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Caspase-8 Activation Independent of CD95/CD95-L Interaction during Paclitaxel-Induced Apoptosis in Human Colon Cancer Cells (HT29-D4)

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ABSTRACT. Antimicrotubule agent-induced apoptosis was examined in the proliferating human colon cancer cell line HT29-D4. G2/M arrest and subsequent apoptosis were dose-dependent, both observed with 100 nM paclitaxel or docetaxel and 10 nM vinorelbine. Bcl-x_L phosphorylation was observed simultaneously with mitotic block, then caspase-3 cleavage and poly(ADP-ribose) polymerase degradation were detected 48 hr later. By using both enzymatic assay and immunoblot detection of cleaved fragments, we showed that caspase-8, a central component of the CD95-induced apoptotic pathway, was significantly activated during paclitaxel exposure, contemporary to apoptosis occurrence. Caspase-8 activation and apoptosis were independent of CD95 ligation and evidenced only for concentrations inducing Bcl-x_L phosphorylation and a decrease in mitochondria permeability. Similar results were obtained with docetaxel and vinca alkaloids. Thus, antimitotic drugs may induce apoptosis via caspase-8 activation independently of CD95/CD95-L. Caspase-8 may be a common mediator of anticancer drug-induced apoptosis that could represent a promising target for future therapies. BIOCHEM PHARMACOL **60**;11:1579–1584, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. paclitaxel; antimicrotubule agents; apoptosis; caspase-8; CD95

Antimicrotubule agents, especially taxoids such as paclitaxel (Taxol®) and docetaxel (Taxotere®), have proven to be highly effective in the treatment of several malignancies and represent a growing class of cytotoxic compounds [1]. At the cellular level, paclitaxel binds to the β-tubulin subunits in microtubules, thus promoting polymerization of tubulin and disrupting microtubule dynamics, leading to a sustained mitotic arrest and ultimately to apoptotic cell death [2]. However, molecular pathways involved in the apoptotic process induced by this agent are still not known. Antimicrotubule agents are known to induce phosphorylation and thus inactivation of the antiapoptotic members of the Bcl family such as Bcl-2 [3–5]. The Bcl protein family acts at multiple levels to regulate mitochondrial integrity and the activity of apoptotic caspases responsible for cell death [6, 7]. Activation of the effector procaspase-3 has been shown with paclitaxel, leading to degradation of PARP† and morphologic features of apoptosis [8, 9]. Cleavage of caspase-7, another effector caspase, was induced with paclitaxel in PC-3 and HeLa cells [10]. However, little is known about other caspases involved in paclitaxel-induced apoptosis and their link to Bcl protein phosphorylation.

Caspase-8 (FLICE/MACH) has been identified as a central component in the CD95 (APO-1/Fas) apoptosis pathway. CD95-mediated cell death is initiated by the CD95 receptor engagement, resulting in aggregation of its intracellular death domain leading to the recruitment of Fas-associated death domain protein/mediator of receptor-induced toxicity-1 (FADD/MORT-1) and procaspase-8. The association of procaspase-8, FADD, and the receptor form the DISC (death-inducing signaling complex), which induces the proteolytic cleavage of procaspase-8 to form the active enzyme. This is the first step of the caspase cascade that triggers activation of several effector procaspases involving caspase-3 [11]. Caspase-8 activation and the involvement of CD95/CD95-L interaction induced by anticancer drugs, including paclitaxel, remain largely controversial [12–16].

We have previously described that paclitaxel and other antimicrotubule agents were able to induce apoptosis in the human colon cancer HT29-D4 cell line [17], which expresses CD95, CD95-L, and procaspase-8 [18, 19]. In the present report, we demonstrate for the first time that antimicrotubule agent-induced apoptosis involves caspase-8 activation following mitotic block, Bcl-x_L phosphorylation, and a loss in mitochondrial transmembrane potential. This apoptotic pathway is independent of CD95

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† *Abbreviations:* PARP, poly(ADP-ribose) polymerase; CD95-L, CD95 ligand; IFN-γ, interferon-γ; mAb, monoclonal antibody; PI, propidium iodide; TUNEL, TdT-mediated dUTP nick end labeling; λ Ptase, λ protein phosphatase; ΔΨm, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; and FLICE, FADD-like ICE.

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ligation in HT29-D4 cells. Thus, caspase-8 activation could be a common signal event of several apoptotic pathways irrespective of the contribution of CD95.

MATERIALS AND METHODS

Cell Line and Treatment

HT29-D4 clone was originally isolated by limited dilution in our laboratory [20]. Doubling time was 20 ± 2 hr [21]. Exponentially growing cells were plated 72 hr before all experiments. Then, cells were treated continuously with paclitaxel (Sigma), docetaxel (gift from Rhône-Poulenc Rorer), or vinorelbine (Pierre Fabre Oncologie) for 24, 48, or 72 hr. The highest concentration of DMSO used was 0.2%. For experiments using CD95-blocking antibody, drugs were added after 1-hr preincubation with 1–5 μg/mL of ZB4 (Immunotech Coulter) and co-incubation was continued for 72 hr.

Cell Cycle Analysis

Cells were stained with PI (Sigma), then DNA content was measured by flow cytometry (FACScan, Becton Dickinson) as previously described [22].

Detection of Apoptosis with the Annexin V–FITC Kit and TUNEL Assay

Surface exposure of phosphatidylserine in apoptotic cells was measured by adding Annexin V–FITC (Immunotech Coulter) before flow cytometry analysis as previously described [23]. Additional exposure to PI made it possible to differentiate apoptotic cells (annexin-positive and PI-negative) from necrotic cells (annexin- and PI-positive). After 72-hr paclitaxel treatment, a TUNEL assay (Apoptag *in situ* fluorescein assay; Oncor) was performed, as previously described [17].

Immunoblots

Cells were lysed in ice-cold lysis buffer (2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate, 1% Triton X-100, and 50 mM Tris pH = 7.5 with protease inhibitors) and an equal amount of protein per lane (30 μg) was fractionated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with polyclonal rabbit antibodies anti-Bcl-X_L (Santa Cruz Biotechnology) and anti-caspase-3 (Pharmingen) or mouse mAb anti-PARP (clone C-2-10, Zymed) and anti-caspase-8 (C15). Then, blots were labeled with an anti-rabbit or anti-mouse antibody conjugated with peroxydase and visualization was performed using the ECL (enhanced chemiluminescence) detection kit (Amersham). To confirm that the mobility shift observed for Bcl-x_L following drug treatment was dependent on phosphorylation, cell lysates were treated with the enzyme λ protein phosphatase (New England Biolabs) as described by Poruchynsky *et al.* [24].

Detection of Caspase-8 Activation

The upstream sequence of the specific site recognized by active caspase-8 is IETD (Ile-Glu-Thr-Asp) [25]. For detection of caspase-8 activation, we used the caspase-8 (FLICE) apoptosis detection kit-colorimetric (US Biological), which is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the substrate IETD-pNA in the presence of cytosolic extracts. Treated or control cells were resuspended in cell lysis buffer and incubated on ice for 10 min. After centrifugation, an equal amount of protein from cytosolic extract (150 μg) was diluted in reaction buffer containing dithiothreitol (DTT) and incubated with IETD-pNA (200 μM final concentration) in a 96-well plate at 37° for 2 hr. The pNA light absorbance was then quantified at 405 nm. As positive control, we used HT29-D4 cells exposed to 500 ng/mL of CD95 agonist mAb (Immunotech Coulter) after a 5-min pretreatment with 40 μg/mL of IFN-γ (Genzyme). Background reading from cell lysates and buffers was subtracted from the readings of each sample. At least three independent experiments were performed using separate cultures.

Determination of Mitochondrial Membrane Potential

For determination of $\Delta\Psi m$, cells (10^6 cells/mL) were incubated with $DiOC_6(3)$ (100 nM) (Molecular Probes) for 15 min at 37° and analyzed on a flow cytometer [26].

RESULTS

Mitotic Block Requirement for Paclitaxel-Induced Apoptosis

Antimicrotubule agents such as paclitaxel, docetaxel, and vinorelbine were cytotoxic against the HT29-D4 cell line by activating an apoptotic process [17]. The observed IC_{50} values for paclitaxel, docetaxel, and vinorelbine were 25, 25, and 10 nM, respectively. In order to accurately describe this phenomenon, cells were continuously treated by paclitaxel and DNA content was quantified by flow cytometry (Table 1). Using paclitaxel at a concentration of 100 nM, the cell population was first strongly arrested in G2/M after 24-hr exposure. Subsequently, a hypodiploid population appeared and was evident after 72- (Table 1) and 96-hr (not shown) exposure. The apoptotic nature of observed cell death was confirmed by Annexin V–FITC binding (Fig. 1C) and TUNEL assay (data not shown). Identical results, i.e. mitotic block and subsequent apoptosis, were obtained with vinorelbine and docetaxel at concentrations at least equal to the IC_{50} values (Fig. 1D and data not shown). Concomitantly to DNA fragmentation, cleavage of caspase-3 and consequently PARP cleavage were detected by Western blot (data not shown). When we used a concentration below the IC_{50} value, no mitotic block and therefore no significant apoptosis were detected (Table 1, Fig. 1B and data not shown for docetaxel and vinorelbine).

TABLE 1. Variation in cell DNA content during paclitaxel exposure

Treatment	G0/G1 (%)	G2/M (%)	Apo (%)
DFM 24 hr	53 ± 1	33 ± 4	4 ± 1
PTX 10 nM 24 hr	51 ± 6	26 ± 1	6 ± 2
PTX 100 nM 24 hr	10 ± 4	65 ± 2	6 ± 4
DFM 48 hr	69 ± 1	22 ± 6	8 ± 3
PTX 10 nM 48 hr	62 ± 2	23 ± 4	12 ± 8
PTX 100 nM 48 hr	14 ± 5	57 ± 6	19 ± 5
DFM 72 hr	66 ± 12	19 ± 6	14 ± 5
PTX 10 nM 72 hr	63 ± 8	18 ± 6	17 ± 4
PTX 100 nM 72 hr	15 ± 5	41 ± 10	58 ± 6
IFN/CH-11 24 hr	23 ± 5	7 ± 3	60 ± 10
IFN/CH-11 24 hr/ZB4	80 ± 3	7 ± 2	11 ± 2
PTX 100 nM 72 hr/ZB4	12 ± 3	32 ± 3	65 ± 7

Cells were incubated in drug-free medium (DFM) or exposed to paclitaxel (PTX) before PI staining and flow cytometry analysis. Involvement of CD95 receptor was investigated by using co-incubation with CD95 antagonist mAb ZB4 (see Materials and Methods). Apoptotic cells (Apo) are located in the sub G0/G1 region. The results are the means ± SD of at least three independent experiments using separate cultures.

Therefore, only concentrations of antimicrotubule agents causing mitotic block were able to induce significant apoptosis in proliferating HT29-D4 cells.

Association of Bcl-x_L Phosphorylation and Mitotic Block in Paclitaxel-Treated Cells

Since Bcl-2 is unexpressed in HT29-D4 cells, we analyzed Bcl-x_L expression during paclitaxel treatment by Western blot. For paclitaxel concentrations causing mitotic block, an electrophoretic mobility shift was detected after 24-hr exposure (Fig. 2A). To demonstrate that such a mobility shift was due to Bcl-x_L phosphorylation, cell lysates were treated with the enzyme λ Ptase, which has specificity for cleavage of phosphate groups appended to the amino acids serine, threonine, or tyrosine [27]. Following λ Ptase treatment, we observed that the more slowly migrating form of Bcl-x_L was absent (Fig. 2A). These results strongly suggest that paclitaxel-induced electrophoretic mobility alteration of Bcl-x_L was due to phosphorylation. No electrophoretic mobility shift was detected when a low dose of paclitaxel unable to induce G2/M arrest and apoptosis (e.g. 10 nM) was used. Maximum Bcl-x_L phosphorylation was observed after 24-hr treatment, preceding occurrence of apoptosis. These results were also noted with vinorelbine 10 nM, a concentration causing mitotic block, as shown in Figure 2A. Therefore, we confirmed that Bcl-x_L phosphorylation is related to G2/M arrest and subsequent apoptosis.

Caspase-8 Activation during Paclitaxel-Induced Apoptosis

CD95-induced apoptosis has been described on HT29 cells following sensitization by cytokine pretreatment including interferon-γ [28]. Indeed, CD95-mediated apoptosis was induced in HT29-D4 cells by using an agonist monoclonal antibody, CH-11, directed to CD95 receptor after brief incubation with IFN-γ. Cell death was present after 24 hr of exposure (Table 1) and massive after 48 hr (data not shown). By using an enzymatic assay, we compared caspase-8 activation during paclitaxel- or IFN-γ/CH-11-induced apoptosis, the latter being used as a positive control (Table 2). In paclitaxel-treated cells, caspase-8 activity was also observed, occurring at 72 hr and coinciding with significant apoptosis. Interestingly, this phenomenon was

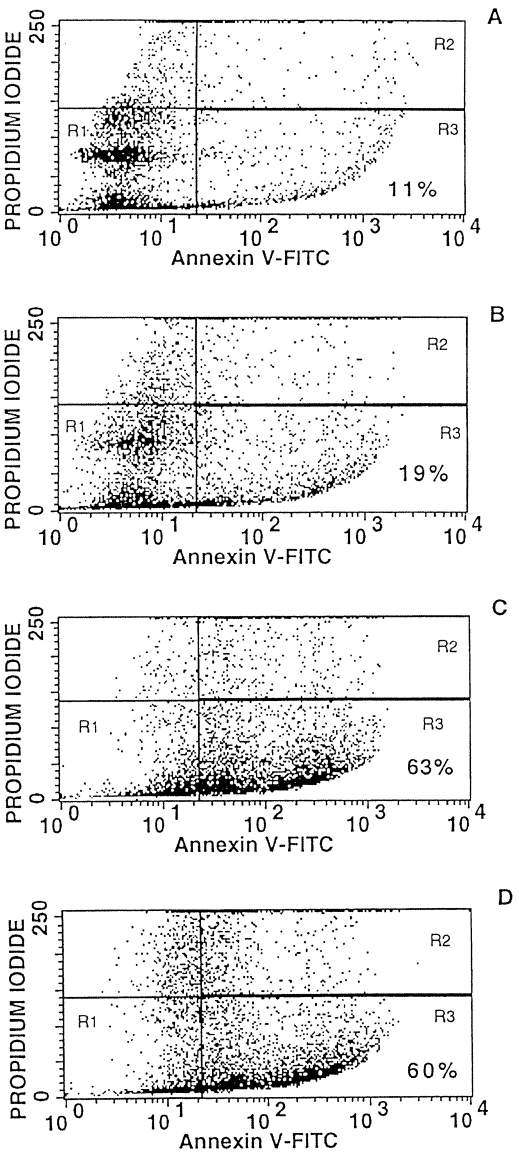

FIG. 1. Detection of apoptosis by the Annexin V–FITC method. Cytograms show Annexin V–FITC binding (abscissa) versus PI uptake (ordinate) in HT29-D4 cells after 72 hr of antimicrotubule agent exposure: untreated cells (A); cells treated with paclitaxel 10 nM (B), 100 nM (C), or vinorelbine 10 nM (D) for 72 hr. Vital, apoptotic, and necrotic/late apoptotic cells are located in gates R1, R2, and R3, respectively. Data are representative of at least three independent experiments using separate cultures.

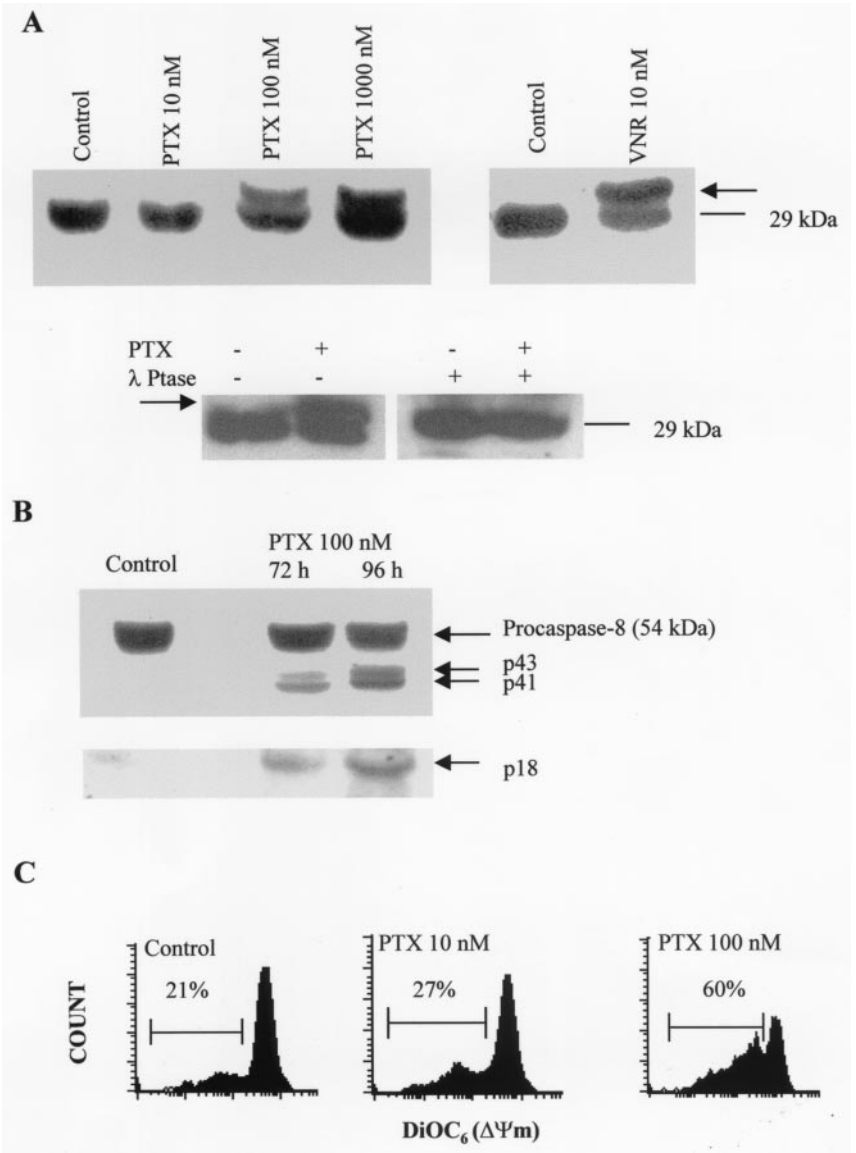

FIG. 2. (A) Bcl-x_L expression during antimicrotubule agent exposure: cells were untreated or treated for 24 hr with paclitaxel (PTX) (10, 100, or 1000 nM) or vinorelbine (VNR) (10 nM) before immunoblotting (top); cell lysates from control or 100-nM paclitaxel-treated cells for 24 hr were incubated without or with 200 Units of λ Ptase before SDS–PAGE and immunoblotting (bottom). Arrows indicate the slower migrating form of Bcl-x_L. (B) Caspase-8 cleavage during paclitaxel exposure: cells were untreated or treated with paclitaxel 100 nM for 72 or 96 hr before immunoblotting. The blots show procaspase-8, the intermediate cleavage products of 43 kDa and 41 kDa, and the active subunit of 18 kDa. (C) Loss of mitochondrial membrane potential during paclitaxel exposure: cells were untreated or treated with paclitaxel (10 or 100 nM) for 72 hr and were monitored for loss of mitochondrial membrane potential ($\Delta\Psi m$) as reflected by reduced uptake of $DiOC_6$. Data are representative of at least three independent experiments.

not detected after 10-nM paclitaxel exposure, an inframitotic concentration. Activation of caspase-8 in paclitaxel-treated cells was confirmed by immunoblot detection of the intermediate cleavage products p43 and p41 as well as the p18 active subunit (Fig. 2B).

Caspase-8 Activation and Apoptosis Independent of CD95 Ligation

Then, we tested if CD95/CD95-L interaction was involved during paclitaxel-induced apoptosis. Following co-incubation with paclitaxel 100 nM and CD95-blocking antibody ZB4 for 72 hr, no decrease in either caspase-8 activation or cell death was observed, even when a high concentration of ZB4 (5 μg/mL) was used (Tables 1 and 2). Identical results (i.e. caspase-8 activation and no effect of CD95-blocking antibody treatment) were obtained by using vinorelbine 10 nM, making it possible to extend these data to other antimicrotubule agents.

Alteration of Mitochondria Permeability Concomitant to Caspase-8 Activation

Available data suggest that caspase-8 can act either upstream or downstream of mitochondria [13]. To investigate

TABLE 2. Detection of cytosolic caspase-8 activity during antimicrotubule agent exposure

Treatment	24 hr	48 hr	72 hr
IFN-γ/CH-11	3.13 ± 0.15*	1.20 ± 0.14	—
Paclitaxel 10 nM	1.06 ± 0.20	0.96 ± 0.15	1.06 ± 0.23
Paclitaxel 100 nM	1.20 ± 0.26	1.26 ± 0.25	3.07 ± 0.15*
Vinorelbine 10 nM	0.90 ± 0.10	1.26 ± 0.25	2.66 ± 0.35*
Paclitaxel 100 nM/ZB4	—	—	2.70 ± 0.30*

Caspase-8 activity is expressed in fold increase by dividing values from treated samples by values from untreated cells. The results are the means ± SD of at least three independent experiments using separate cultures.

* Caspase 8 activity was significantly increased after 72-hr treatment in cells that had been blocked in mitosis by antimicrotubule agents and was not modified by co-incubation with CD95 antagonist mAb ZB4 ($P < 0.01$). This activation was not different from that measured with IFN-γ/CH-11 at 24 hr ($P > 0.10$).

the time relationship between mitochondrial events and activation of caspase-8, we monitored $\Delta\Psi$m after paclitaxel treatment for 24, 48, and 72 hr. The loss of $\Delta\Psi$m was evident at 72-hr treatment for the concentrations of drugs inducing mitotic block and Bcl-x_L phosphorylation (Fig. 2C), concomitantly to caspase-8 activation. This result indicates that the activation of caspase-8 may occur via a mitochondrial pathway.

DISCUSSION

Antimicrotubule agents such as paclitaxel, docetaxel, and vinorelbine were cytotoxic against the proliferating colon cancer cell line HT29-D4 by activating an apoptotic process, as shown in our prior study [17]. In the present study, we demonstrate for the first time that caspase-8 activation is involved in antimicrotubule agent-induced apoptosis, following mitotic block, Bcl-x_L phosphorylation, and a loss in mitochondrial transmembrane potential. This apoptotic pathway is independent of CD95 ligation in HT29-D4 cells.

Only concentrations of antimicrotubule agents able to block cells in G2/M induce apoptosis 48 hr later, demonstrating that mitotic block and subsequent apoptosis are closely related in proliferating HT29-D4 cells, as previously described on several cell lines [9, 29]. Bcl-x_L phosphorylation was observed simultaneously with mitotic block only for concentrations inducing apoptosis, suggesting a mechanistic link between these events that is currently a matter of debate. Moreover, we confirm that Bcl-x_L may substitute for Bcl-2 when the latter is not expressed [24].

Activation of caspase-8 following anticancer drug treatment is an important field of interest at the present time [13, 15, 16, 30, 31]. Recently, Vikhanskaya *et al.* [30] suggested caspase-8 involvement in paclitaxel-induced apoptosis by describing up-regulation of procaspase-8 after 72-hr treatment in a subclone of the A2780 cell line transfected in order to inactivate p53. However, their data remained inconclusive since they failed to detect any evidence of activation (i.e. cleavage into active fragments). Here, we provide evidence that paclitaxel and other antimicrotubule agents activate caspase-8, at the same level as the CD95-directed agonist antibody CH-11 and in a late time–course, following mitotic block and Bcl-x_L phosphorylation. Moreover, such an activation was clearly independent of CD95 ligation. Our results are in agreement with a recent study investigating lung cancer cells [16] and contrast with those published in two recent studies investigating paclitaxel-induced apoptosis in leukemic, breast carcinoma, and osteosarcoma cells [32, 33]. These reports described CD95-L up-regulation after paclitaxel treatment and partial inhibition of apoptosis by using CD95-L-neutralizing and/or CD95-blocking antibody, suggesting that paclitaxel-induced apoptosis is CD95-dependent. While the basis for this difference is unclear, such divergent results suggest that the involvement of CD95 ligation in paclitaxel-induced apoptosis is dispensable since it may vary according to the cell type.

Thus, antimicrotubule agents may activate factors regulating CD95-mediated apoptosis independently of CD95 ligation. The mechanism of caspase-8 activation independent of CD95 ligation is intriguing. Besides the death receptor/Fas-associated death domain (FADD) pathway [14], a mitochondrial pathway could be involved [13, 31, 34]. Indeed, the alteration of mitochondrial permeability leads to cytosolic release of AIF (apoptosis-inducing factor) or cytochrome *c* that may activate caspase-8 [13, 31]. In paclitaxel-treated cells, occurrence of mitochondrial permeability transition, cytosolic accumulation of cytochrome *c*, cleavage of procaspase-9, and subsequent cleavage of procaspase-3 have been described [29], but caspase-8 was not studied. The factors inducing loss of mitochondrial transmembrane potential are not clearly elucidated. Among these, the Bcl protein family is a putative candidate. Antimicrotubule agents induce Bcl-2 phosphorylation, so a decreased amount of Bax is heterodimerized with Bcl-2 [8]. Then, insertion of Bax may be increased at the mitochondria level and facilitate cytochrome *c* release thanks to changes in mitochondria permeability [35].

In conclusion, we report that paclitaxel- and other antimicrotubule agent-induced apoptosis involves caspase-8 (FLICE/MACH) activation independently of CD95 ligation in the HT29-D4 cell line. Such an activation is a late event temporally following mitotic block, Bcl-x_L phosphorylation, and a decrease in mitochondrial transmembrane potential. Finally, caspase-8 activation appears to be a common component in apoptosis induced by cytokines and various anticancer drugs whatever the involvement of CD95/CD95 ligand.

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