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CPP32 inhibition prevents Fas-induced ceramide generation and apoptosis in human cells

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Abstract Intracellular activation of sphingomyelinase, leading to ceramide generation, and ICE-like proteases have been implicated in TNF and Fas-induced apoptosis, but the links between these intracellular apoptotic mediators remain undefined. We show here that a specific peptide inhibitor of the ICE-like protease CPP32/*Yama* (DEVD-CHO) blocks anti-Fas-induced apoptosis in Jurkat and U937 cells, while having no effect on TNF-induced apoptosis in U937 cells. This peptide also prevents ceramide accumulation induced by Fas engagement. Jurkat and U937 cells, as well as their mtDNA-depleted derived lines (ρ^0 cells), were sensitive to ceramide toxicity, which was not prevented by ICE-like protease inhibitors. These results, taken together, suggest that ICE-like protease activation is a prerequisite for ceramide generation and subsequent apoptosis, at least in the case of Fas-induced cell death.

Key words: Fas; Tumor necrosis factor α; Ceramide; ICE-like proteases; Apoptosis

1. Introduction

The intracellular delivery of the apoptotic signal transduced by receptors possessing the so-called death domain [1] is poorly delineated. Recently, the sphingolipid ceramide has been implicated in the apoptosis induced by stimulation of the Fas molecule [2,3] and of tumor necrosis factor (TNF) 55 kDa receptor [4]. In this model, upon occupancy of receptor, an intracellular sphingomyelinase would be activated by an unknown coupling mechanism, causing hydrolysis of sphingomyelin and generation of ceramide [4–6]. Ceramide, in its turn, would bind to and activate several Ser/Thr protein kinases [7] and phosphatases [8] that would connect with the ras/raf/MAP-kinase pathway [9,10]. On the other hand, genetic research in nematodes has led to the discovery of genes which positively and negatively regulate apoptosis. Several Cys-proteases, namely the interleukin-1β converting enzyme (ICE), homologous to the product of *Caenorhabditis elegans ced-3* cell death gene, and other ICE-like proteases have been implicated in different types of apoptosis [11]. In particular, Fas-induced apoptosis is inhibited in ICE-knockout mice [12] and it is also partially inhibited by a tetrapeptide inhibitor (YVAD-cmk) of ICE-like proteases [13]. Moreover, overexpression of the poxvirus-derived serpin *CrmA*, which was shown to inhibit ICE and other ICE-like proteases [14,15], renders cells resistant to Fas- or TNF-induced apoptosis [13,15–17]. However, the precise sites of action of ICE-like proteases in the apoptotic pathway are not known.

We have analyzed here the effect of peptide inhibitors of ICE-like proteases on TNF-, Fas- and ceramide-induced apoptosis. Results indicate that Fas-induced apoptosis and ceramide generation were prevented by DEVD-CHO, a specific inhibitor of the ICE-like protease CPP32/*Yama*, demonstrating that its activation precedes sphingomyelin breakdown in the apoptotic pathway.

2. Materials and methods

2.1. Materials

N-Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk) and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), were from Bachem (Switzerland) and Novosystem (France). Neutral sphingomyelinase from *Staphylococcus aureus*, C_2-ceramide and ceramide type III, from bovine brain, ethidium bromide, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and dodecane were products from Sigma (Spain). Human recombinant TNF-α was kindly provided by The National Biological Standards Board (UK). Mouse monoclonal anti-human Fas IgM antibody (clone CH-11) was from UBI (Lake Placid, USA).

2.2. Cell culture

Human promonocytic leukemia U937 and Jurkat T-cell leukemia (ATCC, clone E6.1) were routinely cultured at 37°C in RPMI 1640 medium (Biowhittaker, Spain) supplemented with 5% fetal calf serum (hereafter, complete medium), using standard cell culture procedures. Two sublines devoid of functional mitochondria (ρ^0 cells) were generated from U937 and Jurkat cells by long-term culture in the presence of 50 ng/ml of ethidium bromide which selectively eliminates mtDNA, as described previously [18]. Selection and culture medium of U937-ρ^0 and Jurkat-ρ^0 cells was also supplemented with glucose (4.5 mg/ml), sodium pyruvate (0.1 mg/ml) and uridine (50 μg/ml). The elimination of mtDNA in ρ^0 cells was monitored by PCR using specific primers, as previously described [18]. After selection for 40 weeks, ρ^0 cells were entirely devoid of mtDNA and ethidium bromide was eliminated from culture media. One year after the establishment of mtDNA-depleted cell lines, no reversion to the ρ^+ phenotype has been observed.

2.3. Cell proliferation assays

U937 and Jurkat cells were seeded in flat-bottom, 96-well plates at an initial density of 2×10^5 cells/ml (100 μl/well), were cultured for 16–20 h in complete medium or in medium containing either anti-Fas antibody (5–100 ng/ml), TNF (5–100 U/ml), C_2-ceramide (10 μM) or natural (C_{18}) ceramide (50–200 nM) and were first dissolved in ethanol:dodecane (98:2, v/v), and then added to serum-free culture medium (RPMI 1640:DMEM:Ham's F12, 2:1:1) as indicated in [19]. At the doses used, ethanol and dodecane had no effect on cell proliferation. The effect of the protease inhibitors YVAD-cmk and/or DEVD-CHO (300–600 μM) on TNF-, Fas- and ceramide-induced apoptosis was also evaluated. Peptide inhibitors were added to cultures dissolved in DMSO, the final DMSO concentration being ≤0.4% (v/v). The addition of YVAD or DEVD to cell cultures at

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Abbreviations: TNF, tumor necrosis factor α; anti-Fas, cytotoxic monoclonal anti-Fas antibody; ICE, interleukin-1β-converting enzyme; PARP, poly(ADP-ribose) polymerase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; C_2-ceramide, *N*-acetyl-ceramide; C_{18}-ceramide, *N*-octadecanoyl-ceramide; PPDA, *p*-phenylenediamine

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concentrations up to 1.2 mM did not affect to the growth rate, cell morphology and Fas expression in Jurkat and U937 cells. Cells were preincubated for 3 h with the protease inhibitors before adding the toxic stimuli to assure a sufficient incorporation by cells [13]. Cell viability was determined by a modification of the MTT reduction method of Mosmann [20] and expressed as percent of the corresponding control cells.

2.4. Fluorescence microscopy

Morphological evaluation of the degree of apoptosis was performed by labelling cells with the nuclear stain *p*-phenylenediamine (PPDA) and visualization by fluorescence microscopy [21]. After incubation in the conditions described in each case, cells were collected, washed with phosphate-buffered saline, pH 7.4 (PBS) and fixed at room temperature with 1% paraformaldehyde in PBS for 15 min. Fixed cells were washed with PBS, centrifuged onto glass coverslips in wells of a 24-well plate and mounted on a glass slide over a drop of PPDA stain (10 mg of PPDA in 1 ml PBS mixed with 9 ml of oxidized glycerol) [22].

2.5. Analysis of ceramide production

Jurkat cells (6×10^6 in 10 ml) were labelled for 48 h with 5 μCi of [1-^{14}C]stearic acid bound to fatty acid-free serum albumin (1:1, molar ratio) in complete medium. Cells were then harvested and resuspended in complete medium at 4×10^5 cells/ml. Prior to the addition of anti-Fas, cells were pretreated with 600 μM DEVD-CHO or with the appropriate amount of DMSO (controls) for 3 h [13]. Anti-Fas antibody (at 20 ng/ml) was added and cells were incubated for another 17 h. Then, cells were harvested, washed with cold RPMI medium and counted. Cell viability was determined in an aliquot of the corresponding cell suspensions by the MTT assay. Total cell lipids were extracted at 4°C with chloroform/methanol 2/1 (v/v) [23]. Radioactivity content of aliquots from $CHCl_3$ phases was determined by liquid scintillation counting and equal amounts of radioactivity in each sample were applied to thin-layer chromatography (TLC) Silica gel G plates (Scharlau, Spain). Plates were pre-washed with chloroform/methanol (1/1, v/v) and heat-activated at 110°C for 1 h. A first development of TLC-loaded samples was performed with chloroform/methanol/water (60/30/5, v/v) up to 10 cm from the bottom of the plate and a second development to its full length with hexane/diethyl ether/acetic acid (80/20/2, v/v). Plates were air-dried and radiolabelled bands located by film autoradiography (Hyperfilm β-max, Amersham) at room temperature for 2–3 days. Then, sample lanes were covered with glass, allowing the marker lanes to be iodine-stained. Authentic standards of ceramide, sphingomyelin, cholesterol, phosphatidylcholine and phosphatidylserine (Sigma) were used as markers. Bands were scraped and transferred to vials, to which 4 ml of scintillation cocktail (Normascint 11, Scharlau), containing 10% (v/v) methanol, was added. Radioactivity in samples was determined by liquid scintillation counting.

3. Results and discussion

3.1. Effect of ICE-like protease inhibitors on TNF- and Fas-induced apoptosis

The peptide chlorometylketone YVAD-cmk, developed initially as an irreversible ICE inhibitor [24], can also inhibit CPP32 and perhaps other ICE-like proteases, at least in in vitro cell-free assays [25]. The aldehyde DEVD-CHO, however, has been proved to be a specific inhibitor of the ICE-like protease CPP32, responsible for the cleavage of the apoptosis-related protein poly(ADP-ribose) polymerase (PARP) [26]. We have used these inhibitors to study the implication of ICE-like protease in Fas-induced apoptosis of sensitive Jurkat cells [27]. Overnight incubation with anti-Fas antibody at 5 and 10 ng/ml induced 50% and 75% cell death, respectively (Fig. 1a). Toxicity caused by anti-Fas at 5 ng/ml was eliminated by YVAD-cmk (Fig. 1a). However, and like the report by Enari et al. [13], at a higher anti-Fas concentration, the protection offered by YVAD-cmk (600 μM) was only partial (Fig. 1a). Using the specific CPP32 inhibitor DEVD-CHO in our in vivo assay, and contrary to results obtained with YVAD-cmk, we found a very efficient protection from anti-Fas-induced toxicity on Jurkat cells, at both concentrations tested (Fig. 1a). Indeed, CPP32 has recently been characterized as the main ICE-like protease implicated in Fas-induced death in Jurkat cells [28]. Fas and the 55 kDa TNF receptor belong to the same family of surface receptors, both express death domains in their cytoplasmic tails, and it has been proposed that they could share the death-inducing mechanism [1]. To study the effect of peptide inhibitors on TNF-induced toxicity we used

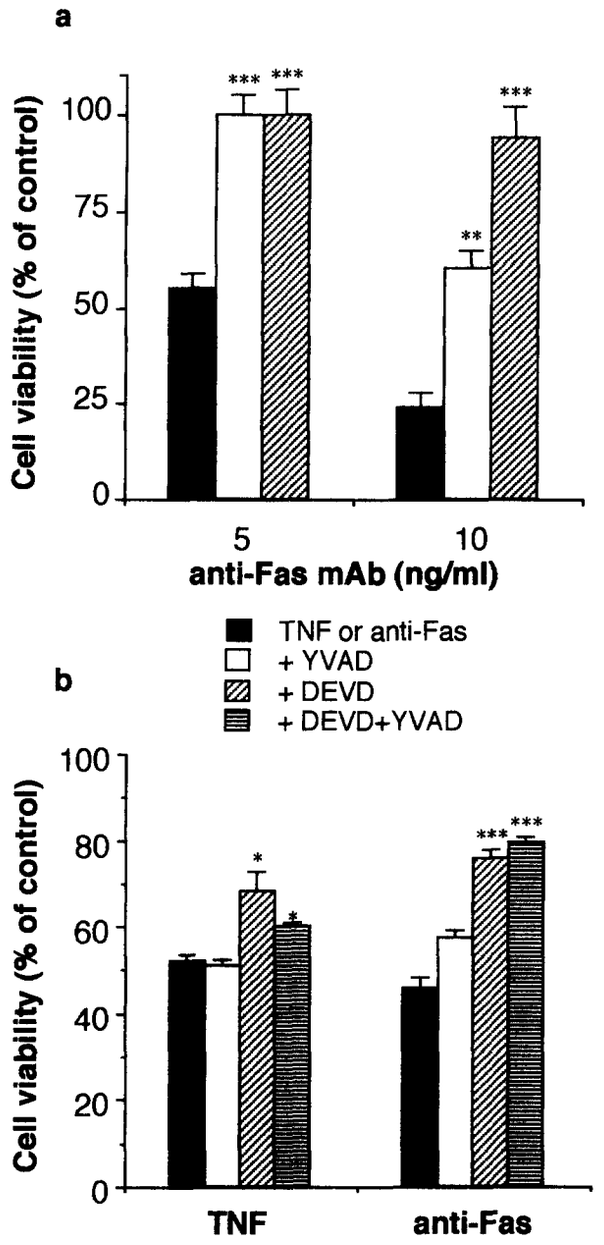

Fig. 1. Effect of ICE-like protease inhibitors on Fas- and TNF-induced cytotoxicity. Jurkat (a) or U937 (b) cells were preincubated at 37°C for 3 h in either control medium, or in medium supplemented with a 600 μM concentration of either YVAD-cmk, DEVD-CHO, or both. Then, anti-Fas (a), and TNF (45 U/ml) or anti-Fas (10 ng/ml) (b), were added and cells incubated for 16 h (anti-Fas) or 24 h (TNF). Cell viability was determined by the MTT assay and expressed as percentage of the values of appropriate controls in each experimental condition. Data are the mean ± S.D. of 4 different determinations. Significant differences from controls are indicated. $^*P<0.05$; $^{**}P<0.005$; $^{***}P<0.0005$.

the TNF- and Fas-sensitive U937 cells, since Jurkat cells are not killed by TNF (data not shown and [29]). As shown in Fig. 1b, TNF toxicity was not prevented by YVAD-cmk. DEVD-CHO offered only a slight protection, not improved by the combination with YVAD-cmk (Fig. 1b). Treatment of U937 cells with anti-Fas at 10 ng/ml induced 55% cell death, which was not prevented by YVAD-cmk, while DEVD-CHO alone or in combination with YVAD-cmk offered significant protection (Fig. 1b). Similar results have been obtained for Fas-based cytotoxicity exerted by T cell effectors on U937, U937-ρ° and L1210Fas cells [22]. These results suggest that, although TN-F and Fas-induced apoptosis could share some biochemical steps, the pathways induced by both receptors should differ beyond a given point. Previous studies have demonstrated that *CrmA*, a cowpox virus-derived serpin, can inhibit both TNF- and Fas-induced apoptosis [15,16]. *CrmA* inhibits ICE [14], and it also inhibits PARP cleavage after Fas or TNF receptor engagement [15]. PARP is not a substrate for ICE [25], while it is a good substrate for CPP32 protease activity [15,26]. These results suggested that *CrmA* blocking of both TNF- and Fas-induced apoptosis was mediated by inhibition of CPP32 activation [15]. By using DEVD-CHO, the direct implication of CPP32 in Fas-induced cytotoxicity is demonstrated in the present and a previous study from our group [22]. Our results also suggest that TNF toxicity could be mediated by a *CrmA*-inhibitable protease different from CPP32.

To analyze the effect of DEVD-CHO on morphological changes induced in Jurkat cells by treatment with anti-Fas antibody, cells were incubated with anti-Fas and/or DEVD, stained with PPDA and analyzed by fluorescence microscopy. Anti-Fas induced in Jurkat cells the typical features of apoptosis, including cell shrinkage, chromatin condensation and extensive nuclear fragmentation (Fig. 2A). However, viability and aspect of cells treated with anti-Fas in the presence of DEVD-CHO was similar to controls and no sign of nuclear fragmentation was detected (Fig. 2B). U937 cells treated with TNF also showed extensive nuclear fragmentation, that was not prevented by either DEVD-CHO, or the combination of YVAD-cmk and DEVD-CHO (data not shown).

3.2. *mtDNA depleted cell lines are sensitive to ceramide-induced apoptosis*

We have previously shown that mitochondrial chain inhibitors or a great reduction in mtDNA content did not prevent TNF and anti-Fas toxicity on U937 cells [18], demonstrating that neither TNF nor Fas-induced toxicity is dependent on intact mitochondrial function. Additionally, U937-ρ° cells lose the expression of the 55 kDa TNF receptor and were no longer sensitive to TNF [18], while in Jurkat-ρ° cells membrane Fas expression was impaired (data not shown). Sphingomyelinase activation and ceramide generation seem to be a common event in TNF- and Fas-induced cytotoxicity [2–4]. We tested therefore if ρ° cells were sensitive to ceramide toxicity. Synthetic (C_2) or natural (C_{18}) ceramide substantially inhibited the growth of normal or ρ° cells after overnight treatment (Fig. 3A). Quantitative differences in the level of toxicity induced by synthetic or natural ceramide were also observed, as shown in Fig. 3A, demonstrating the importance of ceramide hydrophobicity in its function, as already suggested [19]. These results indicate that in spite of the loss of Fas and TNF receptor expression in ρ° cells, the ceramide-activated pathway is intact, and mitochondrial function is not needed for ceramide-induced apoptosis.

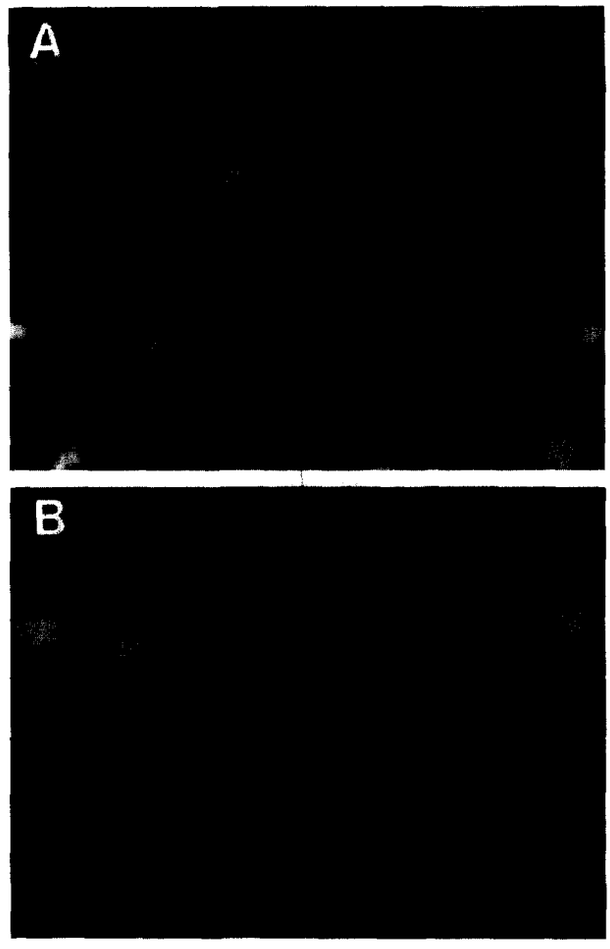

Fig. 2. Fas-induced apoptosis is prevented by CPP32 inhibition. Jurkat cells were treated with anti-Fas (10 ng/ml), as indicated in the legend of Fig. 1, in the absence (A) or in the presence (B) of 600 μM DEVD-CHO. Cell nuclei were stained with PPDA and photographed under epifluorescence illumination. Original magnification, ×400.

Treatment with exogenous ceramide caused morphological features of apoptosis, such as cell shrinkage and nuclear condensation in U937 (Fig. 3B) as well as in U937-ρ° cells (Fig. 3C). However, and contrasting with the extensive nuclear fragmentation observed after anti-Fas treatment of Jurkat cells (Fig. 2A) or TNF treatment of U937 cells [21], ceramide induced a more limited nuclear fragmentation (Fig. 3B,C). Similar results were obtained when comparing normal Jurkat to Jurkat-ρ° cells (data not shown). This result suggests that extensive nuclear fragmentation is mainly dependent on receptor-generated signals other than ceramide production. Nevertheless, nuclear fragmentation is a dispensable event at least in Fas-induced apoptosis [30].

3.3. *ICE-like protease inhibitors do not prevent ceramide-induced apoptosis*

The involvement of sphingomyelinase activation in Fas- and TNF-induced apoptosis is well established [4–6]. Our present data support the previously proposed role of CPP32, at least in Fas-based cytotoxicity [15]. The possibility that sphingomyelinase activation and ceramide generation could

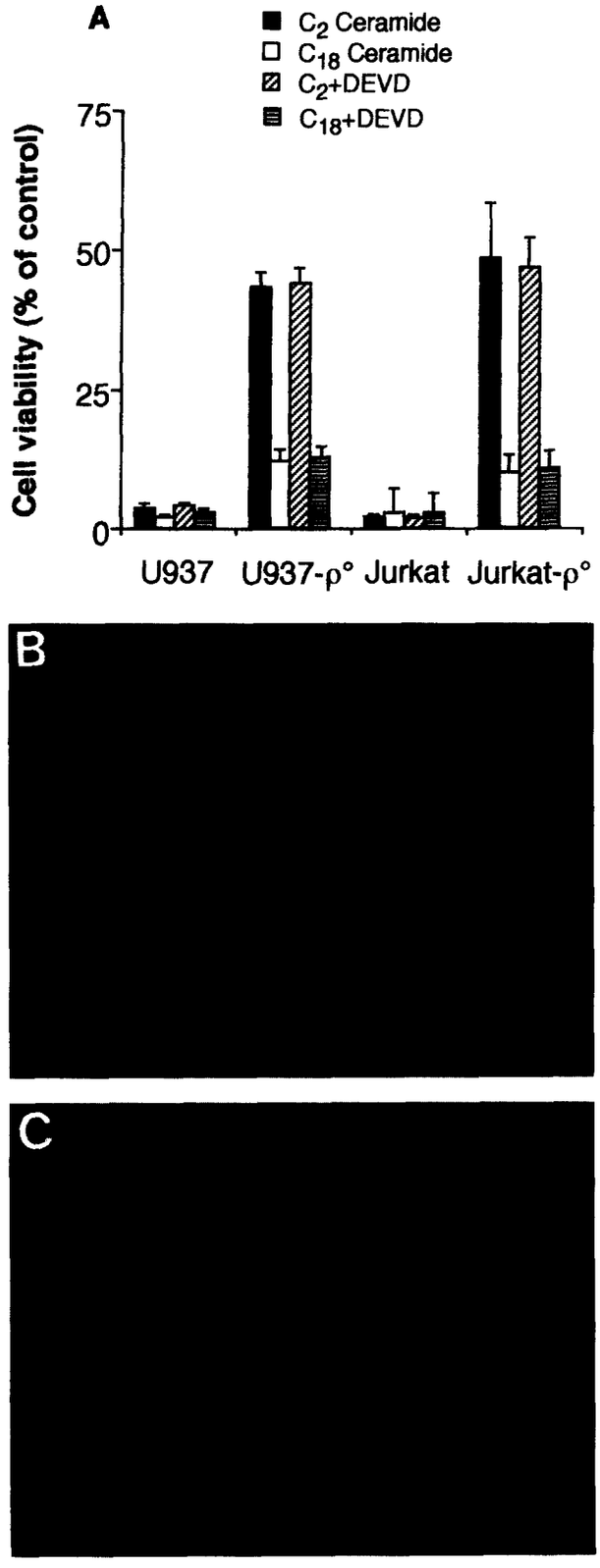

Fig. 3. Ceramide-induced apoptosis in Jurkat, U937 and ρ°-derived cell lines is not prevented by DEVD-CHO. Top: In A, cells were pre-incubated for 3 h either in control medium or in medium containing 600 μM DEVD-CHO. Then, 10 μM C_2-ceramide or 200 nM C_{18}-ceramide, dissolved in ethanol/dodecane 98/2 (v/v), was added and cells were incubated for another 16 h. Final concentration of ethanol/dodecane was 0.4% (v/v). Cell viability was determined by the MTT assay and data are the mean ± S.D. of 4 different determinations. Bottom: Fluorescence micrographs of cells treated for 16 h with 200 nM C_{18}-ceramide and stained with PPDA. B: U937 cells; C: U937-ρ° cells.

lead by a still unidentified mechanism to the activation of ICE-like proteases was explored using U937, Jurkat, and their corresponding ρ°-derived cells. If this was true, ICE-like protease inhibitors should prevent ceramide toxicity. However, as shown in Fig. 3A, ceramide toxicity on all cell types tested was not inhibited by DEVD-CHO, at concentrations that completely blocked anti-Fas induced toxicity on U937 and Jurkat cells (Fig. 1a, Fig. 2). Moreover, DEVD-CHO did not prevent the toxicity caused by the addition to cultures of bacterial sphingomyelinase (data not shown).

3.4. Blocking of anti-Fas-induced ceramide generation by DEVD

The finding that DEVD-CHO did not block ceramide-induced cell death, while it did block Fas-induced apoptosis, suggested that ICE-like protease activation could be an event upstream of sphingomyelinase activation after Fas engagement. To test this possibility, we analysed the effect of DEVD-CHO on Fas-induced ceramide generation. Jurkat cells were labelled with [1-^{14}C]stearic acid for 48 h and treated with anti-Fas antibody in the presence or absence of DEVD-CHO. Cellular lipids were then extracted, separated by TLC and relative levels of ceramide determined as indicated in Section 2. Anti-Fas treatment induced a 2-fold increase in the ceramide to cholesterol ratio and a 2.6-fold increase in the ceramide to sphingomyelin ratio, relative to control cells (Fig. 4). Data were also expressed as ceramide to cholesterol ratio since preliminary experiments indicated that cholesterol labelling remained unchanged, irrespective of cell treatments. Remarkably, DEVD-CHO completely abolished the increase in endogenous ceramide content induced by Fas engagement (Fig. 4). Similar results were obtained when the levels of ceramide were normalised to the cell phospholipid content, as determined by a modified Bartlett's assay [23] (data not shown). In these experiments, the extent of cell death caused by anti-Fas, as monitored by the MTT assay, was around 70% and the protection offered by DEVD-CHO was complete. A similar and parallel increase in ceramide generation and cell death has been recently found in rat SKW6.4 cells treated with anti-Fas [3]. It is also worth noting that the kinetics of Fas-mediated ceramide generation [3] parallels that of PARP cleavage [15].

Sphingomyelinase and ICE-like protease activation have been implicated in several forms of apoptosis and especially in those induced by TNF and Fas [2–5,12,13,15–17,31]. However, the connection between both signalling pathways had not been characterised. Our present results indicate that ICE-like protease(s) activation after Fas engagement is located upstream of sphingomyelinase activation/ceramide generation in human cells. In this respect, it has been recently reported that *reaper*-induced cell death and ceramide generation in insect cells are prevented by ICE-like protease inhibitors [32]. The *reaper* protein from *Drosophila* contains a death domain and is a homologue of the mammalian 55 kDa TNF receptor and Fas [33].

It has been suggested that Fas-induced ceramide generation may be mediated by an acidic sphingomyelinase [2]. However,

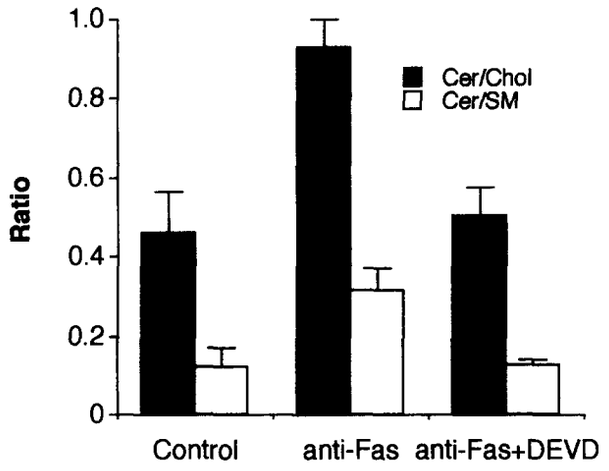

Fig. 4. DEVD-CHO prevents Fas-induced ceramide generation in Jurkat cells. Jurkat cells previously labelled with [1-^{14}C]stearic acid were incubated for 3 h in the presence or absence of DEVD-CHO, as indicated. Then, anti-Fas (20 ng/ml) was added and after incubation for another 16 h, cell lipids were separated by thin-layer chromatography, and radioactivity associated with ceramide (Cer), sphingomyelin (SM) and cholesterol (Chol) determined as indicated in Section 2. Results are expressed as the ratio of radioactivity in the ceramide to cholesterol fractions or ceramide to sphingomyelin fractions. Data are the mean ± S.D. of 3–4 different experiments. Radioactivity recovered in Cer, SM and Chol fractions was greater than 500, 5000 and 1300 cpm, respectively.

another recent and more detailed report demonstrate that ceramide production after Fas engagement is dependent on neutral, and not on acidic, sphingomyelinase [3]. Since ceramide generation by neutral sphingomyelinase takes place presumably at the plasma membrane [4], the present demonstration of CPP32-mediated ceramide generation would suggest a close membrane localisation for this protease. In this respect, it has been reported that ICE is located at the plasma membrane in human monocytes [34]. If CPP32 was also a membrane-associated protein, it could be activated early after Fas crosslinking and the subsequent cleavage of an unidentified substrate would, in turn, stimulate sphingomyelinase. The precise cellular location of CPP32 and the molecular mechanism for CPP32-induced sphingomyelinase activation are not known. In any case, in the present work we demonstrate a close interrelationship between both death-inducing pathways, at least in Fas-induced apoptosis.

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