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Sphingosine generation, cytochrome *c* release, and activation of caspase-7 in doxorubicin-induced apoptosis of MCF7 breast adenocarcinoma cells

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Abstract

Treatment of human breast carcinoma MCF7 cells with doxorubicin, one of the most active antineoplastic agents used in clinical oncology, induces apoptosis and leads to increases in sphingosine levels. The transient generation of this sphingolipid mediator preceded cytochrome *c* release from the mitochondria and activation of the executioner caspase-7 in MCF7 cells which do not express caspase-3. Bcl-x_L overexpression did not affect sphingosine generation whereas it reduced apoptosis triggered by doxorubicin and completely blocked apoptosis triggered by sphingosine. Exogenous sphingosine-induced apoptosis was also accompanied by cytochrome *c* release and activation of caspase-7 in a Bcl-x_L-sensitive manner. Furthermore, neither doxorubicin nor sphingosine treatment affected expression of Fas ligand or induced activation of the apical caspase-8, indicating a Fas/Fas ligand-independent mechanism. Our results suggest that a further metabolite of ceramide, sphingosine, may also be involved in mitochondria-mediated apoptotic signaling induced by doxorubicin in human breast cancer cells. *Cell Death and Differentiation* (2001) **8,** 162–171.

Keywords: apoptosis; sphingosine; doxorubicin; cytochrome *c*; caspases

Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-methylcoumarin; dMAPP, D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol; FB1, fumonisin B1; PARP, poly(ADP-ribose) polymerase

Introduction

The anthracycline antibiotics constitute an important group of drugs that have been used to successfully produce regression in disseminated neoplasias, including acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilm's tumor, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, and many others. The molecular mechanisms responsible for the cytotoxic effects of anthracyclines in malignant cells are not fully understood but are mainly attributed to their ability to intercalate into DNA and cause localized uncoiling of the double helix or by stabilization of the complex between DNA and topoisomerase II.[1] In addition, anthracyclines can mediate oxygen free radical generation originating from quinone-generated redox activity that can also damage DNA.[1]

Ceramide, a sphingolipid produced by the hydrolysis of membrane-associated sphingomyelin, has previously been implicated as a gauge of apoptosis (reviewed in [2]). Diverse apoptotic stimuli, including the anthracycline drug daunorubicin,[3–9] increase ceramide levels, which in turn leads to activation of downstream signals important for the apoptotic process. Daunorubicin triggers apoptosis and ceramide generation by activating a neutral sphingomyelinase in the leukemic cell lines U937 and HL-60,[3] whereas other studies suggest that it stimulates ceramide elevation in lymphocytic P388 and U937 cells by activation of ceramide synthase in the *de novo* pathway, rather than by activation of sphingomyelinase.[4] Although Bcl-2 overexpression inhibits ceramide-mediated apoptosis, it does not intervene in ceramide generation induced by daunorubicin, suggesting that Bcl-2 suppresses apoptosis downstream of ceramide generation.[5] In agreement, doxorubicin, another member of the anthracycline family, induces apoptosis in cardiac myocytes and activates the sphingomyelin-ceramide pathway.[10,11]

Sphingosine, formed from ceramide by ceramidase, has also been implicated in cell growth arrest and apoptosis. Indeed, sphingosine is rapidly produced during TNFα-mediated apoptosis in human neutrophils[12] and in cardiac myocytes,[13] and is capable of inducing apoptosis when added exogenously to many cell types.[12–18] More recently, we found that sphingosine is also transiently produced during the early phase of Fas- and ceramide-induced apoptosis in Jurkat T cells, acting in a mitochondria-dependent manner.[19]

Here, we report that treatment of human breast cancer MCF7 cells with doxorubicin induces transient generation of sphingosine, preceding cytochrome *c* release and activation of the executioner caspase-7. In addition, exogenous sphingosine recapitulates doxorubicin-induced apoptosis, as well as cytochrome *c* redistribution and activation of

caspase-7 in a Bcl-x_L-sensitive fashion. Our findings suggest the potential involvement of sphingosine, in addition to ceramide, in mitochondria-dependent apoptosis triggered by doxorubicin in human breast cancer MCF7 cells.

Results

In agreement with previous studies,[20] we found that treatment of MCF7 cells with doxorubicin caused extensive cell death (Figure 1A). As sphingosine has been implicated in cytokine-induced apoptosis,[12,13,19] it was of interest to determine whether treatment with doxorubicin could also induce apoptosis in a manner dependent on sphingosine generation. After treatment with 1 μg/ml doxorubicin, a therapeutic concentration, sphingosine levels significantly increased by 16 h, peaked at 24 h, and declined thereafter (Figure 1B). Interestingly, sphingosine levels also somewhat increased in untreated cells, probably as a result of changing to fresh serum-free media, in agreement with previous studies demonstrating that sphingolipid synthesis was stimulated by replacement of the media.[21,22] In line with previous studies in other cell types,[12,13,15–19,23,24] addition of exogenous sphingosine also induced apoptosis in MCF7/Fas cells in a time- (Figure 1A) and concentration-dependent manner (data not shown).

To examine the possibility that sphingosine-induced cell death might be due to its conversion to ceramide by the action of ceramide synthase, we investigated the effect of the mycotoxin fumonisin B1, a competitive inhibitor of ceramide synthase.[25] Treatment of MCF7/Fas cells with 25 μM fumonisin B1 had no effect on cell death induced by doxorubicin (Figure 2A) nor did it affect the extent of cell death induced by sphingosine (Figure 2B). However, surprisingly, a higher concentration of fumonisin B1 (100 μM) inhibited cell death in MCF7/Fas cells treated with doxorubicin to a lesser extent than that induced by sphingosine (Figure 2C). These data suggest that sphingosine- and doxorubicin-induced cell death in MCF7/Fas cells is partially dependent on ceramide synthase activity.

The generation of sphingosine during cell death induced by doxorubicin suggests that the increase in sphingosine might result from degradation of ceramide. This is a likely possibility, as sphingosine is not synthesized *de novo* and can only be produced by metabolism of ceramide.[25] To gain further insight into the relative function of endogenous sphingosine, we used the ceramidase inhibitor, dMAPP.[26] Treatment of MCF7/Fas cells with 5 μM dMAPP not only attenuated cell death induced by doxorubicin (Figure 3A), but also surprisingly, that induced by exogenous sphingosine to an even greater extent (Figure 3B).

Because overexpression of Bcl-2 or Bcl-x_L protects against diverse apoptotic stimuli, including ceramide-induced apoptosis,[5,19,27–30] it was important to determine whether the effects of sphingosine were also blocked by these proteins. MCF7/Fas cells stably overexpressing Bcl-x_L were treated with doxorubicin or sphingosine and cell death was measured. In agreement with the anti-apoptotic effects of Bcl-x_L family members in other cell types,

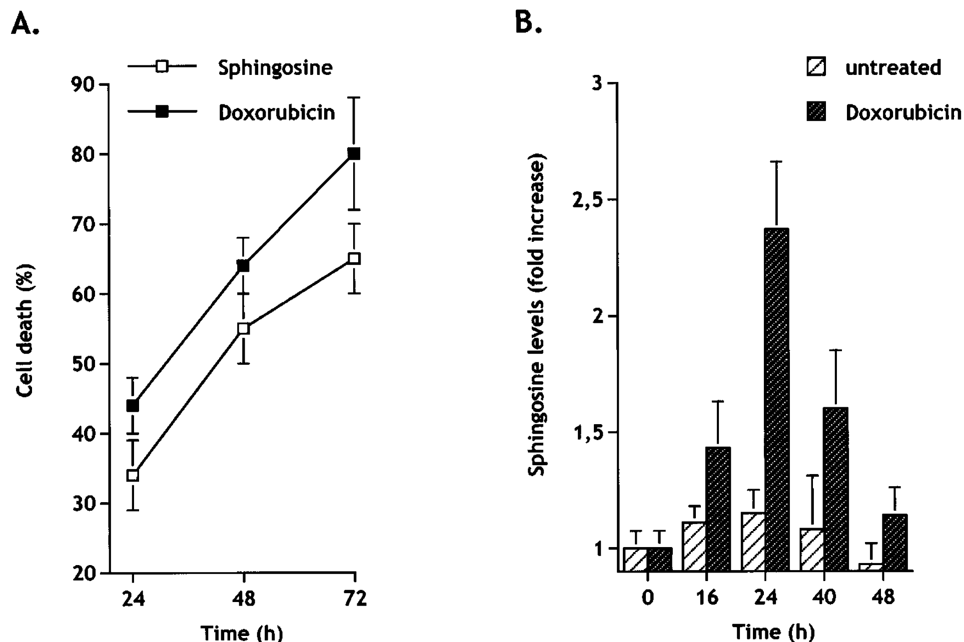

Figure 1 The effect of doxorubicin on cell death and sphingosine levels in MCF7 cells. (**A**) MCF7/Fas cells were treated with 1 μg/ml of doxorubicin (filled squares) or 10 μM sphingosine (open squares) for the indicated times. The percentage of cell death was assessed by the MTT assay. The values are means of triplicate determinations $\pm$ S.D. Similar results were obtained in three additional experiments. (**B**) Sphingosine levels were measured in MCF7/Fas cells cultured in serum-free media without (untreated) or with 1 μg/ml doxorubicin for the indicated times. Results are means $\pm$ S.D. of three independent experiments performed in triplicate

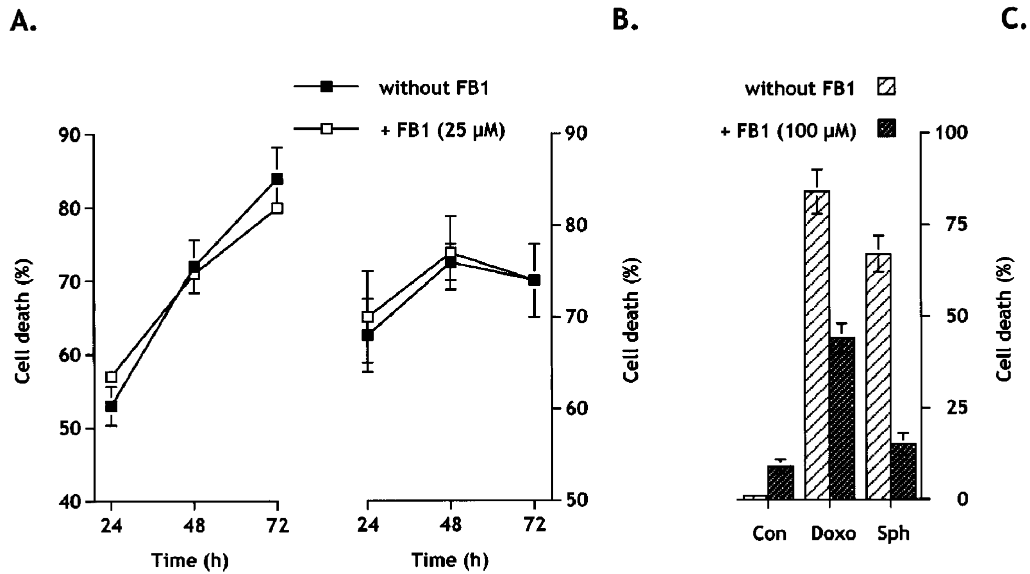

Figure 2 Effects of fumonisin B1 on cell death. (**A**) MCF7/Fas cells were pre-incubated without (filled squares) or with (open squares) 25 μM fumonisin B1 for 30 min in serum-free medium and then 1 μg/ml doxorubicin was added for the indicated times. (**B**) MCF7/Fas cells were pre-incubated without (filled squares) or with (open squares) 25 μM fumonisin B1 for 30 min in serum-free medium and then incubated in the presence of 10 μM sphingosine for the indicated times. (**C**) MCF7/Fas cells were incubated in the presence of 1 μg/ml doxorubicin or 10 μM sphingosine without or with 100 μM fumonisin B1 for 72 h. The percentage of cell death was assessed by the MTT assay. The values are means $\pm$ S.D. of triplicate determinations

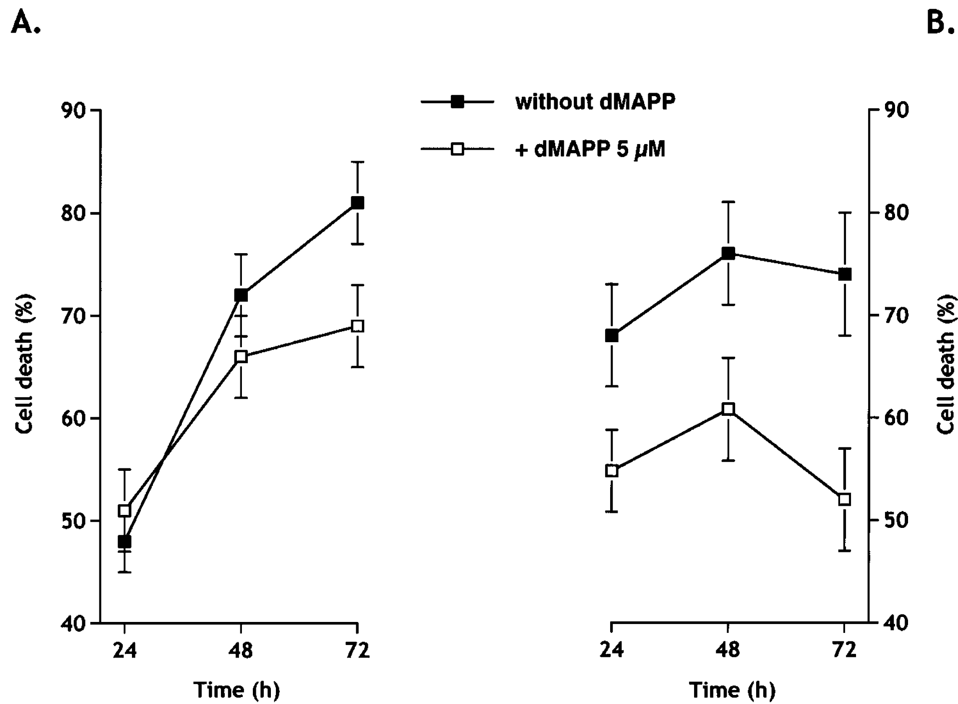

Figure 3 Effects of dMAPP on cell death. MCF7/Fas cells were incubated without (filled squares) or with (open squares) 5 μM dMAPP for 30 min and then 1 μg/ml doxorubicin (**A**) or 10 μM sphingosine (**B**) were added for the indicated times. The percentage of cell death was assessed by the MTT assay. The values are means $\pm$ S.D. of triplicate determinations

including human small cell lung cancer cells,[3] neuroblastoma cells,[32] myeloid leukemia HL-60 cells[33,34] and U937 cells,[35] overexpression of Bcl-x_L inhibited doxorubicin-induced cell death and completely protected against sphingosine-induced cell death (Figure 4A). However, Bcl-x_L expression did not have any significant effect on the

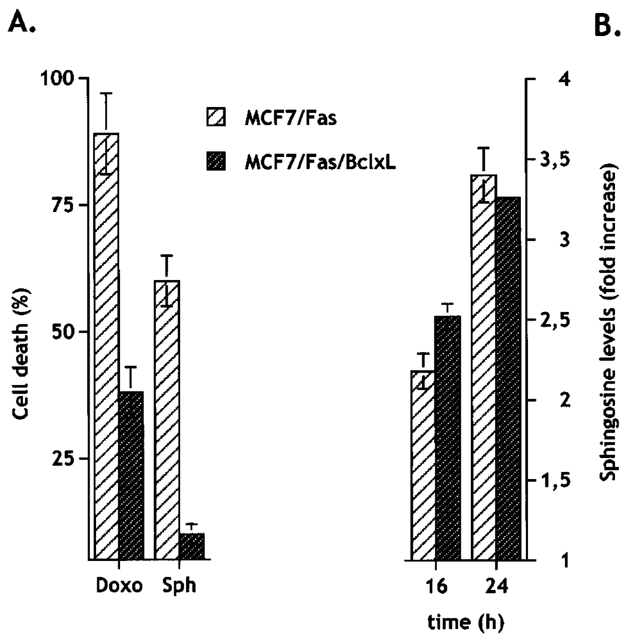

Figure 4 Bcl-x_L overexpression blocks doxorubicin-induced cell death but not sphingosine generation. (**A**) MCF7/Fas and MCF7/Fas/Bcl-x_L cells were treated with 1 μg/ml doxorubicin or 10 μM sphingosine and cell death was assessed by the MTT assay. The values are means $\pm$ S.D. of triplicate determination. This experiment was repeated three times with similar results. (**B**) Sphingosine levels were determined in MCF7/Fas and MCF7/Fas/Bcl-x_L cells treated with 1 μg/ml doxorubicin for the indicated times. Results are means $\pm$ S.D. of three independent experiments performed in triplicate

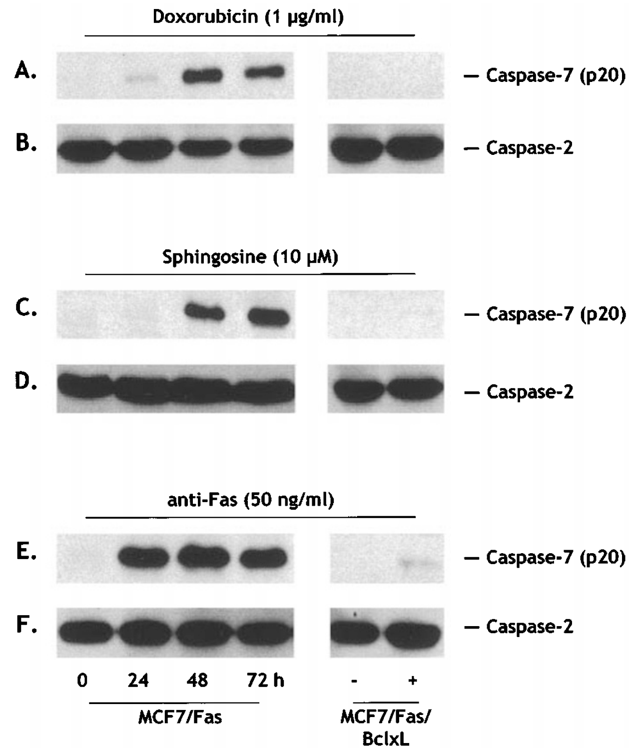

Figure 5 Activation of caspase-7 and caspase-2 in response to doxorubicin, sphingosine, and anti-Fas mAb. MCF7/Fas and MCF7/Fas/Bcl-x_L cells were incubated in serum-free conditions without (−) or with (+) 1 μg/ml doxorubicin (**A, B**), 10 μM sphingosine (**C, D**), or 50 ng/ml anti-Fas mAb (**E, F**) and extracts prepared at the indicated times or 72 h for MCF7/Fas/Bcl-x_L cells. Proteins were resolved by 15% SDS–PAGE, blotted, and probed with anti-caspase-7 antibody (**A,C,E**), or with anti-caspase-2 antibody (**B,D,F**). Migrations indicated: active subunit p20 of caspase-7; full-length caspase-2

increase in sphingosine induced by doxorubicin (Figure 4B). These results suggest that the sphingosine increase is not merely a consequence of cell death as it is also observed in the Bcl-x_L overexpressing cells which do not die and imply that sphingosine may act upstream of Bcl-x_L.

Because our previous findings suggested that sphingosine may function proximal to executioner caspases in Jurkat T lymphoma cells,[19] we next investigated whether doxorubicin and sphingosine activated executioner caspases in MCF7/Fas cells. Based on their substrate specificities, caspase-3, -2, and -7, believed to be the final executioner caspases, belong to the group II subfamily that preferentially cleave the peptide sequence DExD found in numerous death substrates.[36] Proteolytic activation of these caspases was examined by Western blotting analysis. Only caspase-7 was activated by both doxorubicin (Figure 5A) and sphingosine (Figure 5C) in MCF7/Fas cells. Surprisingly, although caspase-2 is clearly expressed in MCF7/Fas cells, it was not cleaved after treatment with doxorubicin (Figure 5B) or sphingosine (Figure 5D). Similarly, extracts from MCF7/Fas cells treated with Fas mAb, a strong inducer of apoptosis in this cell line,[37–40] did not activate caspase-2 (Figure 5F), whereas caspase-7 was processed to the active fragment (Figure 5E). As the MCF7 cell line is devoid of caspase-3 owing to the functional deletion of the *CASP-3* gene,[41] caspase-7 appears to be the only executioner caspase activated in these cells. Despite activation of caspase-7, Ac-DEVD-AMC-cleaving activity was not detectably increased after treatment of MCF7/Fas cells with sphingosine or doxorubicin (data not shown), likely a result of the deficiency of caspase-3 activity.

Having established that doxorubicin- and sphingosine-induced cell death was prevented by Bcl-x_L overexpression, we also examined caspase activation in MCF7/Fas/Bcl-x_L cells. As shown in Figure 5A,C,E, activation of caspase-7 in these cells was markedly inhibited by overexpression of Bcl-x_L.

After initiation of the apoptotic program, release of cytochrome *c* from mitochondria plays an important role in activation of downstream caspases. Cytochrome *c* binds to Apaf-1, a mammalian homologue of the *Caenorhabditis elegans* death-promoting CED-4 protein, inducing it to associate with caspase-9, thereby triggering its autoactivation into a mature form, which in turn can then directly cleave caspase-3 or -7 (reviewed in[42–44]). Because expression of Bcl-x_L has been shown to interfere with cytochrome *c* release,[44] we next determined whether doxorubicin and sphingosine could also trigger mitochondrial cytochrome *c* release. As shown in Figure 6A, treatment with doxorubicin resulted in a significant increase in cytosolic cytochrome *c* levels by 24 h. This is

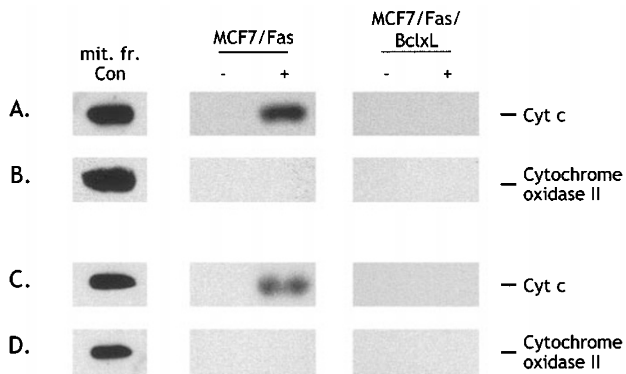

Figure 6 Cytosolic accumulation of cytochrome *c* induced by doxorubicin or sphingosine is inhibited by Bcl-x_L overexpression. MCF7/Fas and MCF7/Fas/Bcl-x_L cells treated without (−) or with (+) 1 μg/ml doxorubicin (**A, B**) or 10 μM sphingosine (**C, D**) were harvested after 48 h, and cytosolic and mitochondrial proteins were separated by 15% SDS−PAGE and analyzed by immunoblotting with anti-cytochrome *c* (**A, C**) or anti-cytochrome oxidase (subunit II; **B, D**). Cytochrome oxidase serves as a marker for mitochondrial contamination of cytosolic extracts. A mitochondrial extract from nontreated cells (mit. Fr. Con) was used as a positive control for cytochrome *c* and cytochrome oxidase (subunit II)

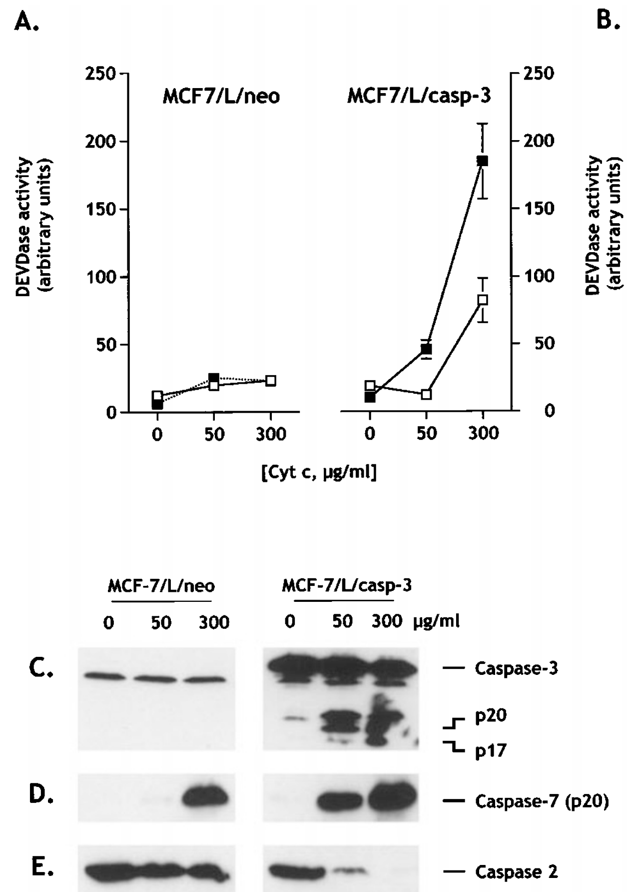

Figure 7 Cytochrome *c*-initiated activation of caspases in a cell-free system from MCF7/L/neo and MCF7/L/casp3 cells. (**A**) DEVDase activity was measured with the fluorogenic substrate Ac-DEVD-AMC in cytosolic extracts from MCF7/L/neo cells treated without or with the indicated concentrations of horse heart cytochrome *c* for 30 min (open squares) or 90 min (filled squares) at 37°C. Results are means $\pm$ S.D. of at least three independent experiments performed in triplicate. (**B**) DEVDase activity was measured in cytosolic extracts from MCF7/L/casp3 cells treated without or with the indicated concentrations of horse heart cytochrome *c* for 30 min (open squares) or 90 min (filled squares) at 37°C. Results are means $\pm$ S.D. of at least three independent experiments. Cytosolic extracts from MCF7/L/neo cells treated without or with the indicated concentrations of horse heart cytochrome *c* for 90 min at 37°C were separated by SDS−PAGE and analyzed by immunoblotting with anti-caspase-3 (**C**), anti-caspase-7 (**D**), or anti-caspase-2 (**E**). Migrations indicated: full-length caspase-3, cleavage intermediate p20; active subunit p17; active subunit p20 of caspase-7; full-length caspase-2

in agreement with a previous study establishing cytochrome *c* release upon doxorubicin treatment in neuroblastoma cells.[32] Similarly, treatment with exogenous sphingosine also caused accumulation of cytochrome *c* in the cytosol (Figure 6C). Cytochrome *c* release induced by doxorubicin or sphingosine was prevented by Bcl-x_L overexpression (Figure 6A,C). The absence of detectable cytochrome oxidase in cytosolic extracts confirmed that our preparations were free of mitochondrial contamination (Figure 6B, D).

As doxorubicin and sphingosine were capable of inducing caspase-7 activation as well as cytochrome *c* translocation to the cytosol, it was important to determine whether cytochrome *c* mediated activation of caspase-7 in caspase-3 null MCF7 cells. When cytochrome *c* was added *in vitro* to cell-free MCF7/L/neo extracts in the presence of 1 mM dATP, cleavage of caspase-7 increased with time of incubation (data not shown) and cytochrome *c* concentration (Figure 7D). However, as recently reported,[45] DEVDase activity was only slightly increased under these conditions (Figure 7A), presumably owing to the absence of caspase-3 in these cells (Figure 7C). Interestingly, but in line with our results showing that caspase-2 was not processed in apoptotic MCF7/Fas cells (Figure 5B,D,F), no activation of caspase-2 was detected in this *in vitro* system (Figure 7E). These results demonstrate that cytochrome *c*-dependent caspase-7 activation can take place in caspase-3 deficient MCF7 cell lines. In a comparative *in vitro* study performed with MCF7/L cells stably expressing caspase-3 (denoted MCF7/L/casp-3), processing of caspase-3 (Figure 7C) was accompanied by increased cleavage of caspase-7 (Figure 7D). Furthermore, increased processing of caspase-2 (Figure 7E) demonstrates that caspase-3 activation is required for proteolysis of caspase-2. Likewise, MCF7/L/casp-3 extracts have several fold higher DEVDase activity than MCF7/L/neo extracts, probably as a result of the combined activity of caspase-3 and caspase-7. In contrast to cytosolic extracts prepared from MCF7 cells and in agreement with previous results, complete processing of caspase-9,[46]-3,[46,47]-7,[46,47] and -2[46] was readily observed in cytosolic extracts prepared from Jurkat cells incubated with a concentration of cytochrome *c* as low as 10 μg/ml within 30 min (Figure 8A−D). Moreover, cytochrome *c*-triggered caspase activation resulted in rapid cleavage of PARP (Figure 8E), confirming that this *in vitro* system recapitulates the apoptotic cascade from initiation of executioner caspases activation to cleavage of death

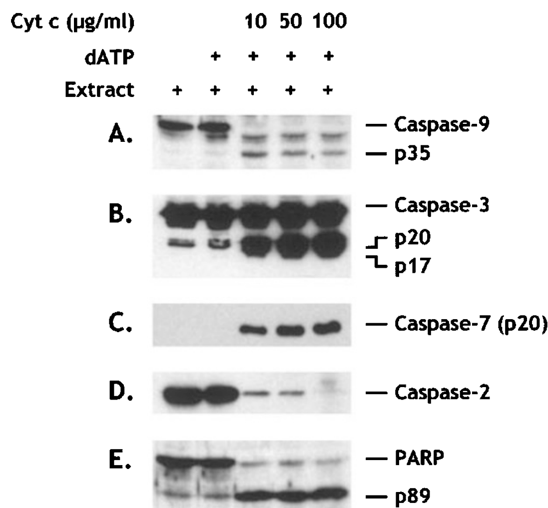

Figure 8 Cytochrome *c*-initiated activation of casp-9, -3, -7,and -2, and cleavage of PARP in Jurkat cell extracts. Cytosolic extracts from Jurkat T cells treated without or with the indicated concentrations of horse heart cytochrome c for 30 min at 37°C were separated by SDS−PAGE and analyzed by immunoblotting with anti-caspase-9 (**A**), anti-caspase-3 (**B**), anti-caspase-7 (**C**), anti-caspase-2 (**D**), anti-PARP (**E**). Migrations indicated: full-length caspase-9, cleavage intermediate p35; full-length caspase-3, cleavage intermediate p20, active subunit p17; active subunit p20 of caspase-7; full-length caspase-2. full-length PARP, active form p89

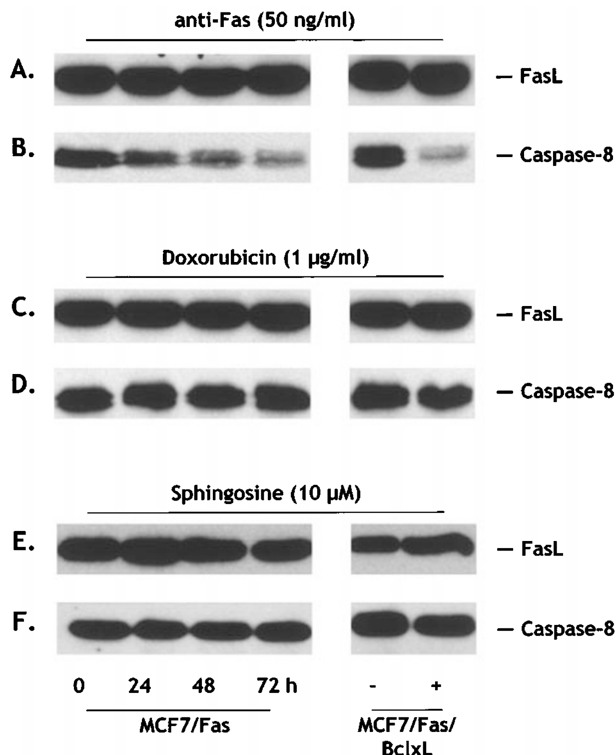

Figure 9 Caspase-8 activation and FasL expression in response to doxorubicin, sphingosine, or anti-Fas mAb. MCF7/Fas and MCF7/Fas/Bcl-x_L cells were incubated in serum-free conditions without (−) or with (+) 50 ng/ml anti-Fas mAb (**A, B**), 1 μg/ml doxorubicin (**C, D**), or 10 μM sphingosine (**E, F**) and extracts prepared at the indicated times or 72 h for MCF7/Fas/Bcl-x_L cells. Proteins were resolved by 15% SDS−PAGE, blotted, and probed with anti-FasL antibody (**A, C, E**), or with anti-caspase-8 (**B, D, F**). Migrations indicated: full-length caspase-8; FasL

substrates. Taken together, these assays demonstrate that caspase-7 does not require the presence of caspase-3 to be activated in MCF7 cells, although overexpression of caspase-3 does enhance its processing.

The Fas receptor/ligand system has been proposed to play a role in doxorubicin-mediated apoptosis.[48−50] Levels of FasL protein were examined by Western blotting to determine whether its expression was increased by doxorubicin or sphingosine treatment. There were no detectable changes in FasL protein expression discernible after doxorubicin (Figure 9C) or sphingosine treatment (Figure 9E). To further examine the possible requirement for Fas signaling in cell death induced by doxorubicin in MCF7/Fas cells, we determined whether caspase-8 could be activated. Caspase-8 cleavage often represents the first detectable event in Fas death receptor-mediated apoptosis.[51] Notwithstanding, in type II cells, such as Jurkat T cells, caspase-8 can be activated in a post-mitochondrial manner, and sphingosine treatment can lead to caspase-8 processing in this cell type.[19] As shown in Figure 7B, caspase-8 was only activated in anti-Fas mAb-treated, but not in doxorubicin- (Figure 9D) or sphingosine-treated (Figure 9F) MCF7/Fas cells. In agreement with previous studies,[37,40,52] Bcl-x_L, which strongly inhibits apoptosis, had no effect on the processing of caspase-8 induced by Fas ligation (Figure 9B). Together, these findings negate a role for the Fas ligand/receptor signaling in doxorubicin-induced cell death in MCF7 carcinoma cells.

Discussion

Accumulating evidence indicates that chemotherapeutic agents such as anthracyclines kill neoplastic cells by the induction of apoptosis. Although the pathways involved in cell death induced by these agents are not fully understood, abundant reports have suggested that the sphingolipid ceramide play an important role in apoptosis initiated by anthracyclines.[3−11]

Accumulation of glucosylceramides, simple glycosylated forms of ceramide, is a feature of some multidrug-resistant cancer cells and tumors from patients who are less responsive to chemotherapy, demonstrating a correlation between cellular drug resistance and alterations in ceramide metabolism.[53] Reduction of ceramide levels by transfection with glucosylceramide synthase, the enzyme that converts ceramide to glucosylceramide, confers doxorubicin resistance in MCF7 cells.[54] Conversely, transfection of antisense glucosylceramide synthase to limit cellular ceramide glycosylation, overcomes doxorubicin resistance.[55] Hence, tumor cells may have reduced sensitivity to chemotherapy because of an inability to produce sufficient apoptotic signal via sphingomyelin hydrolysis to ceramide.[56] Indeed, sphingomyelin co-administration potentiates chemotherapy of human cancer xenografts.[57]

In contrast to doxorubicin, which induces apoptosis only in human epidermoid carcinoma KB-3-1 cells and not in a multidrug-resistant subclone that expresses P-glycoprotein; the ceramide breakdown product, sphingosine, induces apoptosis in both cell types.[14] Furthermore, neither the

protein kinase C (PKC) inhibitor H7 nor staurosporine induced apoptosis in these cell lines, suggesting that PKC-independent signaling is involved in apoptosis induced by sphingosine.[14] An analog of sphingosine, L-threo-dihydrosphingosine (known as safingol), enhanced doxorubicin accumulation and sensitivity of MCF7 drug resistant cells. However, this effect correlated with inhibition of PKC rather than interference with P-glycoprotein drug binding.[58] Because sphingosine and its analogs induce apoptosis regardless of P-glycoprotein expression, they may provide a new strategy for the treatment of anticancer drug-resistant cancers. Indeed, in pilot clinical phase I trials with safingol, which potentiates the effect of doxorubicin in tumor-bearing animals, it was found that safingol can be given safely with doxorubicin at a dose that is potentially pharmacologically active without dose-limiting toxicity.[59]

The results presented here demonstrate that transient generation of endogenous sphingosine also occurs in MCF7 breast cancer cells during apoptosis induced by the anthracycline doxorubicin, preceding release of cytochrome *c* from the mitochondria and activation of the executioner caspase-7. Similar to doxorubicin, exogenous sphingosine induced apoptosis in a caspase-7-dependent manner. Consistent with other studies,[31–35] overexpression of Bcl-x_L protected MCF7 cells from doxorubicin-induced apoptosis and rendered them resistant to sphingosine-induced apoptosis, even though Bcl-x_L overexpression did not inhibit sphingosine generation after doxorubicin treatment. Despite the lack of caspase-3, MCF7 cells underwent apoptosis suggesting that other executioner caspases, such as caspase-7 or -2 may be activated. In this study, we established that caspase-7 is indeed activated during apoptosis of MCF7 cells. Furthermore, even in a cell-free caspase activation assay, caspase-2, which shares the same substrate specificity, was not detectably activated, in agreement with previous studies showing that caspase-2 was not processed in MCF7 cells following TNFα or staurosporine treatment.[60] However, introduction of *CASP-3* cDNA into MCF7 cells restored caspase-2 cleavage. To date, caspase-3-dependent caspase-2 activation has only been reported in Jurkat T cell extracts immunodepleted of caspase-3.[46] Thus our results support the notion that activation of caspase-2 but not -7 depend on the presence of caspase-3.

Our observations establish that cytochrome *c*-inducible caspase-7 activation takes place in caspase-3 null MCF7 cells during apoptosis and raises the possibility that caspase-7 may be able to compensate for the lack of caspase-3, as overexpression of caspase-3 in MCF7 cells did not greatly enhance their apoptotic susceptibility (data not shown). Our results also demonstrate that activation of Fas/FasL signaling is not involved in apoptosis triggered by doxorubicin in MCF7 breast carcinoma cells. In addition, caspase-8, the first caspase to be processed in death receptor-mediated apoptosis,[51] was only proteolyzed when cells were treated with anti-Fas mAb, whereas caspase-8 was not activated during doxorubicin-induced apoptosis.

Interestingly, we have found that the involvement of sphingolipid metabolites in cell death machinery triggered by doxorubicin may be more complex than previously thought. Accumulation of ceramide in anthracycline-mediated cell death has been shown to be due to activation of sphingomyelinase[3] or ceramide synthase.[4] In agreement, our results suggest that doxorubicin might in fact activate both pathways to mediate cell death, *de novo* sphingolipid synthesis[4] and a sphingolipid degradation pathway mediated by a sphingomyelinase[3] and a ceramidase. Unexpectedly, we found that cell death induced by exogenous sphingosine was inhibited by ceramide synthase or ceramidase inhibitors. These findings support the notion that exogenously added sphingosine can first be converted to ceramide, which can then be hydrolyzed to sphingosine by the action of a ceramidase, presumably in another compartment.

In sum, our results suggest that sphingosine, the metabolite of ceramide, is also involved in the doxorubicin-mediated, Bcl-x_L inhibitable pathway of apoptosis in human breast cancer cells.

Materials and Methods

Cell culture and reagents

The MCF7/L cell variant was obtained from the Cell Culture Core Resource (Lombardi Cancer Center, Washington, DC, USA), and was maintained in RPMI 1640 containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. MCF7 cells stably transfected to express the Fas antigen (denoted MCF7/Fas) or both Fas and Bcl-x_L (denoted MCF7/Fas/Bcl-x_L) were a kind gift of Dr. Vishva Dixit (Genentech Inc., San Francisco, CA, USA), and were grown in the same media supplemented with 200 μg/ml G418 and 150 μg/ml hygromycin. The MCF7/L/neo and MCF7/L/casp-3 cell lines were obtained by transfecting MCF7/L cells with either empty pcDNA3 or pcDNA3 engineered to express caspase-3, respectively (the *CASP-3* expression vector was a kind gift of Dr. Donald Nicholson, Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec). These cells were propagated in the same media containing 1 mg/ml G418.

Cells were subcultured twice weekly and 48 h prior to the initiation of an experiment. For induction of apoptosis, cells at $\leqslant$60–75% confluence were treated in serum-free media with either doxorubicin (Sigma), anti-Fas antibody (Upstate Biotechnology, Lake Placid, NY, USA) or sphingosine (Biomol, Plymouth Meeting, PA, USA). Jurkat T cells were cultured in RPMI 1640 containing 10% FBS.

Ac-DEVD-AMC was from Bachem (King of Prussia, PA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), horse heart cytochrome *c* and phosphocreatine were from Sigma. ATP, dATP and rabbit muscle creatine kinase were from Roche Diagnostics. [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN Life Science. FB1 and dMAPP were from Biomol (Plymouth Meeting, PA, USA). Other reagents and solvents were analytical grade.

Mass measurement of sphingosine

Sphingosine was measured after phosphorylation to sphingosine-1-phosphate, using recombinant sphingosine kinase as described by Olivera *et al*.[61] For each experiment, known amounts of sphingosine were used to generate a standard curve. Total phospholipids mass in the lipid extracts was quantified as previously described[62] using a colorimetric phosphate assay.[63]

Cell death assay

The tetrazolium based MTT assay was used to determine cell death as previously described.[64] Briefly, approximately 5000 cells per well were plated in 96-well tissue culture plates as described above, and 24 h later, the medium was replaced with 100 μl serum-free medium containing the indicated amounts of doxorubicin, anti-Fas antibody, or sphingosine. After various times of incubation at 37°C, 25 μl of MTT solution (5 mg/ml) was added and the plates were incubated for approximately 4 h. After solubilization for 16 h with 100 μl of lysis buffer (20% SDS in 50% *N,N*-dimethylformamide), formazan was quantified with a microplate reader at a wavelength of 570 nm.

Preparation of mitochondria and Western blot analysis of cytochrome *c*

Mitochondrial preparations were carried out as previously described.[65] In brief, cells were collected by centrifugation at $600 \times g$ for 5 min at 4°C. After washing once with ice-cold PBS, mitochondrial and cytosolic fractions were prepared by resuspending cell pellets in 5 vol of ice-cold buffer A (20 mM HEPES-KOH pH 7.5, 0.1% BSA, 1 mM sodium EDTA, 1 mM DTT, 0.1 mM PMSF, 20 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin A] containing 250 mM sucrose. After swelling on ice for 15 min, cells were homogenized with 15 to 20 strokes of a number 22 Kontes Dounce homogenizer with a B pestle (Kontes Glass Company, Vineland, NJ, USA), and the homogenates centrifuged at $750 \times g$ for 5 min at 4°C. Supernatants were then centrifuged at $10\ 000 \times g$ for 15 min at 4°C, and the resulting mitochondria pellets resuspended in cold buffer A. For Western blot analysis, equal amounts of mitochondrial and cytosolic proteins were separated on 15% SDS – PAGE and then transblotted to nitrocellulose. Anti-cytochrome *c* mAb (PharMingen) and anti-cytochrome oxidase subunit II mAb (Molecular Probes, Eugene, OR, USA) were used as primary antibodies.

Western blot analysis

Cell lysate preparation and Western blotting were carried out as previously described.[65,66] Rabbit anti-caspase-3 (gift of Dr. Donald Nicholson, Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec), mouse anti-caspase-8 (PharMingen, San Diego, CA, USA), rabbit anti-caspase-7, rabbit anti-caspase-2 (Santa Cruz Biotechnology), rabbit anti-caspase-9 (Research Diagnostic, Flanders, NJ, USA or Oncogene Research), anti-PARP (PharMingen), and anti-FasL (Transduction Laboratories, Lexington, KY, USA) were used as primary antibodies. Proteins were visualized by ECL using anti-rabbit or anti-mouse HRP-conjugated IgG (Bio-Rad).

Fluorogenic DEVD cleavage enzyme assays

Enzyme reactions were performed in 96-well plates with 20 μg of cytosolic proteins and a final concentration of 20 μM Ac-DEVD-AMC substrate as previously described.[65,66]

In vitro assay for cytochrome *c*-dependent activation of caspases

Cytosolic fractions (40 μl, 400 μg proteins), prepared as described above, were incubated at 37°C for various times in 96-well plates in a final volume of 200 μl in buffer B (10 mM HEPES pH 7.4, 2 mM $MgCl_2$, 5 mM sodium EGTA, 5 mM DTT, 1 mM ATP, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 mM phosphocreatine, 150 μg/ml creatine kinase) supplemented without or with 1 mM dATP and horse heart cytochrome *c*. Proteins were then separated by SDS – PAGE, blotted, and probed with anti-caspase and anti-PARP antibodies.

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