**Induction of Apoptosis through B-cell Receptor Cross-linking Occurs via de Novo Generated C16-Ceramide and Involves Mitochondria***

Received for publication, October 18, 2000, and in revised form, January 16, 2001
Published, JBC Papers in Press, January 17, 2001, DOI 10.1074/jbc.M009517200

Bart-Jan Kroesen‡, Benjamin Pettus§, Chiara Luberto§, Mark Busman§, Hannie Sietsema†, Lou de Leij‡, and Yusuf A. Hannun**

*From the Departments of Pathology and Laboratory Medicine and Pediatric Oncology and Hematology, University Hospital Groningen, 9713 GZ Groningen, The Netherlands and the United States Department of Commerce, National Ocean Service, Charleston, South Carolina 29412 and the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

B-cells, triggered via their surface B-cell receptor (BcR), start an apoptotic program known as activation-induced cell death (AICD), and it is widely believed that this phenomenon plays a role in the restriction and focusing of the immune response. Although both ceramide and caspases have been proposed to be involved in AICD, the contribution of either and the exact molecular events through which AICD commences are still unknown. Here we show that in Ramos B-cells, BcR-triggered cell death is associated with an early rise of C16 ceramide that derives from activation of the de novo pathway, as demonstrated using a specific inhibitor of ceramide synthase, fumonisin B1 (FB1), and using pulse labeling with the metabolic sphingolipid precursor, palmitate. There was no evidence for activation of sphingomyelinasises or hydrolysis of sphingomyelin. Importantly, FB1 inhibited several specific apoptotic hallmarks such as poly(A)/DP-ribose polymerase cleavage and DNA fragmentation. Electron microscopy revealed morphological evidence of mitochondrial damage, suggesting the involvement of mitochondria in BcR-triggered apoptosis, and this was inhibited by FB1. Moreover, a loss of mitochondrial membrane potential was observed in Ramos cells after BcR cross-linking, which was inhibited by the addition of FB1. Interestingly, benzoylcarbonyl-Val-Ala-DL-Asp, a broad spectrum caspase inhibitor did not inhibit BcR-induced mitochondrial membrane permeability transition but did block DNA fragmentation. These results suggest a crucial role for de novo generated C16 ceramide in the execution of AICD, and they further suggest an ordered and more specific sequence of biochemical events in which de novo generated C16 ceramide is involved in mitochondrial damage resulting in a downstream activation of caspases and apoptosis.

Apoptosis has been shown to be an important means by which organisms maintain homeostasis in proliferating tissues and systems such as the immune system (1–3). The term apoptosis is commonly used to denote the appearance of one or a combination of, cellular events characterized by nuclear condensation, membrane blebbing, chromatin fragmentation, and loss of membrane integrity resulting in phosphatidylserine exposure and trypan blue uptake (4, 5). Biochemical mechanisms by which each of these cellular characteristics are regulated remain largely unknown. However, it has become evident that the activation of a family of cysteine proteases known as caspases plays an important role in the progression of the apoptotic process (6, 7). Within this caspase family, initiator caspases are activated through an apoptotic stimulus and subsequently activate downstream effector caspases. These effector caspases in turn have a multitude of intracellular substrates, among which are components that are critically needed for cellular homeostasis. Cleavage of one or more of these substrates deregulates cell function and promotes specific morphological characteristics of the apoptotic program (4, 6, 7).

Ceramide accumulation has been widely described to be associated with a number of apoptotic hallmarks such as PARP cleavage, DNA fragmentation, phosphatidylserine exposure, and trypan blue uptake (8–11). Exogenously added ceramides are generally able to mimic stress-induced apoptosis in a stereospecific manner, and inhibition of the formation of ceramide has been shown, at least in some cases, to inhibit progression of apoptosis (12–15). Ceramide can be generated through different metabolic routes in the cell (16). The stress-induced metabolic conversion of sphingomyelin (SM) into ceramide by the enzyme SMase has been described extensively in response to various treatments of cells such as tumor necrosis factor-α, anti-Fas, serum withdrawal, and other agents. However, ceramide can also be generated through the de novo synthesis pathway, in which activation of serine palmitoyl transferase and/or ceramide synthase may play a pivotal role (15, 17). However, how ceramide generated from each pathway is involved in the apoptotic program is as yet largely unknown. In previous studies, using model systems in which apoptosis was induced with tumor necrosis factor or Fas activation via the caspase 8-dependent pathway, it was shown that ceramide formation occurs in between initiator caspases and effector caspases (18, 19).

AICD of B-cells triggered via their BcR provides a physiologically relevant model to study molecular and biochemical events leading to induction of apoptosis. Cross-linking of sur-

---

* This work was supported in part by National Institutes of Health Grants GM43825 and NIGMS GM08716. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Biochemistry & Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425.

---

1 The abbreviations used are: PARP, poly(A)/DP-ribose polymerase; SM, sphingomyelin; SMase, sphingomyelinase; AICD, activation-induced cell death; BcR, B-cell receptor; DiOC6, 3,3′-dihexyloxocarbocyanine iodide; z-VAD, Z-Val-Ala-Asp; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; FB1, fumonisin B1.
face-expressed Ig in B-cells sets off an apoptotic program leading to cell death that is characterized by a number of the above-mentioned apoptotic features including PARP cleavage and DNA fragmentation (13). However, BcR-induced cell death has been shown to commence via an initial caspase-independent step (20). In addition to activation of caspases, ceramide formation has been shown to occur in response to BcR triggering (12, 21). However, the exact metabolic pathway underlying this elevated ceramide, as well as the role of ceramide in the progression of apoptosis, has not been determined.

In this study we have investigated how BcR-induced generation of ceramide is involved in the induction of apoptosis. We first provide evidence that this ceramide appears to arise exclusively from de novo synthesis. We also show that early changes in de novo derived C16 ceramide are linked to a loss of function of mitochondria and subsequent activation of the apoptotic program. We postulate a role for de novo generated C16 ceramide in alterations in the mitochondrial membrane leading to cytochrome c release and subsequent activation of a cascade of downstream effectors involved in the further execution of apoptosis.

MATERIALS AND METHODS

Cell Culture—The Epstein-Barr virus negative Burkitt’s lymphoma Ramos cells were purchased from ATCC (Manassas, VA) and grown in RPMI 1640 supplemented with 25 mM Hepes, 10% fetal calf serum, 2 mM t-glutamine (Life Technologies, Inc.), 1 mM sodium pyruvate (Life Technologies, Inc.), 100 units/ml penicillin (Life Technologies, Inc.), and 50 mM streptomycin (Life Technologies, Inc.) under standard incubator conditions (humidified atmosphere: 95% air, 5% CO2, 37 °C).

Reagents—Polyclonal rabbit anti-PARP antibody and peroxidase-conjugated anti-rabbit antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fumonisin B1 and ribonuclease A were obtained from Phospholipid Source Inc. and Sigma (St. Louis, MO), respectively. Monoclonal mouse anti-CD19 antibody (Becton Dickinson, San Jose, CA) was a gift from Dr. L. Lanier. 

Cell pellets (2 × 106) were lysed in chloroform:methanol (1:2), and lipids were extracted by the method of Bligh and Dyer. Aliquots were dried down under nitrogen and used for ceramide and phosphate measurements. Ceramide levels were measured using the Escherichia coli diacylglycerol kinase assay (22, 23). Briefly, lipids were incubated at room temperature for 30 min in the presence of β-ocytocinolide dioleoylphosphatidyl glycerol micelles, 2 mM diethioletritol, 5 μg of proteins of diacylglycerol kinase membranes, and 2 mM ATP with l-(−)-3H-ATP (in a final volume of 100 μL). Lipids were then fractionated by thin-layer chromatography and visualized by iodine vapor. Ceramide generation was calculated by using RNAse as a reference. TLC plates were sprayed by En3Hance spray, and radiolabeled ceramide spots were scraped, and radioactivity was quantitated by liquid scintillation counting. Ceramide levels were normalized to total lipid phosphate.

Analysis of SMass Activity—The activities of both neutral and acid SMase were determined using radiolabeled substrate in a mixed micelle assay as described (24). For analysis of neutral SMase activity, cells were lysed in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 5 mM diethioletritol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and leupeptin. A cell lysate (50 μL) containing 50 μg of protein was added to a 50-μL reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 0.05% Triton X-100, 5 mM diethioletritol, 5 nMol of [3H]sphingomyelin (100,000 dpm), and 10 nMol of cold SM per reaction. For analysis of acid SMase activity, 50 μL of cell lysate containing 50 μg of protein was added to a 50-μL reaction mixture containing 50 mM sodium acetate, pH 5.0, 5 nMol of [14C]sphingomyelin (100,000 dpm), and 10 nMol of cold SM per reaction. Exogenously added SM was solubilized in the reaction mixture by sonication prior to the addition of enzyme. Reactions were allowed to proceed for 45 min at 37 °C and stopped by the addition of 1.5 ml of chloroform:methanol (2:1) and 0.2 ml of H2O. Separate phases were clarified by centrifugation for 5 min at 3000 rpm, and 0.4 ml of the aqueous upper phase was counted by liquid scintillation counting. Total hydrolyzed SM never exceeded 5% of the total amount of radiolabeled SM added to the assay.

Analysis of Cellular SM Mass—Cells were labeled for 48 h with [methyl-3H]choline chloride (final specific activity, 0.5 mCi/ml). After cell treatment as described, cells were washed with PBS, and lipids were extracted from the cell pellets by the method of Bligh and Dyer. Part of the extracted lipids were used for phosphate measurement. Remaining lipids were subjected to mild base hydrolysis (25), and [3H]sphingomyelin was determined by TLC analysis in chloroform: methanol: acetic acid: H2O (50:30:8:5), followed by scraping and counting radioactivity by liquid scintillation.

Mitochondrial Transmembrane Potential (ΔΨm)—To evaluate changes in Ψm, cells (106) were treated as described. After washing and resuspension in medium without fetal calf serum, cells were labeled with DiOC6 (40 nM) for 15 min at 37 °C. After incubation, cells were washed and analyzed directly on a flow cytometer using extinction and emission wavelengths of 495 and 525 nm, respectively.

DNA Fragmentation Analysis—Cells (0.5 × 107/2 ml) were treated as described. After treatment, cells were pelleted and resuspended in 2 ml of ice-cold Tris HCl buffer containing 0.5% SDS, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cells were digested for 2 h in 4 °C. After incubation, cells were washed twice with PBS and finally resuspended in PBS containing RNase (final concentration, 0.5 mg/ml) and incubated for 30 min at 37 °C. Finally, propidium iodide was added at a final concentration of 50 μg/ml and incubated overnight in the dark at 4 °C. The cell cycle was analyzed by flow cytometry.

Western Blot Analysis—Cells were lysed for 15 min on ice in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 0.05% SDS, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were centrifuged at 10,000 rpm for 10 min and mixed with reducing SDS sample buffer. Proteins (10 μg, as determined by Bio-Rad protein assay) were separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were subsequently blocked by 5% nonfat dry milk in PBS, TWEEN 20 (0.1%, v/v). Blots were incubated with anti-PARP at a 1:1000 dilution with 5% nonfat dry milk. After subsequent incubation with a 1:2000 dilution of horseradish peroxidase anti-rabbit IgG, proteins were visualized by ECL (Amersham Pharmacia Biotech).

RESULTS

BcR Cross-linking Induces Ceramide Generation and Apoptotic Features—BcR cross-linking using anti-IgM antiserum

2B. Pettus, M. Bussmann, and Y. Hannun, manuscript in preparation.
has been shown to induce apoptosis over a time period of ~48 h. As shown in Fig. 1A, PARP cleavage was observed starting at 12 h after BcR cross-linking and gradually increased over the next 36 h. In accordance with this, a gradual increase in DNA fragmentation up to 31% after 48 h was observed in similarly treated cells (Fig. 1B). Similar to BcR cross-linking, direct addition of exogenous ceramide to cells has been shown to induce apoptosis in various cell lines. As shown in Fig. 2, addition of up to 10 μM C6-ceramide to Ramos cells for 24 h induced PARP cleavage in a dose-dependent manner. We next determined ceramide levels in Ramos cells upon BcR cross-linking. As shown in Fig. 3A, ceramide levels increased gradually, reaching a 2.5-fold increase at 48 h after the start of the treatment. These changes are consistent with a previous study in WEHI B-cells (12). The changes in ceramide were further analyzed by TLC. It is important to note that our TLC system resolves ceramide (after derivatization into ceramide phosphate) into two spots (see Fig. 3D). Interestingly, ceramide in the lower spot started to increase as soon as 6 h after BcR cross-linking, whereas an increase in the upper ceramide spot could not be detected until 24 h after treatment (Fig. 3B and C). We find that the derivatized ceramide phosphate migrates with the lower spot, whereas dihydroceramide phosphate migrates with the upper spot (26). In addition, longer chain ceramides migrate with the upper spot as compared with C16-ceramides, which migrate in the lower spot. No changes in ceramide were detected at earlier time points (5 min to 3 h) after BcR cross-linking (data not shown).

Specific Generation of C16-Ceramide—To clarify the molecular nature of these two spots, tandem mass spectrometric analysis was employed. Such analysis has been able to routinely detect 14 ceramide and dihydroceramide subspecies in Ramos cells ranging from C14 to C26 in fatty acid length. Of note was the presence of a double bond on the fatty acid of ceramides longer than C24. C24:0 and C26:0 were also present, but absolute levels of C24:1 to C24:0 were difficult to resolve because, in contrast to the dihydroceramides, there is no difference in column retention with this double bond (ceramides have a double bond in resonance association with a hydroxyl group), and isotope ratios make a m/z of 2 units difficult to quantitate. We speculate that the ratio is nearly 1:1, with this ratio remaining constant during ceramide changes studied thus far.

Analysis by this method under anti-IgM-induced cross-linking of BcR revealed that early ceramide changes were specific to long chain ceramides (C14, C16, and C18 fatty acid lengths) and their respective dihydroceramide counterparts. The composition of the lower spot was overwhelmingly a C16 ceramide, with a 1.5-fold change at 6 h corresponding well to the increase in ceramide seen in the lower spot by diacylglycerol kinase at 6 h (Fig. 4A). Similarly, the composition at 24 h of treatment revealed a nearly 3-fold increase in C16 ceramide, with similar, approximately 2-fold changes in C14, C18, and the dihydroceramides matching the 2.5-fold increase in the lower spot at this time. However, at 24 h the upper spot began to change signif-

---

3 A. Bielawska and Y. Hannun, unpublished observations.
Role of Ceramide and Mitochondria in BcR-induced Apoptosis

Ceramide can be generated from diverse metabolic routes, e.g., from SM through activation of SMase or from activation of the de novo pathway. Thus, the activity of divergent enzymes involved in the generation of ceramide may be regulated differently. Moreover, ceramide derived from different pathways may be involved differently in various cellular processes such as proliferation, differentiation, and apoptosis. To determine the nature of the BcR-induced ceramide formation, we measured SMase activity and determined SM levels in Ramos cells after BcR cross-linking. As shown in Fig. 5, A and B, induction of neither neutral- nor acid-type SMase activity was observed after BcR cross-linking over the time period where ceramide levels increased. In addition, no decrease in SM levels was detected after IgM cross-linking, which would have been indicative of any type of SMase activity. Because SMase activity did not seem to be involved in the formation of ceramide after BcR cross-linking, we next investigated the possibility that the observed ceramide was formed de novo. To this end we made use of fumonisin B1 (FB1), a selective inhibitor of ceramide synthase that catalyzes the acylation step in the de novo pathway of ceramide formation. As shown in Fig. 6 for the lower ceramide spot, treatment of Ramos cells with 50 μM FB1 completely prevented ceramide accumulation in response to anti-IgM-induced BcR cross-linking. This strongly suggested the involvement of ceramide synthase in the formation of ceramide upon BcR ligation. In addition, the increase in ceramide migrating with the upper ceramide phosphate spot was completely inhibited, whereas SM levels remained unchanged (data not shown).

To further substantiate that BcR-induced ceramide accumulation was generated de novo, we performed pulse-labeling experiments using radioactive palmitate, which serves as a substrate for both serine palmitoyl transferase and ceramide synthase in the de novo pathway. As shown in Fig. 7, palmitate added as a pulse label after BcR ligation was incorporated into ceramide, resulting in a 175% increase over control-treated cells 6 h after the start of the treatment. The increase in ceramide from palmitate was nearly totally blocked by adding FB1.

Generation of Ceramide Is Directly Linked to Caspase-dependent Apoptotic Features and Involves Mitochondria—Because ceramide has been proposed to be involved directly in multiple aspects of apoptosis, we determined whether FB1 could inhibit Ramos cells from entering the apoptotic program. As shown in Fig. 8, incubation of Ramos cells with FB1 during BcR cross-linking inhibited both induction of PARP cleavage (Fig. 8A) and DNA fragmentation (Fig. 8B). FB1 by itself did not significantly affect PARP cleavage (Fig. 8A) or DNA fragmentation (data not shown). These results suggest a necessary role for the de novo generated ceramide in activating these downstream effector functions of apoptosis that are mediated by caspases (see below).

It is now generally accepted that mitochondria play key roles in the regulation of apoptosis. To investigate the involvement of mitochondria in BcR cross-linking-induced ceramide formation and apoptosis, we examined the mitochondrial morphology and function of Ramos cells by electron microscopy and flow cytometry, respectively, after anti-IgM treatment in the presence or absence of fumonisin or z-VAD, a broad spectrum caspase inhibitor. BcR cross-linking resulted in distinct morphological changes of mitochondria. Control cells showed a multitude of mostly small intact mitochondria. In contrast, anti-IgM treatment resulted in significant mitochondrial swelling along with extensive disruption of the mitochondrial membranes (Fig. 9). A protective effect on mitochondrial morphology was observed when Ramos cells were treated with anti-IgM in the presence of FB1 (Fig. 9), suggesting the involvement of de novo derived ceramide in the observed mitochondrial damage. Interestingly,
no such protective effect on anti-IgM-induced mitochondrial damage was observed using z-VAD, suggesting that mitochondrial effects of BcR cross-linking are independent of activation of caspases 3, 4, 7, and possibly 9.

Mitochondrial damage can be assessed biochemically by measuring the mitochondrial transmembrane potential ($\Delta V_m$), which has been shown to correlate with the release of cytochrome $c$, giving rise to caspase 9 activation that can then activate caspase 3 (27). DiOC$_6$ is a strong cationic dye that can be used to assay the $\Delta V_m$. Damaged mitochondria lose membrane integrity and as a consequence are no longer able to maintain their transmembrane potential, resulting in a decreased binding of DiOC$_6$. Indeed, a clear decrease in the binding of DiOC$_6$ was observed in anti-IgM-treated Ramos cells (Fig. 10). Moreover, as shown in Fig. 10, the drop in DiOC$_6$ binding was inhibited by FB1 but not by z-VAD, which, along with the results shown in Fig. 8, suggests a direct caspase-independent involvement of anti-IgM-induced ceramide in the loss of function of mitochondria.

In contrast to the inability of z-VAD to protect mitochondrial integrity, anti-IgM-induced PARP cleavage (data not shown), as well as DNA fragmentation (Fig. 10), was almost completely abrogated in the presence of z-VAD, which demonstrates the critical involvement of caspases in the execution phase of BcR-mediated apoptosis.

**DISCUSSION**

Immune homeostasis is maintained by a strictly coordinated regulation of cell proliferation, differentiation, and apoptosis. AICD has been described as serving immune homeostasis, and it occurs in both T- and B-cells to avoid autoimmunity and to self-limit the immune response (1–3). AICD in B-cells occurs after antigenic triggering in the absence of survival signals such as those provided by interaction of CD40 with CD40L, which is expressed by activated Th-cells (28, 29). Although a number of key players involved in BcR-induced cell death have been described, the molecular/biochemical route through which AICD in B-cells proceeds has still not been resolved.

Recently, BcR-induced apoptosis has been shown to involve mitochondrial loss of function, resulting in the activation of the caspase cascade (20, 30). In addition, BcR cross-linking has been postulated to involve the generation of ceramide (13, 21) and reactive oxygen species (31). Earlier it was shown that ceramide directly results in the production of reactive oxygen species by mitochondria in a cell-free system (32), and exogenous ceramide-induced mitochondrial hydrogen peroxide has been suggested to be involved in apoptosis (33, 34).

We therefore investigated the role of ceramide, resulting from BcR cross-linking, in anti-IgM-induced apoptosis. Our results show that BcR cross-linking on Ramos B-cells results in apoptosis, which is preceded by the generation of ceramide. We show that this ceramide response arises from activation of the de novo pathway of sphingolipid biosynthesis. Mass spectrometric data support this by showing significant and specific generation of the long chain ceramide subspecies (mainly C16-ceramide) in early data points prior to apoptotic features. The fact that changes in dihydroceramide subspecies occur is also significant, because it supports the hypothesis of de novo synthesis, which would generate dihydroceramide prior to desaturation to ceramide in that pathway. In addition, the studies described here show that BcR-induced apoptosis proceeds via
mitochondrial loss of function, which in turn is dependent on the accumulation of ceramide, thus linking BcR-induced ceramide formation with mitochondrial damage and progression of apoptosis.

Importantly, our results also provide evidence that BcR-induced apoptosis is dependent on the generation of ceramide derived from the de novo pathway of sphingolipid biosynthesis. In accordance with this was the observation that cotreatment with FB1, a selective ceramide synthase inhibitor, not only blocked ceramide formation but also inhibited PARP cleavage, DNA fragmentation, and changes in mitochondrial Δψ. By contrast, the decrease in mitochondrial Δψ, observed after BcR cross-linking, is not blocked by z-VAD. Taken together, these results place ceramide generation and subsequent loss in mitochondrial Δψ upstream of caspase activation in BcR-induced apoptosis. We therefore postulate a model in which BcR cross-linking induces increased de novo ceramide generation, which is involved directly or indirectly in mitochondrial damage.

There is now a growing body of evidence to implicate ceramide accumulation in induction of apoptosis. Addition of cell-permeable synthetic short chain ceramides closely mimics apoptosis-inducing stimuli, and elevation of endogenous ceramide has been described as a hallmark of the apoptotic death program (10, 12, 35). There are several studies documenting necessary roles for ceramide in regulating various aspects of apoptosis. Mechanistically, ceramide has been implicated as a pleiotropic bioactive molecule giving rise to effector-caspase activation as well as other stress-related biochemical events such as production of reactive oxygen species in mitochondria through yet unidentified mechanisms (32, 35–37).

Mitochondrial damage has been widely accepted as an evolutionary conserved initiating event in the onset of apoptosis.
Loss of mitochondrial function results in the activation of caspase 9 through the release of cytochrome c, which, together with the ubiquitous cellular Apaf-1, has been described to result in activation of pro-caspase 9. The release of cytochrome c can be inhibited by proapoptotic members of the Bcl-2 protein family and can be inhibited by the anti-apoptotic members. Indeed, it has been described that overexpression of Bcl-XL can significantly inhibit BcR-induced apoptosis.

Ceramide generation was completely inhibitable by FB1 and thus seemed to be formed exclusively through the de novo synthesis pathway. This is in contrast to the results reported by Wiesner et al. (12), who described ceramide formation after BcR cross-linking due to increased neutral SMase activity, which was monitored 24 h after BcR cross-linking. No data on cellular SM levels after BcR cross-linking were provided in that study. As shown in the present study, we failed to detect increased neutral or acid SMase activity at any of the assayed time points. In addition, we did not detect even a small decrease in SM levels after BcR cross-linking, which is necessary to result in ceramide formation and which might have been suggestive of additional unassayed SMase activities that we may have failed to detect. In this respect Ramos cells seem to respond differently to BcR cross-linking than WEHI-231 cells, which might reflect differential phases of B-cell maturation. Whereas Ramos cells represent mature germinal B-cells (39), WEHI-231 cells have been reported to represent an immature stage in B-cell development (40).

Our results are the first to implicate de novo ceramide in BcR-induced apoptosis and the first to implicate de novo ceramide in regulation of the mitochondrial response to AICD. Very recently, Kawatani et al. (41) also showed the involvement of protein kinase C-regulated ceramide induction in 

Our studies place ceramide formation upstream of activation of caspases in BcR-induced apoptosis, which is in line with earlier studies demonstrating that z-VAD does not abrogate ceramide formation in response to BcR cross-linking (13, 20). This is also consistent with previous results showing that, with
tumor necrosis factor, ceramide formation is dependent on the upstream, but not downstream/effector, caspases, whereas the action of exogenous ceramide leads to activation of the downstream, but not upstream, caspases (42, 43). In a recent study, Grullich et al. (44) showed that increased ceramide generation occurs early in the apoptotic response to CD95 triggering, prior to the commitment step to the execution phase of apoptosis. Although the increase in ceramide levels as determined by the diacylglycerol kinase assay excellently fitted the elevation of ceramide quantified by mass spectrometry, no specific ceramide species were reported to increase in that study (44). By contrast, we were able to detect early changes of C16 ceramide specifically, followed by later increments of both C16 and C24 ceramides. Earlier changes in ceramide levels (range of minutes to 3 h), measured by the diacylglycerol kinase assay, were not detected. We did not measure SM levels or SMase activity at very early time points after BcR cross-linking. Therefore, we cannot rule out activation of SMase activity at these early time points. However, the lack of detectable levels of ceramide at these time points suggests that early activation of SMase is unlikely.

The nature of ceramide generated in response to a stimulus may hold important clues as to the divergent cell biological effects observed and described to be related to ceramide. In this respect it was important to note the differential kinetics of ceramide generated in the upper versus lower TLC spots. Molecular properties that have been described as determining the separation of ceramide into two spots are the degree of saturation, fatty acyl chain length, and (ω)hydroxylation of the N-linked fatty acids (8, 26). Indeed, this differential response was critical in convincing us of the changes in ceramide preceding any sign of apoptosis by several hours. Confirmation by mass spectrometric analysis, therefore, is significant in several regards. First, it allowed us to identify the ceramide subspecies involved in the early stages of the signal, highlighting the significant and specific way in which these changes occurred.

Fig. 10. Left panel, BcR cross-linking-mediated changes in mitochondrial ΔΨ can be reversed by FB1 but not by z-VAD. Right panel, both FB1 and z-VAD effectively prevent anti-IgM-induced DNA fragmentation. Ramos cells (5 × 10^6/ml) were treated for 24 h with (B–D and F–H) or without (A and E) anti-IgM (10 μg/ml) in the presence of FB1 (50 μM) (C and G) or z-VAD (50 μM) (D and H). After the treatment, cells were harvested and prepared for flow cytometric analysis of mitochondrial ΔΨ (A–D) using staining with DiOC6 (40 nM) or for DNA fragmentation (E–H) as described. Representative results from four independently performed experiments are shown.
Role of Ceramide and Mitochondria in BcR-induced Apoptosis

Second, it added further support to the hypothesized role of de novo synthesis by showing similarly specific changes in the precursor dihydroceramide subtypes. Changes in longer chain (C18–C24) ceramides observed at later time points after BcR cross-linking might be due to metabolic conversion of the initially formed C16-ceramide or other complex sphingolipids. This raises another significant question that deserves further investigation, namely, whether de novo derived and SMase-derived ceramide have fundamentally different roles in cell functioning. It is well established that the generation of ceramide through the de novo and SMase synthesis routes occurs at different cellular sites. Whereas enzymes with SMase-type activity have been shown to be associated with a number of cellular compartments including the lysosomes (acid SMase) and possibly the cell membrane (neutral SMase), the enzymes involved in the de novo synthesis of ceramide are all found in the endoplasmic reticulum (although mitochondrial localization of at least a subpopulation has not been ruled out). This raises another important issue, which relates to the site of generation of sphingolipids and the site of action. To date, no models exist on the specific mitochondrial delivery of naturally formed ceramide or sphingolipids in general. Transport from the endoplasmic reticulum to the trans Golgi network de novo generated ceramide has been reported as not being vesicle-mediated (45) and as not being dependent on protein transport. Whether a similar transport mechanism applies for de novo derived ceramide from endoplasmic reticulum to mitochondria is not known. Theoretically, it may be that a more intricate metabolic network occurs in which de novo formed ceramide is first converted to sphingosine and sphingosine-1-phosphate, which may move more freely compared with ceramide. Alternatively, ceramide might even be generated in situ in mitochondria. In rodent and bovine brain, a mitochondrial localization of a ceramide synthase has been described (46, 47). A very recent study has identified a novel mitochondrial ceramide with reciprocal (ceramide synthase) activity indicative of the existence of a topologically distinct sphingolipid pathway with a direct possible impact on mitochondria (48–50). (49) Whether or not human lymphocytes contain this mitochondrial ceramide synthase activity is unknown. Interestingly, an enzyme, carnitine palmitoyltransferase I, that facilitates passage of long chain fatty acids into mitochondria has been shown to become up-regulated in response to BcR cross-linking. Carnitine palmitoyltransferase I catalyzes the rate-limiting step in the mitochondrial transmembrane transport of fatty acids used for $\beta$-oxidation. Palmitoyl-CoA, an important precursor for de novo sphingolipid synthesis, is a substrate for this enzyme, and BcR cross-linking-induced up-regulation of carnitine palmitoyltransferase I mRNA might thus allow enhanced mitochondrial transmembrane transport of fatty acids that could be used as precursors for ceramide synthesis.

In conclusion, our results demonstrate activation of de novo ceramide synthesis following BcR cross-linking in B-cells. We were able to distinguish a subset of ceramide species centered around C16-ceramide that preceded features of apoptosis. Moreover, this de novo ceramide is important in activating the mitochondrial pathway of apoptosis in response to BcR cross-linking.

Acknowledgments—We acknowledge C. Encock for assistance with flow cytometric analysis and Hazel Martin for assistance with electron microscopic analysis. We also acknowledge Peter Moeller at the United States Department of Commerce, National Ocean Service, Charleston laboratory, for access to the Tandem Mass Spectrometry Facility.