Paclitaxel Triggers Cell Death Primarily via Caspase-independent Routes in the Non-Small Cell Lung Cancer Cell Line NCI-H460

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ABSTRACT

Purpose: Here we report on the role of mitochondria, death receptors (DRs), and caspases in exerting the cytotoxic effect of clinically relevant concentrations of paclitaxel in the non-small cell lung cancer cell line NCI-H460.

Experimental Design: We have characterized paclitaxel-induced cell death with annexin V, propidium iodide staining, and poly(ADP-ribose) polymerase cleavage assays. The involvement of the mitochondria pathway was studied by monitoring cytochrome c release and using H460 cells stable in overexpressing Bcl-2 or Bcl-xl. DR dependency was analyzed in FADD dominant-negative or cytokine response modifier A-overexpressing cells, and a possible role for DR4 and DR5 was investigated by antagonistic antibodies. Caspase activity and cleavage assays and treatment with the synthetic inhibitor zVAD-fmk were used to determine the involvement of caspases.

Results: Paclitaxel-treated cells displayed several features of apoptosis, including annexin V staining and poly(ADP-ribose) polymerase cleavage. The sequence of events suggested the involvement of a DR, as indicated by an early role for Fas-associated death domain and caspase-8, followed by cleavage of Bid and the disruption of mitochondria; nonetheless, we failed to demonstrate the involvement of DR4 and DR5. Interestingly, inhibition of either one of these routes only resulted in a 30% reduction of cell death that was in line with the observed small effect of caspase inhibition by zVAD-fmk on H460 cell survival.

Conclusion: Paclitaxel triggers cell death in H460 cells mainly via a currently unidentified caspase-independent mechanism in which the basic apoptotic machinery is merely coactivated. This finding is in sharp contrast with the largely caspase-dependent response elicited by DNA-damaging agents in these cells. We speculate on therapeutic implications.

INTRODUCTION

Chemotherapy is an established treatment for advanced NSCLC. Anticancer agents exert at least part of their cell killing effects by inducing apoptosis. The taxane paclitaxel is a widely used anticancer agent for the treatment of NSCLC. Paclitaxel stabilizes microtubules and, as a result, interferes with the dynamic changes that occur during the formation of the mitotic spindle, leading to mitotic arrest and subsequent induction of cell death. Its action is different from other anticancer drugs, such as the DNA-damaging agents, and distinct mechanisms underlying the induction of apoptosis and drug resistance have been suggested. Currently, the molecular mechanism by which paclitaxel-induced mitotic arrest leads to apoptosis is not clear, although evidence for the involvement of several signaling pathways has been shown, including the action of various protein kinases such as the mitogen-activated protein kinases, serine/threonine kinase-dependent phosphorylation of Bcl-2, and the p53 pathway (reviewed in Ref. 4). Phosphorylation of Bcl-2 appears to be a hallmark of cell death induced by paclitaxel, but the correlation between this event, mitotic arrest, and apoptosis remains controversial. Initial reports suggested that phosphorylation of Bcl-2 leads to inactivation of its anti-apoptotic function (reviewed in Ref. 5); however, recent data demonstrated that Bcl-2 phosphorylation is a marker of mitotic arrest rather than a determinant of apoptosis (6, 7). p53 also seems to be dispensable for paclitaxel-induced apoptosis (8, 9), which is in agreement with the observation that clinical response rates to treatment with paclitaxel have been shown to be similar between patients with wild-type or mutant p53 cancers (10). As noted by others, the variation in the concentration of paclitaxel used in studies aimed to analyze the mechanism underlying cell death triggered by this agent hampers the comparison and the interpretation of the results (11, 12).

Two major cellular apoptotic pathways have been identified that mediate apoptosis on exposure to different types of...
stimuli, such as anticancer drugs, ionizing radiation, and viruses (13). One route, which is activated by the majority of anticancer drugs, involves the disruption of mitochondria that leads to the release of cytochrome c, which can trigger the activation of caspase-9 in a complex with Apaf-1 and the presence of ATP. Subsequently, additional caspases are activated that take care of the execution phase of the apoptotic process, causing the degradation of cellular proteins and the disassembly of the cell (14). The Bcl-2 family of proteins play a key role in regulating mitochondria stability, and the antiapoptotic members Bcl-2 and Bcl-xL have been extensively characterized; the antiapoptotic function of Bcl-2 and Bcl-xL is opposed by apoptosis-promoting family members such as Bax (15). The pro- and antiapoptotic Bcl-2 homologues can form heterodimers via the so-called Bcl-2 homology domain 3, thereby regulating each other’s activity and, thus, mitochondria integrity. The other apoptosis-mediating route is activated by the ligation of DRs, which belong to the tumor necrosis factor receptor gene superfamily and share a homologous cytoplasmatic sequence termed the death domain that enables engagement of the apoptotic machinery (16). Among the best-characterized ligands that activate DRs are FasL and TRAIL that bind to Fas/CD95 or DR4 and DR5, respectively. On activation, the DRs recruit the adapter molecule FADD by the death domain that is also present on FADD, followed by the activation of caspase-8 (17, 18). The mitochondria route and the DR pathway are linked via the proapoptotic Bcl-2 family member Bid, which can be cleaved by active caspase-8 and subsequently stimulate the release of cytochrome c from mitochondria (19, 20).

In the present study we have examined the contribution of these apoptotic routes to the cell killing effect of physiologically relevant concentrations of paclitaxel in the NSCLC cell line H460. Interestingly, these pathways that form the basic apoptotic machinery in cells played only a minor role in triggering paclitaxel-induced cell death. Nevertheless, the sequence of events that started with partial activation of the DR pathway followed by disruption of mitochondria resembles that of the response induced by DR ligands. The response induced by paclitaxel is clearly different from that induced by DNA-damaging agents that is mediated primarily via mitochondria and caspases (21). On the basis of these findings, we propose that paclitaxel kills H460 cells mainly via an as yet unknown caspase-independent mechanism, possibly in an uncharacterized connection with the DR pathway, and that the observed coactivation of caspases occurs rather as a bystander effect.

MATERIALS AND METHODS

Drugs. Drugs were provided as pure substances. Paclitaxel (Bristol-Myers Squibb, Woerden, The Netherlands) was diluted in ethanol. For each experiment, the stock solutions of the drugs were freshly diluted in culture medium to the indicated final concentration. All of the experiments were performed with IC50 values of paclitaxel, corresponding to clinically relevant concentrations: 50 nM for H460, 10 nM for SW1573, 120 nM for H322, 12 nM for GLC4, and 45 nM for Jurkat cells. The TRAIL/Apo2L protein, the anti-DR4(huTR1-M271)- and anti-DR5(huTRAILR2-M413)-specific mAbs were received from Genentech, Inc. under a joint MTA with Genentech, Inc. and Immunex Corp. The broad-spectrum caspase inhibitor Z-VAD-fmk was diluted to 20 mM in DMSO and added the cells at a final concentration of 50 mM 1 h before addition of paclitaxel.

Cell Lines and Transfectants. The human NSCLC cell lines NCI-H460 (H460), SW1573, NCI-H322 (H322), the SCC cell line GLC4, and Jurkat T-leukemia cells were used in the experiments. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Breda, The Netherlands), 2 mm l-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin, and grown at 37°C in a humidified atmosphere with 5% CO2. Cells from exponentially growing cultures were used in all of the experiments. Stable transfectants of H460 cells expressing Bcl-2, Bcl-xL, CrmA-WT, and FADD-DN were described previously (21). These transfected cell lines were cultured in medium containing a final concentration of 1.5 μg/ml puromycin (Bcl-2, Bcl-xL, and CrmA-WT) or 200 μg/ml neomycin (FADD-DN), respectively. The cell lines were tested regularly for the absence of Mycoplasma infection.

Cell Death Measurement. Cells were plated at a density of 0.5 × 10^5 cells in six-well plates (Costar, Cambridge, MA) 24 h before treatment. Cells were incubated for 8 to 96 h with paclitaxel. For apoptosis experiments with TRAIL/Apo2L and paclitaxel, drugs were added in the presence or absence of antagonistic anti-DR4- and anti-DR5-specific mAbs. The analysis of apoptosis was performed as described previously (21). Briefly, the extent of cell death was determined by PI staining of hypodiploid DNA or by annexin V-FITC and 7-AAD double staining. For the PI staining, 3 × 10^5 cells were resuspended in Nicoletti buffer (50 μg/ml propidium iodide (Sigma Chemicals, St. Louis, MO), 0.1% sodium citrate, 0.1% Triton X-100 and 1 mg/ml RNA-seA (Roche, Basel, Switzerland) in PBS) as described before (21) and analyzed by FACSscan (Becton Dickinson, Mountainview, CA). The fraction of cells with sub-G1 DNA content was assessed using the Lysis program (Becton Dickinson). Annexin V staining was performed according to the manufacturer’s protocol (Nexins Research, Kattendijke, the Netherlands). After incubation with annexin V, 10 μl of 7-AAD (PharMingen, San Diego, CA) was added, and analysis was performed on FACScalibur using CELLQuest software (Becton Dickinson). The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis. For morphological studies, apoptosis was evaluated either by fluorescent microscopic analysis of fragmented nuclei stained with Hoechst 33342 or by light microscopy of cytosin preparations stained with May-Grünwald Giemsa.

Detection of Expression of DR4 and DR5. Cells were stained with DR-4- and DR5-specific mAbs for 45 min at 4°C. Isotype-matched nonbinding antibodies (DAKO, Santa Barbara, CA) were used to control for nonspecific binding. The equivalent to 1 μg of protein of DR4 and DR5 antibodies or the respective control antibody was used in each sample. After the incubation with the primary antibody, cells were washed twice with cold PBS and incubated with FITC secondary antibody at the dilution 1:50 at 4°C in the dark for 30 min. Two new steps of washing with cold PBS were performed before the cells were analyzed by FACScalibur using CELLQuest software. The same instrument settings were used for all of the experiments, and
5000 events were analyzed. MFI ratio was defined as the MFI of gated live cells stained with anti-Fas:MFI of cells stained with isotype-matched antibody.

Electrophoresis and Western Blotting. Western blot analysis was performed essentially as described previously (22). In brief, from each sample, 25 μg of protein/lane were separated on 8–15% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Amersham, Braunschweig, Germany). Subsequently, membranes were incubated overnight at 4°C in solution containing PBS supplemented with 5% nonfat dry milk. For immunodetection, the following antibodies were used: anti-Bcl-2 mAb (Dako); anti-cytochrome c mAb and anticaspase-3 polyclonal antibody (both from PharMingen, San Diego, CA); anticaspase-8 mAb (Immunotech, Marseille, France); anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-PARP (Boehringer Mannheim, Germany); and rabbit polyclonal anti-Bid (provided by Dr. Xiadong Wang). After a 2-h incubation with the primary antibody in dilutions that ranged from 1:500 to 1:2000, membranes were washed in tris-buffered saline [10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.05% Tween 20] followed by horseradish peroxidase-conjugated goat-antimouse or goat-antirabbit antibody. Enhanced chemiluminescence (Amersham) was used for detection, and protein expression was quantified by densitometry of autoradiographs (Model GS-690 imaging densitometer; Bio-Rad, Richmond, CA). Protein loading equivalence was corrected in relation to the expression of β-actin.

Preparation of Cytosol for Measurement of Cytochrome c. Cytosolic extracts were prepared essentially as described before (21). In brief, cells were pelleted by centrifugation after a washing step with ice-cold PBS. Subsequently, cell pellets were resuspended in 2 volumes of buffer A [50 mM Tris, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.2% BSA, 10 mM KH₂PO₄ (pH 7.6), and 0.4 M sucrose], incubated for 20 min on ice, and disrupted by 20 passages through a 26-gauge needle. Cell extracts were clarified of mitochondria by centrifugation at 20,000 × g for 30 min at 4°C. The harvested cytosolic extracts were stored at −80°C and thawed on ice before use.

Fluorimetric Assay for Caspase Activity. Caspase-8- and caspase-3-like enzyme activity was assayed in cells using caspase-8 and caspase-3 activity kits (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer’s instructions. Relative percentage of activity, as measured by DEVD-AFC and IETD-AFC cleavage, respectively, was determined by comparing the levels of treated cells with untreated controls.

Statistics. Quantitative experiments were analyzed by the Student’s t test. All of the Ps resulted from the use of two-sided tests and were considered significant when <0.05.

RESULTS

Paclitaxel Induces Cell Death in H460 Cells. The NSCLC cell line H460 has been extensively characterized previously in our laboratory in regard to its sensitivity to drug- and Fas-induced apoptosis (21, 22). For all of the experiments, IC₅₀ concentrations corresponding to clinically relevant concentrations were used. H460 cells were incubated with 50 nM of paclitaxel for 16, 24, and 48 h, stained with PI, and analyzed by FACS. Cell cycle distribution at these time points showed accumulation of the cells in the G₂-M phases (Fig. 1A). Ap-
proximately 7, 17, and 30%, respectively, of the cells showed a sub-G1 DNA content, indicative of apoptosis (Fig. 1, A and B). These results were confirmed by annexin V-FITC 7-AAD double staining assays, showing the induction of apoptosis already after 8 h of treatment with paclitaxel (Fig. 1B). Morphological features of cells treated with paclitaxel included rounding up, loss of contact with neighboring cells, and detachment from plate. Cytospin preparations demonstrated membrane blebbing, shrinkage, and nuclear condensation, whereas Hoechst 33342 confirmed nuclear condensation and fragmentation (data not shown), consistent with previous reports (12, 23).

As a marker of activation of caspases after treatment, the cleavage of the nuclear protein PARP was determined by monitoring the occurrence of the M, 89,000 cleaved product by Western blotting. PARP cleavage became evident at 24 h after treatment (Fig. 1C), and increased cleavage was observed after 48 h. These results indicate that paclitaxel-induced cell death has several characteristics of apoptotic death.

Mitochondria Play a Minor Role in Paclitaxel-induced Cell Death. The role of mitochondria in mediating cell death in H460 cells exposed to paclitaxel was investigated by analyzing both mitochondria stability as determined by the release of cytochrome c and by studying the involvement of different Bcl-2 family members in this process. As shown in Fig. 2A, the release of cytochrome c by Western blotting in cytosolic extracts derived from paclitaxel-treated H460 cells could not be detected earlier than 48 h after treatment. In addition, paclitaxel treatment resulted in phosphorylation of Bcl-2, characteristic of microtubule-interacting agents, most clearly visible at 16 and 24 h after exposure to the drug as indicated by the occurrence of Bcl-2-specific bands with lower mobility in the gel (Fig. 2B). Bcl-xL phosphorylation was not detected, and the level of expression remained unchanged (Fig. 2B). To additionally examine the role of antiapoptotic Bcl-2 and Bcl-xL, we exploited previously generated and characterized H460 cells stably overexpressing these proteins in which the apoptosis triggering effect of DNA damaging agents such as cisplatin and topotecan was strongly reduced (21). Interestingly, the overexpression of these proteins resulted in only a partial reduction of apoptotic cells by 25% for Bcl-2 (P = 0.15) and 30% for Bcl-xL (P = 0.17) after exposure to paclitaxel (Fig. 2C). Moreover, in time course experiments, the partial inhibitory effect of Bcl-2 and Bcl-xL on apoptosis occurred no earlier than after an exposure time of 48 h, which is in line with the observed cytochrome c release at the same time post-treatment in the parental cells (Fig. 2A). Overexpression of Bcl-2 was effective in preventing the release of cytochrome c, additionally indicating the functional activity of Bcl-2 in these cells (Fig. 2D). Thus, these results show that the role of mitochondria in mediating the cell killing effect of paclitaxel is limited, indicating that alternative mechanisms of cell death must be involved.

The Early Phase of Paclitaxel-induced Cell Death Resembles a DR-mediated Pathway. The findings described above suggest that mitochondria play only a minor and secondary role in mediating the toxic effect of paclitaxel in H460 cells. Involvement of DRs in mediating chemotherapy-induced apoptosis has been described previously (24), and we demonstrated recently the existence of alternative drug-induced routes in H460 cells that trigger DR-independent activation of caspase-8 via an as yet unidentified mechanism (21). Therefore, we examined the possible contribution of the DR pathway to paclitaxel-induced cytotoxicity in H460 cells stably overexpressing FADD-DN that is able to interfere with the function of normal FADD as an adaptor molecule between DRs and caspase-8. This cell line was generated and characterized earlier (21). In H460 cells overexpressing FADD-DN, cell death induced by pacli-
Paclitaxel-induced mode of cell death resembles a death-receptor-mediated pathway. A, H460 cells transfected with FADD-DN or the empty vector were treated with paclitaxel and analyzed for apoptosis by PI staining. B, H460 cells transfected with the caspase-8 inhibitor CrmA or the empty vector were exposed to paclitaxel. After 16, 24, and 48 h, the percentage of apoptotic cells was determined by PI staining. All of the results depicted represent a mean of at least three independent experiments; bars, ± SD. C, Western blot analysis showing the cleavage of Bid on treatment with paclitaxel. β-actin mAb was used as a control for the amount of protein loaded.

The timing of this event parallels the involvement of FADD and caspase-8 in apoptosis induced by paclitaxel at early time points (16 h and 24 h), and the subsequent release of cytochrome c, which does not occur before 48 h of treatment.

The DRs DR4 and DR5 Do Not Mediate Paclitaxel-triggered Apoptosis. We have shown previously that paclitaxel-induced cytotoxicity is not mediated by the Fas system (21). However, other DRs can also act via FADD and may play a role in transducing the cytotoxic effect of paclitaxel. These include DR4 and DR5, which are membrane receptors for TRAIL/Apo2L (25–27). The involvement of DRs in mediating chemotherapy-induced apoptosis has been proposed to occur via the up-regulation of receptor and/or ligand expression (24). Assessment of DR4 and DR5 levels on H460 cells, as evaluated by FACS analysis using specific mAbs against the receptors, showed that DR4 is abundantly expressed, whereas levels of DR5 are lower (Fig. 4A). Apoptosis in H460 cells was readily induced by exposure to TRAIL/Apo2L, as depicted in Fig. 4B: 100 ng/ml TRAIL/Apo2L caused a time-dependent increase in the level of apoptotic cells with an onset at 4 h after treatment. These results indicate the functionality of this pathway in H460 cells. Thus, we investigated the possibility that the early phase of paclitaxel-induced cytotoxicity could be mediated by the TRAIL-receptor pathway. Antagonistic DR4 and/or DR5 mAbs were used to study the cell killing effect of paclitaxel in the absence of a functional DR4/DR5 pathway. As a control for the antagonistic effects, first the potential of anti-DR4 and -DR5 were tested in blocking TRAIL/Apo2L-induced apoptosis, which appeared to be almost complete in case of anti-DR4 (Fig. 4C), whereas anti-DR5 had hardly any effect (data not shown). These results are in agreement with the relative low levels of DR5 expression on these cells. We then additionally studied the potential role of DR4 in mediating the toxic effects of paclitaxel. Antagonistic DR4 antibodies were added 1 h before the addition of paclitaxel, and apoptosis was determined after 16 h, the time point at which the protection provided by FADD-DN was most significant. In contrast to the nearly complete blockade of TRAIL/Apo2L-induced apoptosis, anti-DR4 failed to protect against paclitaxel-induced cell death (Fig. 4C). This result was not affected by varying the concentration of the antibody (10 or 20 μg/ml) or exposure time to paclitaxel (data not shown). In addition, the level of expression of these DRs during paclitaxel-induced apoptosis was examined. Table 1 shows that paclitaxel did not induce an increase in the protein levels of DR4 and DR5 when compared with the levels of untreated controls. Taken together, these observations indicate that the early phase of paclitaxel-induced cell death in H460 cells involves FADD; however, we failed to identify a DR that is responsible for transducing the signal via FADD.

zVAD-fmk Blocks Caspase Activation but Does Not Prevent Paclitaxel-induced Cell Death. Our results indicate that the two major apoptotic pathways only play a minor role in paclitaxel-induced cell death. To additionally substantiate these findings, we studied the contribution of caspases to the cell-killing effects of paclitaxel in more detail. Exposure of H460 cells to 50 nM of paclitaxel for 24 h and 48 h resulted in an increase of caspase-8-like and especially caspase-3-like protease activity as compared with untreated control cells (Fig. 5A) and

Mechanism of Paclitaxel-induced Apoptosis in H460 Cells

taxel at 16 h after treatment was reduced from 10% to 3.5% (P = 0.01). At later time points the apoptosis-protecting effect decreased, with a reduction from 17% to 9% after 24 h (P = 0.02) and a lack of apoptosis inhibition at 48 h (Fig. 3A). Next we used the CrmA derived from cowpox virus, which is a selective inhibitor of caspase-1 and -8 that functions downstream from FADD. Transfectants stably overexpressing CrmA (see Ref. 21) showed 2.3% of death cells at 16 h after treatment versus 7% in cells expressing the empty vector (P = 0.03). After 24 h these numbers were 7.7% versus 14%, respectively (P = 0.03). In line with the results obtained with FADD-DN, CrmA failed to protect at 48 h after exposure to paclitaxel (Fig. 3B).

The results obtained thus far indicate that paclitaxel induces cell death in two phases, an early phase involving the action of FADD and a later phase in which mitochondria contribute to cell death. These two phases resemble the way in which DR ligands trigger apoptosis, involving the initial activation of caspase-8 and at a later step the activation of the Bcl-2-related protein Bid that is cleaved from a precursor by active caspase-8 and subsequently induces mitochondria instability and the release of cytochrome c. In H460 cells exposed to paclitaxel, cleavage of Bid was first detected at 32 h after treatment and increased to near complete cleavage at 48 h (Fig. 3C). The involvement of this event parallels the involvement of FADD and caspase-8 in apoptosis induced by paclitaxel at early time points (16 h and 24 h), and the subsequent release of cytochrome c, which does not occur before 48 h of treatment.
Apo2 L- and paclitaxel-induced apoptosis. H460 cells were incubated with untreated cells. The percentage of apoptotic cells was analyzed by PI staining and compared with controls (MFH). The values represent the mean of three or more independent experiments ± SD.

Table 1  Relative expression of DR4 and DR5 on paclitaxel-treated H460 cells

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<th>Controls</th>
<th>Paclitaxel</th>
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<tr>
<td>DR4</td>
<td>16 h</td>
<td>7.3 ± 1.6</td>
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<td></td>
<td>24 h</td>
<td>5.6 ± 0.8</td>
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<td></td>
<td>48 h</td>
<td>5.6 ± 1.2</td>
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<tr>
<td>DR5</td>
<td>16 h</td>
<td>1.9 ± 0.0</td>
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<td></td>
<td>24 h</td>
<td>2.1 ± 0.1</td>
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<td>48 h</td>
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was accompanied by cleavage of the proforms of caspase-8 as well as caspase-3 to their active products (Fig. 5B). Caspase-9 was not studied here, because we showed previously that this caspase is not activated by chemotherapeutic drugs in H460 cells (21). The processing of cleaved forms of caspase-8 and -3 could be blocked by pretreatment of the cells with 50 μM of the broad-spectrum caspase inhibitor zVAD-fmk (Fig. 5B). Likewise, pretreatment with zVAD-fmk was effective in preventing PARP cleavage (Fig. 5C).

Next, we evaluated the effect of caspase inhibition on paclitaxel-induced cell death. In line with the observations pointing at a minor role for DR and mitochondria pathways, pretreatment with zVAD-fmk hardly affected paclitaxel-induced cell death (Fig. 5D). At 16 h and 24 h after treatment, cells exposed to paclitaxel had a similar percentage of apoptotic cells as cells pretreated with zVAD-fmk, whereas after 48 h, 72 h, and 96 h, zVAD-fmk reduced the percentage of apoptosis only from 34.5% to 30.5%, from 38% to 32%, and from 33% to 28.5%, respectively. Morphologically, cells pretreated with zVAD-fmk could not be distinguished from cells exposed to paclitaxel alone, because they still displayed apoptotic features such as nuclear condensation and apoptotic bodies (data not shown).

In addition, to study the effect of combined inhibition of the mitochondrial and caspase pathways on paclitaxel cytotoxicity, H460 cells stably overexpressing Bcl-2 were treated with zVAD-fmk. Paclitaxel-triggered cell death was somewhat reduced on zVAD-fmk treatment, from 26% to 18% at 48 h after treatment, whereas the empty vector control cells showed a minor reduction from 32% to 27% (Fig. 5E), indicating that simultaneous inhibition of the mitochondria pathway and caspases results in a slightly increased but still incomplete protection against paclitaxel-induced cytotoxicity. Finally, we investigated whether the lack of effect of zVAD-fmk on paclitaxel-induced cell death would also apply to other lung cancer cell lines. The NSCLC cell lines SW1573 and H322, and the SCLC cell line GLC4 were exposed to their respective IC_{50} concentrations of paclitaxel for 48 h and compared with cells pretreated with zVAD-fmk. Jurkat T-leukemia cells were used as control cells in these experiments, because leukemic and lymphoid cells are known to be more apoptosis-prone than most solid tumors (28). As shown in Fig. 5F, zVAD-fmk showed a similar inability to prevent paclitaxel-induced apoptosis in SW1573 and H322 as in H460 cells. In contrast, in Jurkat cells, zVAD-fmk provided a 75% protection, whereas in GLC4 a reduction from 24% to 16.5% was observed. Thus, we conclude that our findings of a caspase-independent mechanism of paclitaxel-induced cell death can likely be extrapolated to other NSCLC cell lines.

**DISCUSSION**

The molecular mechanisms involved in the mediation of cell death induced by the potent anticancer agent paclitaxel are...
only partially understood. In this study, the role of mitochondria- and DR-dependent apoptosis was examined in the NSCLC cell line H460 on treatment with a clinically relevant concentration of paclitaxel. These two distinct routes were found to contribute at two different stages. In an early phase, at 16 to 24 h after treatment, FADD partially mediated paclitaxel-induced cytotoxicity, whereas at a later stage (48 h), the mitochondrial pathway enhanced the triggering of cell death. However, our results indicate that these apoptotic routes do not play an essential role in mediating paclitaxel-induced cell death as demonstrated by the finding that inhibition of these pathways by stable overexpression of FADD DN or Bcl-2 resulted only in a temporary protection until 24 h after exposure and in a 30% reduction in the fraction of death cells after 48 h, respectively. In line with this, the broad-spectrum caspase inhibitor zVAD-fmk did not substantially rescue the cells from cell death, indicating the existence of an alternative, caspase-independent mechanism that is instrumental for the cell killing effect of paclitaxel.
Treatment with paclitaxel elicited several events in H460 cells that have been found to be characteristic for this drug, such as G2-M arrest and the phosphorylation of Bcl-2. Recent studies have demonstrated that Bcl-2 phosphorylation is a marker of mitotic arrest rather than a determinant of apoptosis (6, 7). Therefore, phosphorylation of Bcl-2 at early time points in H460 cells cannot be considered as a key event in mediating cell death, consistent with the lack of effect observed with the overexpression of Bcl-2 and Bcl-xL. Our finding that the anti-apoptotic Bcl-2 family members had only a small protecting effect at later stages of apoptosis in H460 cells is in contrast with reports that have indicated stronger suppression of apoptosis by Bcl-2 and Bcl-xL (29–33). The use of higher concentrations of paclitaxel than in the current study as well as unknown cell line specific factors may account for this discrepancy.

The biphasic response to paclitaxel treatment that we found in H460 cells followed the sequence of a DR pathway: an early involvement of FADD (16–24 h), cleavage of Bid (32–48 h), and at later stages (48 h) an enhancing effect involving mitochondria. The ability of DRs to mediate chemotherapy-induced cytotoxicity, which has been proposed to occur via the up-regulation of receptor and/or ligand expression in treated cells (24), is currently debated. Although several investigators have shown that the Fas/FasL system contributes to chemotherapy-induced apoptosis (reviewed in Ref. 34), genetic studies have demonstrated that the apoptotic response to treatment with cytotoxic drugs is largely intact in FADD knockout mice, arguing against the involvement of DRs (35, 36). In addition, the activation of caspase-8 in colon cancer cells and in B-lymphoma cells by paclitaxel was found to be independent of Fas/FasL (37, 38). We established previously that paclitaxel-induced cytotoxicity is not mediated by the Fas system (22), and in the current study we assessed a possible role for DR4 and DR5, which are membrane receptors for TRAIL/Apo2L. Although H460 cells abundantly express DR4, which effectively mediates TRAIL-induced apoptosis in these cells, antagonistic antibodies did not prevent paclitaxel-triggered cell death. These experiments exclude a role of the TRAIL/Apo2L system in transducing the cytotoxic effect of paclitaxel.

Altogether, the contribution of these caspase-dependent routes to the cytotoxic effects triggered by paclitaxel was not essential. Although overexpression of CrmA rescued H460 cells from early paclitaxel-induced cell death, this protection was lost after 48 h of treatment. These findings are corroborated by the absence of substantial protection against paclitaxel-induced apoptosis provided by zVAD-fmk, implying the existence of caspase-independent routes. Moreover, a double antiapoptotic block in H460 cells, provided by overexpression of Bcl-2 in combination with pretreatment with zVAD-fmk, resulted in only a slightly increased and still incomplete protection against paclitaxel-induced cytotoxicity. Therefore, the activation of caspasas on paclitaxel exposure, as established by caspase-activity assays and cleavage of proforms of caspase-8 and -3, is not instrumental for the execution of cell death and can be considered a bystander effect.

The observation of induction of caspase-independent pathways by paclitaxel is not unprecedented. Okano and Rustgi (12) reported recently that physiologically relevant concentrations of paclitaxel caused cell death via both caspase-dependent and -independent pathways in human esophageal squamous cancer cells. Other studies in H460 and H520 NSCLC cells found a more pronounced protective effect of zVAD-fmk against paclitaxel-induced apoptosis, although the authors mentioned that zVAD-fmk did not reverse paclitaxel-induced inhibition of cell growth but resulted in homeostasis (23). The main cause for the difference with our findings probably is the use of a 10-fold higher concentration of paclitaxel than the one we applied in the current study. Activation of other effector caspasas on paclitaxel-treatment, such as caspase-7, which has been reported previously (23), was not directly evaluated in our study.

Whether apoptosis contributes to drug-induced cell death is a current controversy in the field (39). Evidence has accumulated that apoptosis is not the main mediator of cell killing after anticancer therapy in solid tumors, in contrast to lymphomas and leukemias, although the specific triggering of apoptosis in tumor cells by molecular intervention is a promising novel anticancer approach that is currently being developed and tested in the clinic (40). However, evolving nomenclature as well as the use of different assays to measure cell death has led to substantial increase of confusion in the field. ZVAD-fmk has been widely used to assess caspase-independent cell death but can also inhibit other noncaspase cysteine proteases (41). Chemotherapeutic drugs often cause so-called “slow-cell death” without caspase activation, in which cells die in a protractive and inefficient manner but which may nevertheless be associated with apoptotic features (28). In a recent review, four patterns of cell death mode are described with apoptosis and necrosis on opposite far ends. Apoptosis-like programmed cell death and necrosis-like programmed cell death are used to define the many cases of caspase-independent and specialized caspase-independent signaling pathways, respectively (42). This knowledge substantially weakens the paradigmatic antithesis of apoptosis and necrosis (43). The choice between apoptosis and slow cell death is cell-type dependent, and the availability of classical apoptotic pathways is usually more pronounced in lymphoid and leukemia cells than in solid tumors (28). This is in line with our finding that in contrast to NSCLC cells, zVAD-fmk did rescue Jurkat cells from paclitaxel-induced apoptosis, again emphasizing the role of cell-specific factors in death responses. Because DR and mitochondria-independent pathways can be triggered in Jurkat cells as well, which has been shown recently for the sesquiterpene lactone helenalin (44), stimulus-specific factors are also involved in determining the relative importance of different death mechanisms. It is likely that in many cell types multiple pathways leading to cell death are latently present (45). Participation of other, noncaspase proteases, such as calpains and cathepsins, in chemotherapy-induced apoptosis has been reported (46–49). Whether similar mechanisms could account for paclitaxel-induced apoptosis in NSCLC cells remains to be demonstrated. Additional studies to delineate the signaling pathways triggered by paclitaxel in NSCLC cells leading to cell death are currently being carried out in our laboratory.

The results of our study support the notion that the mechanism underlying the induction of apoptosis by microtubule-interacting agents such as paclitaxel differs in some respects from that of other anticancer drugs (3) and could involve pro-apoptotic factors interacting with microtubules, such as Bim.
Mechanism of Paclitaxel-induced Apoptosis in H460 Cells

Fig. 6 Activation of distinct cell death pathways by DNA-damaging agents and paclitaxel in H460 cells. Whereas DNA-damaging agents clearly trigger apoptosis in H460 cells, paclitaxel induces cell death mainly via caspase-independent pathways. Activation of DR and mitochondria (mito) routes is not instrumental and should be considered a bystander effect.

However, Bim activation is abrogated by Bcl-2 overexpression (50), which makes its involvement in paclitaxel-induced cell death unlikely. In particular, our findings provide a molecular basis for the distinct cytotoxic activities of paclitaxel in comparison with DNA-damaging agents, including cisplatin, gemcitabine, and topotecan. As depicted in Fig. 6, apoptosis triggered by DNA-damaging agents is largely dependent on caspase-8 and the mitochondrial pathway (21). In contrast, activation of caspases and mitochondria seems to be a mere bystander effect on paclitaxel treatment. In H460 cells, paclitaxel induces cell death via an as yet uncharacterized caspase-independent mechanism, which might be connected with FADD, whereas apoptosis induced by DNA-damaging agents does not involve FADD or DRs. These differences seem to be specific for NSCLC cells and could not be demonstrated for Jurkat cells, which suggests that in particular tumor types it is interesting to study the application of cell death mechanisms in relation to the response to chemotherapy and prognosis.

The triggering of distinct apoptotic pathways by paclitaxel in NSCLC cells might have therapeutic implications, because our results were obtained with clinically relevant drug concentrations. The absence of an essential role for mitochondria and caspases in paclitaxel-induced apoptosis in H460 cells could have clinical consequences. Bcl-2 is abnormally expressed in 25% of NSCLCs (51, 52), and on the basis of the current evidence that the relation between expression of inhibitors of apoptosis and chemosensitivity in NSCLC may be more complex than anticipated by in vitro data (53, 54). Finally, paclitaxel is used in several combination regimens, and the combination of paclitaxel and cisplatin or gemcitabine was found to be sequence dependent (55, 56). The current study may open up new ways to study the effect of combination therapy in relation to the apoptotic mechanism activated.

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