage of β-catenin, γ-catenin, and APC protein, but not α-catenin or E-cadherin. These cleavages of β-catenin and γ-catenin were apoptosis-dependent, not mitosis-dependent. Paclitaxel treatment led to the proteolysis and activation of caspase-3 and -7, but not caspase-1. Furthermore, paclitaxel-induced apoptosis and cleavage of β-catenin and γ-catenin were inhibited by the pan-caspase inhibitor Z-VAD-FMK and partially inhibited by the caspase-3 inhibitor Z-DEVD-FMK but were not affected by the caspase-1 inhibitor AC-YVAD-CMK. Although the pan-caspase inhibitor blocked the cleavage of β-catenin as well as DNA fragmentation, it did not affect paclitaxel-induced M-phase arrest and only partially prevented cell-growth inhibition. Biochemical studies revealed that cleaved β-catenin was detected only in the Triton X-100 insoluble fraction, suggesting that it might localize in nuclear and/or membrane structures. Interestingly, the paclitaxel-induced β-catenin fragment lost its ability to bind to E-cadherin, α-catenin, or APC protein and to serve as a substrate for tyrosine kinase. All our data demonstrate that the caspase-mediated cleavage of β-catenin, γ-catenin, and APC protein might contribute to paclitaxel-induced apoptosis.

Apoptosis plays a central role both in development and in homeostasis of all multicellular organisms (Wyllie et al., 1980; Schwartzman and Cidlowski, 1993). It has been demonstrated that apoptosis is induced by various stimuli, including irradiation and chemotherapeutic agents (Bhalla et al., 1993; Kaufman et al., 1993; Solary et al., 1993). It is an active process controlled by a set of genes and gene products, some of which are positive and others negative regulators (Williams and Smith, 1993). ICE, a cysteine protease that cleaves interleukin-1β precursor at two aspartic residues, was the first member to be identified from a large family of caspases that are essential for cells undergoing apoptosis. At least 10 of these caspases have been identified in mammalian cells. Although their contribution to the apoptotic process is not yet fully understood, their functions are linked to initiating the cellular response to apoptotic signals and to cell disassembly (Nicholson and Thornberry, 1997; Wolf and Green, 1999).

The characteristic morphological changes of apoptosis are cellular shrinkage, chromatin condensation, membrane blebbing, and formation of apoptotic bodies. All these changes lead to a loss of cell-cell adhesion and to cellular detachment from substratum (Bannerman et al, 1998; Mills et al, 1999). It is known that cell-cell adhesion is mediated by cadherins, members of the classical cadherin protein family. Cadherins associate with cytoplasmic proteins termed α-catenin, β-catenin, and γ-catenin (plakoglobin) that interact with cytoskeleton proteins (Takeichi, 1991; Kemler, 1993; Gumbiner, 1996). It has been demonstrated that caspase-mediated specific cleavages of cellular components, including cytoskeleton

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ABBREVIATIONS: APC, adenomatous polyposis coli; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NP-40, nonidet P-40 [(octylphenoxy)-polyethoxyethanol]; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; AA, amino acid(s).

proteins, are involved in the apoptotic processes (Cotter et al., 1992; Levkau et al., 1998).

In addition to its presence in cell-cell adherens junctions, β-catenin as well as its homolog Armadillo in the fruit fly *Drosophila melanogaster* are thought to be associated with adenomatous polyposis coli (APC) and GSK-3β proteins in regulation of Wingless/Wnt signal transduction (Smith et al., 1993; Polakis, 1997). Furthermore, β-catenin serves as an oncoprotein by interacting with APC and Tcf/Lef proteins, resulting in enhancement of gene transcription, continuous cell proliferation, and blockade of apoptosis. In contrast, APC protein is a tumor suppressor apparently serving as a cytoplasmic effector of β-catenin, negatively regulating the accumulation of free β-catenin in cytoplasm. Recent reports have revealed that both APC and β-catenin genes are mutated in colon and melanoma cells (Powell et al., 1992; Sparks et al., 1998).

Paclitaxel (Taxol) is a most effective agent for the treatment of breast, ovarian, lung, and head and neck cancers (McGuire et al., 1989). As a microtubule stabilizer, it changes the dynamic equilibrium of assemblage and effectively disrupts the formation of the normal spindle at metaphase, causing the blockade of dividing cells (Schiff et al., 1979; Jordan et al., 1996). We have previously demonstrated that paclitaxel induces M-phase arrest, resulting in morphological changes [i.e., cells become rounded up; they are constricted from their neighboring cells, suggesting that cell-cell adherens junctions are disrupted and that this could lead to cells undergoing apoptosis (Ling et al., 1998)]. In this study, we used human non–small-cell lung cancer cell lines H460 and H520 as models to determine whether paclitaxel-induced apoptosis might be associated with disruption of cell-cell adherens junctions components and β-catenin/APC-related signaling transduction pathways. Our results indicate that paclitaxel treatment causes concentration- and time-dependent cleavage of β- and γ-catenins but not of α-catenin and E-cadherin. These effects are not mitosis arrest-associated but apoptosis-associated. Interestingly, paclitaxel also induces APC protein cleavage, suggesting that paclitaxel-induced apoptosis may be associated with the disruption of Wingless/Wnt signal transduction pathway. Furthermore, the cleavage of β- and γ-catenin and APC protein is associated with the cleavage and activation of caspase-3 and caspase-7. Using several caspase inhibitors, we found that DNA fragmentation and the cleavage of β-catenin are completely inhibited by the pan-caspase inhibitor Z-VAD-FMK and partially prevented by the caspase-3 specific inhibitor Z-DEVD-FMK but are not affected by the caspase-1 inhibitor AC-YVAD-CMK.

Materials and Methods

Chemicals and Drugs. Paclitaxel was purchased from Hande Tech, Inc. (Houston, TX), dissolved in DMSO as stock solution (1 mM). Monoclonal anti-E-cadherin, α-catenin, β-catenin, and γ-catenin antibodies were obtained from Transduction Laboratory, Inc. (Lexington, KY), and monoclonal anti-APC and anti-caspase-3 antibodies from Oncogene Science, Inc. (Cambridge, MA). Monoclonal anti-caspase-7 antibody was purchased from Pharmgene Co. (San Diego, CA). Z-VAD-FMK, Z-DEVD-FMK, and AC-YVAD-CMK were obtained from Bachem Bioscience Inc. (King of Prussia, PA). Other chemicals were purchased from Sigma (St. Louis, MO).

Cell Cultures and Treatment. Human non–small-cell lung cancer cell lines H460 and H520 were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 2 mM pyruvate and 1% L-glutamine. HeLa cells were maintained as monolayer cultures in DMEM with 10% fetal bovine serum. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2.

Cell Synchronization and Cell-Cycle Assay. For synchronization of HeLa cells at M-phase, cells were exposed to 100 ng/ml of nocodazole or 50 ng/ml of paclitaxel for 18 h. M-phase–arrested cells were detached with gentle shaking by hand and collected through centrifugation. After being washed three times with DMEM, they were reincubated in fresh DMEM with 10% fetal bovine serum. For determination of cell cycle distribution, cells were taken from culture and fixed with 75% cold ethanol at −20°C for 30 min, then stained with 5 μg/ml propidium iodide containing 5 μg/ml RNase at 4°C for 1 h. The cellular DNA contents were determined by flow cytometry (Epics Profile Analyzer, Coulter Co., Miami, FL). Mitotic cells were stained for assay with Wright-Giemsa dye solution and were counted by Nikon Diaphot 200 microscopy.

Determination of Apoptosis. For quantitative determination of drug-induced DNA fragmentation, cells were labeled with 10 μCi of [^{3}H]thymidine at 37°C for 24 h and chased in fresh medium without [^{3}H]thymidine at 37°C for another 3 h. Labeled cells were treated with drugs for the indicated times and lysed with 0.5 ml of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100 at room temperature for 1 h. After centrifugation at 14,000*g* for 10 min, the fractions of supernatant and pellet were collected and radioactivities of both fractions were determined with a liquid scintillation counter. DNA fragmentation was calculated as described previously (Ling et al., 1993). For determination of drug-induced DNA laddering, cells were lysed with 0.5 ml of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 100 mM EDTA, 0.5% SDS, and 20 μg/ml of pancreatic RNase at room temperature for 2 h. After incubation with proteinase K (100 μg/ml) at 60°C for 1 h, DNA was extracted from lysate with an equal volume of phenol twice and chloroform once. DNA was precipitated with 100 mM NaCl and an equal volume of isopropanol at −20°C overnight, dissolved in Tris/EDTA buffer and separated by 1% agarose gel electrophoresis. After being stained with ethidium bromide, the resulting DNA fragment ladder was visualized by UV illumination. In addition, apoptotic cells were assessed by a TUNEL reaction kit according to the manufacturer's recommendation (Roche Molecular Biochemicals, Summerville, NJ). After the reaction, the fluorescence-labeled cells were determined by flow cytometry.

Immunoblotting and Immunoprecipitation. For immunoblotting assay, cells were lysed with lysis buffer containing 10 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml each aproptinin and leupeptin, 1% NP-40, and 1% SDS at 4°C for 15 min. After centrifugation at 14,000*g* at 4°C for 10 min, the supernatant fraction was collected and the protein content was measured using a Bio-Rad DC protein assay kit (Hercules, CA). An equal amount of protein in each sample was subjected on a 10% polyacrylamide gel. Protein blots were transferred to a nitrocellulose membrane in Tris/glycine/SDS transfer buffer, and the protein was probed by corresponding antibodies. The protein/antibody complex was detected by enhanced chemiluminescence. The quantitative analysis of proteolytic cleavage was performed by laser scanning densitometry (Bio-Rad GS-670 Imaging Densitometer). In some experiments, cells were separated into Triton X-100 soluble and insoluble fractions as described by Lampugnani et al. (1995). Briefly, cells were extracted with lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml each leupeptin and aprotinin, 1% NP-40, and 1% Triton X-100 for 30 min in an ice bath with gentle agitation. After centrifugation at 14,000*g* at 4°C for 10 min, supernatant was collected as the Triton X-100 soluble fraction and the pellets were extracted as described above with 1% SDS-

containing buffer in an ice bath for 20 min. After centrifugation at 14,000*g* at 4°C for 10 min, the supernatant was collected as a Triton X-100 insoluble fraction. For immunoprecipitation, cells (1×10^6 cells) were solubilized in 0.5 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml each leupeptin and aprotinin, and 1% Triton X-100. After incubation at 4°C for 15 min, the lysate was separated by centrifugation at 4,000 rpm for 10 min at 4°C. The protein content in each sample was determined as described above with a DC protein assay kit and adjusted accordingly before the assay. Lysate (0.5 ml) was incubated with 5 μg of monoclonal anti-E-cadherin, anti-α-catenin and anti-APC antibodies, and 50 μl of protein A/protein G-conjugated agarose (Calbiochem, Cambridge, MA) at 4°C overnight. After being washed three times with lysis buffer, the immunoprecipitated complex was collected by centrifugation at 14,000*g* for 10 min, added to 20 μl of Laemmli sample buffer, and boiled for 5 min. The components were separated by 10% polyacrylamide gel, and the detected proteins were probed with the corresponding antibodies.

Immunocytochemical Studies. Cells were fixed in 4% paraformaldehyde in PBS solution at room temperature for 2 h and then treated with 1% NP-40 in PBS solution for 30 min. After blocking with 1% bovine serum albumin in PBS solution for 1 h, cells were incubated with primary antibodies in 1% bovine serum albumin/PBS solution at room temperature for 1 h. After being washed three times with PBS solution, cells were reincubated with fluorescein isothiocyanate-conjugated secondary antibodies for 30 min in a darkroom. The immunofluorescence complexes were visualized with a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan).

Results

Paclitaxel Induces G_2/M Phase Arrest and Apoptosis in H460 Cells. Initially, we treated human non–small-cell lung cancer cell H460 cells with 0.5 μM paclitaxel for the indicated time and observed drug-induced morphological changes. As shown in Fig. 1A, paclitaxel treatment for 24 h resulted in dramatic morphological changes (i.e., the monolayer cells became rounded up, lost contact with neighboring cells, and were more easily detached from culture). When cells were exposed to paclitaxel for up to 48 h, most became apoptotic; i.e., they displayed shrinkage, nuclear condensation, membrane blebbing, formation of apoptotic bodies, and a tendency to float in the medium. We then used the TUNEL reaction as described under *Materials and Methods* to determine the effect of paclitaxel on cell cycle distribution and apoptosis.

As shown in Fig. 1B, paclitaxel treatment resulted in a gradual increase in cell-cycle arrest at the G_2/M phase; it peaked at ~60% by 24 h and then fell to 7% at 36 h. This drug-induced apoptosis was exposure time-dependent, i.e., only 2% of cells were apoptotic at time 0, ~18% became so at 24 h, and ~48% to ~78% displayed apoptotic death after 48 to 72 h. All results were consistent with the previous report, in which we demonstrated that paclitaxel-induced G_2/M phase arrest before apoptosis (Ling et al., 1998). Furthermore, similar patterns of dose- and time-dependent induction of apoptosis were found in H460 cells after paclitaxel treatment by determining DNA laddering and fragmentation (data not shown).

Much evidence has indicated that the cleavages of some cellular structure proteins are correlated with apoptotic mechanisms. Because of this, we thought it possible that paclitaxel-induced disruption of microtubules and apoptosis could be linked to cleavage of cytoskeleton proteins. To test this hypothesis, we treated H460 cells with 0.5 μM paclitaxel for different periods of time and determined the cleavage of cellular cytoskeleton proteins, actin, α-tubulin, and β-tubulin, by Western blot analysis. Neither actin, α-tubulin, nor β-tubulin was cleaved, even in cells exposed to the drug for 48 to 72 h (data not shown).

Paclitaxel-Induced Apoptosis Is Associated with Cleavages of β-Catenin and γ-Catenin. Increasing evidence has indicated that cell adhesion plays an important role in regulation of cell growth, differentiation, survival, and apoptosis (Takeichi, 1991; Kemler, 1993; Gumbiner, 1996; Wheelock et al., 1996). It is well known that cell-cell adherens junctions are mediated by cadherins, as well as by α-, β-, and γ-catenins. Paclitaxel treatment leads to a striking change in morphology; cells are easily detached from the monolayer culture, suggesting that paclitaxel can affect cell adhesion mechanisms. We therefore determined whether paclitaxel-induced apoptosis could be associated with impairment of cadherins and their related proteins. We used Western blot analysis to investigate the effect of the drug on the expression and cleavage of components of adherens junctions.

As shown in Fig. 2, no band for E-cadherin protein was detected in H460 cells; α-catenin was not reduced or cleaved after paclitaxel treatment at different concentrations and for different times. However, β-catenin and γ-catenin were cleaved in a concentration- and time-dependent manner. About 20% of β-catenin cleavage (a 78-kDa fragment) and ~13% of γ-catenin cleavage (a 76-kDa fragment) were detected after exposure to 0.1 μM paclitaxel for 24 h or exposure to the

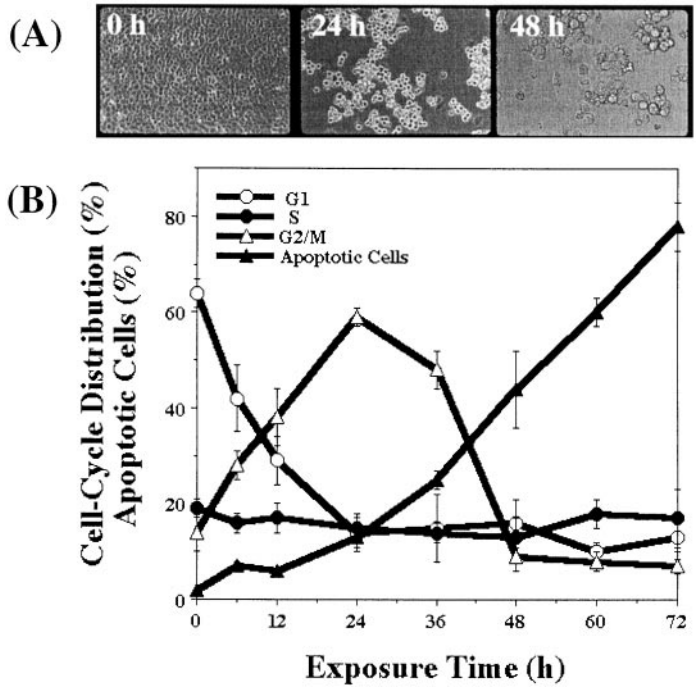

Fig. 1. Paclitaxel-induced G_2/M phase arrest and apoptosis in H460 cells. A, cells exposed to 0.5 μM paclitaxel for the indicated times and taken from culture for determination of morphological changes by Nikon Diaphot 200 microscopy. B, cells exposed to 0.5 μM paclitaxel for the indicated times, harvested, and stained with propidium iodide for determination of cell cycle distribution by flow cytometry. The numbers of apoptotic cells were measured by TUNEL reaction as described under *Materials and Methods*. Each point is the mean ± S.D. of three independent experiments.

drug for 24 h at 0.5 μM, respectively. The amounts of proteolytic fragments induced by paclitaxel were proportional to exposure time and drug concentrations. Interestingly, γ-catenin was cleaved into three distinct fragments of 76, 70, and 65 kDa after 36 to 72 h exposure to 0.5 μM paclitaxel.

In addition, cleavages of β-catenin and γ-catenin were basically correlated with drug-induced DNA fragmentation, indicating that cleavage of these proteins was associated with apoptosis. Because H460 cells displayed no expression of E-cadherin, we used H520 cells, which expressed high levels of E-cadherin, as a model to determine the effect of paclitaxel on cleavage of this protein. As shown in Fig. 3, exposure to 0.5 μM paclitaxel did not induce cleavage of E-cadherin or α-catenin even at 48 to 72 h, by which time ~ 80% of cells were apoptotic. Again, paclitaxel did induce cleavage of β-catenin and γ-catenin in the same time-dependent manner as described for H460 cells.

Cleavage of β-Catenin and γ-Catenin Is Not Associated with M-Phase Arrest. Because paclitaxel-induced M-phase arrest resulted in cells rounding up and becoming easily detached from neighboring cells, those arrested at M-phase could be associated with alteration in cell adherens junction components. We therefore decided to determine whether M-phase arrest could be associated with cleavage of β- or γ-catenins. We synchronized HeLa cells with 100 ng/ml nocodazole or 0.5 μM paclitaxel, as described under *Materials and Methods*. The M-phase synchronized cells were reincubated in drug-free medium, sampled from culture at the indicated times, and lysed with lysis buffer. After protein separation by SDS-PAGE, the proteolytic fragments of β- and γ-catenins were detected by Western blot analysis. In the nocodazole study, the numbers of M-phase cells were gradually reduced to ~14% after 3 h and to ~3% after 6 h of incubation in drug-free fresh medium. The numbers of apoptotic cells remained constant at 2 to 3% during 24 h of incubation, indicating that nocodazole-synchronized M-phase cells easily re-enter the normal cell cycle and do not undergo apoptosis.

In the paclitaxel study, the numbers of M-phase cells remained at ~96 to 80% through 6 h, falling to 56 to 9% after 9 to 24 h. The numbers of apoptotic cells increased from 23 to 81% after 9 to 24 h. Importantly, cleavage of β-catenin and γ-catenin was not found in nocodazole-synchronized cells during 24 h (Fig. 4, A and C). However, ~15% of β-catenin cleavage and ~10% of γ-catenin cleavage were detected in paclitaxel-synchronized cells at 6 h, and the amounts of cleavage fragments were elevated as an increased incubation time. Furthermore, the increase in cleavage of β-catenin and γ-catenin was basically correlated with an increase in the numbers of apoptotic cells, not with the numbers of M-phase cells (Fig. 4, B and D). These results indicate that cleavage of β- and γ-catenins is apoptosis-dependent, not mitosis-dependent.

Paclitaxel Induces APC Protein Cleavage. Recent studies have demonstrated that tumor suppressor protein APC is widely expressed in epithelial and some mesenchymal

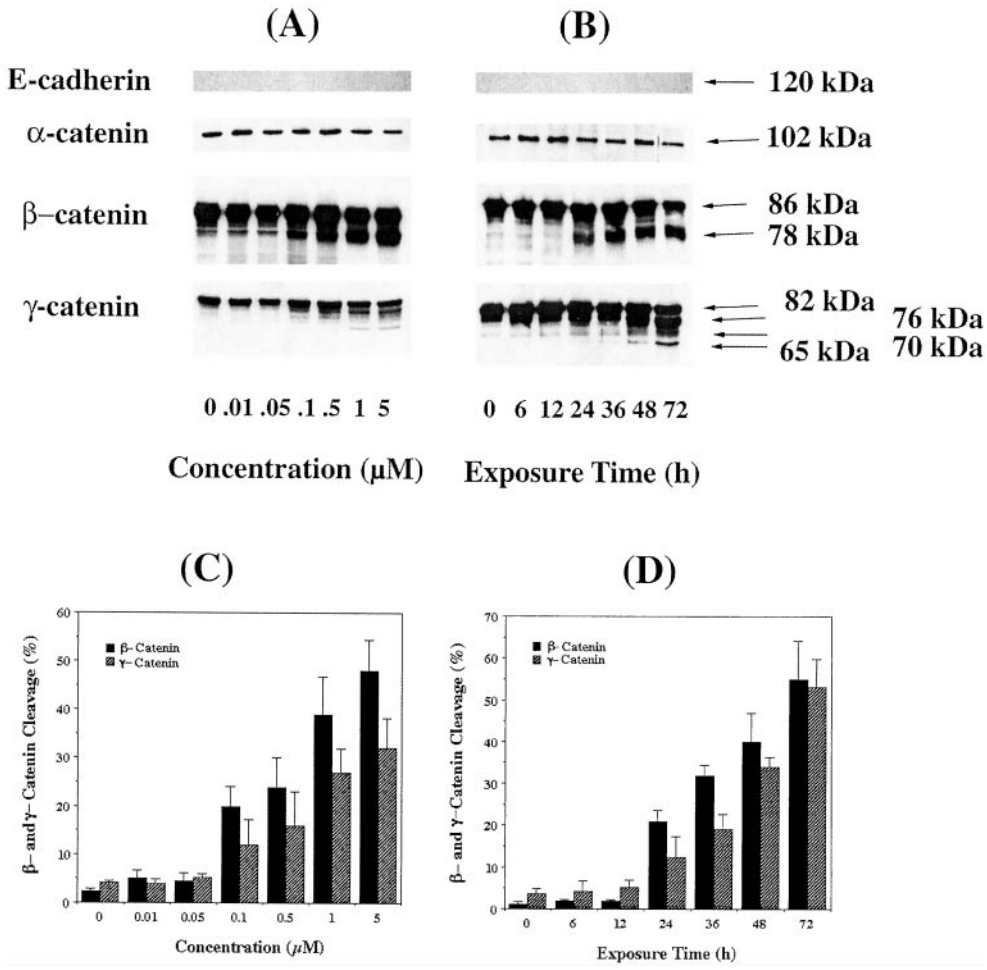

Fig. 2. Concentration- and time-dependent effect of paclitaxel on cleavage of adherens junctions component proteins in H460 cells. Cells were exposed to either increasing concentrations of drug for 24 h (A) or to 0.5 μM drug for increasing exposure times (B). Cells were lysed with lysis buffer, and an equal amount (50 μg of protein) of total cell lysate from each sample was subjected to 10% SDS-PAGE. After electrophoresis, protein blots were transferred to the membrane and probed with the corresponding monoclonal antibodies. Quantification of β- and γ-catenin cleavage caused by paclitaxel at different concentrations (C) and for different exposure time (D), was assessed by laser densitometric scanning as described under *Materials and Methods*. Each bar is the mean ± S.D. of three independent experiments.

cells. Although the precise functions of this protein are still poorly defined, it clearly plays roles in the regulation of cell adhesion, cytoskeletal organization, and cell signaling transduction (Rubinfeld et al., 1995; Barth et al., 1997). We therefore felt it necessary to understand whether paclitaxel-induced disruption of cell adhesion and cleavage of β-catenin was associated with cleavage of APC protein. As shown in Fig. 5, the molecular mass of APC protein seemed to be about 250 kDa rather than the possible full length of 300 kDa, indicating that APC protein in H460 cells is truncated. After 24 h exposure to 0.1 μM paclitaxel, the 250-kDa band of protein was markedly reduced, and a 90-kDa fragment band clearly appeared. The quantitative analysis of APC protein cleavage indicated that only ~6 to 8% of APC protein cleavage was found in cells exposed to 0 to 0.05 μM paclitaxel for 24 h or to 0.5 μM drug for 0 to 12 h; however, ~ 58% of APC protein cleavage was detected at 0.1 μM drug, and the amounts of APC cleavage were elevated as increased concentrations (Fig. 5C). Time-course studies revealed that paclitaxel-induced cleavage of APC protein was also elevated, with an increased exposure time, and essentially coincident with drug-induced β-catenin cleavage and cell apoptotic death (Fig. 5D). In addition, we also examined the effect of paclitaxel on APC protein proteolytic cleavage in H520 and HeLa cells and obtained identical results, indicating that paclitaxel-induced APC and β-catenin protein cleavage are not restricted to H460 cells (data not shown). All our results indicate that cleavage of APC protein is associated with paclitaxel-induced apoptosis.

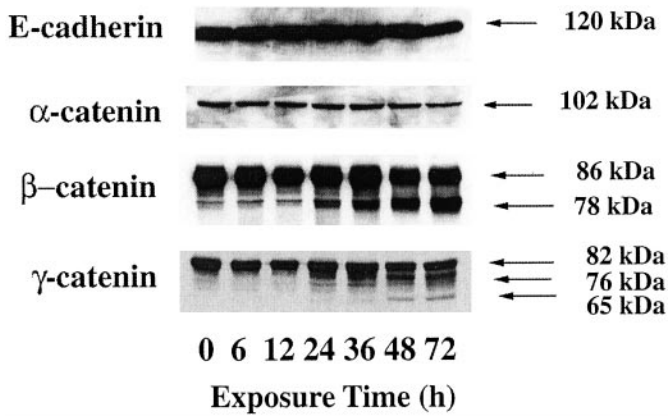

Fig. 3. Paclitaxel-induced cleavage of adherens junctions component proteins in H520 cells. Cells were exposed to 0.5 μM paclitaxel for the indicated times. Cells were lysed with lysis buffer and an equal amount (50 μg of protein) of total lysate was subjected to 10% SDS-PAGE; immunoblots were performed and detected as described in Fig. 2.

Cleavage of β-Catenin, γ-Catenin, and APC Protein Is Linked to the Activation of Caspase-3 and Caspase-7. There are at least 10 members of the caspase family identified as initiators or active executioners in the regulation of apoptosis (Nicholson and Thornberry, 1997; Wolf and Green, 1999), and it was necessary to know which members were involved in paclitaxel-induced apoptosis and cleavage of β-catenin, γ-catenin, and APC protein. Because most members of the caspase family are proenzymes (Martin and Green, 1995), we first determined whether paclitaxel treatment could cleave the proenzymes of caspase into the activated form. As shown in Fig. 6, exposure to 0.5 μM paclitaxel for 24 to 72 h resulted in a time-dependent cleavage of pro-caspase-3 and pro-caspase-7, but not of pro-caspase-1. Importantly, these studies were correlated with induction of apoptosis, as well as cleavage of β-catenin, γ-catenin, and APC protein, suggesting that the activation of caspase-3 and caspase-7 may be involved in the drug-induced cleavage of these proteins and in apoptosis.

We then used the cell-permeable, irreversible caspase inhibitors Z-VAD-FMK, Z-DEVD-FMK, and AC-YVAD-CMK

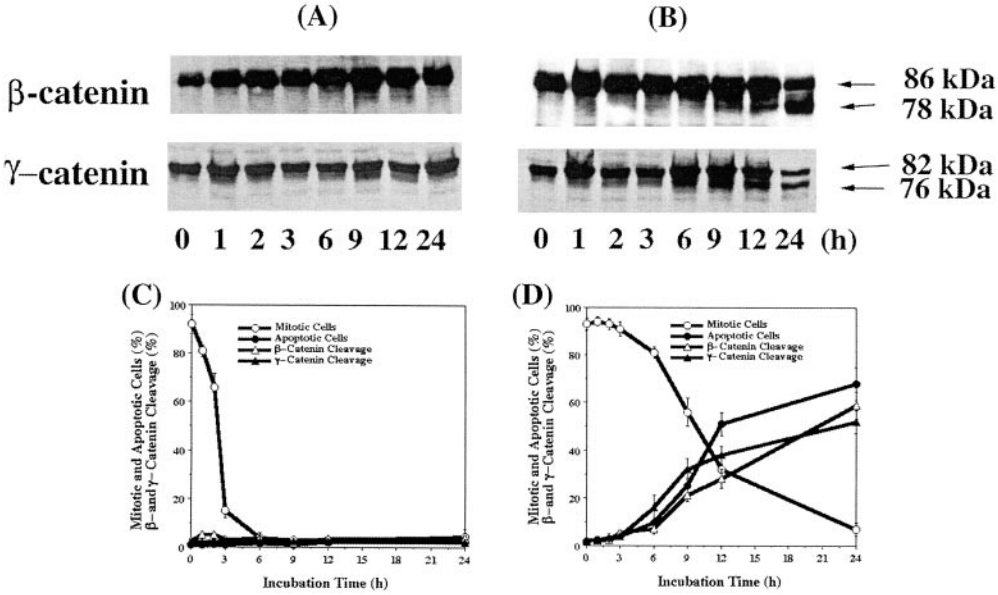

Fig. 4. Cleavage of β- and γ-catenins is apoptosis-dependent, but not mitosis-dependent. HeLa cells were synchronized at M-phase by either (A) treatment with nocodazole (A) or paclitaxel (B). The M-phase synchronized cells were washed three times with medium and incubated in fresh medium for the indicated times. After staining with Wright-Giemsa solution, the numbers of mitotic and apoptotic cells were counted, at least 200 cells, by microscopy. For determination of β- and γ-catenin cleavage, an equal amount (50 μg of protein) of total cell lysate from each sample was subjected to 10% SDS-PAGE. Intact and cleaved β- and γ-catenins were detected by Western blot analysis. Quantification of proteolytic cleavage of β- and γ-catenins was assessed by laser densitometric scanning. C and D, are the correlation of mitosis and apoptosis with cleavage of β- and γ-catenin in synchronized M-phase cells by either treatment with nocodazole, or with paclitaxel. Each point is the mean ± S.D. of three independent experiments.

and examined their effects on paclitaxel-induced DNA fragmentation and cleavage of β-catenin. As shown in Fig. 7C, co-treatment with 50 μM Z-VAD-FMK, a pan-caspase inhibitor, caused inhibition of paclitaxel-induced cleavage of β-catenin. Co-treatment with 50 μM Z-DEVD-FMK, a caspase-3 inhibitor, produced potent but not complete inhibition of β-catenin fragmentation. In contrast, co-treatment with AC-YAED-CMK, a caspase-1 inhibitor, did not block drug-induced β-catenin fragmentation. In addition, we also found that Z-VAD-FMK and Z-DEVD-FMK, but not AC-YVAD-CMK, could prevent paclitaxel-induced cleavage of γ-catenin and APC protein (data not shown).

To further confirm that the blockade of β-catenin cleavage by caspase inhibitors was correlated with the attenuation of drug-induced apoptosis, we determined apoptosis in the presence and the absence of caspase inhibitors. As shown in Fig. 7, A and B, Z-VAD-FMK completely inhibited paclitaxel-induced DNA laddering and fragmentation and Z-DEVD-FMK did likewise, but AC-YVAD-CMK did not affect drug-induced apoptosis. All our data indicate that paclitaxel-induced apoptosis is mediated by some kinds of activated caspases, particularly caspase-3 and caspase-7. In addition, we also determined the effect of the pan-caspase inhibitor Z-VAD-FMK on paclitaxel-induced M-phase arrest and cell survival. As shown in Fig. 8, treatment with 50 μM Z-VAD-FMK did not affect cell growth, cell cycle progression, or cell survival. Co-treatment with this inhibitor did not change paclitaxel-induced M-phase arrest, but it did delay the progression of M-phase arrested cells into apoptosis by at least 12 h. Interestingly, Z-VAD-FMK did not reverse paclitaxel-induced inhibition of cell growth but resulted in homeostasis.

Effect of β-Catenin Cleavage on Complex Formation with E-Cadherin, α-Catenin, and APC Protein. β-Catenin binds directly to the cytoplasmic domain of E-cadherin, α-catenin, and actin to control cell-cell adhesion (Ben-Ze'ev and Geiger, 1998). In addition, β-catenin binding to APC protein is involved in the transcription regulation for some specific gene expression (Hart et al., 1998). We therefore determined whether the cleavage of β-catenin induced by paclitaxel could affect its complex formation with those proteins. H520 cells were treated with 0.5 μM paclitaxel or with the same volume of DMSO as control for 30 h, after which the treated cells and controls were collected as described above. After lysis, the complexes of β-catenin with E-cadherin, α-catenin, and APC protein were immunoprecipitated with specific corresponding antibodies. The members of the β-catenin binding complex were separated by 10% SDS-PAGE, and β-catenin was determined by Western blot analysis. As shown in Fig. 9, β-catenin protein was cleaved into a 78-kDa fragment in the whole-cell lysate after a 30-h exposure to paclitaxel, compared with a full-length band of β-catenin protein in control cells. Interestingly, no 78-kDa fragment of β-catenin was detected in the complexes that were immunoprecipited with E-cadherin, α-catenin, or APC protein, suggesting that the cleaved fragment of β-catenin may not bind to those proteins to form complexes. These results are consistent with reports by Brancolini et al. (1997).

Effect of Paclitaxel on Subcellular Localization of β-Catenins. Some investigations have indicated that the function of β-catenin is dependent on its subcellular localization (Salomon et al., 1997). We therefore determined the effect of paclitaxel on β-catenin's subcellular distribution. We

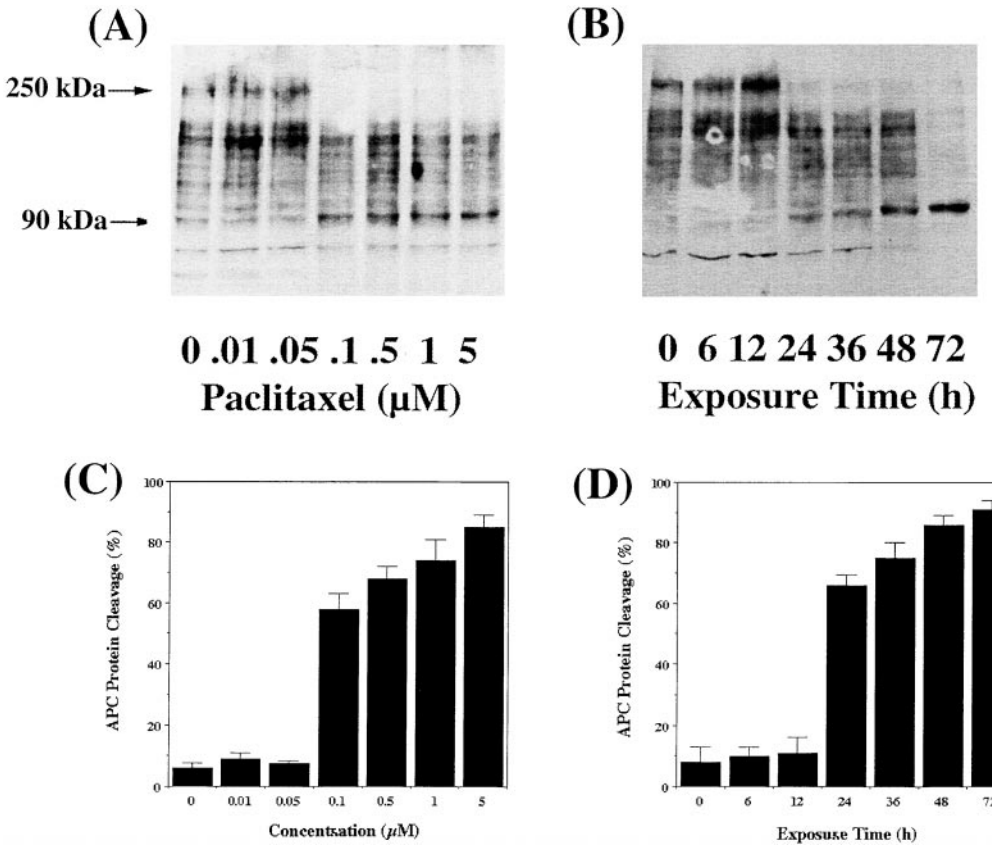

Fig. 5. Paclitaxel-induced concentration- and time-dependent cleavage of APC protein in H460 cells. Cells were exposed to either increasing concentrations of drug (A) or to 0.5 μM of drug for increasing exposure times (B). An equal amount (50 μg of protein) of total cell lysate was subjected to 7% SDS-PAGE. Intact and cleaved APC protein was analyzed by Western blot analysis. Quantitation of proteolytic cleavage of APC protein caused by paclitaxel at different concentration (C) or for different exposure time (D) was assessed by laser densitometric scanning as described under *Materials and Methods*. Each point is the mean ± S.D. of three independent experiments.

used immunocytochemical techniques to observe the alteration in the subcellular localization of β-catenin in H460 cells after 30 h of drug treatment. As shown in Fig. 10A, β-catenin in control cells localized predominantly around the plasma membrane, and treatment with paclitaxel did not markedly alter the cellular distribution of this protein. However, biochemical studies using detergent Triton X-100 to separate the cells into detergent-soluble and -insoluble fractions revealed that a 78-kDa fragment of β-catenin in paclitaxel-treated cells was detected only in the Triton X-100 insoluble fraction (Fig. 10B), suggesting that this fragment might localize in membrane and nuclear structures (Lampugnani et al., 1995).

Effect of Paclitaxel on β-Catenin Phosphorylation. It has been reported that β-catenin is targeted by some tyrosine kinases, although the precise role of this tyrosine phosphorylation remains to be further defined (Hamaguchi et al., 1993; Muller et al., 1999). In this study, we have demonstrated that paclitaxel-induced apoptosis is associated with the caspase-mediated cleavage of β-catenin. It was therefore necessary to determine whether paclitaxel treatment could affect the tyrosine phosphorylation of β-catenin. H460 cells were exposed to 0.5 μM paclitaxel or to the same volume of DMSO as control for 30 h and then incubated in the presence or the absence of 1 mM pervanadate for 1 h to inhibit tyrosine phosphatases before cell lysis. Immunoprecipitate was prepared by a monoclonal anti-β-catenin antibody, separated by 10% SDS-PAGE, and probed with either anti-β-catenin or anti-phosphotyrosine antibodies, as described under *Materials and Methods*.

Western blot analysis using anti-β-catenin antibody revealed that the bands of β-catenin in control cells pretreated with or without pervanadate presented similar intensities, and the cleaved fragments of β-catenin in paclitaxel-treated cells showed the same amounts in cells pretreated with or without pervanadate (Fig. 11A). In the Western blot analysis using anti-phosphotyrosine antibody as a probe, the phosphorylated β-catenin was detected in lysate from cells pretreated with the tyrosine inhibitor pervanadate, but no band of phosphorylated β-catenin was found in cells without pervanadate pretreatment. The intensity of full-length tyrosine phosphorylated β-catenin in paclitaxel-induced apoptotic cells was similar to that in controls. Interestingly, the band for tyrosine phosphorylation of cleaved fragments of β-catenin could not be detected (Fig. 11B), indicating that these fragments do not serve as a substrate for tyrosine kinase.

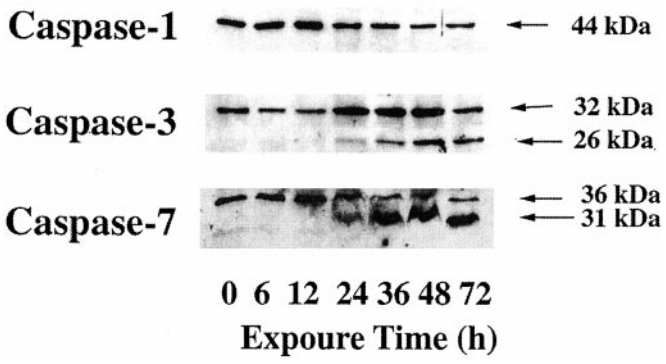

Fig. 6. Paclitaxel-induced cleavage of caspase-3 and caspase-7 in H460 cells. Cells were exposed to 0.5 μM drug for the indicated times. After lysis, an equal amount of cell lysate (50 μg of protein) from each sample was subjected to 4 to 15% SDS-polyacrylamide gradient gel. After electrophoresis, the intact and cleaved caspase proteins were detected by Western blot analysis.

Discussion

Cell adhesion plays a critical role in regulation of cell migration, proliferation, differentiation, survival, and death (Raff, 1992; Day et al., 1999). The present experiments, as well as previous studies by us and by others, have demonstrated that paclitaxel treatment disturbs the dynamic equilibrium between assembling and disassembling the microtubules and blocks cell cycle progression at M-phase. Morphologically, paclitaxel-treated cells become rounded up

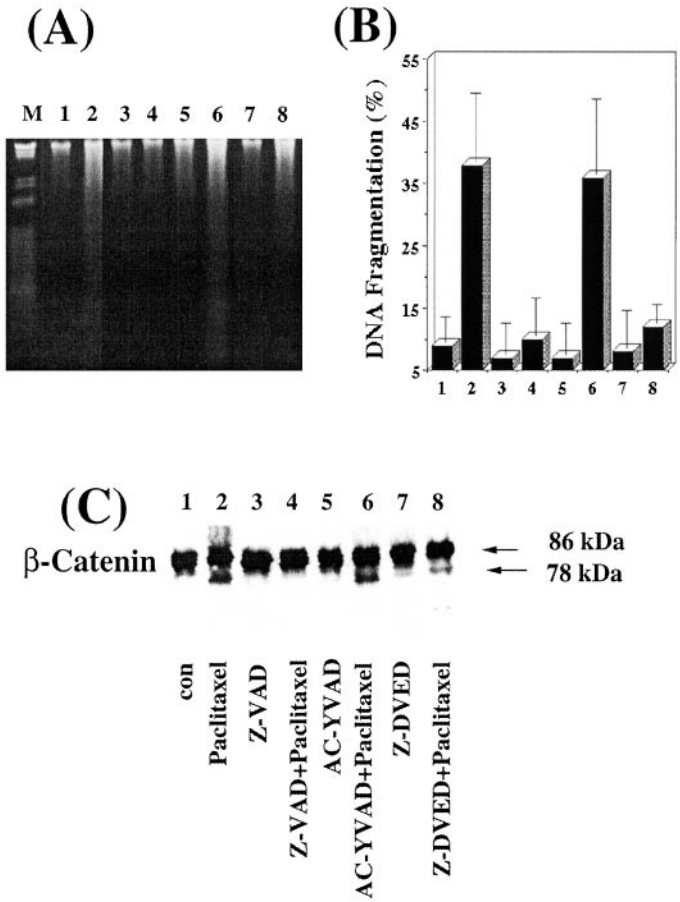

Fig. 7. Effects of caspase inhibitors on paclitaxel-induced DNA fragmentation and cleavage of β-catenin in H460 cells. Cells were treated with 0.5 μM paclitaxel (lane 2), 50 μM Z-VAD-FMK (lane 3), 50 μM AC-YVAD-CMK (lane 5), 50 μM Z-DEVD-FMK (lane 7), or with paclitaxel plus caspase inhibitors (lanes 4, 6, and 8), or with the same volume of DMSO as control (lane 1). A, after 30-h incubation, cells were harvested and DNA was extracted and subjected to electrophoresis on 1% agarose gel. After staining with ethidium bromide, the fragmented DNA was visualized with UV light. B, for quantitative determination of DNA fragmentation, cells were prelabeled with 10 μCi of [^{3}H]thymidine for 24 h and chased in fresh medium for another 3 h. The labeled cells were treated as described above. After treatment, cells were lysed with lysis buffer and the DNA fragmentation was determined as described under *Materials and Methods*. Each bar is the mean ± S.D. of three independent experiments. C, for assay of β-catenin cleavage, cells were treated with paclitaxel, with caspase inhibitors alone, or with drug plus caspase inhibitors as described above. After treatment, cells were lysed with lysis buffer and an equal amount of total cell lysate (50 μg of protein) from each sample was subjected to 12% SDS-PAGE. The intact and cleaved β-catenin was detected by a monoclonal anti-β-catenin antibody.

and loose cell-cell contacts, thus indicating that drug-induced disruption of the microtubules causes disruption of cell-cell adhesion and their related events. In this study, we have examined in more detail whether paclitaxel-induced apoptosis is associated with the cleavage of components of cell adherens junctions. The results demonstrate that paclitaxel treatment leads to a concentration- and time-dependent cleavage of β-catenin and γ-catenin but not α-catenin or E-cadherin; this was true even if cells were exposed to the drug for 48 to 72 h, by which time ~78% of cells had undergone apoptosis.

These results are consistent with reports by Broncolini et al. (1997) and Schmeiser et al. (1998), who found that treatment with the antitumor agent cisplatin [*cis*-diamminedichloroplatinum(II)] resulted in the cleavage of β- and γ-catenins in human 3T3 fibroblasts and human embryo retinoblasts. Based on their chemical structure, α-, β-, and γ-catenins contain some domains that could potentially be cleaved by caspases (Sacco et al., 1995; Miller and Moon, 1996). However, our results indicate that paclitaxel treatment produced only one major cleaved fragment of β-catenin (78 kDa), one that might correspond to the motif containing AA 760 to 764: DLMDG. No additional cleavage products of β-catenin were detected even when cells were exposed to paclitaxel for 72 h. Under the same experimental conditions, we found that the cleavage of γ-catenin was dependent on paclitaxel exposure time. The first and major cleaved product, with a molecular mass of 76 kDa, might correspond to AA 693 to 696 (DDMD), and was observed after 30 h of drug exposure. The additional proteolytic fragments of 70 kDa and 65 kDa, corresponding to the potential caspase cleavage sites AA 617 to 620 (DAID) and AA 445 to 448 (DKDD, were gradually detected after 36 and 72 h of exposure, respectively. These results indicate that the caspase sensitivity of the cleavage sites in γ-catenin might be different, and/or that different members of caspase that require paclitaxel activation for different periods of time might mediate these cleavages. In addition, other proteolytic enzymes, such as the calcium-dependent cysteine protease calpain, could also be involved in paclitaxel-induced cleavage of β- and γ-catenin (Wood et al., 1998). However, no cleavage product of α-catenin was observed in this study, although there were at least three potential caspase cleavage sites in this protein (Schmeiser et al., 1998). One possible explanation for these results is that the structural conformation of α-catenin in whole cells protects its caspase binding sites. Another may be some unidentified cofactors by which α-catenin could lose its sensitivity to caspase. Finally, it may be that the monoclonal antibody used in this experiment could not recognize the cleavage products of α-catenin.

In this study, we also found that β- and γ-catenins were intact in M-phase cells synchronized either by nocodazole or by paclitaxel, although morphological changes in the M-phase cells suggest that they were starting to undergo apoptosis. These results are consistent with reports by Bauer et al. (1998), who found that only changes in subcellular localization of β-catenin, not cleavage of β-catenin, were associated with cell cycle arrest at M-phase. Samejima et al. (1998) proposed that the apoptotic process can be conceptually divided into at least three stages: the condemned, committed, and execution stages. Based on this concept, paclitaxel-induced M-phase arrest could correspond to the committed stage, during which various caspases are activated to cleave some of the substrates (Samejima et al., 1998). However, our results indicate that the proteolysis of β- and γ-catenins in paclitaxel-synchronized M-phase cells was not detected until 7 to 12 h after incubation in fresh medium, at which time M-phase cells gradually underwent the execution phase of apoptosis. Thus, we suggest that the cleavage of β- and γ-catenins occurs at the execution stage of the apoptotic process.

A considerable amount of evidence demonstrates that caspases are important for the regulation of apoptosis. It is known that there are two main categories of caspases (i.e., initiators, such as caspase-2, -8, -9, and -10, which act upstream of apoptosis, and executioners, such as caspase-3, -6, and -7, which act downstream to cleave the apoptotic substrates). Most caspase family members are proenzymes, and these must be cleaved into activated forms by autoproteolysis or by other proteinases (Martin and Green, 1995). In our experiments, we found that paclitaxel treatment resulted in cleavage of caspase-3 and -7, but not of caspase-1. Kinetic studies showed that the time points of drug-induced cleavage of caspase-3 and -7 were coincident with the drug-induced DNA fragmentation and cleavage of β- and γ-catenins and APC protein. We also found that the pan-caspase inhibitor Z-VAD and the caspase-3 inhibitor Z-DEVD were able to block drug-induced cleavage of β-catenin, but the caspase-1

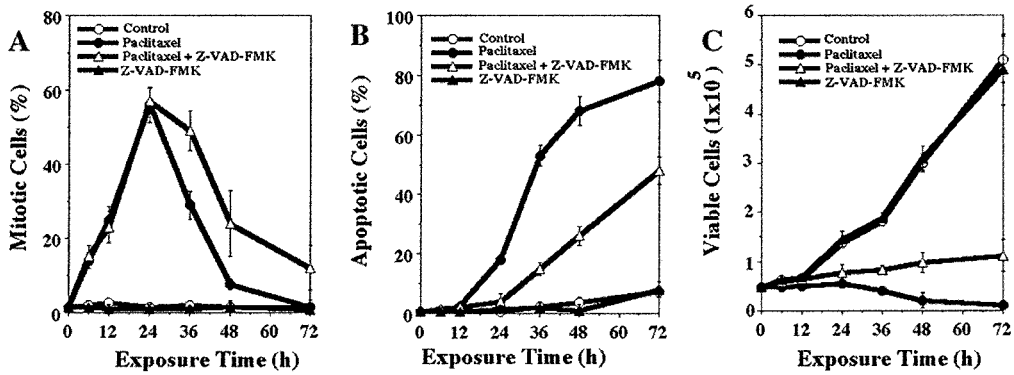

Fig. 8. Effect of pan-caspase inhibitor Z-VAD-FMK on paclitaxel-induced M-phase arrest, apoptosis, and cell growth in H460 cells. Cells were treated with 0.5 μM paclitaxel, 50 μM Z-VAD-FMK alone, or with paclitaxel plus Z-VAD-FMK, or with the same volume of DMSO as control. After treatment for the indicated times, cells were harvested and stained with Wright-Giemsa solution. The numbers of mitotic and apoptotic cells from each sample (A, B) were counted at least 200 cells by microscopy. C, viable cells from each sample were counted by trypan blue exclusion assay. Each point is the mean ± S.D. of three independent experiments.

inhibitor AC-YAVD did not affect it. In addition, the caspase-3 inhibitor Z-DEVD only partially inhibited drug-induced DNA fragmentation in H460 cells. However, complete inhibition of DNA fragmentation in H460 cells was achieved with the use of the pan-caspase inhibitor, Z-VAD. Thus, other caspases, in addition to caspase-3, might be involved in paclitaxel-induced apoptosis and cleavage of adherens junction proteins in H460 cells. Previous studies have shown that the proteolysis of β- and γ-catenins is mediated by caspase-3 in vivo and in vitro (Brancolini et al., 1997; Schmeiser et al., 1998). In this study, we have demonstrated that, as in caspase-3, caspase-7 is cleaved and activated by paclitaxel. The time course study showed that drug-induced caspase-7 cleavage is essentially coincident with the cleavage of β- and γ-catenins. Therefore, both β- and γ-catenins may serve as substrates for caspase-7, although this remains to be further defined by the use of purified caspase-7 to cleave the recombinant β- and γ-catenins in vitro. Furthermore, we have determined the effect of the pan-caspase inhibitor Z-VAD on paclitaxel-induced M-phase arrest and inhibition of cell growth. The results indicated that Z-VAD did not markedly affect drug-induced M-phase arrest during 24 h of incubation, but could delay drug-induced apoptosis of M-phase cells for at least 12 h. These data suggest that the pan-caspase inhibitor does not affect the entry of paclitaxel into cells or disturb the drug's binding to the microtubules. Interestingly, Z-VAD-induced delay of M-phase arrest is essentially coincident with the attenuation of drug-induced apoptosis. We consequently presumed that disproportionate paclitaxel-induced M-phase arrest might be an initial event in the apoptotic process. Only further exposure to paclitaxel could lead to triggering of execution events, such as the cleavage and activation of effector caspases, as well as the proteolysis of some targeting proteins. The caspase inhibitor prevented the activation of some kinds of caspases and the cleavage of targeting proteins and also protected paclitaxel-induced M-phase arrested cells from apoptosis.

Although DNA fragmentation and cleavage of β-catenin was completely inhibited by Z-VAD in cells exposed to paclitaxel for 30 h, ~52% of the cells became apoptotic after 72 h of incubation with paclitaxel and pan-caspase inhibitor, indicating that the inhibitor did not prevent eventual drug-

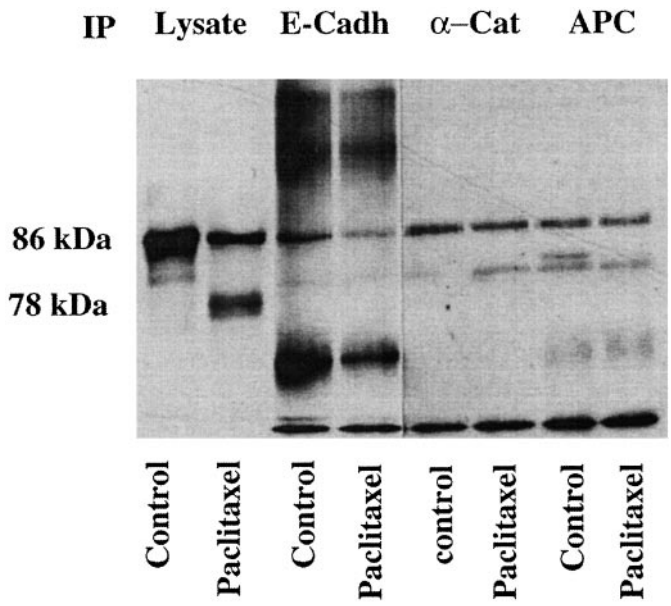

Fig. 9. Effect of paclitaxel-induced β-catenin cleavage on the formation of complexes with E-cadherin, α-catenin, and APC protein. H460 cells were exposed to 0.5 paclitaxel or with same volume of DMSO as a control. After 30 h of exposure, cells were lysed with lysis buffer and immunoprecipitated with anti-E-cadherin, α-catenin, and APC protein antibodies. The immunoprecipitants were separated by 12% SDS-PAGE and β-catenin was detected by a monoclonal anti-β-catenin antibody.

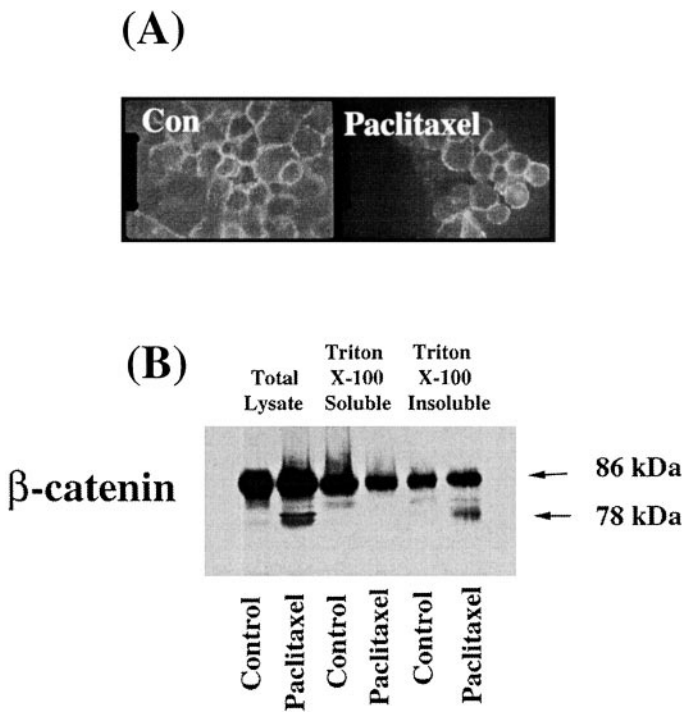

Fig. 10. Immunocytochemical and biochemical studies on subcellular localization of paclitaxel-induced cleavage of β-catenin. A, H460 cells treated with 0.5 μM paclitaxel or the same volume of DMSO as control. After 30 h of exposure, cells were fixed with 4% paraformaldehyde in PBS solution for 2 h, treated with 1% NP-40 in PBS solution for 10 min, and stained with monoclonal anti-β-catenin antibody at room temperature for 1 h. After incubation with fluorescein isothiocyanate-anti-mouse IgG second antibody in a darkroom for 30 min, the localization of β-catenin was detected with a Nikon fluorescence microscope. B, cells treated with 0.5 μM paclitaxel or with the same volume of DMSO as control as described above. After treatment, cells were separated into Triton X-100 soluble and insoluble fractions. The intact and cleaved β-catenin was detected by monoclonal anti-β-catenin antibody.

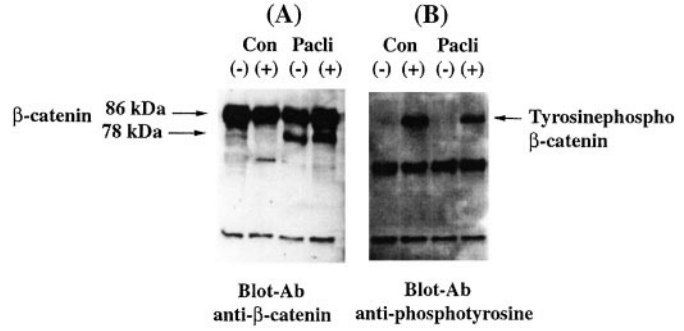

Fig. 11. Tyrosine phosphorylation of β-catenin in H460 cells treated with 0.5 μM paclitaxel or with the same volume of DMSO as control for 30 h. Before lysis, cells were incubated with (+) or without (−) 1 mM pervanadate for 1 h. After lysis, immunoprecipitation was performed with monoclonal anti-β-catenin antibody. The immunoprecipitants were separated by 10% SDS-PAGE, and β-catenin (A) and tyrosine-phosphorylated β-catenin (B) were detected by monoclonal anti-β-catenin and anti-phosphotyrosine antibodies, respectively.

induced cell death. To explain these results, we presume two possibilities: 1) the pan-caspase inhibitor loses its ability to inhibit the apoptotic mechanism after a long period of incubation or 2) the prolonged exposure to paclitaxel triggered caspase-independent mechanisms of apoptosis (Miller et al., 1997). Furthermore, we found that the pan-caspase inhibitor could not completely reverse paclitaxel-induced cytotoxicity and had a cytostatic effect during the 72-h incubation, indicating that these cells were still alive but had lost their proliferating capability. We therefore suggest that this loss or reduction of proliferating capability caused by the pan-caspase inhibitor could be a considerable mechanism of escape from drug-induced cell death, although its precise operation requires further investigation.

A large amount of evidence indicates that cell-cell adhesion depends on the cellular localization of E-cadherin and α-, β- and γ-catenins, as well as formation of complexes with those components (Ozawa et al., 1998). In addition, the modification of E-cadherin and α-, β-, and γ-catenins is implicated in the regulation of their functions. In this study, we have demonstrated that the cleavage fragment of β-catenin induced by paclitaxel was not bound to E-cadherin, α-catenin, or APC protein to form complexes. These results suggest that the cleavage of β-catenin certainly interfered with cell adhesion. In addition, we examined the effect of paclitaxel on the subcellular localization of β-catenin, both by immunocytochemical observation and biochemical analysis. The immunocytochemical results showed that paclitaxel did not strikingly alter the localization of β-catenin; however, the biochemical studies indicated that the cleavage fragments of β-catenin were only detected in the detergent-insoluble fractions. These data suggest that the cleaved products might localize only in membrane and/or nuclear structures. The significance of such subcellular distribution remains to be further defined.

A number of reports have indicated that APC protein is associated with β-catenin, GSK3β, and axin to form the complexes involved in the regulation of Wingless/Wnt signaling transduction pathways (Rubinfeld et al., 1995; Barth et al., 1997). In this study, we found that the APC protein (~250 kDa) in H460 and H520 cells was shorter than full-length protein (300 kDa) and was cleaved into a fragment of ~90 kDa after 30 h of exposure to paclitaxel. Time course studies have also demonstrated that paclitaxel-induced cleavage of APC protein was closely coincident with the cleavage of β- and γ-catenins. Caspase inhibitors Z-VAD and Z-DEVD, but not AC-YAVD, were able to prevent the cleavage of APC protein (data not shown). Thus, as in the cleavage of β- and γ-catenin, the proteolysis of APC protein is mediated by caspase-3 and caspase-7. These results are in agreement with the study by Webb et al. (1999). Although there is no direct evidence to confirm an alteration in β-catenin/APC-related signaling pathways after treatment with paclitaxel, further investigation of such novel mechanisms may provide a new insight into the elucidation of anti-microtubule agent-induced programmed cell death.

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