Ceramides induce a form of apoptosis in human acute lymphoblastic leukemia cells that is inhibited by Bcl-2, but not by CrmA

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Abstract **The generation of ceramides by the action of acidic and/or neutral sphingomyelinases has been implicated in many forms of apoptosis. We investigated whether exposure to ceramides is sufficient to induce apoptosis in human leukemia cells and, if so, what the characteristics of this form of apoptosis might be. Treatment of the acute lymphoblastic T-cell line CEM-C7H2 with short- and medium-chain ceramide analogs (C2-, C6-, and C8-ceramide) resulted in apoptosis, whereas the inactive C2-dihydroceramide had no effect on cell survival. Induction of apoptosis was relatively slow (~40% after 24 h) and required high concentrations of ceramide analogs (40–100 μM). To investigate a possible involvement of interleukin 1-β-converting enzyme (ICE) or ICE-related proteases, we treated CEM-C7H2 sublines constitutively expressing the vaccinia virus protease inhibitor crmA with ceramide analogs. Although such cells were completely resistant to apoptosis induced by antibodies to the Apo-1/Fas surface receptor (a form of apoptosis known to be inhibitable by CrmA), they were not protected from ceramide-induced cell death. In contrast, tetracycline-regulated overexpression of Bcl-2 protected CEM-C7H2 sublines stably transfected with corresponding constructs from ceramide-induced apoptosis. Thus, in these human leukemia cells, ceramides induce a relatively slow death response that can be prevented by Bcl-2, but is independent of CrmA-inhibitable proteases. These characteristics distinguish ceramide-induced from other forms of apoptosis, such as Apo-1/Fas-induced cell death where ceramide production has been causally implicated.**

Key words: Human leukemia; Apoptosis; Fas antigen; Ceramide; CrmA; Bcl-2

1. Introduction

Programmed cell death (apoptosis) has been recognized as an essential mechanism by which multicellular organisms dispose of unnecessary or potentially harmful cells (reviewed in) [1–4]. An increasing number of genes contributing to, or protecting from, apoptosis induction has been identified, and numerous laboratories are dedicated to the study of signal transduction pathways by which the various apoptosis inducers lead to ultimate cell death. Analyses of programmed cell death in *C. elegans* have identified two crucial genes (reviewed in [5]). First, ced-9 and its mammalian homolog, bcl-2, that prevent apoptosis in many systems by poorly understood mechanisms (reviewed in [6,7]). Second, ced-3 that is homologous to proteases of the interleukin-1β-converting enzyme (ICE) family. These molecules are thought to mediate the terminal effector phase of most, if not all, forms of apoptosis by cleaving proteins essential for cell survival [8].

Elevation of ceramides has been observed in many different forms of apoptosis (reviewed in [9–15]), a phenomenon attributed to the action of primarily neutral sphingomyelinases that become activated during apoptosis and liberate ceramide from membrane lipid sphingomyelin [13]. Elevation of ceramide may activate ceramide-dependent phosphatase [16] or kinase [17]. Other possible targets include some protein kinase C isoforms [18] and members of the mitogen-activated protein (MAP) kinases [19] which, in turn, may pass on the death signal. Moreover, treatment of various cells with ceramide analogs carrying short- or medium-sized fatty acid chains are sufficient to induce apoptosis (see above reviews). These results suggest a causal role of ceramide generation in many forms of apoptosis, such as that triggered by activation of the Apo-1/fas surface receptor [15,20,21].

Our laboratory has been studying different forms of apoptosis in the human acute T-cell leukemia model CCRF-CEM [22], including cell death induced by glucocorticoids, antibodies to Apo-1/Fas, DNA-damaging agents and others [23–26]. Since ceramide has been implicated as a second messenger in several of these responses (see above), we analysed the effect of direct ceramide application in our system. In the human acute lymphoblastic cell line CEM-C7H2, ceramide analogs induced a relatively slow apoptotic response (~40% apoptotic cells 24 h after initiation of treatment) that required high concentrations of ceramide analogs (40–100 μM). Ceramide-induced cell death could not be prevented by the vaccinia virus protease inhibitor crmA, while overexpression of Bcl-2 protected CEM-C7H2 cells from this form of apoptosis. In conclusion, our study represents a first characterization of ceramide-induced apoptosis in the widely-used CCRF-CEM human leukemia model and, as discussed below, provides evidence against the proposed causal role of ceramides in Apo-1/Fas-induced apoptosis [27].

2. Material and methods

2.1. Cell lines and cell culture

The GC-sensitive cell line CEM-C7H2 [23], a subclone of the CCRF-CEM-C7 line [22], crmA-transfected CEM-C7H2 subclones 2E8, 2G10 and 2H10 [26], and cell line CEM-C7H2-9F3 expressing transgenic bcl-2 under the control of a tetracycline responsive promoter [28] have been described. In brief, crmA-expressing lines 2E8, 2G10, and 2H10 were produced by transfecting CEM-C7H2 with a construct containing crmA cDNA driven by a β-actin promoter [29]. For generation of bcl-2-expressing cell lines, CEM-C7H2 cells were first stably transfected with a construct constitutively expressing a tetracycline repressor — herpes simplex VP16 transactivator fusion protein (tTA) [30] from a human elongation factor 1α (EF-1) promo-

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Abbreviations: ICE, interleukin 1-β-converting enzyme

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ter. tTA-expressing CEM-C7H2 were subsequently stably transfected with a construct expressing bcl-2 cDNA [31] under the control of a tTA-responsive fusion promoter. The production of the sublines expressing crmA under the control of the human EF-1 promoter will be detailed below. All cell lines were grown in 5% CO_2, saturated humidity, at 37°C in RPMI-1640 supplemented with 10% bovine calf serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

2.2. Generation of CEM-C7H2 subclones expressing crmA under the control of the EF-1 promoter

Plasmid pEF1-crmA was generated by PCR amplification of crmA cDNA from pHD1.2 [29] using primers ON361 (5′-TAT*GAATTCAC-CATGG*ATATCTTCAGGGAAATCG-3′) and 362 (5′-ACTTA*A-GATCT*TTAATTAGTTGTTGGAGAGCAATATC-3′). ON361 introduced a partial Kozak translation consensus sequence (underlined) and *Eco*RI and *Nco*I restriction sites (italics), while ON362 introduced a *Bgl*II restriction site (italics). After amplification and purification, the DNA fragment was digested using *Eco*RI and *Bgl*II resriction enzymes and subcloned into the *Eco*RI and *Bam*HI sites of pKS^+ (Stratagene, La Jolla, CA) to generate pKS-crmA. CrmA was then released from pKS by digestion with *Eco*RI and *Not*I followed by subcloning into the *Eco*RI and *Not*I sites of the expression vector pEF1-Neo (kindly provided by G. Baier, Innsbruck, Austria). For stable transfections, logarithmically growing CEM-C7H2 cells were washed in PBS, pelleted at 300×*g* and resuspended at a density of 1×10^7 cells/400 μl PBS. Cells were mixed with 20 μg of either pSTneoB (the empty β-actin promoter-based expression vector), pEF1-crmA, or pEF1-Neo, incubated for 10 min on ice, and electroporated (Biorad Lab., Vienna, Austria) at 500 μF and 300 V. Thereafter, cells were again placed on ice for 10 min, diluted in 20 ml of growth medium and seeded on 96-well flat-bottom plates. Selection of stably transfected cells was initiated 48 h after electroporation using 1 mg/ml G418 (bioactivity 70%). G418-resistant clones were cultured and frozen in liquid nitrogen for further analyses.

2.3. Reagents, induction and analysis of apoptosis

All reagents were from Sigma (Vienna, Austria) unless otherwise indicated. To study induction of apoptosis through Apo1/fas, 2×10^5 cells/ml were cultured with 0.1 μg/ml mouse monoclonal IgM antibody to Apo1/fas (Immunotech, Marseille, France; clone CH11) or an isotype-matched control monoclonal for 6 h. Ceramides (Calbiochem, La Jolla, CA) were dissolved in DMSO (5 mg/ml) and used in final concentrations of 50 and 100 μM for C2-, C6-, and C2-dihydroceramide (*N*-acetyl,*N*-hexoyl,*N*-acetyldihydrosphingosine, respectively), and 10 and 80 μM for C8-ceramide (*N*-octoylsphingosine) for 6–48 h. Degree of apoptosis was determined as previously detailed [23]. Briefly, 2.5×10^5 cells/ml were cultured in the presence or absence of apoptosis-inducing substances in 24-well flat-bottom plates for the indicated time, lysed with Triton X-100, stained with propidium iodide, and fluorescence determined by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

3. Results and discussion

To investigate whether ceramides can induce apoptosis in human lymphoblastic leukemia cells, CEM-C7H2 were treated with ceramide analogs at various concentrations over 6–48 h. Apoptosis was induced using 50–100 μM C2-, C6-, and C8-ceramide, whereas 100 μM C2-dihydroceramide, used as an ineffective control substance, had no effect on cell survival. Fig. 1 shows a representative experiment using 50 μM C2-ceramide and 100 μM C2-dihydroceramide as controls. After incubation of CEM-C7H2 cells with 50 μM C2-ceramide, apoptosis was evident at around 24 h (~40%) and reached ~70% after 48 h. In contrast, cells incubated with 100 μM dihydroceramide did not differ from untreated cells (5–10% apoptosis).

Ceramides have been implicated in various forms of apoptosis, including Apo-1/Fas-induced apoptosis (see Section 1). The latter has been demonstrated to depend on the activity of

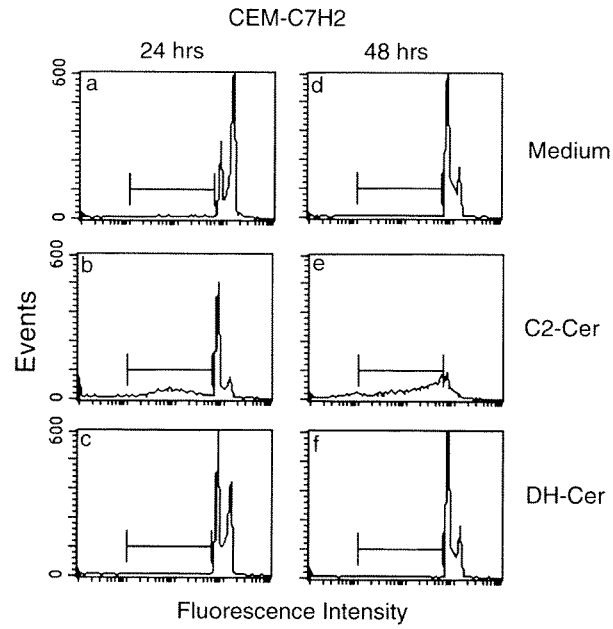


Fig. 1. Representative example of flow cytometric apoptosis analysis of untreated CEM-C7H2 (top panel) or cells treated with 50 μM C2-ceramide (middle) or 100 μM C2-dihydroceramide as ineffective control (bottom) for 24 (a–c) and 48 (d–f) h. The percentages of apoptotic nuclei were 8, 40, 6, 4, 73, and 4%, for panels a,b,c,d,e and f, respectively.

ICE and ICE-like proteases [26,32–34] that may be triggered in a cascade-like fashion by an Apo-1/Fas receptor-bound protease [35,36]. It is currently unclear whether ceramide production contributes to, or results from, activation of this pathway. To investigate whether ceramide-induced apoptosis depends on the activity of ICE or ICE-like proteases, we treated CEM-C7H2 cell lines stably transfected with the ICE inhibitor crmA with ceramides. In one set of cell lines (exemplified by line 2E8), crmA-cDNA was expressed from a chicken β-actin promoter. In an additional transfected CEM-C7H2 subline (2C11), crmA was expressed from the human EF-1 promoter using a construct that also contained a partial Kozak consensus sequence to optimize translational efficiency. As exemplified in Fig. 2, crmA overexpression did not protect CEM-C7H2 sublines from ceramide-induced apoptosis. Thus, crmA overexpressing sublines 2E8 and 2C11 underwent apoptosis with the same characteristics as two control lines (stably transfected with the 'empty' plasmids STNeoB and pEF-1-Neo, respectively) and the parental untransfected CEM-C7H2 line.

To rule out the possibility that ceramide induced a long-lasting activation of crmA-sensitive ICE-like proteases that would consume all protective crmA, we treated the crmA-expressing cell lines with 50 μM C2-ceramide (or the ineffective control substance dihydroceramide) for 24 h and added 100 ng/ml anti-Apo-1/Fas antibody for an additional 8 h (Fig. 3). The anti-Apo-1/Fas antibody caused a marked increase in apoptosis in the parental CEM-C7H2 line and in sublines stably transfected with control plasmids pStNeoB and pEF-Neo, whereas crmA-transfected sublines (2E8 and 2C11) were completely protected from increased apoptosis caused by the antibody. The low level of apoptosis seen in 2E8 and 2C11 cells treated with C2-ceramide and anti-Apo-1/Fas antibody

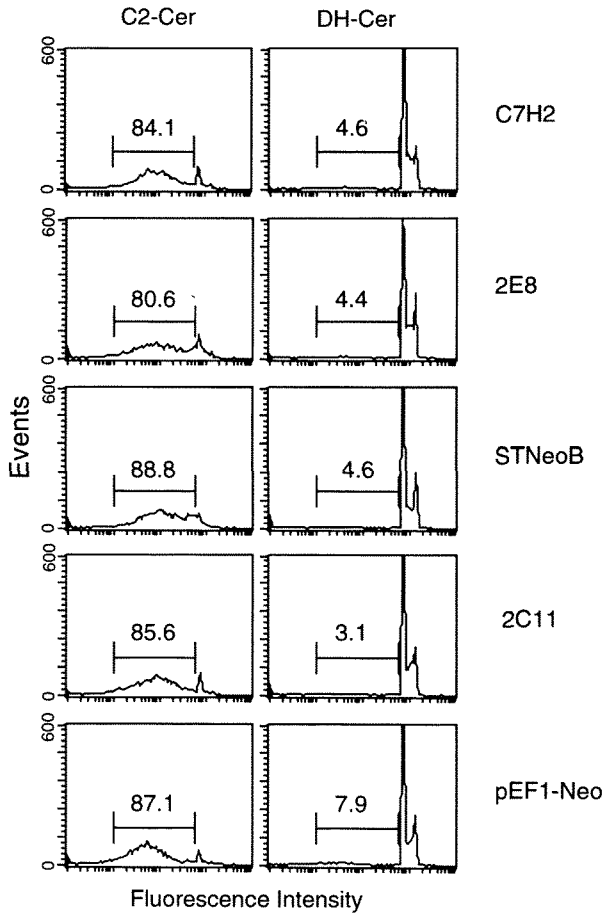


Fig. 2. Representative example of flow cytometric apoptosis analysis of parental CEM-C7H2, CEM-C7H2 sublines transfected with constructs expressing crmA-cDNA from a chicken β-actin (2E8) or a human EF-1 (2C11) promoter, and corresponding control lines (transfected with the 'empty' vectors StNeoB and pEF1-Neo, respectively). All cells were treated with 100 μM C2-ceramide (C2-Cer) or C2-dihydroceramide (DH-Cer) for ~24 h. The percentage of apoptotic nuclei is indicated in each panel.

was due to the crmA-independent effect of C2-ceramides, since cells treated with the ineffective control substance dihydroceramide and anti-Apo-1/Fas showed essentially no apoptosis. These data clearly showed that sufficient amounts of bioactive crmA were present after ceramide treatment to protect cells from anti-Apo-1/Fas-induced apoptosis. Thus, Apo-1/Fas-induced apoptosis and ceramide-induced apoptosis trigger different cell death pathways, the former depending on activation of crmA-sensitive ICE-like proteases and the latter not.

To investigate whether ceramide-induced apoptosis of human leukemia cells is sensitive to bcl-2 expression, we incubated the CEM-C7H2 subline 9F3 that expresses human bcl-2 cDNA under the control of a tetracycline-regulated promoter with ceramide. As shown in Fig. 4, in the presence of the tetracycline analog doxycycline, i.e., when bcl-2 expression is repressed, ceramide induced significant apoptosis in 9F3 cells. However, in the absence of doxycycline, i.e., when transcriptional repression is released and bcl-2 is expressed, ceramide-triggered apoptosis was completely prevented. Thus, ceramide-induced apoptosis in these human leukemia cells occurs via a Bcl-2-sensitive pathway.

In conclusion, our analyses provide the first characterization of ceramide-induced apoptosis in a human T-cell lymphoblastic leukemia line. These features can now be compared to cell death induced by other means and may provide evidence against, or in support of, a role of ceramide generation in the respective types of apoptosis. One particularly interesting example concerns Apo-1/Fas-induced apoptosis which has been associated with ceramide production in various cell types (see Section 1). Crosslinking of Apo-1/Fas or the related TNFα receptor leads to rapid, macromolecule neosynthesis-independent apoptosis. The cytosolic domains of both receptors share a homologous region, called death domain, that presumably associates with death domain-containing factors (e.g., FADD, TRADD, TRAF, and possibly others) thereby transducing the death signal [37]. In the effector phase, ICE-like proteases appear to be critical. Thus, Apo-1/Fas-induced cell death is dependent on expression of crmA-inhibitable ICE-like proteases in human leukemia [26] and HeLa [32,33] cells, and ICE-deficient mice do not undergo Apo-1/Fas or

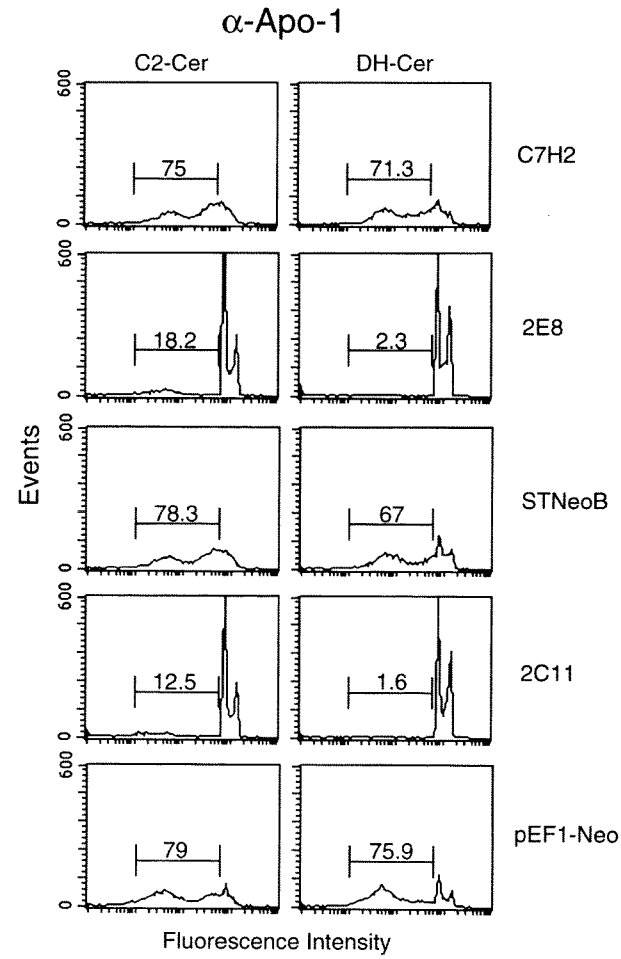
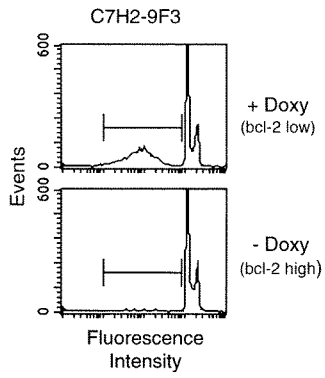


Fig. 3. Representative example of flow cytometric apoptosis analysis of parental CEM-C7H2, CEM-C7H2 sublines transfected with constructs expressing crmA-cDNA from a chicken β-actin (2E8) or a human EF-1 (2C11) promoter, and corresponding control lines (transfected with the 'empty' vectors StNeoB and pEF1-Neo, respectively). All cells were incubated with 50 μM C2-ceramide (C2-Cer, left panels) or C2-dihydroceramide (DH-Cer, right panels) and, after 24 h, 100 ng anti-Apo-1/Fas antibody CH111 was added for another 8 h. The percentage of apoptotic nuclei is indicated in each panel.



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