Distinct sites of action of Bcl-2 and Bcl-x_L in the ceramide pathway of apoptosis

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We studied the inhibition of tumour necrosis factor α (TNFα)- and camptothecin-induced apoptosis by Bcl-2 and Bcl-x_L as they relate to the ceramide pathway. Expression of either Bcl-2 or Bcl-x_L provided significant protection from the apoptotic effects of TNFα or camptothecin. In contrast to Bcl-2, Bcl-x_L overexpression did not protect cells from ceramide-induced apoptosis. On the other hand, Bcl-x_L prevented the accumulation of endogenous ceramide in response to TNFα or camptothecin, whereas Bcl-2 showed little effect on ceramide formation. Moreover, Bcl-x_L, but not Bcl-2, totally inhibited a caspase-8-like activity in cell lysates stimulated with TNFα. These results identify a different mechanism of action for Bcl-x_L compared with Bcl-2 and they demonstrate that Bcl-x_L targets a point upstream of ceramide generation, whereas Bcl-2 functions downstream of ceramide in the TNFα- and camptothecin-activated pathways of apoptosis.

INTRODUCTION

The Bcl-2 family of proteins plays an important role in the regulation of apoptosis [1]. This family includes both anti-apoptotic and pro-apoptotic members that are differentially regulated but share sequence-homology domains [2,3]. Bcl-2 and its homologue, Bcl-x_L, inhibit apoptosis induced with a wide variety of stimuli by mechanisms that remain unidentified. Although Bcl-2 and Bcl-x_L are frequently considered to be functionally indistinguishable [4,5], evidence in support of distinct functions has been building over the past few years. These distinctions include the superior ability of Bcl-x_L to inhibit apoptosis induced by the chemotherapeutic agents etoposide, teniposide and cisplatin, and the immunosuppressive agents FK506, cyclosporin A and rapamycin, as well as anti-immunoglobulin-induced apoptosis [6–8]. In contrast, Bcl-2 and Bcl-x_L appear equipotent in inhibiting apoptosis induced by tumour necrosis factor α (TNFα), Fas ligation, staurosporine, gamma irradiation, and the chemotherapeutic agents vincristine and vinblastine [7–9]. The reasons for these differential effects are unknown.

Caspases are a family of cysteine proteases that are involved in the signalling and execution of apoptosis in response to a variety of stimuli [10]. Stimulation of the TNFα receptor results in the rapid recruitment of caspase-8 to the receptor and its activation. Distal caspases (e.g. caspase-3 or caspase-7) are not activated until several hours later [11]. This interim period witnesses the gradual accumulation of ceramide, a sphingolipid break-down product [11]. Ceramide has been shown to be an important component of the TNFα and other signalling pathways, such as those activated by chemotherapeutic agents [12–15]. TNFα causes activation of sphingomyelinases that can hydrolyse membrane sphingomyelin to produce phosphocholine and ceramide. Addition of exogenous, cell-permeable, ceramide analogues reproduces a number of TNFα actions, including the activation of downstream caspases, leading to DNA fragmentation and the induction of apoptosis [11,12,16–18]. Recently, we showed that the cowpox virus protein cytokine-response-modifier A (CrmA), a selective caspase inhibitor, blocks TNFα-induced apoptosis by inhibiting ceramide accumulation without interfering with ceramide-induced caspase activation [11]. In contrast, Bcl-2 blocked the activation of caspase(s) by ceramide without significantly interfering with ceramide accumulation. These studies suggested that, in the TNFα pathway of apoptosis, caspases belonging to different subfamilies may function either upstream or downstream of ceramide accumulation.

In this study we investigated whether Bcl-2 and Bcl-x_L modulate the TNFα- and camptothecin-activated ceramide pathways of apoptosis. We utilized MCF7 breast carcinoma cells overexpressing either Bcl-x_L or Bcl-2. We present evidence that Bcl-2 and Bcl-x_L modulate the apoptotic response of these cells to TNFα and camptothecin by targeting distinct components of this pathway defined by the cellular accumulation of ceramide. Whereas Bcl-2 appears to function at a site distal to ceramide accumulation, Bcl-x_L acts 'upstream' of the formation of ceramide. These results suggest that Bcl-x_L and Bcl-2 have distinct mechanisms of action.

MATERIALS AND METHODS

Cell lines and cultivation

The previously described cell lines overexpressing Bcl-2 or Bcl-x_L were derived from a TNFα-sensitive MCF7 parental line [9] and were a kind gift from Dr. Muneesh Tewari and Dr. Vishva Dixit (University of Michigan, Ann Arbor, MI, U.S.A.). Cells were grown in RPMI 1640 medium supplemented with 10% fetal

Abbreviations used: TNFα, tumour necrosis factor α; CrmA, cytokine-response-modifier A; C_2-ceramide, *N*-acetylsphingosine; C_6-ceramide, *N*-hexanoylsphingosine; IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide.

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bovine serum and 0.2% sodium bicarbonate. Hygromycin (150 μg/ml; Calbiochem, La Jolla, CA, U.S.A.) was added to the Bcl-2 and Bcl-x_L cell lines and their vector to maintain selection. Experiments were done in the absence of hygromycin. Cell viability was determined by the ability to exclude Trypan Blue.

Quantification of apoptosis

The fluorescent DNA-staining dye, propidium iodide, was used to evaluate nuclear morphology. MCF7 cells from the different cell lines were plated on 13 mm Thermanox cover slips (Nunc, Rochester, NY, U.S.A.) placed in 15.5 mm wells of a 24 well plate (Falcon, Becton-Dickinson, Falcon Lakes, NJ, U.S.A.) at a density of 5×10^4 cells/well and incubated until they reached 60–70% confluence. Cells were then treated with TNFα (1 nM) or various concentrations of synthetic cell-permeable ceramides (kind gifts from Dr. Yusuf Hannun and Dr. Alicja Bielawska, Duke University, Durham, NC, U.S.A.). Treatments were for 20 h, after which the cells were washed once with PBS and then fixed overnight in 100% methanol at -20 °C. The cells were then stained at room temperature in a 50 μg/ml solution of propidium iodide (Sigma, St. Louis, MO, U.S.A.) for 10 min and washed in 5 ml of PBS. The cover slips were then removed and mounted on glass slides and the stained nuclei were visualized by fluorescence microscopy using an FITC range filter on a Zeiss confocal microscope fitted with a Nikon 6006 camera. Apoptotic cells were defined by the condensation of nuclear chromatin, its fragmentation, or its margination to the nuclear membrane. The percentage of non-apoptotic cells was determined after counting 100–250 cells. Each experiment was done in duplicate at least twice.

Annexin V labelling was performed using a commercially available kit from Boehringer-Mannheim (Mannheim, Germany) as directed by the manufacturer. The number of annexin V-labelled cells that did not simultaneously take up propidium iodide was quantified by flow cytometry.

Ceramide measurement

Cells were seeded at 1×10^6 cells/ml in a 10 ml volume, rested overnight, then treated with TNFα (1 nM). Lipids were collected according to the method of Bligh and Dyer [19]. Briefly, cells were trypsinized, pelleted, washed once with PBS and extracted with 3 ml of chloroform/methanol (1:2, v/v) in 13×100 mm screw-top glass tubes. The monophase was mixed and 0.7 ml of water was added, and the samples were left to settle for 10 min. The organic and aqueous phases were subsequently separated by the addition of 1 ml of chloroform and 1 ml of water followed by vigorous shaking and centrifugation at 300 ***g***. The organic phase was carefully removed and transferred to a new tube and the samples were dried under N_2. Lipids were then resuspended in 1 ml of chloroform. Ceramide levels were measured using a modified diacylglycerol kinase assay [20,21] using external standards. Briefly, 80% of the lipid sample was dried under N_2. The dried lipid was solubilized in 20 μl of an octyl-β-D-glucoside/dioleoyl phosphatidylglycerol micellar solution (7.5% octyl-β-D-glucoside/25 mM dioleoyl phosphatidylglycerol) by two cycles of sonication in a bath sonicator for 60 s followed by resting at room temperature for 15–20 min. The reaction buffer was prepared as a 2× solution, containing 100 mM imidazole/HCl, pH 6.6, 100 mM LiCl, 25 mM $MgCl_2$ and 2 mM EGTA. To the lipid micelles, 50 μl of 2× reaction buffer, 0.2 μl of 1 M dithiothreitol, 5 μg of diglycerol kinase membranes, and dilution buffer [10 mM imidazole (pH 6.6)/1 mM diethylenetriamine-penta-acetic acid (pH 7)] were added to a final volume of 90 μl. The reaction was started by adding 10 μl of 2.5 mM [γ-^{32}P]ATP solution (specific activity of 75000–200000 c.p.m./nmol). The reaction was allowed to proceed at 25 °C for 30 min. Lipids were extracted as described above and a 1.5 ml aliquot of the organic phase was dried under N_2. Lipids were then resuspended in 100 μl of methanol/chloroform (1:20, v/v), and 20 μl were spotted on a 20 cm silica gel thin-layer chromatography plate. Plates were developed with chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, by vol.), air dried, and subjected to autoradiography. The radioactive spots corresponding to ceramide phosphate, the phosphorylated product of ceramide, were scraped into a scintillation vial containing 4 ml of scintillation fluid and counted on a scintillation counter. Linear curves of phosphorylation were produced over a concentration range of 0–640 pM of external standards (CIII ceramide; Sigma). Ceramide levels were always normalized to lipid phosphate, which was measured according to the method of Rouser et al. [22]. Briefly, 20% of the lipid sample was dried down under N_2 and oxidized with 150 μl of 70% perchloric acid on a heating block at 160 °C for 45 min. The tubes were allowed to cool, and then 830 μl of water was added, followed by 170 μl of 2.5% ammonium molybdate and 170 μl of 10% ascorbic acid, with vortexing after each addition. The tubes were then incubated at 50 °C for 15 min, allowed to cool and absorbance read at 820 nm and compared with standard.

Cleavage of caspase-8 substrate

Cells were seeded at 2×10^6 cells in a 10 cm Petri dish and rested overnight. The cells were then treated with TNFα at 1 nM for 14 h. Subsequently, cells were harvested by scraping, washed with PBS, and then suspended in lysis buffer [50 mM Hepes (pH 7.4)/0.1% CHAPS/1 mM dithiothreitol/0.1 mM EDTA] and then lysed by one cycle of freeze–thawing. The lysate was cleared by spinning at 9000 ***g*** for 10 min. Protein concentration in the supernatant was determined by the Bradford assay. Caspase activity was determined by incubating 100 μg of protein with 200 μM of the caspase-8-specific colorimetric peptide substrate *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (IETD-pNA; Biomol, Plymouth Meeting, PA, U.S.A.) in a 200 μl final volume using reaction buffer [50 mM Hepes (pH 7.4)/100 mM NaCl/0.1% CHAPS/10 mM dithiothreitol/1 mM EDTA/10% glycerol]. Cleavage of the substrate was quantified by measuring the absorbance at 405 nm in an ELISA plate reader.

Western blotting

For experiments analysing caspase-8 proteolysis, cells were seeded and treated as described for the ceramide-measurement experiments. At 24 h following treatment, cells were harvested by treatment with trypsin, centrifuged (800 ***g***) at 4 °C and washed once with ice-cold PBS. The cell pellet was then resuspended in 50 μl of PBS, lysed with sample buffer [30 mM Tris/HCl (pH 6.8)/10% (v/v) glycerol/4% (w/v) SDS], and boiled for 10 min. Protein concentrations were determined using the detergent-compatible Bio-Rad assay (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts of protein, usually 100 μg, were resolved by 12% SDS/PAGE and transferred to a nitrocellulose membrane. The p20 subunit of cleaved caspase-8 was identified by the specific goat anti-human polyclonal antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), used at a dilution of 1:200, and a rabbit anti-goat secondary antibody at a dilution of 1:5000. The signal was visualized by enhanced chemiluminescence (Amersham-Pharmacia, Bucks., U.K.).

Statistical analyses

Quantitative data are expressed as means ± S.E.M. Significant differences among groups were examined by one-way analysis of variance followed by Bonferroni's *post hoc* test. $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS PC plus software (Statistical Product and Service Solutions Inc., Chicago, IL, U.S.A.).

RESULTS

TNFα treatment induces cell death in the breast carcinoma cell line, MCF7, with reproducible morphological and biochemical changes typical of apoptosis [11,23]. The effects of overexpression of Bcl-x_L or Bcl-2 on TNFα-induced apoptosis in this cell line were examined. We utilized MCF7 cell lines that overexpress Bcl-x_L (MCF7-Bcl-x_L) or Bcl-2 (MCF7-Bcl-2), which were described previously [9]. Bcl-2 and Bcl-x_L expression was confirmed by Western blotting using specific antibodies ([9], and results not shown). Wild-type MCF7 and vector-transfected cells expressed low levels of Bcl-2 and Bcl-x_L, whereas MCF7-Bcl-x_L and MCF7-Bcl-2 showed 10–15-fold overexpression of Bcl-x_L and Bcl-2 respectively.

Treatment of vector-transfected MCF7 (MCF7-V) cells with TNFα at a concentration of 1 nM resulted in progressive morphological changes typical of apoptosis, including cell shrinkage, rounding and detachment from the plate, as observed with phase-contrast microscopy. Examination of these cells under a fluorescent microscope after staining with the DNA-binding dye, propidium iodide, showed chromatin condensation and margination to the inner leaflet of the nuclear membrane (Figures 1A and 1D). These characteristic features differentiate death by apoptosis from death by necrosis [24]. Although cell-membrane integrity in apoptosis is maintained *in vivo* in situations where these cells are phagocytosed before they completely disintegrate, the absence of phagocytes under tissue-culture conditions results in the gradual loss of membrane integrity, leading to Trypan Blue permeability, which is seen several hours after biochemical evidence of apoptosis, such as cleavage of the death substrate poly(ADP)-ribose polymerase [11]. Quantification of cell death after TNFα treatment was achieved by counting cells permeable to Trypan Blue (Figure 2). Whereas only 18% of cells treated with TNFα were permeable to Trypan Blue at 24 h, most of the remaining cells exhibited cell shrinkage and rounding, and were detaching from the plate, indicating onset of the apoptotic

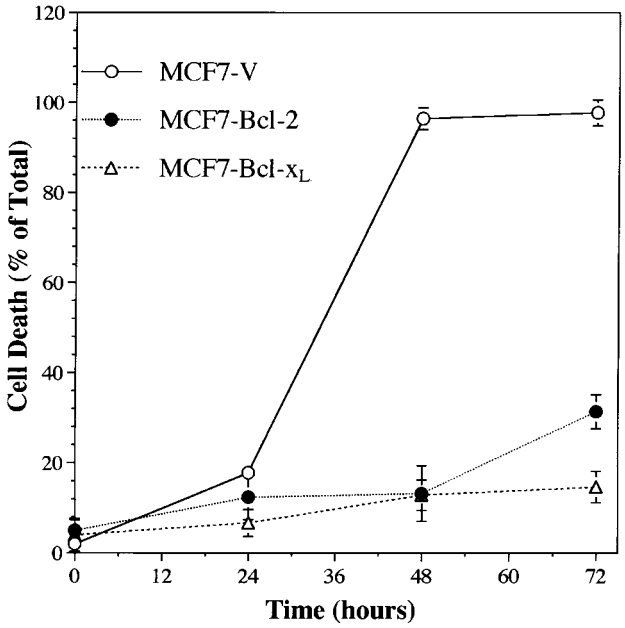

Figure 2 Inhibition of TNFα-induced cell death by Bcl-2 and Bcl-x_L

Cells were seeded at 5×10^4 cells/well of three 24-well plates and incubated overnight. Cells were then treated with 1 nM TNFα in triplicate. Cells from one of the plates were harvested at the indicated time-points and Trypan Blue-positive cells from each well were counted and are presented as a percentage of the total. A representative experiment out of two is shown, the bars represent S.E.M.

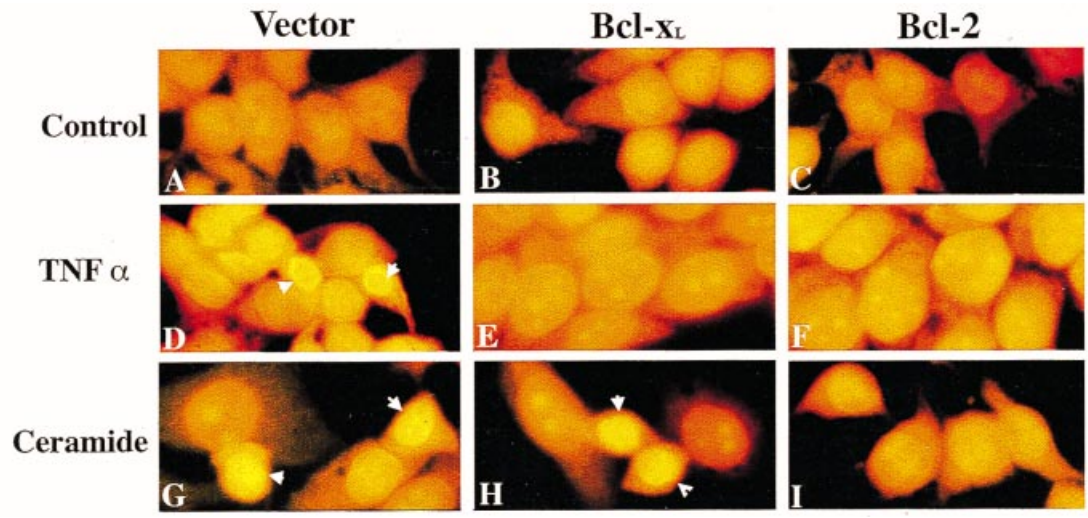

Figure 1 The effects of TNFα or *N*-acetylsphingosine (C_2-ceramide) on vector- (A,D,G), Bcl-x_L- (B,E,H) and Bcl-2-expressing (C,F,I) cells

Cells were plated on Thermanox cover slips placed in a 24-well plate at a concentration of 5×10^4 cells/well and incubated overnight. They were then treated, as indicated, with TNFα (1 nM) or C_2-ceramide (20 μM) for 24 h. Cells were fixed and stained with propidium iodide, and then visualized by fluorescence microscopy as described in the Materials and methods. (**A**,**B**,**C**) Untreated cells; (**D**,**E**,**F**) TNFα-treated cells; (**G**,**H**,**I**) C_2-ceramide-treated cells. Examples of apoptotic cells are indicated by arrows.

Table 1 Induction of apoptosis by campothecin or C_6-ceramide in MCF7 cells

Cells were seeded at 6×10^5 cells/well in a 6-well plate, left overnight, then treated as indicated. Annexin V labelling was performed as discussed in the Materials and methods. Annexin V-positive cells were quantified by flow cytometry. The data presented are representative of three different experiments with similar results.

	Annexin V-positive cells (% of total)		
	MCF7-V	MCF7-Bcl-2	MCF7-Bcl-x_L
Vehicle control	5	4	6
C_6-ceramide (10 μM)	10	5	12
C_6-ceramide (40 μM)	32	7	35
Campothecin (10 μM)	24	11	7

programme. By 48 h, most of these cells were dead, as judged by their permeability to Trypan Blue. In contrast, cells over-expressing Bcl-x_L or Bcl-2 were protected from the cytotoxic effects of TNFα. These cells exhibited normal morphology with only a minority of the cells showing apoptotic changes and Trypan Blue permeability at 24 h after treatment (Figures 1B, 1C, 1E and 1F, Figure 2). Moreover, protection from TNFα-induced cell death lasted for 72 h after treatment with only a slight loss of protection seen in MCF7-Bcl-2 cells, compared with MCF7-Bcl-x_L cells, at the later time-points. These experiments demonstrate that overexpression of either Bcl-2 or Bcl-x_L can protect MCF7 cells from TNFα-induced cell death.

In order to determine whether these observations were specific to TNFα, we examined the effects of the chemotherapeutic agent camptothecin on MCF7 cells. Camptothecin is a topoisomerase I inhibitor that is used in the treatment of a number of human cancers and that can induce apoptosis in many tumour cell lines [25]. Treatment of MCF7 cells with camptothecin at a concentration of 10 μM for 24 h resulted in morphological changes typical of apoptosis and similar to those induced by TNFα. To confirm that camptothecin was inducing apoptosis in these cells we assayed for annexin V binding, which indicates phosphatidylserine exposure and the onset of apoptosis [26]. Following treatment with camptothecin, MCF7-V cells underwent apoptosis, as judged by their annexin V binding, whereas MCF7-Bcl-2 and MCF7-Bcl-x_L cells were more resistant (Table 1).

We decided to investigate the interaction of the anti-apoptotic molecules, Bcl-x_L and Bcl-2, with the ceramide pathway of apoptosis that is activated in response to TNFα and several chemotherapeutic agents. We examined the effects of exogenous, cell-permeable, ceramides on the induction of apoptosis in the cell lines overexpressing Bcl-x_L or Bcl-2 as compared with wild-type cells. Naturally occurring ceramides have a long fatty-acid chain (18–22 carbons) in addition to the 18-carbon sphingosine backbone. This structure results in very hydrophobic molecules, which makes their exogenous delivery to cells extremely difficult. We utilized synthetic ceramides that have a short fatty-acid side chain that allows adequate water solubility. Treating MCF7-V cells with 20 μM *N*-acetylsphingosine (C_2-ceramide) resulted in an increase in the percentage of apoptotic cells, as determined by propidium iodide staining of methanol-fixed cells 24 h following treatment (Figures 1G–I). Similar results were obtained when cells were treated with increasing concentrations of *N*-hexanoylsphingosine (C_6-ceramide; Figure 3). Treatment of MCF7-Bcl-2 cells with similar concentrations of C_2-ceramide (Figure 1I) or C_6-ceramide (Figure 3) resulted in the induction of apoptosis in only a small percentage of cells, indicating that Bcl-2 can protect

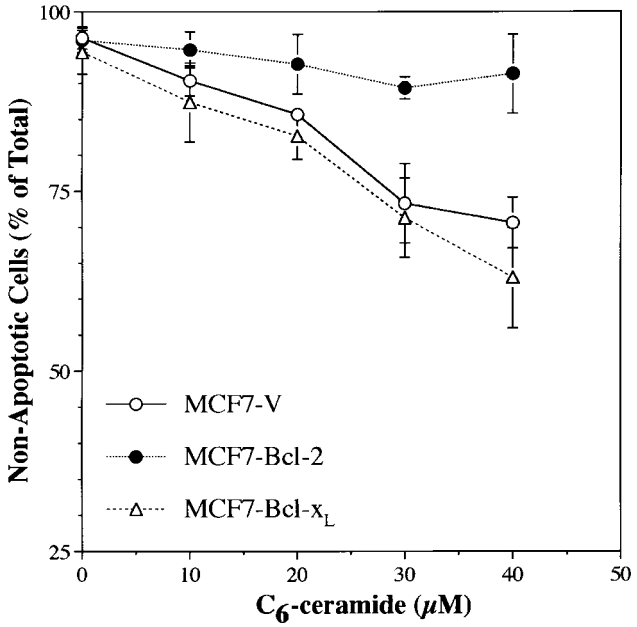

Figure 3 Differential inhibition of ceramide-induced apoptosis by Bcl-2 and Bcl-x_L

Cells were seeded and treated in duplicate with the indicated concentrations of C_6-ceramide for 24 h as in Figure 1. Apoptotic cells were scored as described in the Materials and methods. Non-apoptotic cells are shown as a percentage of the total cells. A representative experiment (with S.E.M), out of three, is shown.

from ceramide-induced apoptosis. In contrast, treatment of MCF7-Bcl-x_L cells with C_2-ceramide (Figure 1H) or C_6-ceramide (Figure 3) resulted in typical apoptotic changes in a large percentage of cells, which is comparable with what occurs in similarly treated vector cells. To confirm that these cells were dying by apoptosis, we also assayed for annexin V binding following treatment with C_6-ceramide. As shown in Table 1, quantification of apoptotic cells by flow-cytometric detection of annexin V-binding cells revealed that treatment of MCF7-V or MCF7-Bcl-x_L cells with C_6-ceramide induced significant apoptosis as compared with MCF7-Bcl-2 cells, which were more resistant. These results suggest that, whereas both Bcl-2 and Bcl-x_L can protect from TNFα- and camptothecin-induced apoptosis, only Bcl-2 protects from ceramide-induced apoptosis.

We then examined the effects of Bcl-x_L or Bcl-2 expression on ceramide accumulation, in response to TNFα and camptothecin. In MCF7 cells, treatment with TNFα resulted in the gradual accumulation of ceramide over several hours. By 24 h, these levels had increased 3–4-fold over baseline levels, as compared with time-matched control cells (Figure 4, top panel). As shown in previous studies [11,27–29], Bcl-2 overexpression did not interfere significantly with ceramide accumulation, particularly at early time-points. In contrast, Bcl-x_L overexpression completely inhibited ceramide accumulation in response to TNFα (Figure 4, top panel). Similarly, in response to treatment with camptothecin, Bcl-2 overexpression did not interfere with ceramide accumulation, whereas Bcl-x_L overexpression significantly blunted, although it did not totally inhibit, ceramide accumulation (Figure 4, lower panel).

To determine the possible mechanisms by which Bcl-2 and Bcl-x_L differ in their interactions with the ceramide pathway, as initiated by TNFα, we examined their interference with TNFα-

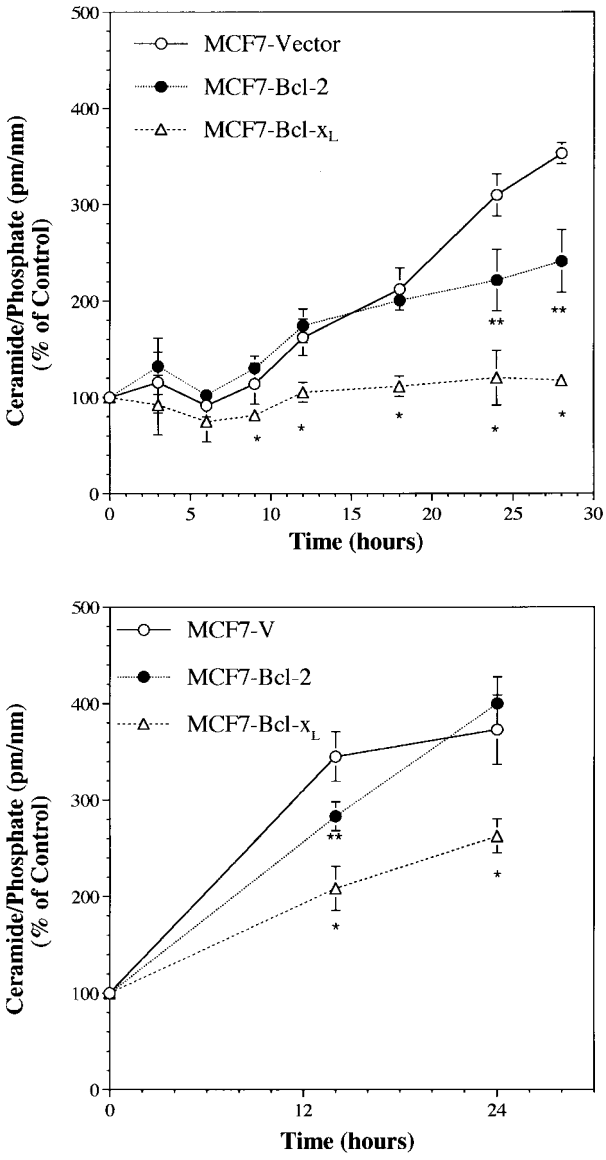

Figure 4 Effects of Bcl-2 or Bcl-x_L on ceramide accumulation following TNFα (top panel) or camptothecin (lower panel) treatment

Cells were seeded at 1×10^6 cells/well in 6-well plates and incubated overnight. Cells were then treated with TNFα at 1 nM (top panel) or camptothecin at 10 μM (lower panel) in duplicates. Treated and untreated cells from the three cell lines were harvested at the indicated time-points and lipids were extracted. Ceramide was measured and was normalized to lipid phosphate content as described in the Materials and methods. Ceramide content in untreated cells was 2–3 pmol (pm)/nmol (nm) of lipid phosphate. Results from treated cells are presented as the percentage of time-matched control cells. The data shown are the averages of three independent experiments done in duplicate for each treatment. * indicates statistical significance when compared with time-matched, similarly treated, control cells; ** indicates statistically significant differences as compared with either time-matched control or Bcl-x_L-expressing cells.

induced caspase activation. Recruitment of caspase-8 to the TNF receptor and its activation are thought to be at the apex of a signalling cascade leading to apoptosis that can be blocked by specific protease inhibitors, such as CrmA. We hypothesized that Bcl-x_L may function similarly to CrmA by inhibiting the activation of an upstream caspase related to caspase-8. We assayed for the activation of caspase-8-like protease by quantifying the cleavage of the specific colorimetric peptide substrate IETD-pNA. We found that treatment of MCF7 cells with TNFα resulted in the activation of a caspase-8-like protease, as evidenced by cleavage of IETD-pNA. The activity in MCF7-V cells was increased to $173 \pm 22\%$ of activity in control cells. Overexpression of Bcl-2 in these cells does not interfere significantly with the appearance of this activity ($145 \pm 26\%$ of control), whereas Bcl-x_L overexpression completely inhibits it ($100 \pm 4\%$ of control, $P < 0.005$). In order to confirm whether caspase-8 activation was inhibited by Bcl-x_L, we examined by Western blotting the cleavage of the caspase-8 zymogen to its active subunits, using an antibody specific for the p20 subunit of caspase-8 (Santa Cruz Biotechnology). Following treatment with TNFα, caspase-8 was cleaved in MCF7-V and MCF7-Bcl-2 cells, but cleavage was completely inhibited in MCF7-Bcl-x_L cells. Interestingly, Bcl-2 partially inhibited the cleavage of caspase-8, which is compatible with its inhibitory effects on ceramide accumulation observed at later time-points (Figure 5). These studies suggest that Bcl-2 and Bcl-x_L have differential effects on caspase activation and may explain their distinct functions in the TNFα-induced ceramide pathway of apoptosis.

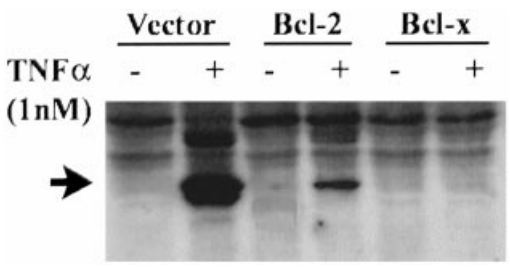

Figure 5 Inhibition of caspase-8 cleavage by Bcl-x_L

MCF7-V, MCF7-Bcl-2 and MCF7-Bcl-x_L cells were seeded and treated with TNFα or cultivation medium as described in the Materials and methods. The p20 cleavage product of caspase-8 detected by specific antibody in control or TNFα-treated cells is shown.

DISCUSSION

The biochemical mechanisms by which members of the Bcl-2 family of proteins inhibit apoptosis remain enigmatic [1]. Early studies suggested that Bcl-2 functions as an anti-oxidant by regulating the generation of reactive oxygen species [30], but this was subsequently challenged [31,32]. More recently, other properties of the Bcl-2 family of proteins were proposed to play a role in the modulation of apoptosis [1]. First, the ability of some members to form ion channels with selective permeability, similar to some pore-forming bacterial toxins, which may control the flow of regulatory molecules involved in apoptosis [33,34]. Second, the ability of the different members of this family to function as docking proteins able to bind each other to form homo- or heterodimers as well as bind other proteins [35–37]. In this regard, the interactions between the anti-apoptotic members, e.g. Bcl-2 and Bcl-x_L, with the pro-apoptotic members, e.g. Bax and Bak, have been demonstrated to be critical in determining the fate of the cell. The ratio of Bcl-2/Bax in a particular cell may then determine the response to an apoptotic stimulus. Whereas it was shown that the interaction of Bcl-x_L with Bax is important in its anti-apoptotic activity [35,38], loss of this interaction does not significantly compromise its anti-apoptotic activity [39]. The latter studies indicated that, in some systems, Bcl-x_L may have Bax-independent, apoptosis-inhibiting, functions. Importantly,

Bcl-2 and Bcl-x_L may modulate the activity of different caspases via interaction with homologues of CED-4, the proapoptotic *Caenorhabditis elegans* protein that can simultaneously and selectively interact with caspases [40].

Ceramide has been proposed as a co-ordinator of the cellular stress response [41,42]. In apoptosis, ceramide appears to be involved in the mid-stages of activation and perhaps perpetuation of the death signal [43]. Bcl-2 has been shown by several groups to block ceramide-induced apoptosis without significantly interfering with ceramide accumulation [11,27–29]. This evidence suggests that ceramide accumulation is not a terminal event and that it occurs before the 'point of no return' is reached.

In our present study, we focused on TNFα-induced apoptosis in MCF7 cells. Whereas both Bcl-2 and Bcl-x_L can inhibit TNFα-induced apoptosis, they modulate the ceramide pathway differently. Our first observation is that Bcl-x_L does not protect cells from ceramide-induced apoptosis, which is in contrast with Bcl-2. This suggests that the downstream targets that are activated by ceramide are not susceptible to Bcl-x_L inhibition. Since both Bcl-2 and Bcl-x_L are equally effective in inhibiting TNFα-induced apoptosis in MCF7 cells, our second observation, the inhibition of ceramide accumulation in response to TNFα by Bcl-x_L, indicates that Bcl-x_L blocks a target whose activation precedes, and may be necessary for, ceramide accumulation. This is analogous to CrmA which, in response to TNFα, similarly blocks the accumulation of ceramide [11]. In the case of CrmA, it is hypothesized that this occurs because of the ability of CrmA to preferentially inhibit caspase-8, which is recruited to the TNFα receptor [44]. Our third observation that Bcl-x_L, but not Bcl-2, may inhibit caspase-8 or a related protease may support this hypothesis.

Our results showing inhibition of ceramide accumulation by Bcl-x_L are in contrast with a recent study showing that Bcl-x_L does not inhibit ceramide accumulation following anti-immunoglobulin treatment, which induces apoptosis in the B-lymphocytic cell line WEHI 231 [45]. These differences could represent the involvement of different caspases in these two pathways or, alternatively, different mechanisms of ceramide accumulation.

Whereas our present study does not elucidate the biochemical mechanisms by which Bcl-x_L or Bcl-2 modulate the TNFα/ceramide pathway of apoptosis, it clearly indicates that these two molecules are not always interchangeable but that each of them may have well-defined, but distinctive, functions that remain to be discovered. However, it is important to note that the differences in function observed in our study may not be applicable to all cell types and that in some systems Bcl-2 and Bcl-x_L may indeed be interchangeable.

Our results are not consistent with a simple model of dimerization of the anti- and pro-apoptotic members of the Bcl-2 family. This is also supported by emerging evidence from the literature that indicates the presence of functions that are independent of interactions with other family members [1,33,39]. In this regard, the relevant target for Bcl-x_L in the regulation of apoptosis is different from that for Bcl-2 and, based on our data, operates upstream of the accumulation of ceramide. Thus, Bcl-x_L may inhibit apoptosis induced by stimuli other than TNFα that share the relevant Bcl-x_L target (e.g. caspase) as a common component of their apoptotic pathways. Bcl-x_L would not be expected to inhibit other stimuli that bypass this target. On the other hand, Bcl-2 functions further downstream and would be expected to inhibit other pathways of apoptosis that converge on its relevant target which, based on our data, is activated downstream of ceramide accumulation. Further studies should help identify the specific targets of Bcl-x_L and Bcl-2 and their interactions with the ceramide pathway.

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REFERENCES

1 Reed, J. C. (1997) Nature (London) **387**, 773–776
2 Oltvai, Z. N. and Korsmeyer, S. J. (1994) Cell **79**, 189–192
3 Yin, X.-M, Oltvai, Z. N. and Korsmeyer, S. J. (1994) Nature (London) **369**, 321–323
4 Huang, D. C., Cory, S. and Strasser, A. (1997) Oncogene **14**, 405–414
5 Chao, D. T., Linette, G. P., Boise, L. H., White, L. S., Thompson, C. B. and Korsmeyer, S. J. (1995) J. Exp. Med. **182**, 821–828
6 Gottschalk, A. R., Boise, L. H., Oltvai, Z. N., Accavitti, M. A., Korsmeyer, S. J., Quintáns, J. and Thompson, C. B. (1996) Cell Death Differ. **3**, 113–118
7 Gottschalk, A. R., Boise, L. H., Thompson, C. B. and Quintáns, J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 7350–7354
8 Simonian, P. L., Grillot, D. A. M. and Nuñez, G. (1997) Blood **90**, 1208–1216
9 Jäättelä, M., Benedict, M., Tewari, M., Shayman, J. A. and Dixit, V. M. (1995) Oncogene **10**, 2297–2305
10 Cohen, G. M. (1997) Biochem. J. **326**, 1–16
11 Dbaibo, G. S., Perry, D. K., Gamard, C. J., Platt, R., Poirier, G. G., Obeid, L. M. and Hannun, Y. A. (1997) J. Exp. Med. **185**, 481–490
12 Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannun, Y. A. (1993) Science **259**, 1769–1771
13 Dbaibo, G. S., Obeid, L. M. and Hannun, Y. A. (1993) J. Biol. Chem. **268**, 17762–17766
14 Hannun, Y. A. (1994) J. Biol. Chem. **269**, 3125–3128
15 Kolesnick, R. and Golde, D. W. (1994) Cell **77**, 325–328
16 Smyth, M. J., Perry, D. K., Zhang, J., Poirier, G. G., Hannun, Y. A. and Obeid, L. M. (1996) Biochem. J. **316**, 25–28
17 Mizushima, N., Koike, R., Kohsaka, H., Kushi, Y., Handa, S., Yagita, H. and Miyasaka, N. (1996) FEBS Lett. **395**, 267–271
18 Kojima, H. and Datta, R. (1996) Oncol. Res. **8**, 497–501
19 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
20 Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. and Bell, R. M. (1986) J. Biol. Chem. **261**, 8597–8600
21 Okazaki, T., Bielawska, A., Bell, R. M. and Hannun, Y. A. (1990) J. Biol. Chem. **265**, 15823–15831
22 Rouser, G., Siakotos, A. N. and Fleischer, S. (1966) Lipids **1**, 85–86
23 Tewari, M. and Dixit, V. M. (1995) J. Biol. Chem. **270**, 3255–3260
24 Arends, M. J. and Wyllie, A. H. (1991) Int. Rev. Exp. Pathol. **32**, 223–254
25 Shimizu, T. and Pommier, Y. (1996) Exp. Cell Res. **226**, 292–301
26 Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. (1995) J. Immunol. Methods **184**, 39–51
27 Allouche, M., Bettaieb, A., Vindis, C., Rousse, A., Grignon, C. and Laurent, G. (1997) Oncogene **14**, 1837–1845
28 Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M. and Hannun, Y. A. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 5325–5328
29 Martin, S. J., Takayama, S., McGahon, A. J., Miyashita, T., Corbeil, J., Kolesnick, R. N., Reed, J. C. and Green, D. R. (1995) Cell Death Differ. **2**, 253–257
30 Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990) Nature (London) **348**, 334–336
31 Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H. and Tsujimoto, Y. (1995) Nature (London) **374**, 811–813
32 Jacobson, M. D. and Raff, M. C. (1995) Nature (London) **374**, 814–816
33 Minn, A. J., Vélez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M. and Thompson, C. B. (1997) Nature (London) **385**, 353–357
34 Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M. and Reed, J. C. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 5113–5118
35 Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B. and Korsmeyer, S. J. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7834–7838
36 Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H. G. and Reed, J. C. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 9238–9242
37 Hacker, G. and Vaux, D. L. (1995) Curr. Biol. **5**, 622–624
38 Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B. and Korsmeyer, S. J. (1995) Cell **80**, 285–291

39 Cheng, E. H., Levine, B., Boise, L. H., Thompson, C. B. and Hardwick, J. M. (1996) Nature (London) **379**, 554–556
40 Chinnaiyan, A. M., O'Rourke, K., Lane, B. R. and Dixit, V. M. (1997) Science **275**, 1122–1126
41 Hannun, Y. A. (1996) Science **274**, 1855–1859
42 Dbaibo, G. S. (1997) Biochem. Soc. Trans. **25**, 557–561
43 Farschon, D. M., Couture, C., Mustelin, T. and Newmeyer, D. D. (1997) J. Cell. Biol. **137**, 1117–1125
44 Peter, M. E., Kischkel, F. C., Scheuerpflug, C. G., Medema, J. P., Debatin, K. M. and Krammer, P. H. (1997) Eur. J. Immunol. **27**, 1207–1212
45 Wiesner, D. A., Kilkus, J. P., Gottschalk, A. R., Quintáns, J. and Dawson, G. (1997) J. Biol. Chem. **272**, 9868–9876

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