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Susceptibility to drug-induced apoptosis correlates with differential modulation of Bad, Bcl-2 and Bcl-x_L protein levels

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Abstract

To define the responses of apoptotic regulatory proteins to different chemotherapeutic agents, we investigated the expression of Bcl-2 family gene products, the release of cytochrome c, and the activation of pro-caspase-3 during apoptosis induced by Taxol and Thiotepa, in the MCF-7 breast carcinoma and the HL-60 leukemia cell lines. The earliest event induced by drug exposure was increase in Bad protein levels, followed by Bcl-2 down-regulation, cytochrome c release, and Bcl-x_L and Bax up-regulation. Bak accumulation was a late event. Activation of pro-caspase-3 and cleavage of Bcl-2 protein occurred in the HL-60 cells only, and followed the cytochrome c release. The overall responses were qualitatively similar in both cell types, but MCF-7 cells treated with Taxol showed a significant delay in apoptosis, correlating with early up-regulation of Bcl-2 and delayed release of cytochrome c. We conclude that Bad up-regulation is an early indicator of a cellular response that will lead to cell death, but may be modulated by survival mechanisms, which cumulatively govern the ultimate susceptibility to apoptosis. *Cell Death and Differentiation* (2000) **7,** 574–586.

Keywords: apoptosis; taxol (paclitaxel); thiotepa; Bad; Bcl-2; Bcl-2 cleavage; Bcl-x_L

Introduction

Apoptosis is a highly regulated process that results physiologically in the selective death of cells. The cell death pathway may be conceived as a series of intracellular decision points where death regulatory genes are activated in response to physiologic or pathologic signals. The Bcl-2 family of genes is a major component of this pathway, comprising both positive and negative regulators of cell death, which share conserved Bcl-2 homology domains and interact with each other to form a dynamic equilibrium of homo- and hetero-dimers. The intracellular level and the ratio of members of the family dictate the efficiency of engagement in the apoptotic program.[1]

The proto-oncogene Bcl-2[2] and its homolog Bcl-x_L[3] have the ability to inhibit apoptosis induced by a variety of stimuli, but the biochemical mechanism of their function is continuing to be elicited. Bcl-2 is an integral membrane protein located on the outer membrane of mitochondria and other organelles.[2,4] The Bcl-x gene generates two alternatively spliced transcripts: Bcl-x_L, known to promote survival and the shorter Bcl-x_S, which antagonizes the Bcl-2 effect.[5] Bcl-x_L is an integral membrane protein, again predominantly localized to mitochondria.[6] Low basal levels of Bcl-x_L correlated with a greater tendency to undergo apoptosis, while cells with higher basal levels of Bcl-x_L correlated with resistance to apoptosis.[7] Bcl-2 and Bcl-x_L tend to prevent the loss of membrane potential, the efflux of cytochrome c from the mitochondria and the initiation of apoptosis.[8–10]

Bad, a Bcl-2 related family member, promotes cell death apparently by hetero-dimerization with Bcl-2 or Bcl-x_L death antagonists, which eventually results in the release of free Bax and apoptosis.[11,12] Bad can be phosphorylated by the serine/threonine kinase Akt (and other kinases), and in the phosphorylated state binds to 14.3.3 proteins in the cytosol and only the unphosphorylated form binds to the Bcl-x_L at membrane sites.[13,14]

Bak[15] and Bax,[16] two other members of the Bcl-2 family, have been shown to accelerate apoptosis. Bax protein can induce both membrane potential dissipation and cytochrome c release, suggesting that Bax acts to destabilize mitochondrial membrane function.[17,18] Suppression of survival signals is the result of complex formation between Bcl-2 or Bcl-x_L and apoptosis inducing proteins like Bax and Bak,[5,19] where a high Bcl-2/Bax ratio favors cell survival whereas a low ratio promotes apoptosis.[20]

Post-translational modifications of Bcl-2 have also been proposed as a regulatory mechanism of apoptosis. Phosphorylation of Bcl-2 has been reported, although it is still unclear whether Bcl-2 phosphorylation is activating or inactivating[21–24] and whether this is a general phenomenon since it appears to occur prominently in M-phase arrested cells[25,26] More recently, Bcl-2 was reported to be a substrate for cleavage by activated caspase-3 with the carboxyl-terminal Bcl-2 cleavage product triggering apoptosis.[27]

Apoptotic pathways activated by various signals, converge into a common event controlled by the ICE family proteases or caspases, which are antagonized by the anti-apoptotic members of the Bcl-2 family.[28] Caspases are present in the cytosol as inactive precursors and cleaved to an active form during apoptosis.[29] The mammalian caspase-3 plays a critical role in the apoptotic

pathway, and cytochrome c is necessary for its activation through a complex formation with Apaf-1 and caspase-9.[30]

A key question, which we address in this paper, is how chemotherapeutic agents with diverse molecular targets interact with the bcl-2 family-regulated survival system. Thiotepa is a classical DNA directed alkylating agent[31,32] currently used in 'high dose' therapy approaches to the treatment of both hematopoietic and solid tumors. Thiotepa induces S-phase cell cycle arrest and cell death as a result of DNA cross-links or adducts formation. Taxol (paclitaxel) has demonstrated a broad spectrum of activity against both solid tumor and leukemia cell lines and it is established that it binds to and stabilizes the microtubules, which results in mitotic disruption and subsequent block in G2/M.[33–38] The lag between mitotic block and cell death varies depending on the cell line. The signal transduction pathways involved in cell cycle arrest and apoptosis induced by Taxol continue to be subject of investigation.[39–44]

In the present study, we show that HL-60 cells respond to Taxol and Thiotepa treatment by showing early initiation of apoptosis. In contrast, MCF-7 cells show delayed apoptosis in response to Taxol but not to Thiotepa. Despite these differences in time course, the two cell lines show qualitatively very similar patterns of apoptotic regulation in response to the two drugs. The efficiency and timing of apoptosis depend on the degree to which cytochrome c release occurs. We detect Bad up-regulation as an early response to drug treatment, which precedes the release of cytochrome c into the cytosol in both HL-60 and MCF-7 cells. We also detect an early increase in Bad protein levels in different other cell lines belonging to the leukemia/lymphoma and breast sub-panels, and certain lung carcinoma cells from the NCI *in vitro* drug screen. The release of cytochrome c is greatly delayed in the MCF-7 cells in response to Taxol but not to Thiotepa, and in comparison to the HL-60 cells. This delay correlates with the up-regulation of Bcl-2. Activation of pro-caspase-3 occurs after cytochrome c release in the HL-60 cells. Bad and Bax down-regulation and accumulation of Bak are late events, occurring in the context of increasing cell death. Our results suggest that in response to drug treatment, cells can undergo delayed or accelerated apoptosis based on the differential regulation of Bad, Bcl-2 and Bcl-x_L expression, and underscores the basis for interest in defining therapeutic strategies, which might modulate these targets.

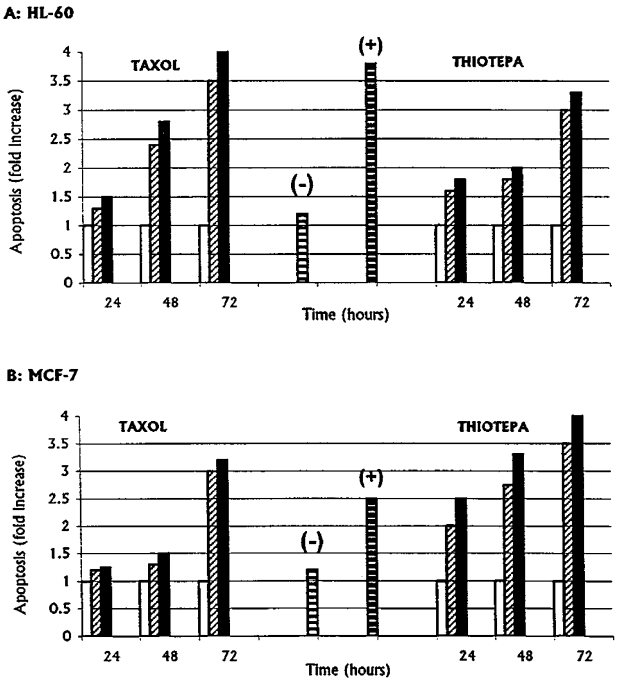

Figure 1 Quantitative ELISA for DNA fragmentation in MCF-7 and HL-60 cells after Taxol and Thiotepa treatment. Fold increase in apoptosis expressed as amount of cytoplasmic DNA-histone in treated cells compared to untreated cells. HL-60 cells **(A)** and MCF-7 cells **(B)** were treated with 0 (empty bar), 50 (hatched bar) and 100 (filled bar) nM Taxol or with 0 (empty bar), 100 (hatched bar) and 200 (filled bar) μM Thiotepa, in a 96-well plate. Cytoplasmic extracts were prepared after 0, 24, 48 and 72 h of drug treatment, according to the manufacture's instructions (Cell Death ELISA, Boehringer Manheim, Indianapolis, IN, USA). Positive controls for apoptosis (+) were HL-60 cells treated with 150 nM Camtothecin for 4 h (**A**) and MCF-7 cells treated with 10 μM Calcium Ionophore A 23187 for 24 h **(B)**. The control for nectotic death (−) consisted of HL-60 and MCF-7 cells treated with 20 mM KCN for 4 h, as described elsewhere.[8] The enrichment of nucleosomes in the cytoplasm of treated cells is expressed as fold increase, compared to untreated cells. Each treatment was performed in triplicate and the results shown are representative of three separate experiments (standard deviation was less than 5%)

Table 1

(A) Basal levels of expression of Bcl-2 family members, capase-3 and p53 status

Cell line	BAD[a]	BCL-2[a]	BCL-xL[a]	BAX[a]	BAK[a]	Capase-3	P53[b]
HL-60	0	16	2	10	18	+	–/–
MCF-7	14	11	16	13	21	–	w/w

(B) Cytotoxicity of Taxol and Thiotepa

	Taxol			Thiotepa		
Cell line	GI50	TGI	LC50	GI50	TGI	LC50
HL-60	0.005	0.05	0.5–1	10	200	$\leqslant$500
MCF-7	0.005	0.5–1	50–100	30	300	$\leqslant$500

1A –/–=null, w/w-wild-type. Summary of basal levels of apoptosis-regulating proteins and other targets from the molecular targets database at NCI. [a]Determined by relative expression in Immunoblots as ng/50 μg of total protein.[57] [b]Determined through complete bi-directional p53 cDNA sequencing.[58] 1B Average μM concentrations necessary to induce: GI50 (50% cell growth inhibition), TGI (total cell growth inhibition) and LC50 (50% cell death) in a 48 h cytotoxicity assay as described[59]

Results

Effects of Taxol and Thiotepa on cell survival and apoptosis

The HL-60 and the MCF-7 cell lines were selected from the 60-cell line panel of the NCI anticancer drug screen. These cells were chosen in an effort to highlight differences in potential apoptotic protein modulation in response to drug action. HL-60 is a myeloid hematopoietic cell line, while MCF-7 is from an epithelial breast cancer. As shown in Table 1A, the HL-60 cells have undetectable basal levels of Bad protein and low levels of Bcl-x_L protein while MCF-7 cells have high basal levels of Bad and Bcl-x_L. The two cell types have comparable levels of Bcl-2, Bax and Bak. MCF-7 cells have undetectable levels of caspase-3 protein and have a wt p53 status, while HL-60 cells express caspase-3 and have a null

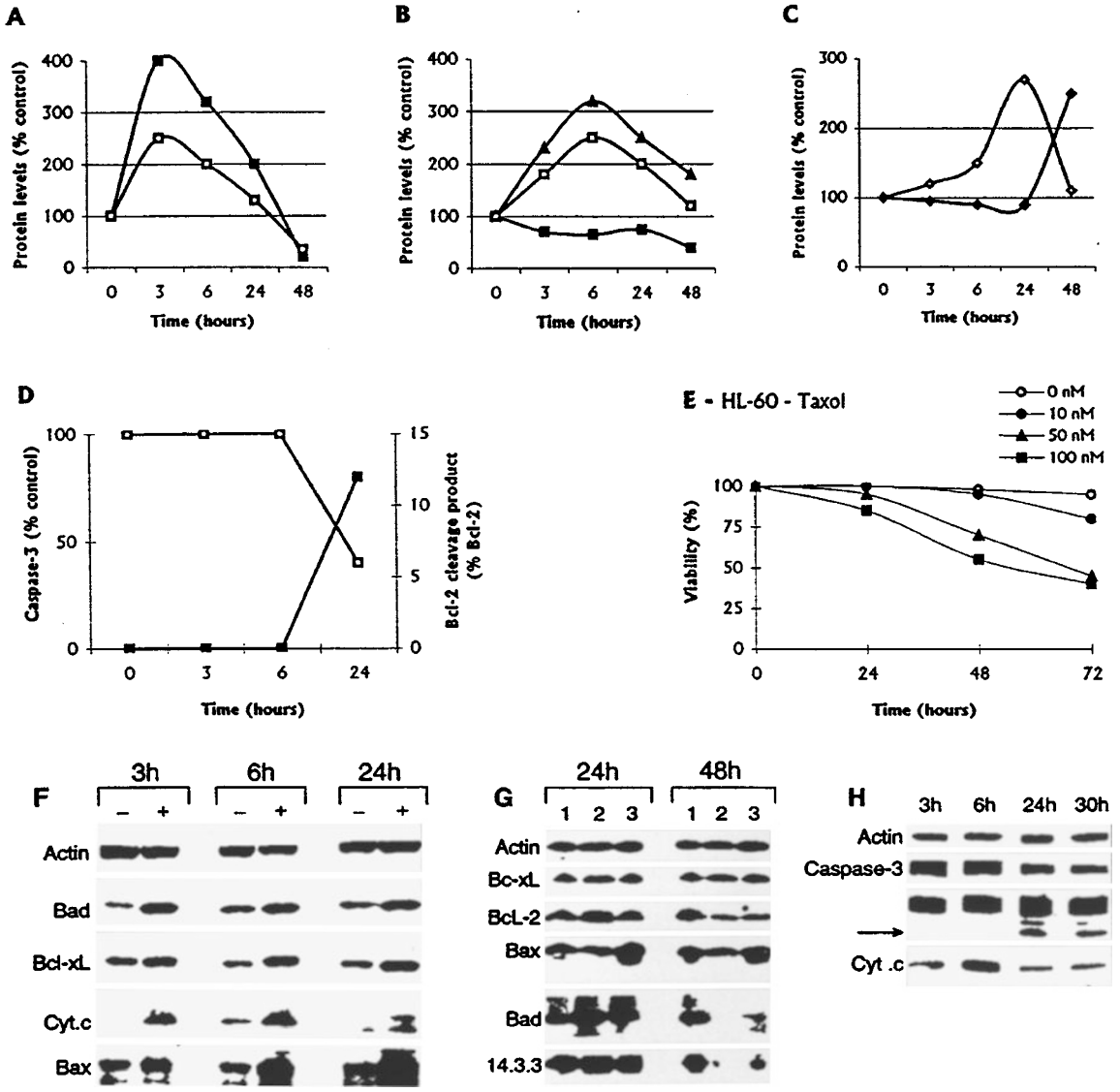

Figure 2 Expression and viability analysis of HL-60 cells treated with Taxol. Total proteins (50 μg/lane) from HL-60 cells, either treated or not treated with Taxol for 0, 3, 6, 24 and 48 h, were resolved on 12% SDS-polyacrylamide gels. The protein levels were assessed by immunoblot analysis with antibodies that detect human Bad, 14.3.3β, Bcl-2, Bcl-x_L, Bax, Bak, cytochrome c and caspase-3 and measured by densitometric scanning. Anti-β-actin mAb was used to assess equal protein loading and the relative ratios of 'protein investigated : β-actin' were calculated by densitometric measurements. Results are expressed as protein levels relative to untreated controls (percentage of control). The results shown are representative of three independent experiments. (**A**) Bad (■), and 14.3.3 (□) protein levels with 50 nM Taxol; (**B**) Bcl2 (■), (Bcl-x_L(□) and cytochrome c (▲) protein levels with 50 nM Taxol; (**C**) Bax (◇) and Bak (◆) protein levels with 50 nM Taxol; (**D**) Processing of caspase-3 (□) at 50 nM Taxol coincides with the appearance of the Bcl-2 cleavage product (■); (**E**) Viability of the HL-60 cells treated with 0, 10, 50 and 100 nM Taxol. Cells were collected at intervals, rinsed and scored for viability by Trypan blue staining. Triplicate samples were used for each treatment; (**F**) Cells were treated with (+) or without (−) 50 nM Taxol and aliquots, taken at intervals, were analyzed by immunoblot, as described; (**G**) Cells untreated (lane 1) or treated with 10 nM Taxol (lane 2) and 50 nM Taxol (lane 3) were analyzed by immunoblot, as described; (**H**) Cells were treated with 50 nM Taxol and aliquots, taken at intervals, were analyzed by immunoblot, as described. The (→) indicates the Bcl-2 cleavage product

p53. This choice of cells was anticipated to reveal different apoptotic modulatory protein responses to drug. Table 1B shows the cytotoxicity profile for the HL-60 and MCF-7 cells in response to Taxol and Thiotepa. HL-60 cells are more susceptible to Taxol than MCF-7 in screening assays, and both cell types have analogous susceptibility to Thiotepa.

We selected equitoxic concentrations of drug, which completely inhibited cell growth and induced limited cell death at 24 h. This was in an effort to define equivalent degrees of cellular response to the drug, resulting from different ways of potentially modulating responses to apoptotic stimuli. Asynchronous populations of cells were treated with drug for increasing periods of time and evaluated for apoptosis and cell survival, as described in Materials and Methods. Figure 1 shows the fold increases in apoptosis, relative to untreated controls. HL-60 cells, exposed to 50 and 100 nM Taxol (Figure 1A, left), show notable occurrence of apoptosis by 48 h, whereas MCF-7 cells, treated with 100 and 500 nM Taxol (Figure 1B, left), do not display major evidence of apoptosis until 72 h of drug exposure. In contrast, both cell types show similar patterns of sensitivity to 100–200 μM Thiotepa (Figure 1A,B, right), with a 2–3-fold increase in the proportion of apoptotic cells by 48 h, and 3–3.5-fold increase in apoptotic cells by 72 h. Noticeably, the MCF-7 cells are more sensitive to Thiotepa than the HL-60 cells.

Expression of apoptotic regulatory proteins, cytochrome c release and activation of pro-caspase-3 in response to Taxol and Thiotepa

To study the temporal relationship between protein expression and apoptosis, cells were continuously exposed to drug for different periods of time and analyzed by Western blot and semi-quantitative RT–PCR. The level of expression in parallel cultures, without drug treatment, was considered to be the basal level of expression or 100% control.

The HL-60 cells were treated with 10, 50 and 100 nM Taxol and the changes in apoptosis-related protein expression were monitored in an interval between 0 to 48 h. Figure 2 (A–D) shows these changes in response to 50 nM Taxol, which causes total growth inhibition and 95% cell survival at 24 h (Figure 2E). In the present study we detect low base-line levels of Bad protein in the HL-60 cells. At 3 h (Figure 2A,F), there is increased expression of Bad protein (fourfold), accompanied by an increase in 14-3-3 protein levels and followed by a steady decline. At 48 h, Bad and 14-3-3 protein levels are below baseline levels

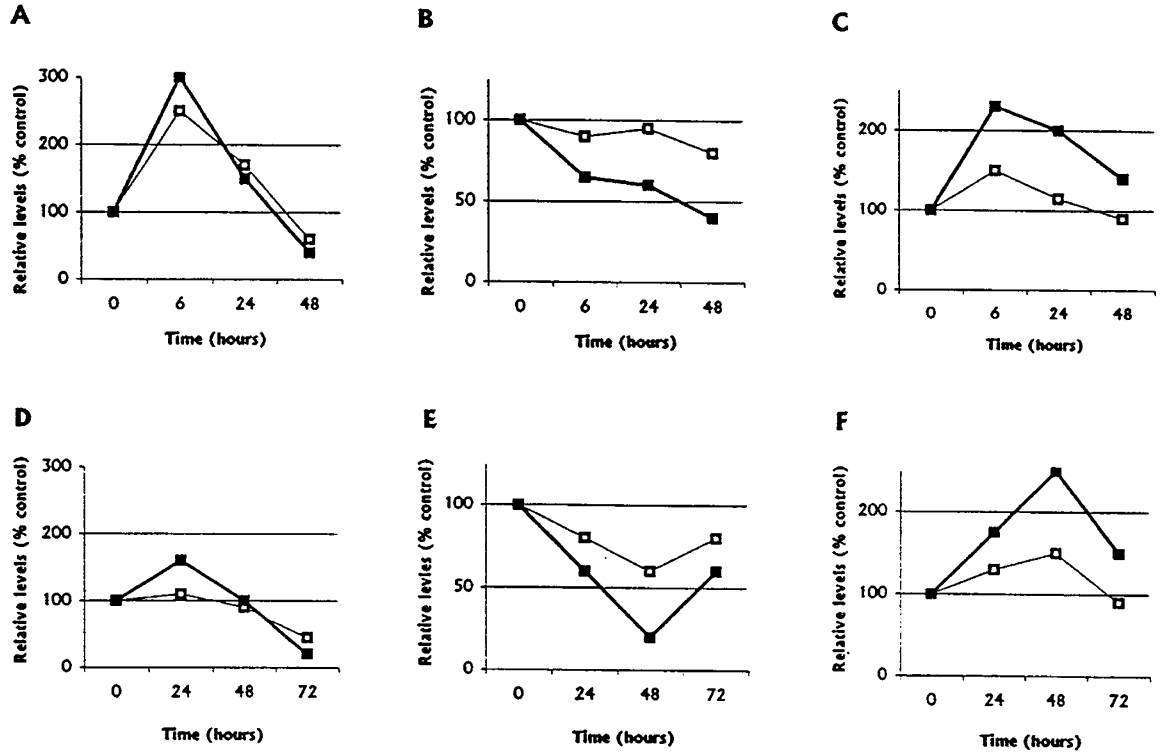

Figure 3 mRNA expression levels in HL-60 and MCF-7 cells treated with Taxol. Total RNA from HL-60 and MCF-7 cells treated with 50 nM and 500 nM Taxol respectively was isolated at different time points, as described. The mRNA levels were assessed by semi-quantitative RT–PCR as described and quantified by densitometric scanning of the amplified products. Results are expressed as mRNA levels relative to untreated controls (percentage of control). The results shown are representative of three independent experiments. (**A**) Bad mRNA (□) and Bad protein (■) levels in the HL-60 cells; (**B**) Bcl-2 mRNA (□) and Bcl-2 protein (■) levels in the HL-60 cells; (**C**) Bcl-x_L mRNA (□) and Bcl-x_L protein (■) levels in the HL-60 cells; (**D**) Bad mRNA (□) and Bad protein (■) levels in the MCF-7 cells; (**E**) Bcl-2 mRNA (□) and Bcl-2 protein (■) levels in the MCF-7 cells; (**F**) Bcl-x_L mRNA (□) and Bcl-x_L protein (■) levels in the MCF-7 cells

(Figure 2A,G) although, at this point, a notable degree of cell death has occurred. The increase in Bad protein is paralleled by a similar increase in Bad mRNA (Figure 3A), which suggests a transcriptional control of this gene product in response to Taxol treatment. At 3 h cytochrome c is being released from the mitochondria and reaches a peak at 6 h. This is accompanied by the progressive down-regulation of Bcl-2 and a steady increase in Bcl-x_L protein levels (Figure 2B,F). Bax protein accumulates at 6 h and reaches a maximal level at 24 h, followed by a drop to base-line levels at 48 h (Figure 2C,F,G). Bax mRNA levels follow very closely the Bax protein levels. The normalization of Bax levels at 48 h, coincides with accumulation of high levels of Bak protein (Figure 2C) and mRNA. We detected procaspase-3 in the HL-60 lysates as a band at 32 kDa, corresponding to the full-length pro-caspase-3. Its activation through cleavage begins after 6 h (Figure 2D,H) and continues through 48 h. It has been shown that activated caspase-3 initiates the cleavage of Bcl-2 and the generation of a Bcl-2 fragment with apoptotic properties. We notice this Bcl-2 cleavage product being generated at 24 h (Figure 2D,H), concomitant with caspase-3 activation.

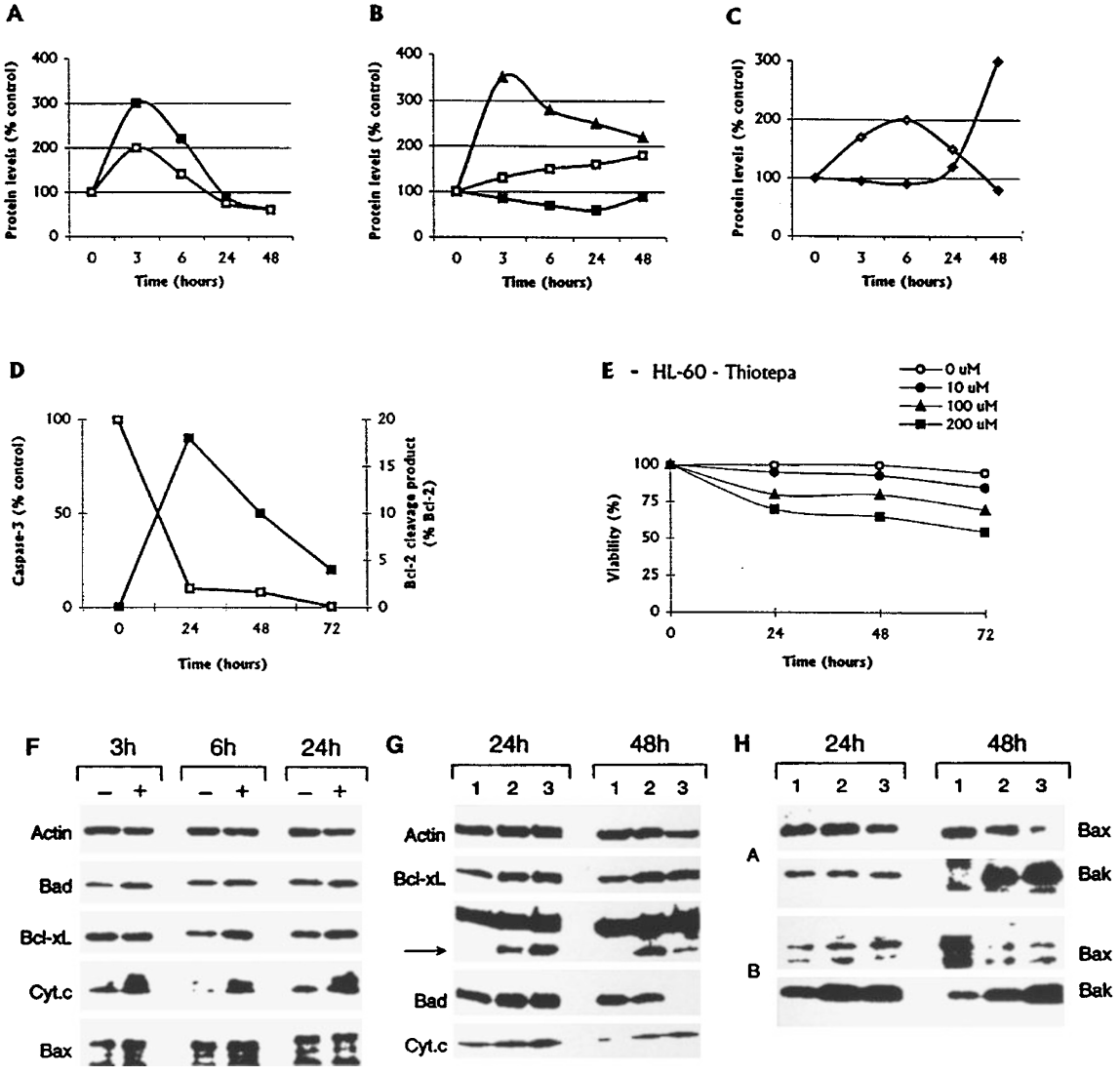

Figure 4 Expression and viability analysis of HL-60 cells treated with Thiotepa. Total proteins (50 μg/lane) from MCF-7 cells, either treated or not treated with Thiotepa for 0, 3, 6, 24 and 48 h. The overall methodology is similar to Figure 2. The results shown are representative of three independent experiments. (**A**) Bad (■) and 14.3.3 (□) protein levels with 100 μM Thiotepa; (**B**) Bcl-2 (■), Bcl-x_L (□) and cytochrome c (▲) protein levels with 100 μM Thiotepa; (**C**) Bax (◇) and Bak (◆) protein levels with 100 μM Thiotepa; (**D**) Processing of caspase-3 (□) at 100 μM Thiotepa coincides with the appearance of the Bcl-2 cleavage product (■); (**E**) Viability of the HL-60 cells treated with 0, 10, 100 and 200 μM Thiotepa. Cells were collected at intervals, rinsed and scored for viability by Trypan blue staining. Triplicate samples were used for each treatment; (**F**) Cells were treated with (+) or without (−) 100 μM Thiotepa and aliquots, taken at intervals, were analyzed by immunoblot, as described; (**G**) Cells untreated (lane 1) or treated with 100 μM (lane 2) or 200 μM (lane 3) Thiotepa were analyzed by immunoblot, as described. (**H**) Row A: Cells untreated (lane 1) or treated with 50 nM Taxol (lane 2) and 100 nM Taxol (lane 3) were analyzed by immunoblot, as described; Row B: Cells untreated (lane 1) or treated with 100 μM Thiotepa (lane 2) and 200 μM Thiotepa (lane 3) were analyzed by immunoblot, as described

However, the limited extent of its occurrence ($\leqslant$15% of the total Bcl-2 protein) is of uncertain significance in affecting the overall Bcl-2 function. The level of phosphorylated Bcl-2 is also high by 24 h and declines thereafter, but again, its abundance is very limited. These post-translational changes of the Bcl-2 protein could eventually account for its decrease. Alternatively, the reduction in Bcl-2 protein levels and only the limited decrease in mRNA levels (Figure 3B) would suggest post-transcriptional mechanisms, such as inefficient translation or changes in protein stability. The discrepancy between the Bcl-x_L protein and mRNA levels (Figure 3C) could also be due to post-translational changes or to a delay in the Bcl-x_L protein synthesis. At 48 h, both survival members Bcl-2 and Bcl-x_L are down-regulated, whereas the death inducing protein Bak shows a 2.5-fold increase, which corresponds to high levels of apoptosis (2.5-fold increase).

A similar and more rapid response occurs in the HL-60 cells treated with 100 μM Thiotepa (Figure 4), which causes 80% cell survival at 24 h (Figure 4E). This is shown by the earlier down-regulation of Bad at 24 h (Figure 4A,F), the faster increase in cytochrome c release (Figure 4B,F), and

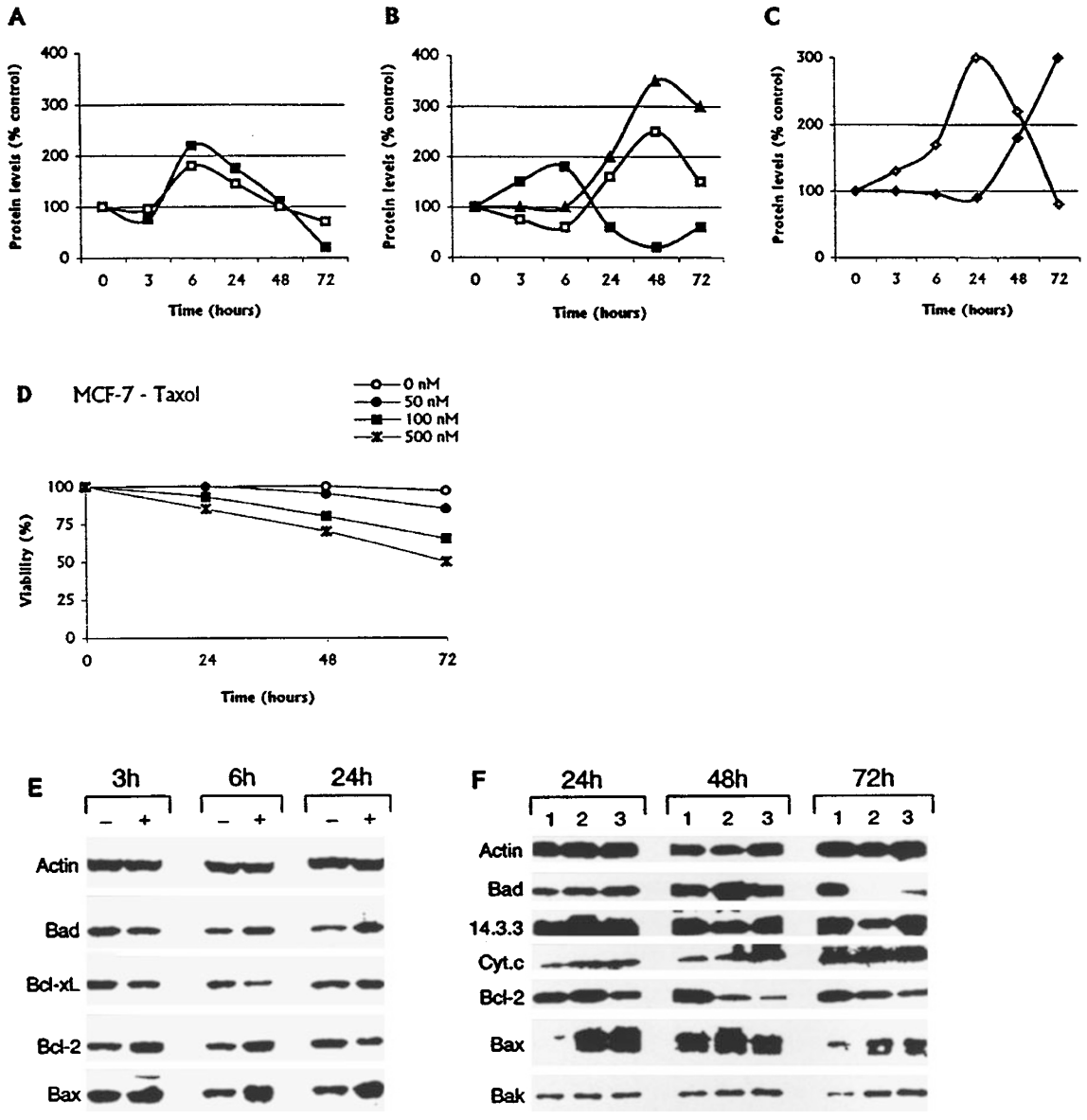

Figure 5 Expression and viability analysis of MCF-7 cells treated with Taxol. Total proteins (50 μg/lane) from MCF-7 cells, either treated or not treated with Taxol for 0, 3, 6, 24, 48 and 72 h. The overall methodology is similar to Figure 2. The results shown are representative of three independent experiments. (**A**) Bad (■) and 14.3.3 (□) protein levels with 500 nM Taxol; (**B**) Bcl-2 (■), Bcl-x_L (□) and cytochrome c (▲) protein levels with 500 nM Taxol; (**C**) Bax (◇) and Bak (◆) protein levels with 500 nM Taxol; (**D**) Viability of the MCF-7 cells treated with 0, 50, 100 and 500 nM Taxol. Cells were collected at intervals, rinsed and scored for viability by Trypan blue staining. Triplicate samples were used for each treatment; (**E**) Cells were treated with (+) or without (−) 500 nM Taxol and aliquots, taken at intervals, were analyzed by immunoblot, as described; (**F**) Cells untreated (lane 1) or treated with 100 nM (lane 2) or 500 nM (lane 3) Taxol were analyzed by immunoblot, as described

the complete processing of caspase-3 by 24 h (Figure 4D). Bax protein also shows a faster accumulation by 6 h. This is in agreement with the higher rate of apoptosis at 24 h for the HL-60 cells treated with Thiotepa as compared to Taxol. The down-regulation of Bax by 48 h, is accompanied by rapid accumulation of Bak, similar to the Taxol treated cells (Figure 4H). We have not detected phosphorylated Bcl-2 in response to Thiotepa treatment, which would suggest, as reported by others, that phosphorylated Bcl-2 is a marker of M-phase events. Interestingly, the Bcl-x_L protein remains above the base-line level up to 48 h and the Bcl-2 level is also stable. This could account for the slower progression of apoptosis in the HL-60 cells treated with Thiotepa, at 48 h, as compared to the MCF-7 cells or to the HL-60 cells treated with Taxol (Figure 1A,B).

An early increase in Bad and 14-3-3 protein levels was also detected in the MCF-7 cells treated with Taxol (Figure 5A,E), but to a lesser extent (twofold) than the increase detected in the HL-60 cells, and at a higher concentration of Taxol, 500 nM, which causes 85% cell survival at 24 h (Figure 5D). This might be related to the higher baseline level of Bad protein in these cells. At 72 h Bad protein level drops below the baseline level (Figure 4A,F) and this is paralleled by a similar reduction in mRNA level (Figure 3D).

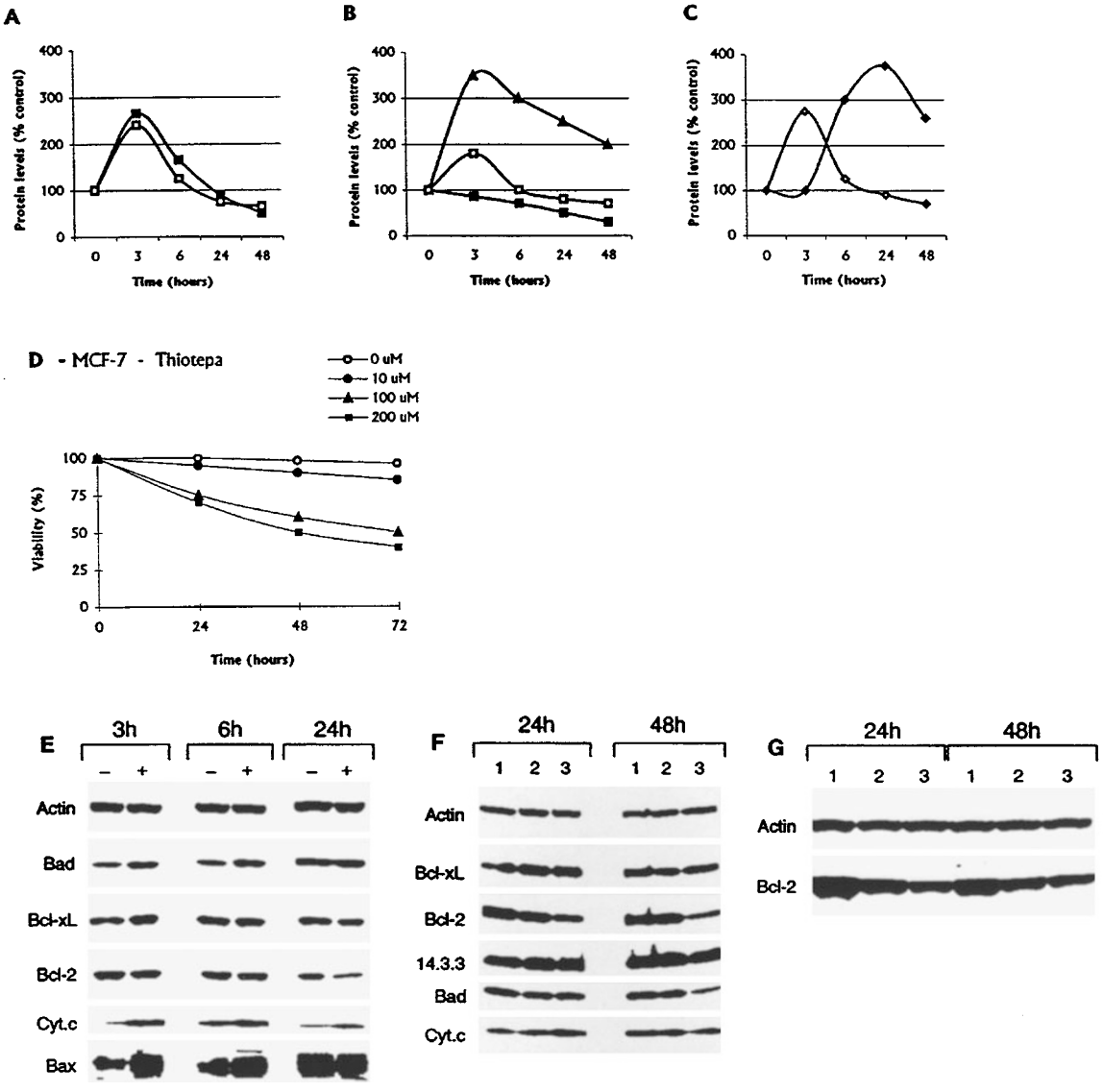

Figure 6 Expression and viability analysis of MCF-7 cells treated with Thiotepa. Total proteins (50 μg/lane) from MCF-7 cells, either treated or not treated with Thiotepa for 0, 3, 6, 24, 48 and 72 h. The overall methodology is similar to Figure 2. The results shown are representative of three independent experiments. (**A**) Bad (■) and 14.3.3 (□) protein levels with 100 μM Thiotepa; (**B**) Bcl-2 (■), Bcl-x_L (□) and cytochrome c (▲) protein levels with 100 μM Thiotepa; (**C**) Bax (◇) and Bak (◆) protein levels with 100 μM Thiotepa; (**D**) Viability of the MCF-7 cells treated with 0, 10, 100 and 200 μM Thiotepa. Cells were collected at intervals, rinsed and scored for viability by Trypan blue staining. Triplicate samples were used for each treatment; (**E**) Cells were treated with (+) or without (−) 100 μM Thiotepa and aliquots taken at intervals, were analyzed by immunoblot, as described; (**F**) Cells untreated (lane 1) or treated with 10 μM (lane 2) or 100 μM Thiotepa (lane 3) were analyzed by immunoblot, as described; (**G**) Cells untreated (lane 1) or treated with 100 μM (lane 2) or 200 μM (lane 3) Thiotepa were analyzed by immunoblot, as described

Table 2. Bad protein expression in different tumor cell lines

	Bad protein (% control)[a]		Drug conc.	Viability (%)[b]		
Cell line	**3 h**	**6 h**	**[μM]**	**3 h**	**6 h**	**24 h**
Leukemia						
			Taxol			
CEM	85	90	0.1	97	90	40
SR	100	175	0.025	86	70	60
RPMI	100	180	0.1	90	85	70
MOLT-4	100	100	0.25	95	95	90
K562	150	220	0.25	95	92	90
			Thiotepa			
CEM	170	90	100	90	90	80
RPMI	100	115	100	90	90	86
MOLT-4	100	160	100	95	90	80
K562	100	100	500	95	95	95
Breast carcinoma			Taxol			
T47-D	135	75	0.5	95	95	75
MDA-231	135	75	0.5	90	85	70
BT549	180	80	0.5	95	80	70
MDA-MB-435	140	115	0.5	95	93	90
HS-578	280	160	0.025	85	75	60
MDA-N	100	130	0.5	95	95	95
NSCLC (Non Small Cell Lung Carcinoma)			Taxol			
H-322	135	240	2.5	98	98	90
H-226	135	160	2.5	98	90	90
H-460	75	80	2.5	95	90	80

Bad protein levels in leukemia/lymphoma and breast and lung carcinoma cell lines from the 60 cell line-panel at NCI. [a]Cells were incubated with or without drug for 3 h and 6 h and Bad protein levels were determined by immunoblot as described in Materials and Methods. The results are expressed as protein levels relative to untreated control (percentage of control). [b]Cells were incubated with the indicated amounts of drug for 3 h and 6 h and the cell viability was assessed by Trypan blue staining

The increase in Bad at 6 h is followed by cytochrome c release, which is first detected at 24 h. The delay in cytochrome c release is consistent with the idea that Bcl-2 prevents the release of cytochrome c from the mitochondria and inhibits apoptosis. In fact, Bcl-2 accumulates very early after exposure to Taxol and reaches a maximal level at 6 h. Subsequently, the down-regulation of Bcl-2 at 24 h, is accompanied by the release of cytochrome c, which reaches a peak at 48 h (Figure 5B,E,F). The release of cytochrome c is preceded by accumulation of Bax protein. There is no noticeable delay in the accumulation of Bax protein when compared to the HL-60 cells treated with Taxol, but the amplitude is higher. Again, Bax down-regulation coincides with Bak accumulation (Figure 5C,F). We have not detected caspase-3 or the Bcl-2 cleavage product in the MCF-7 cells, even after prolonged exposure to high concentrations of Taxol. The phosphorylated Bcl-2 band is first seen at 24 h. Our study shows that the early response to Taxol is delayed and blunted in the MCF-7 cells, with smaller increase in Bad protein levels and delayed cytochrome c release. This is in agreement with the detection of apoptosis as late as 72 h, when Bcl-2 and Bcl-x_L levels are low and Bak levels are high.

In contrast to Taxol, the response of the MCF-7 cells to 100 μM Thiotepa is very rapid, as shown by early detection of apoptosis (Figure 1) and only 75% viability at 24 h (Figure 6D). The early Bad induction ($\leqslant$3 h) (Figure 6A,E) is again accompanied by a transient increase in 14-3-3 protein levels and followed by the release of cytochrome c. The up-regulation of Bax and the down-regulation of Bcl-2 and Bcl-x_L occur very early and, by 24 h, both survival proteins are below baseline, whereas Bak levels are rapidly increasing (Figure 6B,C,E–G). This would explain the early apoptotic peak in these cells in response to Thiotepa.

We conclude that the early response to Taxol and Thiotepa treatment in both cell types consists of increased Bad expression, followed by cytochrome c release, Bcl-2 down-regulation and Bcl-x_L and Bax up-regulation. In both cell lines, with both drugs, the early events are similar, except for the delay seen in the MCF-7 cells in response to Taxol, in a way that correlates with Bcl-2 accumulation and block of cytochrome c release. The late increase in Bak is common to both cell lines. The delayed apoptosis of the MCF-7 cells in response to Taxol does not seem to be related to the absence of caspase-3 in these cells, since the response of the MCF-7 cells to Thiotepa is very rapid, as shown by the early increase in cytoplasmic histone-bound DNA fragments (Figure 1).

The time interval between 24–72 h for the MCF-7 cells treated with Taxol is strikingly similar to the interval between 3–24 h for the HL-60 cells treated with Taxol or Thiotepa and to the interval between 0–6 h for the MCF-7 cells treated with Thiotepa. These time intervals correspond to early events leading to apoptosis, which becomes evident only when both survival members, Bcl-2 and Bcl-x_L are down-regulated. Bcl-2 and Bcl-x_L expression levels are reciprocal during these early time intervals. Similarly, Bax and Bak protein levels appear to be inversely correlated during the entire duration of drug treatment.

Bad protein expression in different cell lines in response to Taxol and Thiotepa treatment

In order to see if these cytotoxic signals in other cancer cell types could also involve Bad, we looked at early Bad expression in 14 different cell lines belonging to the leukemia/lymphoma and breast sub-panels and certain lung carcinoma cell types in the NCI *in vitro* cell line cancer drug screen (Table 2). After Taxol treatment, three of five leukemia/lymphoma cell lines show increased Bad expression at 3 or 6 h after treatment and sooner in the CEM cells; all breast carcinoma cells examined (six of six) also show some degree of Bad increase when examined at 3 or 6 h after drug exposure. All cell types retain viability during this period. In the case of Thiotepa, three of four leukemia/lymphoma cell lines show evidence of Bad increase during the same period after exposure to the agent, although in the case of RPMI the increase is minimal at 6 h. It is further noteworthy that the magnitude or early timing of the Bad increase correlates in a general way with a greater reduction in viability at 24 h.

Discussion

We hypothesized that different cellular responses leading to apoptosis might be evident in cell types exposed to different chemotherapeutic agents. We recognize that this response may or may not have involved changes in apoptotic regulatory protein levels, as there is no necessary reason to expect that such changes would be required for cell death. However, our results indicate that in two very different human cancer cell types (the MCF-7 breast carcinoma and the HL-60 myeloid leukemia cell lines), a qualitatively similar set of changes in apoptotic protein expression occurs after exposure to two agents with different mechanisms of action: Taxol, which stabilizes microtubules and Thiotepa, which is a DNA-directed alkylating agent. While the MCF-7 response to Taxol is greatly delayed and requires a higher drug concentration, analogous changes to those shown by the very sensitive HL-60 cells, are ultimately manifested.

Specifically, we have found that Bad protein increases at early times after drug exposure in both cell types, and this is accompanied by a similar increase in 14-3-3 protein. Our data demonstrate a coordinated increase in Bad and 14-3-3 proteins by 3 h after exposure to Taxol or Thiotepa in the HL-60 cells, or to Thiotepa in the MCF-7 cells, and a blunted and delayed response to Taxol in the MCF-7 cells. While we did not specifically assess phosphorylated Bad here, the coordinate increase in 14-3-3 with Bad suggests at least some level of signaling to Bad to promote downstream effects that will affect the survival of cells. Additionally, it is apparent that Bad mRNA also increases at a time when the cells are still largely viable, supporting the existence of regulation of either transcription or mRNA stability to promote Bad accumulation. This response appears to be temporally followed by increase in Bcl-x_L and decrease in Bcl-2 levels at a time when Bax and cytochrome c are accumulating as an expression of mitochondrial injury and commitment to apoptosis. Again, the elevation of cytochrome c and Bcl-x_L appears delayed in Taxol-treated MCF-7 cells. Increase in Bak occurs as a relatively late event in both cell types in response to drug treatment. In the HL-60 (but not the MCF-7) cells, pro-caspase-3 is detected and evidence of processing occurs after cytochrome c release. These events and their temporal sequence therefore focus attention on the early response of Bad to the toxic stimulus, as potentially a key regulator of downstream steps in the commitment to apoptosis. In support of this hypothesis, we also detect an early increase in Bad protein levels in different other breast and lung carcinoma and leukemia/lymphoma cell lines.

Bad is known to exist in a phosphorylated state in the cytoplasm, bound to 14-3-3, and a non-phosphorylated state at the mitochondrial surface, where it is hypothesized to antagonize the Bcl-2/Bcl-x_L survival function.[13,45] The phosphorylated and non-phosphorylated forms of Bad might be involved in communicating transduced signals from the cytosol to the membrane-bound Bcl-2-like proteins, but the steps involved in the subcellular redistribution of Bad during apoptosis are not yet defined. It has been shown that phosphorylation of Bad by Akt results in its binding to 14-3-3 in the cytosol,[13,14] which would suggest that 14-3-3 might function as a protective element in preventing dephosphorylation of Bad and assure its transport to the mitochondria. Alternatively, the phosphorylation of Bad and its binding to 14-3-3 would allow its relocation to the cytosol and preclude its binding to Bcl-x_L in the mitochondrial membrane. Therefore, 14-3-3 might serve as a competitor of Bad, keeping it sequestered in the cytosol and preventing it from reaching the mitochondria. Since in our study, Bad mRNA increases in both cell-lines, we can further hypothesize as a result of these experiments that alteration of Bad transcription or mRNA stability is an early cellular response to stress or drug-induced injury. Furthermore, the increase in 14-3-3 level, would agree with the model suggesting that 14-3-3 associates with Bad at a pre-mitochondrial step. The evident injury to mitochondria through increase of detectable cytochrome c supports the participation of Bad in mitochondrial injury, as non-phosphorylated Bad, which binds to Bcl-x_L, thus freeing BAX and inducing apoptosis. This temporal sequence is also clearly supported by our data.

We notice that cytochrome c release correlates inversely with the amount of Bcl-2 protein in the cell, as might be expected if up-regulation of Bcl-2 retards the release of cytochrome c and inhibits apoptosis. This is most clearly suggested by observations in the MCF-7 cells treated with Taxol but not in the same cells treated with Thiotepa and leads to the hypothesis that the timing plus the amount of cytochrome c released might be good early indicators of the efficacy of a cytotoxic agent. We also notice that down-regulation of Bcl-2 is accompanied by increase of Bcl-x_L. An inverse correlation between Bcl-2 and Bcl-x_L persists during the early stages of the apoptotic pathway. The modulation of the cytochrome c release would offer a rationale for the balance between the two survival proteins, which complement each other in the attempt to suppress death and this could result in a very fine control of the cytochrome c release at the mitochondrial level.

It has been shown that Bcl-x_L mRNA and protein levels increased in a variety of cells upon exposure to ionizing radiation (IR) and this was accompanied by Bax up-regulation,[7] suggesting that Bcl-x_L is an early response gene to genotoxic stress and apoptosis induced by IR through the p53-dependent pathway. Instead, we detect an increase in Bcl-x_L and Bax levels independently of the p53 status. Also, the up-regulation of Bax is accompanied by the down-regulation of Bcl-2. This would promote apoptotis as a result of decrease in the free homodimeric form of Bcl-2 and an increase in the free, homodimeric form of Bax. Bax up-regulation is more pronounced in the MCF-7 cells, which would be in agreement with the reports correlating it to the p53 status in the cells.[46–48] It has been shown that the down-regulation of Bcl-2 mRNA and protein levels and the increase in Bax protein levels in the MCF-7 cells was associated with Basic Fibroblast Growth factor (bFGF)-induced apoptosis in these cells.[49] Down-regulation of Bcl-2 mRNA and protein levels was also reported in connection with the induction of apoptosis in MCF-7 by sodium butyrate.[50]

We notice, as a later event, the down-regulation of Bax, which always coincides with a significant increase in Bak protein levels. The ability of Bax and Bak to induce cell death has been attributed to their heterodimerization with the death-suppressing Bcl-2 members. However, recent studies have shown that Bax and Bak do not necessarily require heterodimerization with Bcl-x_L and Bcl-2 to promote cell death.[51–53] We show that in both cell lines and both drugs, Bax and Bak show a very similar pattern of expression, independently of the Bcl-2 and Bcl-x_L protein levels, which would suggest that some of their functions might not be dependent on dimerizations.

The presence of cytochrome c in the cytosol has been reported to be necessary for activation of pro-caspases. Pro-caspase-9 and Apaf-1 (apoptotic protease activating factor 1) bind to each other via their respective NH2-terminal CED-3 homologous domains in the presence of cytochrome c and dATP, an event that leads to pro-caspase-9 activation. Activated caspase-9 in turn, cleaves and activates pro-caspase-3.[54,55] It has been shown that Apaf-1 functions downstream of Bcl-2, but upstream of caspase-3 and that Bcl-2 functions upstream of Apaf-1 by regulating the release of cytochrome c from mitochondria. According to this hypothesis, overexpression of Bcl-2, or its close family member Bcl-x_L, blocks the release of cytochrome c from mitochondria. Our study shows that the activation of pro-caspase-3 occurs downstream from the cytochrome c release.[56] Furthermore, the MCF-7 cells, which have undetectable levels of caspase-3, show early and significant release of cytochrome c in response to Thiotepa treatment.

The present experiments are phenomenological in nature and clearly do not address the mechanism underlying the very similar responses to very mechanistically different antiproliferative agents in two different cell types. The results presented here lead to the hypothesis that up-regulation of Bad protein expression, which correlates with Bcl-2 and Bcl-x_L protein levels, may be an important mediation of cellular response to anticancer agents, and is also found in different histologic types of tumor cells.

Bax increase, activation of caspases, post-transcriptional modifications of Bcl-2 and finally Bak increase, represent clearly later steps. Our experiments also strikingly illustrate the general applicability of this pathway to two very different non-transfected or otherwise manipulated cell types (including wild-type and mutant p53 status) in response to drugs acting by different mechanisms. Further experiments will be of great interest in trying to understand the mechanisms leading to increase in Bad protein, and the manipulation of that response in modulating susceptibility to chemotherapeutic agents.

Materials and Methods

Chemicals and cell culture

Taxol (paclitaxel) (NSC-125973) and Thiotepa (NSC-6396) were obtained from the Drug Synthesis and Chemistry Branch, NCI Screen Program. Stock solutions were made in dimethyl sulfoxide, so that the highest concentration of DMSO used for cells treatment was less than 0.1%. HL-60 promyelocytic and MCF-7 breast carcinoma cells were obtained from the NCI anticancer drug screen and grown in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) with 10% FBS, 5 mM L-Glutamine, 100 ng/ml penicillin and 100 ng/ml streptomycin at 37°C in 5% CO_2.

Drug treatment

Asynchronous and exponentially growing cells were treated with 0, 10, 50, 100 and 500 nM Taxol or with 0, 10, 100 and 200 μM Thiotepa. Cells were collected after removal of the drugs, at 0, 24, 48 and 72 h for long term and at 0, 3, 6 and 24 h for short term time-course studies. Cells were then separated into aliquots and used for viability assay, protein and total RNA extraction.

Apoptotic cell death and viability assays

For the quantification of apoptotic cell death, we used a 'Cell Death Detection ELISA^{PLUS}' (Boehringer Mannheim, Indianapolis, IN, USA) that measures the cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) generated during apoptosis. Briefly, 2×10^4 cells were plated into 96-well plates, in triplicates, and different concentrations of Taxol or Thiotepa were added for 0, 24, 48 and 72 h. After drug treatment, plates were spun, washed and the pellets lysed according to the manufacturer. The enrichment of nucleosomes in the cytoplasm of treated cells was expressed as fold increase in apoptosis, compared to untreated cells. To evaluate the levels of cell death due to necrosis, triplicate control wells were set up, consisting in cells treated with 20 mM KCN for 4 h, as described elsewhere.[8] Positive controls for apoptosis consisted in triplicates of MCF-7 cells treated with 10 μM Calcium Ionophor A 23187 (Sigma) for 24 h, and HL-60 cells treated with 150 nM Camtothecin for 4 h, respectively. The results represent three independent experiments and are expressed as fold increase relative to untreated cells.

For the viability assay, 2×10^6 cells were treated with 0, 10, 50, 100 and 500 nM Taxol and 0, 10, 100 and 200 μM Thiotepa and cell viability was assayed by trypan blue exclusion at 0, 24, 48 and 72 h. Triplicate samples were used for each treatment.

Western blot analysis

Total proteins were isolated from $1-2\times10^7$ cells, lysed in 0.5–1 ml lysis buffer (50 mM HEPES [pH 7.4]; 150 mM KCl; 5 mM $MgCl_2$; 1 mM EGTA; 0.5% NP-40; 0.5 mM phenylmethylsulfonyl fluoride; 20 μM leupeptine; 20 μM aprotinin; 14 μM pepstatin A) by repeated freezing and thawing. Cell homogenates were centrifuged in an Eppendorf centrifuge at 4°C, for 15 min. Lysates for the immunodetection of cytochrome c were prepared using the same lysis buffer, followed by quick mechanical disruption on ice, and spun at 14 000 $\times$ *g* for 30 min at 4°C. Supernatants were removed and stored at −70°C, until analysis by gel electrophoresis. These lysates contained the cytosolic and light membrane fractions but not nuclei or mitochondria. Protein content in each lysate was determined by using the 'BCA Protein Assay' (Pierce, Rockford, IL, USA). Fifty μg of protein/lane were loaded on 12% Tris-glycine precast gels (Novex) and transferred in a submerged transfer unit (NOVEX Xcell II) to polyvynildifluoride (PVDF) membrane, in Tris-glycine transfer buffer (NOVEX) containing 5% methanol. Membranes were then blocked in TBS containing 0.1% Tween-20 and 10% dry milk, at room temperature for 1 h and used for immunoblotting. The following primary antibodies were used: *Bcl-2*, 124 mouse monoclonal (Dako, Copenhagen, Denmark, # MO887), *Bax* N-20 rabbit polyclonal (Calbiochem, #PC66); *Bcl-x_L*, rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-634), *BAD*, rabbit polyclonal (Transduction Laboratories, #B31420); *Bak*, mouse monoclonal (Pharmingen, #65371); *Caspase-3*, rabbit polyclonal (Pharmingen, #65906E); *β-actin*, mouse monoclonal (Sigma, #A5441); *Cytochrome c*, mouse monoclonal (Pharmingen); *14-3-3 β* rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-629). Immunoblotting was performed using 2–5 μg of primary antibody and 1 : 2500 dilution of secondary antibody, in 10 ml TBS containing 0.1% Tween 20 and 10% dry milk. Proteins were visualized using peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and the ECL Western blotting detection reagents (Amersham, Arlington Heights, IL, USA) as recommended by the manufacturer. The resulting immunoblot signals were quantified by densitometric scanning (Digital Imaging System from Alpha Innotech Co.) The β-actin signal was used to normalize for the amount of protein loaded in each lane and the results were expressed as protein levels relative to untreated control (percentage of control).

Isolation of total RNA and RT–PCR analysis

Total RNA was isolated from 1×10^7 cells following treatment with different concentrations of drugs, by using the TRI REAGENT method (Molecular Research Center, Inc., Cincinnati, OH, USA). The reverse transcription (RT) reactions were done using the GeneAmp/RNA PCR reagents (Perkin Elmer #N808-0017) on 5 μg of DNAse treated total RNA, following the manufacturer's procedure. The resulting reverse transcribed product was divided into seven equal aliquots (for the seven sets of primers), in duplicate, and each sample was subsequently serially diluted in 1 : 2, 1 : 5 and 1 : 10. The primers were quantified prior to their use by agarose gel electrophoresis, ethidium bromide staining and densitometric scanning. Consistent amounts of primers and template were used for all experiments. The subsequent DNA amplification step was performed according to the manufacturer's recommendations, in a Perkin Elmer 9600 PCR machine (95°C for 45 s, 65°C for 45 s and 72°C for 2 min for 35 cycles). Amplification cycles were preceded by a denaturation step (95°C for 2 min) and followed by an elongation step (72°C for 10 min). The amplified products were analyzed on a 2% agarose gel by ethidium bromide staining and the signal was quantified by densitometric scanning (Digital Imaging System, Alpha Innotech Co.). The following sets of primers were used: Bad forward: 5′-gagccgagtgagcaggaagac, Bad reverse: 5′-cactcatcctccggagctcgc; Bcl-x forward: 5′-cgggcattcagtgacctgac, Bcl-x reverse: 5-′tcaggaac-cagcggttgaag; Bcl-2 forward: 5′-cgacttcgccgagatgtccagccag, Bcl-2 reverse: 5′-cttgtggctcagataggcacccagg; Bak forward: 5′-gtcctcccagg-caggagtgcggag, Bak reverse: 5′-gtcgaccacgaagcgggtcacctgg; Bax forward: 5′-cagctctgagcagatcatgaagaca, Bax reverse: 5′-gcccatcttcttc-cagatggtgagc; β-actin forward: 5′-tgacggggtcacccacactgtgcccatcta, β-actin reverse: 5′-ctagaagcatttgcggtggacgatggaggg; 18S forward: 5′-tcaagaacgaaagtcggagg, 18S reverse: 5′-ggacatctaagggcatcaca. Twenty μl of the final amplified DNA were run on 2% agarose gels, stained with ethidium bromide and the signal quantified by densitometric scanning. β-actin and 18S amplified products were quantified and these values were used to normalize for the amount of template used in each reaction. The results are expressed as mRNA levels relative to untreated control (percentage of control).

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