GENERAL THORACIC SURGERY

PACLITAXEL-INDUCED APOPTOSIS IN NON-SMALL CELL LUNG CANCER CELL LINES IS ASSOCIATED WITH INCREASED CASPASE-3 ACTIVITY

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Objective: Our objective was to determine whether paclitaxel-induced apoptosis in human lung cancer cells is Fas dependent.

Methods: Human lung cancer cell lines were evaluated for morphologic evidence of apoptosis, DNA fragmentation (TUNEL positivity), and caspase-3 activation after paclitaxel treatment. Human lung adenocarcinoma, squamous cell carcinoma, undifferentiated lung carcinoma, and bronchoalveolar carcinoma cell lines were each cultured in 10 μ mol/L paclitaxel.

Results: After 24 hours of culture in paclitaxel, a 22% to 69% increase in the number of apoptotic cells was evident by means of methylene blue-azure A-eosin staining with characteristic blebbing and nuclear condensation. TUNEL assay also confirmed an increase of 19.9% to 73.0% of cells with nuclear fragmentation. Caspase-3 activity, assayed by Z-DEVD cleavage, increased from 20% to 215% ($P < .05$). ZB4, an antagonistic anti-Fas antibody, did not block paclitaxel induction of caspase-3 activity (155.8 vs 165.8 U, not significant). Apoptotic morphologic changes were inhibited in cells cultured in the presence of paclitaxel and Ac-DEVD-CHO, a caspase-3 inhibitor.

Conclusions: Paclitaxel induces apoptosis in lung cancer cell lines, as assessed by a consistent increase in caspase-3 activity, DNA laddering, and characteristic morphologic changes. Paclitaxel-induced apoptosis in human lung cancer cells is associated with caspase-3 activation but is not Fas dependent. (J Thorac Cardiovasc Surg 2000;119:795-803)

Lung carcinoma is the most important cause of malignancy-related mortality in the United States, with 170,000 new cases and more than 160,000 deaths in

1998 alone. The 5-year survival for non-small cell lung cancer remains 14% despite recent advancements in chemotherapy and radiotherapy. Paclitaxel (Taxol) is an antineoplastic agent that has shown promise in the treatment of non-small cell lung cancer. Paclitaxel prevents microtubule disaggregation and induces tumor cell death by apoptosis. Paclitaxel-treated tumor cells arrest in the G₂-M phase of the cell cycle, leading to apoptotic cell death.¹ Apoptosis is a morphologically distinct form of programmed cell death that is associated with chromatin condensation, cytoskeletal alterations, and membrane blebbing. Mitochondrial function is irreversibly impaired early in apoptosis.² In a later stage of apoptosis, nuclear fragmentation becomes evident (karyorrhexis), the cytoplasm condenses progressively, and one or more apoptotic bodies are formed from each dying cell. The events occurring between the stabilization of microtubules and the initiation of apoptosis are unclear, but downstream intracellular events include cleavage of DNA³ and activation of several caspases.⁴ At least 14 separate caspases have been identified and

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form a multigene family containing 4 distinct domains: an amino-terminal domain, a large subunit, a small subunit, and a linker region between the large and small subunits. Activation of each caspase is induced by proteolytic cleavage between domains.⁵ Mammalian cell death proteases have been divided into upstream (initiator) and downstream (effector) caspases on the basis of their sites of action in the proteolytic caspase cascade. Initiator caspases have long prodomains, and effector caspases have characteristic short prodomains. Caspase-3 (CPP32), an effector caspase, is a key member of the caspase family. Poly (ADP-ribose) polymerase, an enzyme involved in DNA repair, is cleaved during apoptosis³ and serves as a substrate for caspase-3.⁶

Several anticancer drugs applied in the treatment of lung cancer, including bleomycin, doxorubicin, etoposide (VP-16), cisplatin, and methotrexate, have been reported to enhance Fas ligand (FasL) expression on the surface of Fas receptor-expressing cells, suggesting that apoptosis caused by these drugs may be mediated by means of Fas cross-linking.^{7,8} Fas (CD95) is a tumor necrosis factor (TNF) receptor family member containing a death effector domain. FasL (CD95L) cross-linking of Fas induces apoptotic cell death in both lymphoid and nonlymphoid cells.⁹ Fas is constitutively expressed on a variety of normal and tumor cells,¹⁰⁻¹² including human lung carcinoma cells.^{13,14} Downstream signaling from the Fas receptor is initiated by the recruitment of proteins to form a death-inducing signaling complex.¹⁵ Binding of FasL to Fas results in the recruitment of Fas-associating protein with death domain, which in turn interacts with the death domain of the trimerized Fas receptor. Fas-associating protein with death domain is responsible for the cleavage of the first of the series of caspases, caspase-8.

Activation of caspase-3 and caspase-9 has been recently demonstrated in HL-60 leukemia cells after paclitaxel treatment, with no significant alteration of HL-60 Fas or FasL expression.¹⁶ The mechanism of paclitaxel-induced lung carcinoma cell death and the intracellular events associated with paclitaxel treatment have not been elucidated. We thus studied the apoptotic cell death in paclitaxel-treated lung carcinoma cells, examining both caspase-3 activity and the role of the Fas-FasL pathway.

Material and methods

Reagents. The drug paclitaxel was purchased from Sigma Chemical Company (St Louis, Mo) as a pure substance and was dissolved and stored in dimethyl sulfoxide before use in individual experiments. Culture media (Dulbecco's modified Eagle's medium and Roswell Park Memorial Institute medium), fetal bovine serum (FBS), streptomycin, and penicillin were obtained from Gibco BRL (Grand Island, NY).

The monoclonal agonistic anti-human Fas immunoglobulin (Ig) M (clone CH11) and the monoclonal anti-human Fas IgG1 (clone ZB4) were obtained from Upstate Biotechnology (Lake Placid, NY). The F(ab')₂ fragment of goat anti-mouse IgG (Fc specific) used as a secondary antibody for clone ZB4 was obtained from Sigma.

Tumor cell lines and cell culture. Human lung carcinoma cell lines 201T and 84T were previously established from endobronchial biopsy specimens from patients with lung cancer. The 84T cell line was derived from a bronchogenic squamous cell carcinoma and initially cultured in A549-conditioned medium and 1% bovine serum, as was 201T, an undifferentiated lung carcinoma cell line.¹⁷ Both 201T and 84T cells were grown in basal medium Eagle with 100 µg/mL streptomycin, 100 U/mL penicillin, and 10% FBS. A549, H226, H596, and H358 cell lines were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, Md; ATCC No.: CCL185, CRL-5826, HTB 178, and CRL-5807, respectively) and are bronchogenic adenocarcinoma,¹⁸ squamous cell carcinoma, adenosquamous carcinoma, and bronchoalveolar carcinoma, respectively. These cell lines were each cultured in Roswell Park Memorial Institute medium supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, and 10% FBS (complete medium) and grown until 80% confluence before paclitaxel treatment.

Morphologic analysis of paclitaxel-treated cells. Cell morphology was examined by using methylene blue-azure A-eosin staining of cells with a leukostat stain kit (Fisher, Pittsburgh, Pa). After a 24-hour incubation period with or without paclitaxel (10 µmol/L), cells were cytopun, fixed, and stained with eosin and methylene blue-azure A. Viability, paclitaxel-induced cytotoxicity, or both were assessed by means of trypan blue exclusion.

TUNEL on cytopun slides. Cell morphology and apoptotic cell death can be simultaneously examined by TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) performed on cytopun slides. DNA fragmentation, present in apoptotic cells, can be quantitated by the identification of multiple DNA 3'-hydroxy termini. This property can be used to identify apoptotic cells by labeling the DNA breaks with fluorescein isothiocyanate-tagged deoxyuridine triphosphate nucleotides. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes template-independent addition of deoxyribonucleoside triphosphate to the 3'-hydroxyl ends of double- or single-stranded DNA. Cytopun preparations of lung carcinoma cells were made after 24 hours of incubation with or without paclitaxel (10 µmol/L) and assessed by means of TUNEL staining with a TdT FragEL fragmentation detection kit (Oncogene Research Products, Cambridge, Mass). Briefly, cells were permeabilized with proteinase K (20 µg/mL) and treated with 30% H₂O₂ to inactivate endogenous peroxidases. DNA fragments were then labeled by means of fluorescein isothiocyanate-tagged deoxyuridine triphosphate nucleotides by using the TdT enzyme for 1.5 hours at 37°C before termination of the labeling with 0.5 mol/L ethylenediamine tetraacetic acid (pH 8). A blocking step with 4% bovine serum albumin in phos-

phate-buffered saline solution was followed by detection with peroxidase streptavidin and 3,3'-diaminobenzidine staining. Slides were counterstained with a methyl green solution and fixed in 100% ethanol before microscopic examination.

Caspase-3 activity assay. Caspase-3 is a marker for cells entering apoptosis. Caspase-3 activity can be measured in an assay on the basis of the cleavage of its substrate, the fluorogenic peptide Z-DEVD-AFC (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; Biorad, Hercules, Calif). After paclitaxel treatment, cells were trypsinized, washed, and adjusted to the same number of cells for each culture condition tested. Pelleted cells were resuspended in lysis buffer and submitted to 4 freeze-thaw steps before the addition of the substrate Z-DEVD-AFC. Luminescence was assessed at time 0 and time 1 hour by fluorometry (luminescence fluorometer Perkin Elmer LS50B; PE Corporation, Norwalk, Conn). Caspase-3 activity values, calculated from a standard curve and generated by using varying concentrations of Z-DEVD-AFC, were expressed in units. Apoptosis was also assessed in lung cancer cells cultured with paclitaxel (10 μ mol/L) in the presence or absence of a caspase-3 inhibitor (Ac-DEVD-CHO; Pharmingen, San Diego, Calif). Ac-DEVD-CHO was added to cell cultures 24 hours before paclitaxel treatment at a concentration of 500 ng/mL.

Treatment of cells with paclitaxel and an anti-Fas antibody (ZB4). To determine whether paclitaxel induces apoptosis through the activation of the Fas receptor in a functional assay, we cultured lung carcinoma cells in the presence of paclitaxel (10 μ mol/L) with and without ZB4 (500 ng/mL), an anti-Fas antibody that blocks Fas receptor activity.⁹ ZB4 was added 1 hour before treatment with paclitaxel. Cells were harvested at 24 hours, and apoptosis was quantified by measurement of caspase-3 activity, as previously described.

Statistical analysis. Data were expressed as means $\pm$ SE and were obtained from an average of 3 to 5 different experiments per cell line. Each experiment included internal controls with the same pool of cells. Derived values were expressed as a percentage of the respective controls for individual experiments. The nonparametric Mann-Whitney *U* test was used for comparison to derive *P* values. A standard linear regression was used for all correlations (*R* values). Calculations and statistical analysis were performed by using the Statview SE II statistical package (Abacus Concepts, Berkeley, Calif).

Results

Paclitaxel induces apoptosis in cultured human lung cancer cells. As evidenced by morphologic and biochemical assays, paclitaxel (10 μ mol/L) induced apoptotic cell death in human lung cancer cells. Optimal cytotoxicity occurred 24 hours after paclitaxel treatment, as detected by trypan blue exclusion with 10 μ mol/L paclitaxel (data not shown). At this concentration, paclitaxel arrested cells in the G₂-M phase of the cell cycle (data not shown). In the basal state cultured lung cancer cells (A549, H226, H358,

H596, 84T, and 201T) exhibited little apoptosis (0.7%-5% of cells). The addition of paclitaxel to tumor cell cultures for 24 hours increased the relative number of apoptotic cells, as assessed by morphologic analysis, by 22% to 69% (*P* < .05; Fig 1). TUNEL assay confirmed an increase in apoptotic cells of 19% to 73%, depending on the individual cell line tested (*P* < .05; Fig 2).

Paclitaxel increases caspase-3 activity. Caspase-3 activity was assayed by using a Z-DEVD cleavage assay. Basal caspase-3 activity in controls (untreated cells) was between 13 and 83 units, depending on the cell line tested. Culture in paclitaxel for 24 hours resulted in a significant increase in caspase-3 activity in A549, H226, H358, H596, and 84T cell lines (*P* < .05; range, *P* = .01-.03). This increase in caspase-3 activity was reproducible for multiple experiments performed on 5 of 6 cell lines tested (*R* = 0.94-0.99; Fig 3).

Paclitaxel-induced apoptosis is reversed by Ac-DEVD-CHO, a caspase-3 inhibitor. Caspase-3 activity was assayed in 3 different lung cancer cell lines (A549, H358, and H596) cultured in paclitaxel for 24 hours in the presence or absence of a caspase-3 inhibitor (Ac-DEVD-CHO). Induction of caspase-3 activity by paclitaxel in the presence of Ac-DEVD-CHO was decreased by 10.5% to 786.8% when compared with control cells (paclitaxel alone; Table I). The addition of Ac-DEVD-CHO also prevented the characteristic morphologic changes associated with apoptosis, such as membrane blebbing and nuclear condensation.

Paclitaxel-induced apoptosis is not blocked by the anti-Fas antibody ZB4. A549, H226, and H358 cell lines were cultured for 24 hours in paclitaxel (10 μ mol/L) or ZB4 (500 ng/mL), an antagonistic anti-Fas antibody that blocks the activity of Fas receptor, alone. In addition, A549, H226, and H358 cell lines were cultured in the presence of both paclitaxel (10 μ mol/L) and ZB4 (500 ng/mL). The addition of ZB4 did not block paclitaxel induction of caspase-3 activity in these cell lines (Fig 4). Furthermore, the addition of a secondary F(ab')₂ fragment, Fc-specific, cross-linking antibody to ZB4 did not block paclitaxel induction of caspase-3 activity (data not shown).

Discussion

In the present study we investigated the intracellular mechanisms involved in paclitaxel-induced apoptosis in human lung cancer cell lines. We showed that paclitaxel induced the characteristic morphologic changes observed in apoptosis, including membrane blebbing and nuclear condensation, as well as DNA fragmentation. Culture in paclitaxel for 24 hours induced a sig-

Fig 1. Paclitaxel-treated human lung carcinoma cells undergo characteristic changes of apoptotic cell death with condensation of nuclear chromatin, membrane blebbing, and karyorrhexis. Cells were cultured for 24 hours in the presence or absence of paclitaxel (10 $\mu\text{mol/L}$). At the end of the incubation period, cells were harvested and stained with methylene blue–azure A–eosin dye (magnification $\times 40$). *PA*, Paclitaxel; *A549*, lung adenocarcinoma cells; *H358*, lung bronchoalveolar carcinoma cells; *201T*, undifferentiated lung carcinoma cells; *H226*, squamous cell lung carcinoma cells; *84T*, squamous cell lung carcinoma; *H596*, adenosquamous lung carcinoma.

Fig 2. Paclitaxel-treated lung cancer cell lines demonstrate TUNEL positivity. After 24 hours of culture in paclitaxel (10 $\mu\text{mol/L}$), cells were harvested and assessed for TUNEL positivity (magnification $\times 10$ and $\times 40$). PA, Paclitaxel; A549, lung adenocarcinoma cells; H358, lung bronchoalveolar carcinoma cells; 201T, undifferentiated lung carcinoma cells; H226, squamous cell lung carcinoma cells; 84T, squamous cell lung carcinoma; H596, adenosquamous lung carcinoma.

Fig 3. Paclitaxel increased caspase-3 activity in all lung cancer cell lines tested. Tumor cells were cultured for 24 hours with or without paclitaxel (10 $\mu\text{mol/L}$) and were assessed for caspase-3 activity by means of a Z-DEVD-AFC fluorometric cleavage assay. Each box represents a separate cell line. Experiments were repeated 3 to 5 times per cell line (as indicated by the individual series shown), with good correlation between experiments ($R = 0.94$ – 0.99 in 5 of 6 cell lines studied). PA, Paclitaxel; A549, lung adenocarcinoma cells; H358, lung bronchoalveolar carcinoma cells; 201T, undifferentiated lung carcinoma cells; H226, squamous cell lung carcinoma cells; 84T, squamous cell lung carcinoma; H596, adenosquamous lung carcinoma.

Fig 4. Paclitaxel-induced apoptosis is not blocked by the antagonistic anti-FAS antibody ZB4. **A**, A549, H358, and H596 cells were cultured in 10 μ mol/L paclitaxel for 24 hours in the presence or absence of the antagonistic anti-FAS antibody (ZB4). Experiments were performed in duplicate for each of the 3 cell lines studied. PA, Paclitaxel. **B**, CH11 (agonistic Fas antibody)-induced caspase-3 activity was inhibited by ZB4. Fas-sensitive Jurkat cells were used as positive controls to test the ability of ZB4 to block caspase-3 activity induced by CH11, an agonistic FAS antibody. 0, No antibody treatment; A549, lung adenocarcinoma cells; H358, lung bronchoalveolar carcinoma cells; H226, squamous cell lung carcinoma cells.

Table I. Paclitaxel-induced apoptosis is blocked by a caspase-3 inhibitor

Cell lines	Controls	Caspase-3 inhibitor	Paclitaxel	Paclitaxel + caspase-3 inhibitor	Percentage inhibition*
H358	37.3	33.9	1085.8	298.9	786.84
A549	42.2	30.6	68.3	50.4	42.4
A549	62.1	49.6	69.4	62.9	10.5
H596	19.9	17.8	57.1	24.2	164.3

Cell lines (A549, H358, and H596) were treated for 24 hours with dimethyl sulfoxide (controls); paclitaxel (10 μ mol/L); caspase-3 inhibitor (Ac-DEVD-CHO; 500 ng/mL); or paclitaxel (10 μ mol/L) plus Ac-DEVD-CHO (500 ng/mL). Caspase-3 activity in cells was assayed by Z-DEVD-AFC cleavage and expressed in units.

*Paclitaxel alone versus paclitaxel plus caspase-3 inhibitor.

nificant increase in caspase-3 activity in all lung cancer cell lines tested. In addition, the addition of an anti-Fas antagonistic antibody failed to block the induction of apoptosis by paclitaxel.

Upregulation of Fas and FasL on the surface of tumor cells after chemotherapy treatment has been demonstrated.¹⁹ Several investigators have reported that drug-induced apoptosis can involve Fas cross-linking.^{12,19}

Others have shown that Fas ligation is not involved in apoptosis induced by etoposide in Jurkat T cells despite significant activation of both caspase-3 and caspase-7.⁹ Similarly, Fas ligation did not induce apoptosis in human renal tubular cells, even though cell-surface Fas expression was demonstrated.²⁰ Recently, Chlichlia and colleagues²¹ postulated that apoptosis' dependency on Fas ligation by FasL is cell-line specific. Cell-surface Fas expression has been reported to be very low, even in Fas-positive lung tumors, and may be primarily cytosolic.²² Our data suggest that Fas cross-linking is not required for paclitaxel-induced apoptosis in lung cancer cell lines.

The finding that paclitaxel induces apoptotic cell death in a non-Fas-dependent mechanism suggests that paclitaxel might be synergistically combined with other, Fas-dependent, apoptotic treatments to enhance tumor regression. Many agents have been shown to induce Fas-dependent apoptotic cell death, including radiation therapy, TNF family members (including TNF- α), lymphotoxin, and FasL. Potentially, paclitaxel could also be used synergistically with immunotherapeutic approaches that enhance T or natural killer cell-mediated death and promote apoptotic death through perforin-mediated delivery of granzymes that activate intracellular caspases.⁹ We have shown similar induction of Fas-independent, apoptotic cell death with other chemotherapeutic agents, including cis-platinum and etoposide (Odoux and associates unpublished data).

The sensitivity of freshly cultured (unpassaged) tumor cells obtained from human lung carcinomas to paclitaxel-induced apoptosis needs to be assessed, and these studies are ongoing in our laboratory. Recently, we have demonstrated that freshly cultured, normal human, bronchial epithelial cells express both Fas and FasL, suggesting that this is a normal host mechanism for induction of autocrine or paracrine cell death and may not be intact in tumor cell lines (data not shown). Attempts to exploit the differential sensitivity of normal and tumor cells to Fas-mediated apoptosis in combination with non-Fas-dependent apoptotic agents, such as paclitaxel, may be important for the design of future antineoplastic therapeutic strategies.

We have been particularly intrigued with the observations of others that apoptotic cells serve as a preferred antigen source for dendritic cells.²³ Dendritic cells are the proximate cells driving immune reactivity, capturing antigen in the periphery, and delivering it to naive T cells within draining lymph nodes to elicit a primary immune response to viral, bacterial, tumor, and alloantigens. Their application in tumor therapies²⁴ in

murine lung cancers, including the 3LL tumor, and potential application in human clinical trials suggest that coupling chemotherapeutic agents (as a source of apoptotic bodies) with immunotherapies (dendritic cells) may be possible. Means to enhance delivery of dendritic cells to sites of tumor undergoing paclitaxel-induced apoptotic death then becomes an exciting anti-tumor strategy. We are in the process of performing studies in vitro to assess the ability of paclitaxel to promote apoptotic death of tumor cells capable of being taken up by dendritic cells and contrasting this with that of other inducers of apoptosis, including gamma radiation, ultraviolet irradiation, serum starvation, FasL or TNF cross-linking, natural killer-induced lysis, and photodynamic therapy. We hope to use this information to initiate a new generation of adjuvant and possibly neoadjuvant strategies in patients with lung cancer.

In conclusion, paclitaxel-induced apoptosis is not mediated by Fas in the lung cancer cell lines tested. Caspase-3 activity is significantly increased after culture in the presence of paclitaxel, and lung cancer cells demonstrate the characteristic morphologic features of apoptotic cell death.

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Discussion

Dr Dao M. Nguyen (*Bethesda, Md*). Have you demonstrated or do you have any idea whether Fas pathway is functional in non-small cell lung cancer?

Dr Tracey L. Weigel. That is actually a very good question. Around 1997, there were many reports suddenly that Fas and FasL were on lung cancer cell lines, and most of these studies were done by using immunostaining. Further work has shown that actually this is probably a cytosolic expression of the Fas receptor, and many postulate now that there is actually a defect in the exteriorization or the localization from the cytosol to the surface of the Fas receptor, and that this, in addition to the expression of FasL on the cell surface, may actually be a way of immune escape by non-small cell lung cancers. We were not able to demonstrate a role with respect to Taxol for Fas receptor.

Dr Nguyen. Have you looked at Fas expression on the surface by using FACS (fluorescence-activated cell sorter)?

Dr Weigel. Yes, we did. Actually, I did not have time to present that, but we did not confirm what has been reported by others with respect to the almost uniform expression of Fas on the surface. We did see it on permeated cells (ie, in the cytosol) but not on the cell surface.

Dr Nguyen. My previous work shows that about 30% to 40% of the lung cancer cell lines expressed variable degrees of Fas, but whether the Fas pathway is functional is unclear. The question of FasL expression on the surface of cells is still a controversial issue at this point because FasL antibodies are known to be unreliable in detecting FasL expression on the surface of cells.

Dr Mark K. Ferguson (*Chicago, Ill*). I noticed that there was some difference among the cell lines with regard to paclitaxel-induced caspase-3 activity. Do you have some explanation?

Dr Weigel. No, we do not. We did not see any consistent difference. We used a variety of histologies in our cell lines, but we did not see any consistent differences with respect to histology. The cell line that really had no effect was 84T, which was a primary tumor-derived culture, and this may be because that cell line has been passaged quite a bit. It is hard to say.