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*J Immunol* 1998; 160:3528-3533; 
http://www.jimmunol.org/content/160/7/3528

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Dual Role of Ceramide in the Control of Apoptosis Following IL-2 Withdrawal

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Ceramide is largely known as a lipid second messenger with pleiotropic effects. Increases in ceramide levels have been related to the onset of apoptosis, terminal differentiation, or growth suppression. In this study, addition of exogenous C2-ceramide to CTLL-2 cells is found to block IL-2-induced cell cycle entry, as well as the apoptosis triggered by IL-2 deprivation. The protective effect of C2-ceramide is achieved only in the early stages following cytokine deprivation and is related to the inhibition of bc1-xL degradation and the induction of a G1 arrest of cells. The same treatment over a longer time when, as we demonstrate, ceramide is produced physiologically, enhances cell death by apoptosis. The dual effect of ceramide both in protecting from or inducing apoptosis is discussed further. The Journal of Immunology, 1998, 160: 3528–3533.

Stimulation of quiescent T lymphocytes by cell-bound Ags triggers a complex activation program that results in cell cycle entry (G0 to G1 phase transition) and the expression of high affinity IL-2R (1). The subsequent binding of IL-2 to its high affinity receptor drives activated T cells through a late G1 phase restriction point, after which the cells are committed to complete a relatively autonomous program of DNA replication and, ultimately, mitosis (2). The maintenance of correct homeostasis requires that the activated T cells be removed. This is achieved by cytokine deprivation or by religation of the TCR, which activates a mechanism known as activation-induced cell death (AICD)3 (3, 4). While AICD requires the interaction of CD95 (Fas) and its ligand (5–7), expressed in activated T cells, apoptosis following cytokine deprivation is due to the down-regulation of the survival factors bc1-2 and bc1-xL (8). IL-2 binding to its high affinity receptor, therefore, regulates signaling events that control both lymphocyte cell survival and cell cycle progression. Although both processes occur simultaneously following IL-2 binding, the two mechanisms can be separated. For instance, treatment of IL-2-dependent cell lines with immunosuppressants prevents IL-2-induced proliferation without affecting cell survival (9). IL-2 has also recently been shown to regulate cell survival in the absence of cell proliferation (10).

Many studies have demonstrated the essential role of certain glycerol- and sphingolipids, not only as second messengers that can activate molecular targets, but also as biosensors whose concentration determines the control of processes such as differentiation, proliferation, and apoptosis. In this regard, ceramide is widely considered an antimitogenic lipid, since increased levels have been correlated to cell cycle arrest (11), terminal differentiation (12), cellular senescence (13), and apoptotic processes (14). We thus reasoned that modulation of ceramide levels might affect IL-2-regulated processes such as IL-2-induced proliferation and cell survival. To test this hypothesis, we added exogenous ceramide to synchronized CTLL-2 cells and evaluated its effects on the IL-2-induced S phase entry, as well as on the apoptosis triggered by IL-2 deprivation. Our data demonstrate that addition of the cell-permeable ceramide analogue, C2-ceramide, blocks IL-2-induced G1 to S transition, preventing IL-2 induction of c-myc and c-fos proto-oncogenes. Addition of exogenous C2-ceramide to arrested T cells maintains elevated bc1-xL levels and prevents the apoptosis observed following cytokine deprivation. When endogenous concentrations of DAG and ceramide were evaluated, we found that IL-2 deprivation induces a rapid and sustained decrease in the ratio of DAG/ceramide that correlated with the onset of apoptosis. The physiologic implications of modulation of ceramide concentration in IL-2-regulated cell survival mechanisms are discussed.

Materials and Methods

Cells and cell culture

CTLL-2 cells (clone G7) were maintained in basal medium (RPMI 1640, 2 mM L-glutamine, 50 μM 2-ME, buffered to pH 7.2 with 10 mM HEPES) supplemented with 10% (v/v) FCS and 20 U/ml recombinant human IL-2. To obtain maximal synchronization, cells were washed extensively and incubated for 8 h in IL-2, serum-free RPMI medium. After this period of incubation the majority of the cells were found in G1 phase and no apoptosis was observed.

Reagents and Abs

Recombinant human IL-2 was generously donated by Hoffmann-La Roche (Nutley, NJ). C2-ceramide and C2-dihydroceramide were from Biomol (Plymouth Meeting, PA). Anti-c-myc, anti-c-fos, anti-cyclin A, and anti-raf 1 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-bcl-2 and anti-PARP (poly[adenosine diphosphate-ribose] polymerase) were from PharMingen (San Diego, CA); and anti-bcl-xL Ab was from Transduction Laboratories (Lexington, KY). Anti-rabbit and anti-mouse Ig horseradish peroxidase-linked whole Ab Abs were from Amersham Corp. (Aylesbury, U.K.). Primary Abs were used at 1/1000 dilution.

Cell cycle analysis

Cells were harvested by centrifugation and washed in PBS. After centrifugation, cells were resuspended in permeabilization solution (0.1% sodium
Results and Discussion

For the studies presented here, we used CTLL-2 cells, an IL-2-dependent mouse T cell line in which cytokine deprivation induces cell growth arrest followed by the rapid onset of apoptosis. In arrested cells, addition of IL-2 induces the entry of cells into S phase. CTLL-2 cells, therefore, constitute an ideal system for studying the effect of the cell-permeable analogue of ceramide, C₄-ceramide, in IL-2-induced proliferation, as well as apoptosis induced by IL-2 deprivation. To better define how ceramide affects the pathways implicated in both mechanisms (survival and proliferation), we sought to obtain maximal cell cycle arrest, assessed by propidium iodide staining and cell cycle distribution analysis by flow cytometry. Following 8 h of culture in basal medium (no IL-2, no serum), a marked increase in the percentage of G₁ phase cells was found (see Ref. 18 and Fig. 5). Under these conditions, arrested CTLL-2 cells entered S phase in a synchronous fashion at 11 to 12 h after restimulation with IL-2. Following 40 h of IL-2 addition, the cells were in exponential growth, with 60% of the cells in S phase. CTLL-2 cell growth arrest followed by the rapid onset of apoptosis. In arrested cells, C₂-ceramide, the cell fraction in G₀/G₁ was 65% of the total, and only 18% were in S phase. 60% in the presence of IL-2 alone. Addition of C₂-ceramide in the absence of IL-2 was no longer observed. These effects were specific to ceramide, since C₄-dihydroceramide, a closely related structural analogue (19), was used at the same concentration (5 μM) as a,
IL-2 binding to the high affinity IL-2R is known to initiate a complex signaling cascade that ultimately results in the expression of the proto-oncogenes c-myc, c-fos, bcl-xL, and bcl-2 (20). Addition of C2-ceramide to arrested CTLL-2 cells prevented IL-2-induction of c-myc and c-fos gene products, while bcl-2 or bcl-xL expression was not modified (Fig. 2). Addition of dihydroceramide did not affect IL-2 induction of any of these genes. These data concur with the cell cycle analysis of IL-2- and ceramide-treated cells, in which C2-ceramide prevents IL-2-induced proliferation with no changes in the sub-G1 percentage (see Fig. 1). When CTLL-2 cells were deprived of IL-2, there was a minor decrease in bcl-2 protein levels, whereas bcl-xL protein levels were dramatically reduced. This finding is in agreement with the studies by Broome et al., who demonstrated that the down-regulation of bcl-xL levels, and not of bcl-2, is responsible for T cell death by apoptosis following IL-2 deprivation (21). The addition of C2-ceramide in the absence of IL-2 prevented bcl-xL down-regulation (Fig. 2). The levels of bcl-xL 48 h after C2-ceramide addition were, thus, equivalent to those expressed in exponentially growing cells.

To further evaluate the effect of C2-ceramide addition on apoptosis initiated after IL-2 deprivation, activation of p-rICE proteases following IL-2 removal was determined in the presence and absence of C2-ceramide. IL-2 deprivation-induced activation of p-rICE, the protease responsible for cleavage of poly(adenosine diphosphate-ribose) polymerase (PARP). Addition of C2-ceramide to arrested CTLL-2 cells prevented PARP proteolysis. Addition of permeable analogues of ceramide has been demonstrated to activate PARP cleavage in MOLT-4 cells, but ectopic expression of survival genes such as bcl-2 prevents this activation (22) and also prevents cytokine deprivation-induced cell death (21). We therefore speculate that, by preventing the degradation of bcl-xL, a member of the bcl-2 family, C2-ceramide inhibits PARP cleavage and the death by apoptosis that follows IL-2 deprivation. Again, C2-dihydroceramide, used as a negative control, had no effect in preventing PARP hydrolysis or in maintaining elevated bcl-xL levels following cytokine deprivation.

Following deprivation of serum and IL-2, CTLL-2 cells are transiently arrested and undergo apoptosis. However, in the presence of C2-ceramide, cells were arrested for >1 wk (data not shown). We speculated that ceramide addition does not induce cell cycle arrest in the G1 phase, but rather drives cells into G0. We focused our attention, therefore, on resting T lymphocytes, since primary T cells have been shown to represent a particularly useful model system for cell growth regulation. Unstimulated cells are found physiologically in a noncycling or quiescent state and only enter the cell cycle following TCR/CD3 complex activation and the triggering of the costimulatory signals. Like most types of cells, T lymphocytes require more than one signal to reenter the cell cycle from a quiescent state. In the specific case of T cells, these signals are provided, sequentially, by Ag receptor stimulation and mitogenic cytokines. Stimulation of the T cell Ag receptor promotes synthesis of the cyclins and CDKs that are necessary for G1 progression and entry into S phase. Ag receptor stimulation is not sufficient to promote formation of active cyclin/CDK complexes, and kinase activity is undetectable until cells have received the full complement of mitogenic stimuli provided by IL-2. Elegant studies by Roberts and coworkers previously demonstrated that quiescent lymphocytes do not express cyclin E nor cyclin A, but interestingly, following stimulation of the Ag receptor, both cyclins were expressed (23). These observations indicate that, in T cells, cyclin A expression is initiated in the G1 stage, although a significant increase in expression of this cyclin does occur after cells are stimulated by IL-2. In the same way, other proteins necessary to transduce the mitogenic signal of IL-2 such as raf-1 are also barely detectable in G0 lymphocytes and are expressed following T cell activation (24).

As shown in Figure 3, in the absence of serum and IL-2, arrested CTLL2 cells expressed low levels of both cyclin E and cyclin A and also c-raf-1, corresponding to G0 lymphocytes. In agreement with the data reported for activated T cells, addition of IL-2 to arrested CTLL2 cells enhanced cyclin A, cyclin E, and c-raf-1 expression (Fig. 3). Interestingly, addition of C2-ceramide at a final concentration of 5 μM decreased the expression level of the three proteins either in IL-2-stimulated or unstimulated cells.
suggesting that ceramide treatment conducts the cells not just to G, but to a G0 stage resembling that described for resting T cells. As we have previously shown (see Fig. 1), ceramide treatment of CTLL-2 cells did not affect cell viability.

Growth factor deprivation has been related to increases in endogenous ceramide levels. For example, ceramide concentration is elevated following serum deprivation in MOLT-4 cells, a situation that involves predominantly cell cycle arrest with a small component of apoptosis (11). Our studies show that long term IL-2 deprivation induces apoptosis in CTLL-2 cells and that this mechanism is prevented by addition of C2-ceramide, which apparently causes cell arrest in a G0 stage resembling that of resting T cells. We therefore decided to analyze whether the cytokine deprivation-induced initiation of the apoptotic signaling program correlated with increases in ceramide by measuring the endogenous levels of this lipid. Since it has been reported that elevation in DAG levels can counteract the effect of ceramide in the induction of apoptosis (11), we also measured changes in DAG concentration after IL-2 addition or deprivation. Previous reports by us and other authors have described that IL-2 binding to its receptor induces early, transient changes in DAG concentration after IL-2 addition or deprivation. Previous reports by us and other authors have described that IL-2 binding to its receptor induces early, transient changes in DAG concentration after IL-2 addition or deprivation. Previous reports by us and other authors have described that IL-2 binding to its receptor induces early, transient changes in DAG concentration after IL-2 addition or deprivation. Previous reports by us and other authors have described that IL-2 binding to its receptor induces early, transient changes in DAG concentration after IL-2 addition or deprivation. Previous reports by us and other authors have described that IL-2 binding to its receptor induces early, transient changes in DAG concentration after IL-2 addition or deprivation. Previous reports by us and other authors have described that IL-2 binding to its receptor induces early, transient changes in DAG concentration after IL-2 addition or deprivation.

Increases in ceramide levels were not fully observed until 12 h after cytokine deprivation and reached their maximum level (approximately fourfold) at 48 h after IL-2 removal. Delayed and sustained kinetics of ceramide accumulation have been described in other apoptotic processes such as treatment of MCF-7 cells with TNF-α (27) or cross-linking of surface IgM receptors in the murine B lymphoma WEHI 231 (28).

Analysis of endogenous production of ceramide and DAG indicates that, in CTLL-2 cells, deprivation of IL-2 induces a rapid decrease in DAG levels and a delayed but sustained increase in ceramide concentration. This situation differs from that described for MOLT-4 cells, in which serum withdrawal results in a three- to fourfold elevation in endogenous diacylglycerol levels (11). This difference in the DAG/ceramide ratio could explain the different effects that growth factor deprivation has in MOLT-4 vs CTLL-2 cells, inducing predominantly cell cycle arrest in MOLT-4 and mainly apoptosis in CTLL-2 cells. At the same time points at which changes in endogenous DAG and ceramide levels were measured, we also studied cell cycle arrest and apoptosis events such as Rb dephosphorylation and PARP proteolysis using Western blot analysis (Fig. 4C). These analyses indicate that, at the initial stages following IL-2-deprivation, ceramide increases are not detected, and they become elevated only after the apoptotic program is initiated. One possible explanation for this observation is that ceramide is not the only lipid second messenger that plays a role in the activation of the apoptotic program in CTLL-2 cells and the rapid decrease in the ratio DAG/ceramide could also be responsible for the activation of cell cycle arrest and apoptosis events.
Addition of exogenous C2-ceramide to arrested CTLL-2 cells prevents apoptosis, but there is a sustained accumulation of ceramide levels at longer times following IL-2 deprivation. To test whether physiologic increases in ceramide levels had a role in the activation of programmed cell death, we assessed the effect of exogenous ceramide addition at the onset of ceramide production on cell survival. As shown in Figure 5, a dramatic difference was observed in the cell response depending on the time at which C2-ceramide was added after IL-2 deprivation. While C2-ceramide addition 8 h after IL-2 deprivation prevented apoptosis, addition of C2-ceramide 4 h later (when physiologic production begins) did not prevent but rather accelerated cell death by apoptosis.

The results presented here indicate that, as has been shown in other systems, changes in the concentration of lipid second messengers such as DAG and ceramide can act not only as activators of early signaling events but also as biosensors of the cellular state. As we demonstrate, IL-2-induced cell cycle entry is accompanied by long term increases in DAG levels, while cytokine deprivation induces DAG levels decrease and a steady increase in ceramide. Although DAG decreases are detected rapidly following IL-2 removal, ceramide increases do not take place immediately, but are augmented following a longer starvation period. Increases in ceramide levels have been described as inducing states such as differentiation, senescence, or apoptosis. It is not clear, however, what determines one effect or the other. Our experiments concur with the current hypothesis that mitogenic lipids such as DAG are directly related to the final effects of ceramide and can prevent its apoptotic effect but not the effect of growth arrest. The different effects of ceramide in the literature are described in different systems or cell types. Using the same cells and the same stimulus (IL-2 deprivation), we demonstrate here that ceramide can control cell fate, and the final effect (cell arrest or apoptosis) is determined by the balance with other lipids as well as the genetic background of the cells, something that is generally determined by the cell cycle or differentiation status. In this system, when the levels of mitogenic lipids such as DAG decrease and bcl-xL levels are down-regulated, an increase in ceramide levels accelerates cell death. In contrast, when bcl-xL protein levels are high, addition of exogenous ceramide can prevent the onset of apoptosis through a mechanism that inhibits bcl-xL degradation.

Our experiments demonstrate for the first time that bcl-2 family members not only protect from ceramide effect, but ceramide itself can maintain the endogenous level of one of these proteins, bcl-xL. Future work will be aimed at determining the physiologic significance of ceramide regulation of cell survival genes. Changes in ceramide levels, for instance, have been described in response to CD28 triggering (29), a mechanism that prevents apoptosis by up-regulating bcl-xL levels during the primary T cell response (30). Regulation of ceramide concentration in T cells can also be envisioned as a key event in the maintenance of T cell homeostasis, coupled to the existence of memory after immune response. The majority of activated cells die, but the rescue of some activated T cells from apoptosis is essential for the persistence of memory. To avoid apoptosis, either through AICD or cytokine deprivation, the memory T cell population must remain in a quiescent state that permits subsequent reactivation and clonal expansion upon antigenic encounter. In this regard, in vitro experiments have demonstrated that, when cytokines that signal through the IL-2R γ-chain (IL-2, IL-4, IL-7, and IL-15) are removed, activated T cells can survive in a resting state by interaction with monolayers of fibroblasts, epithelial cells, or endothelial cells (31). This stromal cell-mediated rescue is mediated by the selective induction of bcl-xL (32). Therefore, although ceramide increases have been described as part of the apoptosis mechanisms induced following Fas ligation (33) or cytokine deprivation (our results here), it can be hypothesized that ceramide could increase in response to signals generated by certain microenvironments. Elevation in ceramide levels at early times following cytokine deprivation, according to our results here, would allow the cells to exit the cell cycle and achieve quiescence while maintaining elevated levels of bcl-xL, a situation necessary for the cells to escape apoptosis and undergo subsequent reactivation.
Acknowledgments

We thank M. C. Moreno for help with the flow cytometer. We also extend our appreciation to Hoffmann-La Roche for the kind gift of recombinant human IL-2. Finally, we thank Dr. Ana Carrera for helpful discussion and advice. Drs. David Jones and Emilio Diez for critical reading of the manuscript, and C. Mark for editorial assistance.

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