Bax- α promotes apoptosis induced by cancer chemotherapy and accelerates the activation of caspase 3-like cysteine proteases in p53 double mutant B lymphoma Namalwa cells

Estelle Schmitt¹, Alain Steyaert¹, Guido Cimoli¹ and Richard Bertrand^{1,2}

¹ Hospital Research Center of University of Montreal, Montreal Cancer Institute Montreal (Que) H2L 2M1 Canada

² corresponding author: CHUM, Notre-Dame Pavilion, 1560 Sherbrooke St. East, Room Y-5634, Montreal (Que) H2L 4M1 Canada. Tel: (514) 281 6000 ext 6615; fax: (514) 896 4689; Email: bertrand@ere.umontreal.ca

Received 1.10.97; revised 9.12.97; accepted 19.1.98
Edited by J. Cidlowski

Keywords: Bax; cancer chemotherapy; caspase; lymphoma; apoptosis

Abbreviations: CPT, camptothecin; VP16, etoposide; DABCYL-YVADAPV-EDANS, 4-(4-dimethylaminophenylazo)benzoyl-Tyr-Val-Ala-Asp-Ala-ProVal-5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Ha-tag, hemagglutinin epitope tag; PARP, poly(ADPribose) polymerase.

Abstract

Stable transfected human p53 (mt/mt) B lymphoma Namalwa variant lines showing differential expression of the Bax- α protein were derived under hygromycin selection. Overexpression of Bax- α in these variant cells accelerates cell death induced by short or continuous treatments with various concentrations of camptothecin, etoposide, vinblastine and shows no accelerating cell death activity in cis-platinum and paclitaxel-treated cells. Activation of apoptosis and oligonucleosome-sized DNA fragmentation was observed in the variant lines with more pronounced effect in cells containing high level of Bax- α protein. These results suggest that increased cell death mediated by anticancer drugs correlates with Bax- α level of expression and that Bax- α sensitizes Namalwa cells treated at low drug concentrations. The extent of DNA synthesis inhibition following DNA topoisomerase inhibitor treatments was similar in control and all transfected Namalwa cells suggesting that Bax- α acts downstream of DNA topoisomerase-mediated DNA strand breaks. To define further the relation between Bax- α expression and apoptosis activation, kinetics of caspase activation was measured in drug-treated cells. Caspase activities were measured using specific fluorogenic peptide derivatives DABCYL-YVADAPV-EDANS and Ac-DEVD-AMC, substrates of the caspase 1-like and caspase 3-like families, respectively. In control and Bax- α transfected Namalwa cells no increase in caspase 1-like activity was detected following camptothecin and etoposide treatments. In contrast, a significant difference in Ac-DEVD-AMC hydrolysis activity was observed in Bax- α transfected Namalwa cells compared to that of control Namalwa cells after camptothecin and etoposide treatment. Increased caspase 3-like activity correlated also with poly(ADPribose) polymerase cleavage. Taken together, these results suggest that Bax- α sensitize B lymphoma cells to series of anticancer drugs and accelerates the activation of apoptotic protease cascade.

Introduction

Programmed cell death (apoptosis) is a fundamental process essential for normal regulation of cell homeostasis and seems to play an important role in tumor development and progression (Raff *et al*, 1994; Thompson, 1995; Wyllie, 1997). All of the anticancer drugs used in clinic can induce apoptosis in various cell lines but the mechanisms by which these drugs activate programmed cell death are just beginning to be examined (Hickman, 1996; Reed, 1995). Several studies have provided major evidence that specific genes can act either as inhibitor or activator in the apoptotic process. The human Ced-9/Bcl-like family includes a series of related gene products that either inhibits apoptosis such as Bcl-2 (Hockenbery *et al*, 1990; Korsmeyer, 1992; Reed, 1997; Vaux *et al*, 1988), Bcl-xL (Boise *et al*, 1993), Mcl-1 (Kozopas *et al*, 1993), A1 (Lin *et al*, 1993), Bag (Takayama *et al*, 1995, 1996), Bfl-1 (Choi *et al*, 1995), Bcl-w (Gibson *et al*, 1996) and Brag-1 (Das *et al*, 1996), while others such as Bax (Oltvai *et al*, 1993), Bcl-xS (Boise *et al*, 1993), Bak (Chittenden *et al*, 1995; Farrow *et al*, 1995; Kiefer *et al*, 1995), Bad (Yang *et al*, 1995), Bik (Boyd *et al*, 1995), and Bid (Wang *et al*, 1996) are associated with the activation of apoptosis. Bcl-2 has been found translocated [t(14:18)] in human B cell lymphoma and is thought to contribute to the development of B-cell neoplasia (Clarke *et al*, 1986; Tsujimoto *et al*, 1984), while bcl-xL is found often highly expressed in B lymphoma tumor cells (Xerri *et al*, 1996). Bcl-2 and Bcl-xL are also associated with cell death protection after treatment with anticancer compounds (Datta *et al*, 1995; Dole *et al*, 1995; Fang *et al*, 1995; Hockenbery, 1995; Ibrado *et al*, 1996; Korsmeyer, 1995; Reed, 1995) and recent observations suggested that Bax- α (Chresta *et al*, 1996; Krajewski *et al*, 1995; Sakakura *et al*, 1996; Thomas *et al*, 1996; Wagener *et al*, 1996) and Bcl-xS (Ealovega *et al*, 1996; Sumantran *et al*, 1995) may inhibit protective effects conferred by Bcl-2 and Bcl-xL and accelerate cell death induced by some anticancer drugs.

An emerging family of Ced-3/Ice-like cysteine proteases (caspases) has been also identified and several studies have revealed their importance in executing the process of cell death itself. The caspases are separated in three subfamilies based on their degrees of homology and by phylogenetic analysis; the ICE-related family includes Ice/caspase-1 itself with its spliced isoforms (Alnemri *et al*, 1995; Yuan, 1993), Tx/Ich-2/Ice rel-II/caspase-4 (Faucheu *et al*, 1995; Kamens *et al*, 1995; Munday *et al*, 1995), Ty/Ice rel-III/caspase-5 (Faucheu *et al*, 1996; Munday *et al*, 1995) and Ich-3/caspase-11 (Wang *et al*, 1996); the CPP32-related family includes CPP32/Yama/Apopain/caspase-3 (Fernandes-Alnemri *et al*, 1994; Nicholson *et al*, 1995; Tewari *et al*, 1995), Mch-2/caspase-6 (Fernandes-Alnemri *et al*, 1995), Mch-3/Ice-Lap3/Cmh-1/caspase-7 (Duan *et al*, 1996a; Fernandes-Alnemri *et al*, 1995; Lippke *et al*, 1996), Mch-4/caspase-10 (Fernandes-Alnemri *et al*, 1996), Mch-5/Flice/Mach/caspase-8 (Boldin *et al*, 1996; Fernandes-Alnemri *et al*, 1996; Muzio *et al*, 1996) and the third group includes Ich-1[and s]/caspase-2 (Wang *et al*, 1994) and Ice-Lap6/Mch6/caspase-9 (Duan *et al*, 1996b).

Chemotherapeutic drugs such as DNA topoisomerase I (top 1) and II (top 2) inhibitors (camptothecin, CPT; etoposide, VP16, respectively) trigger apoptosis in various cell lines (Barry *et al*, 1990; Bertrand *et al*, 1991, 1993; Del Bino *et al*, 1992; Jaxel *et al*, 1988; Kaufmann, 1989; Solary *et al*, 1993; Walker *et al*, 1991). These drugs are known to stabilize a transient intermediate of topoisomerase reactions where enzymes are linked to the 3' (top 1) or 5' (top 2) terminus of a DNA duplex producing DNA single- or double-strand breaks (Gupta *et al*, 1995; Pommier, 1997). The unknown signals emerging from DNA damage induced by chemotherapy drugs like DNA topoisomerase inhibitors are influenced by a series of checkpoints and are related to specific gene expression. Although it has been proposed that Bax- α is a wild-type p53 response gene, DNA topoisomerase inhibitors are able to trigger apoptosis in p53 mutated and null cells. Thus, to analyze further the relation between Bax- α expression, drug sensitivity and caspase activation, we have developed human B lymphoma Namalwa variant lines that express differential levels of Bax- α protein.

In this paper we show that Bax- α accelerates apoptosis induced by CPT (DNA topoisomerase I inhibitor), VP16 (DNA topoisomerase II inhibitor) and vinblastine (a tubulin polymerization inhibitor) even at very low drug concentration. The promoting cell death activity of Bax- α correlates also with its level of expression. Bax- α does not interfere with primary mechanism of action of DNA topoisomerase inhibitor CPT and acts downstream of DNA damage-mediated DNA synthesis inhibition. However, Bax- α acts upstream of caspase and DNA fragmentation induction and accelerates the activation of caspase 3-like at low drug concentration. Taken together, these results suggest that Bax- α acts primarily downstream of the primary effect of chemotherapy drugs such as CPT and sensitizes B lymphoma cells to a series of anticancer drugs. Bax- α functions also upstream of caspase 3-like in the apoptotic process and accelerates their activation.

Results

Expression of Bax- α and Bcl-xL in Namalwa cell lines

The human B lymphoma Namalwa cells are p53 double mutant cells (O'Connor *et al*, 1993) that contain two copies of the Epstein-Barr virus (EBV) genome integrated in opposite orientations on chromosome 1 (Lawrence *et al*, 1988). The Namalwa cells are characterized by their absence of EBV episomes and the lack of virus replication in response to inducers (Klein and Dombos, 1973; Matsuo *et al*, 1984). These cells express nuclear antigen proteins belonging to the EBNA-3 family, which expression was proposed to play a role to maintain the lymphoblastoid cells in an immortalized state (Luka *et al*, 1978; Sawada *et al*, 1989) although a causal role for EBV in tumor development remains obscure (Horner *et al*, 1995). However, these cells do not express the latent membrane proteins of the LMP family (Sample *et al*, 1989) and the Bcl-2 homolog protein BHFR1, which expression is primarily linked with virus replication and lytic induction (Pearson *et al*, 1987) and more often associated with nasopharyngeal carcinoma (Horner *et al*, 1995). Moreover, these cells show high molecular weight and oligonucleosome-sized DNA fragmentation patterns typical of apoptosis after short DNA damaging agent treatments (Sane *et al*, 1997) and express members of the Ced-9/Bcl-like family, such as Bcl-2, Bax- α and Bcl-xL (Figure 1, lower panels). To analyze the effect of Bax- α expression on drug response and caspase activation in human B lymphoma cells, we have developed Namalwa variant lines that express differential levels of Bax- α protein. A Namalwa variant line that overexpress Bcl-xL was also derived to compare their effects. The human Bax- α and Bcl-xL cDNAs were first amplified by RT-PCR and inserted in a modified pCEP4 vector containing Kosak and hemagglutinin epitope tag sequences (HA-tag). After transfection, Namalwa cells were selected as bulk culture under hygromycin (500 μ g/ml, 1.5 mg/ml) and selection were maintained for about 2 months to obtain stable lines prior to perform the experiments. Control Namalwa cells did not survive under hygromycin selection. Western blot analysis indicates that the HA-Bax- α protein is expressed at different levels with respect to hygromycin concentration (Figure 1, upper left panel). The highest expression of HA-Bax- α has been found in transfected cells selected at 1.5 mg/ml hygromycin. High level of expression of HA-Bcl-xL was also achieved in cells selected at 1.5 mg/ml hygromycin (Figure 1, upper right panel). Protein expression was also visualized by immunohistochemical staining and micrographs show that all cells express the HA-epitope tagged Bax- α and Bcl-xL proteins (Figure 2).

Effect of Bax- α upon apoptosis and DNA fragmentation

To test Bax- α activity in the selected transfected cell lines, we first determined HA-Bax- α potency to promote cell death in cells grown in low serum concentration (0.1%) or triggered with an anti-CD95/Fas antibodies for 24 h (Figure 3). We routinely used a filter DNA binding assay to monitor and quantitate kinetics of DNA fragmentation in cells undergoing apoptosis (Bertrand *et al*, 1991, 1995; Bertrand and Pommier,

Figure 1 Expression of Bcl-related proteins in Namalwa cells. Upper panels. Cytosolic extracts were prepared as described in Materials and Methods from control, HA-Bax- α transfected Namalwa cells selected at 0.50 mg/ml and 1.5 mg/ml hygromycin (left panel) and HA-Bcl-xL transfected cells selected at 1.5 mg/ml hygromycin (right panel). Following SDS-polyacrylamide gel electrophoresis and electrophoretic transfer, HA-Bax- α and HA-Bcl-xL proteins were detected using anti-HA peptide monoclonal antibodies. Visualization of signals was performed by ECL and autoradiography (lower panels). Cytosolic extracts were prepared from control Namalwa cells. Following SDS-polyacrylamide gel electrophoresis and electrophoretic transfer, Bcl-2, Bax- α and Bcl-xL proteins were detected using specific antibodies. Visualization of signals was performed by ECL and autoradiography

1995). Higher expression of Bax- α in Namalwa cells increases rates of DNA fragmentation both in cells grown in low serum concentration (0.1%) and in the presence of anti-CD95/Fas antibodies for 24 h (Figure 3). The amount of DNA fragmentation has been found higher in cells expressing the highest level of Bax- α protein.

To analyze the effect of Bax- α upon anticancer drug-induced apoptosis we first evaluated the effect of CPT, a DNA topoisomerase I inhibitor, in control and Bax- α transfected Namalwa variant lines. Several studies have shown that CPT, a DNA topoisomerase I inhibitor, activates the morphological changes and internucleosomal DNA fragmentation associated with apoptosis. Short treatments

(30 min) with CPT at moderate concentrations (0.1 μ M–0.5 μ M) induced apoptosis in control Namalwa cells while low concentration of CPT such as 0.01 μ M does not trigger apoptosis significantly (Figure 4). However in the Bax- α transfected Namalwa cells, both the kinetic and extent of DNA fragmentation were more pronounced after CPT treatment at low concentration (Figure 4). Moreover, the promoting cell death activity of Bax- α after such treatment was more pronounced in cells selected at 1.5 mg/ml hygromycin, expressing the highest level of Bax- α . At 0.01 μ M and 0.05 μ M CPT, the promoting cell death effect of Bax- α in the transfected lines was relatively more pronounced compared to the effect of CPT in control Namalwa cells. At higher CPT concentration (0.1 μ M and 0.5 μ M) the difference between the CPT-treated control and Bax- α transfected Namalwa cell line selected at 1.5 mg/ml hygromycin seems to be less pronounced (Figure 4) and no significant differences were detected in the Bax- α variant line selected at 0.5 mg/ml hygromycin when compared to Namalwa cells (data not shown). These results indicate a direct relation between the amount of Bax- α and the degree of apoptosis induced by low concentration CPT. Thus, higher level of Bax- α sensitizes these cells to low drug concentration. For comparison, Namalwa cells transfected with HA-Bcl-xL and selected at 1.5 mg/ml hygromycin, are completely resistant to CPT-induced apoptosis (Figure 4; open circles).

CPT traps and stabilizes an intermediate of DNA topoisomerase I reaction when the enzyme is covalently linked to DNA. Stabilization of these cleavable complexes induces DNA strand breaks. These enzyme-linked DNA adducts on active replication forks inhibit DNA synthesis. DNA replication inhibition is one of the most widely explored processes to be implicated in the cytotoxic mechanism of action of DNA topoisomerase I inhibitors (Gupta *et al*, 1995). To determine whether Bax- α acts primarily upstream or downstream of CPT translated effect on DNA synthesis, rates of DNA synthesis were measured by thymidine pulse incorporation immediately after CPT treatments. Table 1 shows that in Namalwa control cells, immediately following CPT treatment at 0.01 μ M, 0.05 μ M, 0.1 μ M and 0.5 μ M, DNA synthesis rates were reduced to approximately 85, 75, 65 and 59% to that of control untreated cells, respectively. The extent of DNA synthesis reduction was similar in the two transfected Namalwa cells treated with the same concentrations of CPT with rates of DNA synthesis of approximately 80, 72, 64 and 57% to that of Bax- α transfected untreated cells, respectively. These results suggest that Bax- α does not interfere with the primary mechanism of action of CPT and does not amplify CPT-induced DNA synthesis inhibition.

Modulation of cell death by Bax- α was also observed in cells treated with VP-16 (DNA topoisomerase II inhibitor) and vinblastine (tubulin polymerization inhibitor). The promoting death activity of Bax- α was more pronounced in the variant line expressing the highest level of Bax- α (Figure 5) at low VP-16 and vinblastine concentrations. While the control Namalwa cells do not show significant DNA fragmentation when treated at or below 20 μ M VP-16 and vinblastine, in the high Bax- α expressing cells more

Figure 2 Immunohistochemical revelation of the HA-Bax- α and HA-Bcl-xL proteins in transfected Namalwa variant cells. Upper panels are control Namalwa (left) and HA-Bax- α transfected Namalwa cells (right). Lower panels are control Namalwa (left) and HA-Bcl-xL transfected Namalwa cells (right)

than 70% DNA fragmentation was detected 8 h after drug treatment (Figure 5). As with CPT, these results show that higher level of Bax- α sensitizes these cells to low VP-16 and vinblastine concentrations. Conversely, overexpression of Bax- α did not significantly sensitize these cells to paclitaxel and cis-platinum(II) diammine dichloride treatment neither 8 h nor 24 h after drug treatment (Figure 5). To confirm the specificity of the DNA fragmentation observed by the filter assay, we visualized the intensity of the oligonucleosome-sized DNA fragments caused by these drugs in Namalwa and Namalwa-Bax cells (Figure 6). Overexpression of Bcl-xL in these cells completely abrogates the occurrence of DNA fragmentation (Figure 6).

Effect of Bax- α on caspase activation

A series of caspases are involved in the apoptotic process (Kumar and Lavin, 1996; Patel *et al*, 1996). To determine whether Bax- α accelerates caspase activation, we monitored caspase 1-like and caspase 3-like activities using specific fluorogenic peptide substrates. In cytosolic extracts obtained from Namalwa cells, a slow increase in caspase 3-like activity

was detected after 0.05 μ M CPT or 10 μ M VP16 treatment (Figure 7). The kinetic of caspase 3-like activity correlates with the slow activation of DNA fragmentation following CPT and VP-16 treatment in control Namalwa cells (Figures 4 and 5). In contrast, in Bax- α transfected Namalwa cells a rapid increase in caspase 3-like activity was detected following CPT and VP-16 treatment (Figure 7). These results suggest that caspase 3-like activity correlates with occurrence of DNA fragmentation and that Bax- α accelerates caspase 3-like activation. No increase in DABCYL-YVADAPV-EDANS hydrolysis was detected following CPT and VP-16 treatment in both cell lines suggesting that caspase 1-like activity be neither activated nor involved in the apoptotic process induced by CPT and VP-16 in these cells. Moreover, caspase 3 activation was not detected in cells overexpressing the HA-Bcl-xL protein after CPT and VP-16 treatment (data not shown). These observations are consistent with the protective effect conferred by Bcl-xL in these cells.

To validate these findings, kinetic of poly(ADPribosyl) polymerase (PARP) cleavage, a known substrate of caspase 3-like activity (Lazebnik *et al*, 1994), was monitored in control and Bax- α transfected Namalwa cells

Figure 3 Bax- α promotes DNA fragmentation in cells grown in low serum and in presence of CD95/Fas antibodies. 14 C-Thymidine-labeled cells were grown for 24 h in 0.1% heat inactivated serum (close bars) and in the presence of anti-CD95/FAS antibodies (open bars). DNA fragmentation was determined by filter elution assays. Results are expressed as percentage DNA fragmentation relative to untreated cells grown in 10% heat inactivated serum. Bars represent the means $\pm$ S.E. of a triplicate experiment

treated with VP-16. Figure 8 shows that PARP cleavage and the appearance of the small 24 kDa fragment occurs much more extensively in VP-16-treated Bax- α transfected Namalwa cells compared to that of VP-16-treated control Namalwa cells. Taken together, our results suggest that Bax- α accelerate the processing and activation of caspase 3-like at low drug concentrations.

Discussion

The human Bax gene belongs to the Ced-9/Bcl-like family that regulates cell survival and cell death. Several mRNA isoforms have been isolated and derived likely from alternative splicing of a single Bax gene mapped to human chromosome 19q13.3-q13.4 (Apte *et al*, 1995; Oltvai *et al*, 1993). The first study with Bax gene product indicated that Bax- α has a cell death promoting activity, inhibiting the protective effect conferred by Bcl-2 in an interleukine (IL)-3 dependent cell line upon cytokine removal (Oltvai *et al*, 1993). Immunohistochemical analysis of Bax expression in mice showed that Bax proteins are expressed in a wide variety of cell types and predominantly in a variety of epithelial cells, in the thymic medulla and in the germinal center lymphocytes of lymph nodes and in several population of reproductive and neuronal cells (Krajewski *et al*, 1994). Yeast two-hybrid assays indicated that Bax- α interacts and heterodimerizes with itself, Bcl-2, Bcl-xL, Mcl-1 and A-1 (Sedlak *et al*, 1995). Bax-deficient mice displayed thymocytes and B cells hyperplasia and disordered ovaries granulosa cells, atypical male germ cells and no mature haploid sperm (Knudson *et al*,

Figure 4 The effects of overexpression of Bax- α in Namalwa cells treated at various CPT concentrations. 14 C-Thymidine-labeled cells were treated for 30 min with 0.01 (upper left panel), 0.05 (upper right panel), 0.1 (lower left panel) and 0.5 μ M CPT (lower right panel). After drug removal, cells were incubated in drug free-medium and at the indicated times (x axis; hour), DNA fragmentation was determined by filter elution assays. Results are expressed as percentage DNA fragmentation relative to that of untreated cells. Points represent the means $\pm$ S.E. of four independent experiments performed in duplicate. Symbols are Namalwa cells (●); Bax- α transfected Namalwa cells selected at 0.50 mg/ml (▼) and 1.5 mg/ml hygromycin (■). For comparison, Bcl-xL transfected cells selected at 1.5 mg/ml hygromycin (○). The difference in rate of DNA fragmentation between the variant lines and control cells was statistically significant at 4, 8, 16 and 24 h after drug treatment (0.001 < P < 0.03; student t -test values; except for values at 24 h in HA-Bax selected at 0.5 mg/ml hygromycin in the upper right panel)

Table 1 Rates of DNA synthesis after camptothecin in control and transfected Namalwa cells

CPT	Namalwa	Nam-bax ^{0.50}	Nam-bax ^{1.50}
0.01 μ M	85.0 $\pm$ 9.9	80.5 $\pm$ 9.8	80.7 $\pm$ 3.8
0.05 μ M	74.7 $\pm$ 5.8	73.7 $\pm$ 5.2	71.2 $\pm$ 10.9
0.10 μ M	65.5 $\pm$ 6.4	64.7 $\pm$ 2.9	63.5 $\pm$ 8.2
0.50 μ M	59.2 $\pm$ 7.5	56.2 $\pm$ 4.8	57.5 $\pm$ 4.8

Cells were treated with various concentrations of CPT for 30 min. 3 H-thymidine labeled incorporation were determined by 10 min pulse experiments. Values are expressed as % of DNA synthesis relative to control untreated cells and represent the means $\pm$ S.E. of two independent experiments performed in triplicate ($n=6$). Nam-bax^{0.50} and Nam-bax^{1.50} mean Namalwa cells transfected with Bax and selected at 0.50 mg/ml and 1.5 mg/ml hygromycin, respectively

Figure 5 Activation of DNA fragmentation in control and Bax- α transfected Namalwa cells following VP-16, vinblastine, paclitaxel and cis-platinum treatments. ^{14}C -Thymidine-labeled cells were treated for 8 h (close symbols) or 24 h (open symbols) with various concentrations of vinblastine (upper left panel), VP-16 (lower left panel), paclitaxel (upper right panel) and cis-platinum (lower right panel). DNA fragmentation was determined by filter elution assays. Results are expressed as percentage DNA fragmentation relative to that of untreated cells. Points represent the means $\pm$ S.E. of three experiments performed in triplicate. Symbols are Namalwa control cells ($\bullet$, $\circ$) and Bax- α transfected Namalwa cells selected at 0.5 mg/ml hygromycin ($\blacktriangle$, $\triangle$) and 1.5 mg/ml hygromycin ($\blacksquare$, $\square$). The difference in rate of DNA fragmentation between the variant lines and control cells was statistically significant for vinblastine and etoposide treated cells ($0.0001 < P < 0.03$; student *t*-test values)

1995). Thus, low Bax expression may result in hyperplasia and plays a role in tumor development.

In this study, by selecting transfected B-lymphoma Namalwa variant cells that express differential level of Bax- α , we show that its effect is directly related to its level of expression. Reduced expression of Bax was associated recently with poor response rates to chemotherapy in women with metastatic breast adenocarcinoma (Bargou *et al*, 1995). Gene transfer studies in human breast cell lines showed that Bax- α sensitized these cells to radiation- and drug-induced apoptosis (Sakakura *et al*, 1997; Wagener *et al*, 1996). Other studies have indicated that the Bax:Bcl-2 ratio in human testicular tumors and B-cell chronic lymphocytic leukemia is related to cell susceptibility to

Figure 6 Analysis of DNA fragmentation by agarose gel electrophoresis. Oligonucleosome-sized DNA fragments were visualized following standard agarose gel electrophoresis by ethidium bromide staining. (1) untreated cells; (2) 0.05 μM CPT; (3) 1.0 μM VP-16; (4) 1.0 μM cisplatin; (5) 1.0 μM vinblastine; (6) 1.0 μM paclitaxel. Treatments were of 16 h for Namalwa cells, 8 h for HA-Bax- α and 24 h for HA-Bcl-xL variant Namalwa cells

Figure 7 Caspase 1-like and caspase 3-like activities in CPT and VP-16 treated control and Bax- α transfected Namalwa cells. Cytosolic extracts were prepared from control (open symbols) and Bax- α transfected Namalwa cells (close symbols) at the indicated times following CPT (0.05 μM ; left panel) and VP-16 (10 μM ; right panel) treatment. Caspase activities were monitored continuously at 37°C by detecting fluorescence emission in the presence of DABCYL-YVADAPV-EDANS using excitation wavelength of 360 nm and emission wavelength of 490 nm and Ac-DEVD-AMC at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Enzyme activities were measured as initial velocities and expressed as relative intensity/min/mg. Points represent the means $\pm$ S.E. of three independent determinations. Symbols used are ($\blacktriangle$, $\triangle$) caspase 1-like activity; ($\bullet$, $\circ$) caspase 3-like activity

undergo apoptosis (Chresta *et al*, 1996; McConkey *et al*, 1996; Thomas *et al*, 1996). Ordered balance within the ratio of effector and repressor proteins regulating the decision of a cell to survive or die has already been suggested to form an important checkpoint for cell death (Oltvai and Korsmeyer, 1994). Our results are consistent with that model and indicate that the level of expression of Bax- α might be an important control point regulating anticancer drug sensitivity in tumor cells. Others have reported also that Bax- α can antagonize the protective effects of Bcl-xL during VP-16-induced apoptosis in FL5.12 cells and

Figure 8 PARP cleavage in control and Bax- α -transfected Namalwa cells following VP-16 treatment. Whole-cell extracts were prepared as described in Materials and Methods at the indicated times after VP-16 treatment (10 μ M) from Namalwa (left panel) and Bax- α transfected Namalwa cells selected at 1.5 mg/ml hygromycin (right panel). Following SDS-polyacrylamide gel electrophoresis (12%) and electrophoretic transfer, HA-Bax- α expression and PARP cleavage were detected using specific antibodies. Visualization of signals was performed by ECL and autoradiography represent different exposition

interestingly in these cells, Bax- α was also much less potent to promote cell death induced by cisplatin consistent with our observations in Namalwa cells (Simonian *et al*, 1996). Although our observations suggest that Bax- α does not promote paclitaxel-induced apoptosis in the B lymphoma Namalwa cells, others have reported that a mean of tenfold increased level of murine Bax expression in human SW626 ovarian cancer cells promotes cell death induced by paclitaxel (Strobel *et al*, 1996). No rationale explanation could be provided to explain the different observations, but the difference in paclitaxel sensitivity between these cell lines may reflect the presence or absence of other unknown factors that are involved in paclitaxel-induced apoptosis.

Our results indicate that Bax- α does not interfere with the primary mechanism of action of anticancer drugs such as CPT, since extent of DNA synthesis reduction is found similar in all Bax- α transfected and control cells following CPT treatment. Although signals emerging from unfinished replication and damaged DNA are not yet fully understood, this study and others, indicate that Bax- α is a control point and influences the decision of a cell to undergo apoptosis. It has been proposed that Bax- α is a wild-type p53 primary response gene, in p53-regulated pathway for apoptosis activation (Miyashita *et al*, 1994; Miyashita and Reed, 1995). The Namalwa cells are p53 double mutant cells (codon 248/exon 7; Arg to Gln/Arg to Trp) (O'Connor *et al*, 1993) and it is unlikely that mutated p53 plays a role in the induction of apoptosis in these lines. Nevertheless, gene transfer-mediated elevation of Bax- α sensitizes these cells to some but not all anticancer agents in a p53-independent manner. Moreover, the expression of EBNA-3 protein family in the Namalwa cells are unlikely to play a role in apoptosis regulation (Horner *et al*, 1995).

Activation of apoptotic proteases are known to be a critical step in dying cells. Series of caspases were reported to play a central role in executing apoptosis. In this study, we investigate the relation between Bax- α and the activation of caspases. We examined whether Bax- α accelerates the activation of the proteases required in the execution phase of apoptosis. Our experiments using fluorogenic substrates provide evidence that in cells

undergoing apoptosis induced by CPT and VP-16, the activation of caspase-3 like was accelerated in Bax- α transfected cells. No evidence of caspase 1-like activation was detected in these cells treated with CPT and VP-16. Caspase 3-like activation was also associated with PARP cleavage, a known substrate for these proteases (Lazebnik *et al*, 1994). Interestingly, the effect of Bax- α on caspase activation is opposite to that of Bcl-2 and Bcl-xL. Recent studies have reported that Bcl-2 like Bcl-xL prevents the activation of caspases in cultured cells (Monney *et al*, 1996; Shimizu *et al*, 1996). Activation of caspase 3 in Bax-induced cell death was reported recently in several studies (Deveraux *et al*, 1997; Kitanaka *et al*, 1997; Xiang *et al*, 1996). Interestingly, blocking caspase 3-like activities by the protease inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone prevents the cleavage of nuclear and cytosolic substrates and DNA fragmentation but does not prevent cells to die by a necrosis-like mode of cell death (Hirsch *et al*, 1997; Xiang *et al*, 1996).

In summary, our results indicate that Bax- α is a primary controlpoint that promotes transmission of cell death signals emerging from DNA damage in a p53-independent manner and accelerates activation of an apoptotic protease cascade. The identification of cell death signals triggered by DNA damage in p53 mutant cells and understanding how Bax- α promotes caspase activation and DNA fragmentation, will certainly help to design in future new therapeutic strategies to circumvent drug resistance mechanisms in p53-independent pathway of apoptosis.

Materials and Methods

Chemicals

Radioactive precursors [2- 14 C]thymidine (59 mCi/mmol), and [methyl- 3 H]thymidine (78 Ci/mmol) were obtained from ICN BioMedicals (Costa Mesa, CA). 20-S-camptothecin lactone, etoposide (VP-16), vinblastine, paclitaxel and cis-platinum(II) diammine dichloride were purchased from Sigma Chemicals Co. (St Louis, MO) and dissolved in dimethyl-sulphoxide (at 10 mmol/l) prior to each experiment. The fluorogenic peptide derivatives 4-(4-dimethylamino-

phenylazo)benzoyl-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (DABCYL-YVADAPV-EDANS) and Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Antibodies to human CD95/Fas receptor were obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of reagent grade and purchased either from Sigma, ICN and Boehringer-Mannheim or from other local sources.

Cell culture and DNA labeling

The human Namalwa cell line obtained from the American Type Culture Collection (Rockville, MD), were grown in suspension culture at 37°C in the presence of 5% CO₂ in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell culture products were purchased from Gibco BRL Life Technologies (Grand Island, NY). For DNA labeling, Namalwa cells were grown with [¹⁴C]thymidine (0.02 µCi/ml) for 24 h and then chased in isotope-free medium overnight prior to drug treatment.

cDNA cloning and transfection

The human Bax- α and Bcl-xL cDNAs were cloned by RT-PCR from total RNA using specific adapter primers containing *NotI* sequences at the ATG start codon and TAA stop codon. The amplified fragments were first cloned in pCRII vector (TA cloning system; Invitrogen, San Diego, CA) and then subcloned at the *NotI* restriction site in the eucaryotic expression vector pCEP4 (Invitrogen) that has been modified to include consensus Kosak and hemagglutinin epitope Tag sequences (HA-Tag) between the *BamHI* and *NotI* restriction sites. Vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech, Upsalla, Sweden) using both vector and internal primers. Purified pCEP4-HA-Bax, pCEP4-HA-Bcl-xL constructs and pCEP4-mock vector were transfected in Namalwa cells by electroporation at 0.27 kVolts (Gene Pulser, BioRad, Hercules, CA). Transfected cells were grown under hygromycin selection for 2–3 months to obtain stable lines prior to perform the experiments. Control Namalwa cells do not survive under hygromycin selection. Drug sensitivity of control and pCEP4-mock transfected variant lines were identical. Restriction and modification enzymes were purchased either from Pharmacia or Gibco BRL.

Western blot and immunohistochemistry analysis

For Western blot, control and transfected Namalwa cells were collected by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS) and then homogenized in a lysis buffer containing 5 mM HEPES pH 7.4, 160 mM KCl, 40 mM NaCl, 10 mM MgCl₂, 1 mM PMSF, 0.5% NP40 and a cocktail of protease inhibitors (Complete™, Boehringer-Mannheim) at 4°C for 30 min with agitation. After centrifugation, supernatants were collected as cytosolic protein extracts. Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard. Antibodies to the peptide epitope derived from the hemagglutinin protein of human influenza virus (HA epitope tag) and to human PARP protein were obtained from Boehringer-Mannheim. Affinity purified antibodies to human Bcl-2 and Bax were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and antibodies to Bcl-xL were obtained from CalBiochem (San Diego, CA). Enhanced chemiluminescence detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents. Expression of HA-Bax and -Bcl-xL by immunohistochemistry was visualized using anti-HA mouse

monoclonal antibodies (Boehringer-Mannheim) as primary antibody (10 µg/ml), a biotinylated goat anti-mouse (Sigma) as secondary antibody (10 µg/ml) followed by a streptavidin-alkaline phosphatase complex (Sigma) and colorimetric reaction using FastRed TR/Naphthol AS-MX (Sigma). Briefly, cells were spread by centrifugation on microscope slides and fixed in a solution containing 50% acetone and 50% methanol. Permeable cells were then incubated for 30 min in PBS buffered solution containing 10% (v/v) goat serum and 5% (w/v) bovine serum albumin (Sol A). Cells were then incubated with the primary antibody diluted in Sol A for 30 min, followed by several washings with Sol A and then incubated with the secondary antibodies diluted in Sol A. After several washes in Sol A, cells were incubated with a streptavidin-alkaline complex, washed again and incubated with a solution of FastRed TR/Naphthol As-MX according to the manufacturer's instructions (Sigma). Cells were then analyzed under an Axioscop camera-and equipped microscope (Carl Zeiss, West Germany).

DNA filter elution assay

DNA filter elution assays were designed to monitor and quantitate DNA fragmentation associated with apoptosis (Bertrand and Pommier, 1995). Each sample (approximately 0.5×10^6 ¹⁴C-thymidine-labeled cells) is loaded onto a protein-adsorbing filter (vinyl/acrylic copolymers filters, Metrical membrane, 0.8 µm pore size, 25 mm diameter, Gelman Sciences Inc., Ann Arbor, MI) mounted in a Millipore filter holder connected to a 50 ml syringe. Cells are then washed with 5 ml of phosphate buffered saline (PBS). As soon as the washing solution has dripped through by gravity, lysis is performed with 5 ml of lysis solution (0.2% sodium sarkosyl – 2 M NaCl – 0.04 M EDTA, pH 10.0). After the lysis has dripped through by gravity, it is washed from the filters with 5 ml of 0.02 M EDTA (pH 10.0). The filter is then processed as described (Bertrand and Pommier, 1995). Briefly, it was placed in a scintillation vial to which 1.0 ml of 1 N HCl is added. The vial is sealed and heated for 1 h at 60°C to depurinate the DNA. Then, 2.5 ml of 0.4 N NaOH is added and allowed to stand 1 h at room temperature to release the labeled DNA from the filters. Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading wash, lysis, EDTA wash, filter). DNA fragmentation was determined as the fraction of DNA in the loading wash fraction+lysis fraction+EDTA wash fraction relative to total DNA (loading wash+lysis+EDTA wash+filter). Results are expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in control untreated cells (background) using the formula:

$$(F - F_0/1 - F_0) \times 100$$

where F and F₀ represent DNA fragmentation in treated and control cells, respectively.

Analysis of DNA fragmentation by agarose gel electrophoresis

To visualize the oligonucleosome-sized DNA fragments, at specified times after drug treatment cellular DNA was extracted by a salting-out procedure as described previously (Miller *et al.*, 1988). Electrophoresis was done in 1.6% agarose gel in Tris-acetate buffer (pH 8.0). Following electrophoresis DNA was visualized by ethidium bromide staining.

Measurement of thymidine incorporation

Control and transfected Namalwa cells were prelabeled with ¹⁴C-thymidine (0.02 µCi/ml) for 24 h and then chased in isotope-free

medium overnight prior to drug treatment. Cells were incubated in CPT containing medium for 30 min. After drug treatment, cells were washed twice with complete medium and rates of nucleotide incorporation were measured by 10 min pulse experiments with [3 H]thymidine (10 μ Ci/ml) as described (Bertrand *et al*, 1992). Nucleotide incorporation was stopped by adding 10 ml of ice-cold PBS and cells were quickly pelleted by centrifugation (1000 g, 3 min, 4°C). Acid-insoluble nucleotides were precipitated on ice with 10% trichloroacetic acid. The precipitates were dissolved in 0.4 N NaOH and radioactivity was monitored by scintillation spectrometry. Results were expressed as the ratio of [3 -H]/[14 -C] for treated cells over the ratio of [3 -H]/[14 -C] of untreated cells (Bertrand *et al*, 1992).

Caspase activity determination

Cytosolic extracts are prepared by washing control and transfected Namalwa cells twice by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca $^{2+}$ and Mg $^{2+}$). Cells are then homogenized at 4°C for 30 min in a lysis buffer containing 100 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM DTT, 20% glycerol and 0.5% NP-40. Samples are then centrifuged (13 000 g for 10 min at 4°C) and supernatants collected as cytosolic extracts. Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence fluorometer (LS 50B Perkin Elmer) using excitation wavelength of 360 nm and emission wavelength of 490 nm for the substrate DABCYL-YVADAPV-EDANS and excitation wavelength of 380 nm and emission wavelength of 460 nm for the substrate Ac-DEVD-AMC. Reactions were carried out in cuvettes and temperature maintained at 37°C using a water-jacketed sample compartment. The assay mixture contained 100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 5 mM EDTA and 200 μ M fluorogenic peptides. Enzyme activities were determined as initial velocities and expressed as relative intensity/min/mg.

Acknowledgements

RB is supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society (grant # 006254) and by the Medical Research Council of Canada (grant # MT 12125). ES is a recipient of a Canderel fellowship. GC was supported by a fellowship from the Ministère de l'Éducation du Québec. RB obtained scholarship from the Medical Research Council of Canada in association with the Research Cancer Society Inc. We would like to thank Dr AH Greenberg (Manitoba Institute of Cell Biology, Winnipeg, Manitoba) for providing the HA epitope sequences and Nathalie Gaudreault for performing the DNA sequencing analysis. GC is now at Laboratory of Chemical Carcinogenesis, National Institute for Cancer Research, Viale Benedetto XV, 10, I-16132 Genova, Italy.

References

Alnemri ES, Fernandes-Alnemri T and Litwack G (1995) Cloning and expression of four novel isoforms of human interleukin-1 beta converting enzyme with different apoptotic activities. *Journal of Biological Chemistry* 270: 4313–4317

Apte SS, Mattei MG and Olsen BR (1995) Mapping of the Human Bax gene to chromosome 19q13.3-q13.4 and isolation of a novel alternatively spliced transcript, Bax δ . *Genomics* 26: 529–594

Bargou RC, Daniel PT, Mapara MY, Bommert K, Wagener C, Kallinich B, Royer HD and Dorken B (1995) Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-alpha expression in tumor cells correlates with resistance towards apoptosis. *International Journal of Cancer* 60: 854–859

Barry MA, Behnke CA and Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hypothermia. *Biochem. Pharmacol.* 40: 2353–2362

Bertrand R, Kohn K, Solary E and Pommier Y (1995) Detection of apoptosis-associated DNA fragmentation using a rapid and quantitative filter elution assay. *Drug Development Res.* 34: 138–144

Bertrand R, O'Connor P, Kerrigan D and Pommier Y (1992) Sequential administration of camptothecin and etoposide circumvents the antagonistic cytotoxicity of simultaneous drug administration in slowly growing human carcinoma, HT-29 cells. *Eur. J. Cancer* 28A: 743–748

Bertrand R and Pommier Y (1995) Assessment of DNA damage in Mammalian cells by DNA filter elution methodology. In GP Studzinski (Ed.), *Cell growth and apoptosis: A practical approach*, (Vol. 159, pp. 97–118). New York: IRL Press

Bertrand R, Sarang M, Jenkin J, Kerrigan D and Pommier Y (1991) Differential induction of secondary DNA fragmentation by topoisomerase II inhibitors in human tumor cell lines with amplified c-myc expression. *Cancer Research* 51: 6280–6285

Bertrand R, Solary E, Jenkins J and Pommier Y (1993) Apoptosis and its modulation in human promyelocytic HL-60 cells treated with DNA topoisomerase I and II inhibitors. *Experimental Cell Research* 207: 388–397

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G and Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74: 597–608

Boldin MP, Goncharov TM, Goltsev YV and Wallach D (1996) Involvement of mach, a novel mort1/fadd-interacting protease, in fas/Apo-1- and Tnf receptor-induced cell death. *Cell* 85: 803–815

Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, Ebb RG, Subramanian T, Chittenden T, Lutz RJ and Chinnadurai G (1995) Bcl-2, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* 11: 1921–1928

Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI and Guild BC (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature* 374: 733–736

Choi SS, Park IC, Yun JW, Sung YC, Hong SI and Shin HS (1995) A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. *Oncogene* 11: 1693–1698

Chresta CM, Masters JRW and Hickman JA (1996) Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high Bax-Bcl-2 ratio. *Cancer Research* 56: 1834–1841

Clarke ML, Smith SD and Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 47: 19–28

Das R, Reddy EP, Chatterjee D and Andrews DW (1996) Identification of a novel Bcl-2 related gene, Breg-1, in human glioma. *Oncogene* 12: 947–951

Datta R, Manome Y, Taneja N, Boise L, Weichselbaum R, Thompson C and Kufe D (1995) Overexpression of Bcl-xL by cytotoxic drug exposure confers resistance to ionizing radiation-induced internucleosomal DNA fragmentation. *Cell Growth and Differentiation* 6: 363–370

Del Bino G, Bruno S, Yi PN and Darzynkiewicz Z (1992) Apoptotic cell death triggered by camptothecin or teniposide. The cell cycle specificity and effects of ionizing radiation. *Cell Proliferation* 25: 537–548

Deveraux QL, Takahashi R, Salvesen GS and Reed JC (1997) X-linked iap is a direct inhibitor of cell-death proteases. *Nature* 388: 300–304

Dole MG, Jasty R, Cooper MJ, Thompson CB, Nunez G and Castle VP (1995) Bcl-X(L) is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. *Cancer Research* 55: 2576–2582

Duan HJ, Chinnaiyan AM, Hudson PL, Wing JP, He WW and Dixit V (1996a) Ice-Lap3, a novel mammalian homologue of the caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *Journal of Biological Chemistry* 271: 1621–1625

Duan HJ, Orth K, Chinnaiyan AM, Poirier GG, Froelich CJ, He WW and Dixit V (1996b) Ice-Lap3, a novel member of the Ice/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *Journal of Biological Chemistry* 271: 16720–16724

Ealovega MW, McGinnis PK, Sumantran VN, Clarke MF and Wicha MS (1996) Bcl-X(S) gene therapy induces apoptosis of human mammary tumors in nude mice. *Cancer Research* 56: 1965–1969

Fang W, Rivard JJ, Ganter JA, Leblen TW, Nath KA, Mueller DL and Behrens TW (1995) Bcl-X(L) rescues Wehi 231 B lymphocytes from oxidant-mediated death following diverse apoptotic stimuli. *Journal of Immunology* 155: 66–75

- Farrow SN, White JH, Martinou I, Raven T, Pun KT, Grinham CJ, Martinou JC and Brown R (1995) Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature* 374: 731–733
- Faucheu C, Blanchet AM, Collarddutilleul V, Lalanne JL and Diuhercend A (1996) Identification of a cysteine protease closely related to interleukin-1-beta-converting enzyme. *European Journal of Biochemistry* 236: 207–213
- Faucheu C, Diu A, Chan AW, Blanchet AM, Miossec C, Herve F, Collard-Dutilleul V, Gu Y, Aldape RA, Lippke JA et al (1995) A novel human protease similar to the interleukin-1 beta converting enzyme induces apoptosis in transfected cells. *EMBO Journal* 14: 1914–1922
- Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G and Alnemri ES (1996) In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FDD-like domains. *Proc. Natl. Acad. Sci. USA* 93: 7464–7469
- Fernandes-Alnemri T, Litwack G and Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *Journal of Biological Chemistry* 269: 30761–30764
- Fernandes-Alnemri T, Litwack G and Alnemri E (1995) Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. *Cancer Research* 55: 2737–2742
- Fernandes-Alnemri T, Takahashi A, Armstrong R, Krebs J, Fritz L, Tomaselli KJ, Wang L, Yu Z, Croce CM, Salveson G, Earnshaw WC, Litwack G and Alnemri ES (1995) Mch3, a novel human apoptotic cysteine protease highly related to Cpp32. *Cancer Research* 55: 6045–6052
- Gibson L, Holmgren SP, Huang DCS, Bernand O, Copeland NG, Jenkins NA, Sutherland GR, Baker E, Adams JM and Cory S (1996) Bcl-W, a novel member of the Bcl-2 family, promotes cell survival. *Oncogene* 13: 665–675
- Gupta M, Fujimori A and Pommier Y (1995) Eukaryotic DNA topoisomerase I. *Biochim. Biophys. Acta* 1262: 1–14
- Hickman JA (1996) Apoptosis and chemotherapy resistance. *European Journal of Cancer* 32A: 921–926
- Hirsch T, Marchetti P, Susin SA, Dallaporta B, Zamzami N, Marzo I, Geuskens M and Kroemer G (1997) The apoptosis-necrosis paradox – apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene* 15: 1573–1581
- Hockenbery D, Nunez G, Millman C, Schreiber RD and Korsmeyer SJ (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348: 334–336
- Hockenbery DM (1995) BCL-2, a novel regulator of cell death. *Bioessays* 17: 631–638
- Horner D, Lewis M and Farrell PJ (1995) Novel hypotheses for the roles of EBNA-1 and BHRF1 in EBV-related cancers. *Intervirology* 38: 195–205
- Ibrado AM, Huang Y, Fang GF and Bhalla K (1996) Bcl-X(L) overexpression inhibits taxol-induced yama protease activity and apoptosis. *Cell Growth and Differentiation* 7: 1087–1094
- Jaxel C, Taudou G, Portemer C, Mirambeau G, Panijel J and Duguët M (1988) Topoisomerase inhibitors induce irreversible fragmentation of replicated DNA in concanavalin A stimulated splenocytes. *Biochemistry* 27: 95–99
- Kamens J, Paskind M, Hugunin M, Talanian RV, Allen H, Banach D, Bump N, Hackett M, Johnston CG, Li P, Mankovich JA, Terranova M and Ghayur T (1995) Identification and characterization of Ich-2, a novel member of the interleukin-1-beta-converting enzyme family of cysteine proteases. *Journal of Biological Chemistry* 270: 15250–15256
- Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Research* 49: 5870–5878
- Kiefer MC, Brauer MJ, Powers VC, Wu JJ, Umansky SR, Tomei LD and Barr PJ (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature* 374: 736–739
- Kitanaka C, Namiki T, Noguchi K, Mochizuki T, Kagaya S, Chi S, Hayashi A, Asai A, Tsujimoto Y and Kuchino Y (1997) Caspase-dependent apoptosis of Cos-7 cells induced by Bax overexpression – differential effects of Bcl-2 and Bcl-X(L) on Bax-induced caspase activation and apoptosis. *Oncogene* 15: 1763–1772
- Klein G and Dombos L (1973) Relationship between the sensitivity of EBV-carrying lymphoblastoid lines to superinfection and the inducibility of the resident viral genome. *Int. J. Cancer* 11: 327
- Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ and Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 270: 96–99
- Korsmeyer SJ (1992) Bcl-2 initiates a new category of oncogenes: Regulators of cell death. *Blood* 80: 879–886
- Korsmeyer SJ (1995) Regulators of cell death. [Review]. *Trends in Genetics* 11: 101–105
- Kozopas KM, Yang T, Buchan HL, Zhou P and Craig RW (1995) MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to Bcl-2. *Proc. Natl. Acad. Sci. USA* 90: 3516–3520
- Krajewski S, Blomqvist C, Franssila K, Krajewska M, Wasenius VM, Niskanen E, Nordling S and Reed JC (1995) Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. *Cancer Research* 55: 4471–4478
- Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG and Reed JC (1994) Immunohistochemical determination of In Vivo distribution of Bax, a dominant inhibitor of Bcl2. *American Journal of Pathology* 145: 1323–1336
- Kumar S and Lavin MF (1996) The ICE family of cysteine proteases as effectors of cell death. *Cell Death Differ.* 3: 255–267
- Lawrence JB, Villave CA and Singer RH (1988) Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma cell line. *Cell* 52: 51–61
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG and Earnshaw WC (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371: 346–347
- Lin EY, Orlofsky A, Berger MS and Prystowsky MB (1993) Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J. Immunol.* 151: 1979–1988
- Lippke JA, Gu Y, Sarnacke C, Caron PR and Su MSS (1996) Identification and characterization of Cpp32/2mch2 homologue 1, a novel cysteine protease similar to Cpp32. *Journal of Biological Chemistry* 271: 1825–1828
- Luka J, Lindahl T and Klein G (1978) Purification of the Epstein–Barr virus-determined nuclear antigen from Epstein–Barr virus-transformed human lymphoid cell lines. *Journal of Virology* 27: 604–611
- Matsuo T, Heller M, Petti L, O'Shiro E and Kieff E (1984) Persistence of the entire Epstein–Barr virus genome integrated into human lymphocyte DNA. *Science* 226: 1322–1325
- McConkey DJ, Chandra J, Wright S, Plunkett W, McDonnell TJ, Reed JC and Keating M (1996) Apoptosis sensitivity in chronic lymphocytic leukemia is determined by endogenous endonuclease content and relative expression of Bcl-2 and Bax. *Journal of Immunology* 156: 2624–2630
- Miller SA, Dykes DD and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16: 1215
- Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 9: 1799–1805
- Miyashita T and Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80: 293–299
- Monney L, Otter I, Olivier R, Ravn U, Mirzazadeh H, Fellay I, Poirier GG and Borner C (1996) Bcl-2 overexpression blocks activation of the death protease Cpp32/Yama/Apopain. *Biochemical & Biophysical Research Communications* 221: 340–345
- Munday NA, Vaillancourt JP, Ali A, Casano FJ, Miller DK, Molineaux SM, Yamin TT, Yu VL and Nicholson DW (1995) Molecular cloning and pro-apoptotic activity of ICErelIII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. *Journal of Biological Chemistry* 270: 15870–15876
- Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME and Dixit VM (1996) Flice, a novel Fadd-homologous Ice/Ced-3-like protease, is recruited to the Cd95 (Fas/Apo-1) death-inducing signaling complex. *Cell* 85: 817–827
- Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA et al (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376: 37–43
- O'Connor PM, Jackman J, Jondle D, Bhatia K, Magrath I and Kohn KW (1993) Role of the p53 tumor suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. *Cancer Research* 53: 4776–4780

- Oltvai ZN and Korsmeyer SJ (1994) Checkpoints of dueling dimers foil death wishes. *Cell* 79: 189–192
- Oltvai ZN, Millman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74: 609–619
- Patel T, Gores GJ and Kaufmann SH (1996) The role of proteases during apoptosis. *FASEB Journal* 10: 587–597
- Pearson GR, Luka J, Petti L, Sample J, Birkenbach M, Braun D and Kieff E (1987) Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. *Virology* 160: 151–161
- Pommier Y (1997) DNA topoisomerase II inhibitors. In BA Teicher (Ed.), *Cancer Therapeutics: Experimental and Clinical Agents*, (pp. 153–174). Totowa, New Jersey: Humana Press Inc
- Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y and Jacobson MD (1994) Programmed cell death and the control of cell survival. *Philosophical Transactions of the Royal Society of London – Series B. Biological Sciences* 345: 265–268
- Reed JC (1995) Bcl-2 family proteins – regulators of chemoresistance in cancer. *Toxicology Letters* 3: 155–158
- Reed JC (1997) Double identity for proteins of the Bcl-2 family. *Nature* 387: 773–776
- Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Nakatani H, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K and Takahashi T (1996) Overexpression of Bax sensitizes human breast cancer MCF-7 cells to radiation-induced apoptosis. *International Journal of Cancer* 67: 101–105
- Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K and Takahashi T (1997) Overexpression of Bax sensitizes breast cancer MCF-7 cells to cisplatin and etoposide. *Surgery Today* 27: 676–679
- Sample J, Liebowitz D and Kieff E (1989) Two related Epstein–Barr virus membrane proteins are encoded by separate genes. *Journal of Virology* 63: 933–937
- Sané AT, Schmitt E, Steyaert A, Meyer D and Bertrand R (1997) The CrmA- and TPCK-sensitive pathways that trigger the oligonucleosome-sized DNA fragmentation in camptothecin-induced apoptosis: relation to caspase activation and high molecular weight DNA fragmentation. *Biochemical and Cellular Biology* 75: 359–368
- Sawada K, Yamamoto M, Tabata T, Smith M, Tanaka A and Nonoyama M (1989) Expression of EBNA-3 family in fresh B lymphocytes infected with Epstein–Barr virus. *Virology* 168: 22–30
- Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB and Korsmeyer SJ (1995) Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc. Natl. Acad. Sci. USA* 92: 7834–7838
- Shimizu S, Eguchi Y, Kamiike W, Matsuda H and Tsujimoto Y (1996) Bcl-2 expression prevents activation of the ICE protease cascade. *Oncogene* 12: 2251–2257
- Simonian PL, Grillot DAM, Merino R and Nunez G (1996) Bax can antagonize Bcl-X(L) during etoposide and cisplatin-induced cell death independently of its heterodimerization with Bcl-X(L). *Journal of Biological Chemistry* 271: 22764–22772
- Solary E, Bertrand R, Kohn KW and Pommier Y (1993) Differential induction of apoptosis in undifferentiated and differentiated HL-60 cells by DNA topoisomerase I and II inhibitors. *Blood* 81: 1359–1368
- Strobel T, Swanson L, Korsmeyer S and Cannistra SA (1996) Bax enhances paclitaxel-induced apoptosis through a p53-independent pathway. *Proc. Natl. Acad. Sci. USA* 93: 14094–14099
- Sumantran VN, Ealovega MW, Nunez G, Clarke MF and Wicha MS (1995) Overexpression of Bcl-X(S), sensitizes MCF-7 cells to chemotherapy-induced apoptosis. *Cancer Research* 55: 2507–2510
- Takayama S, Kochel K, Irie S, Inazawa J, Abe J, Sato T, Druck T, Huebner K and Reed JC (1996) Cloning of Cdnas encoding the human Bag 1 protein and localization of the human Bag1 gene to chromosome 9p12. *Genomics* 35: 494–498
- Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA and JC R (1995) Cloning and functional analysis of Bag-1; a novel Bcl-2 binding protein with anti-cell death activity. *Cell* 80: 279–284
- Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM (1995) Yama/Cpp32-Beta, a mammalian homolog of Ced-3, is a CrmA-inhibitable protease that cleaves the death substrate Poly(ADP-ribose) polymerase. *Cell* 81: 801–809
- Thomas A, Elrouby S, Reed JC, Krajewski S, Silber R, Potmesil M and Newcomb EW (1996) Drug-induced apoptosis in B-cell chronic lymphocytic leukemia – relationship between p53 gene mutation and Bcl-2/Bax proteins in drug resistance. *Oncogene* 12: 1055–1062
- Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456–1462
- Tsujimoto Y, Finger LR, Yunis J, Nowell PC and Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14:18) chromosome translocation. *Science* 226: 1097–1099
- Vaux DL, Cory S and Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335: 440–442
- Wagener C, Bargou RC, Daniel PT, Bommert K, Mapara MY, Royer HD and Dorken B (1996) Induction of the death-promoting gene Bax- α sensitizes cultured breast cancer cells to drug-induced apoptosis. *International Journal of Cancer* 67: 138–141
- Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF and Sikorska M (1991) Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Research* 51: 1078–1085
- Wang L, Miura M, Bergeron L, Zhu H and Yuan J (1994) Ich-1, an Ice/Ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78: 739–750
- Wang K, Yin XM, Chao DT, Millman CL and Korsmeyer SJ (1996) Bid – a novel Bcl2 domain-only agonist. *Genes & Development* 10: 2859–2869
- Wang SY, Miura M, Jung YK, Zhu H, Gagliardini V, Shi LF, Greenberg AH and Yuan JY (1996) Identification and characterization of Ich-3, a member of the interleukin-1-beta converting enzyme (Ice)/Ced-3 family and an upstream regulator of Ice. *Journal of Biological Chemistry* 271: 20580–20587
- Wyllie AH (1997) Apoptosis and carcinogenesis. *European Journal of Cell Biology* 73: 189–197
- Xerri L, Parc P, Brousset P, Schlaifer D, Hassoun J, Reed JC, Krajewski S and Birnbaum D (1996) Predominant expression of the long isoform of Bcl-X (Bcl-XL) in human lymphomas. *British Journal of Haematology* 92: 900–906
- Xiang JL, Chao DT and Korsmeyer SJ (1996) Bax-induced cell death may not require interleukin 1-beta-converting enzyme-like proteases. *Proc. Natl. Acad. Sci. USA* 93: 14559–14563
- Yang E, Zha J, Jockel J, Boise LH, Thompson CB and Korsmeyer SJ (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80: 285–291
- Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz R (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1B-converting enzyme. *Cell* 75: 641–652