



# Ceramide-induced apoptosis occurs independently of caspases and is decreased by leupeptin

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## Abstract

Caspase activation is currently proposed as a common feature of apoptosis. However, although the apoptotic events triggered by Fas ligation are well documented, the terminal effectors of the ceramide-induced pathway are not completely identified. In this work, we found that C2-ceramide (Cer)-induced apoptosis was antagonised by leupeptin while Fas-triggered cell death occurred in the presence of this protease inhibitor. Nevertheless, this Cer-induced apoptosis could not be attributed to chymotrypsin, calpain or proteasome activation. In addition, the caspase inhibitor Z-VAD-fmk suppressed Fas-triggered death but did not prevent ceramide-induced apoptosis. In MCF7, a caspase-3-deficient cell line, Cer has been found to induce cell death whereas an anti-Fas IgM (7C11) treatment was inefficient. Moreover, Cer induced apoptosis without DEVDase activation in U937 cell line. Finally, Cer induced an intracytoplasmic calcium release while Fas ligation remained without effect. These results are consistent with the notion that Cer acts, at least in part, independently of Fas signalling, and sheds light on a new caspase 3-free apoptotic pathway triggered by ceramide.

**Keywords:** apoptosis; ceramide; caspase; calpain

**Abbreviations:** 7C11, Fas agonists IgM antibody; Calp, calpeptin; Cer, C2-ceramide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FADD, Fas-associating protein with death domain; HEPES, hydroxyethylpiperazineethanesulfonic acid; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; TPCK N<sup>z</sup>-tosyl-Phe chloromethylketone; UV, ultra-violet; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

## Introduction

Apoptosis is a controlled form of cell suicide that can be triggered by a variety of internal or external signals.<sup>1,2</sup> Ceramide has emerged as a potentially important mediator involved in the effect of a number of natural or pharmacological agents on cell growth, viability, and differentiation.<sup>3,4</sup> This lipid second messenger is generated from sphingomyelin by the activation of a neutral and/or an acidic sphingomyelinase. Agonists of the sphingomyelin-ceramide pathway include cytokines or growth factors such as tumour necrosis factor  $\alpha$ ,<sup>5</sup> interleukin-1- $\beta$ ,<sup>6</sup>  $\gamma$ -interferon,<sup>7</sup> nerve growth factor,<sup>8</sup> and stress-inducing agents such as UV<sup>9</sup> or ionising radiation,<sup>10,11</sup> and antileukemic agents such as daunorubicin.<sup>12–14</sup> The observation that cell-permeant ceramides could mimic the biological effects of most sphingomyelin–ceramide cycle agonists has provided significant weight to the role of ceramide in signal transduction. Ceramide has been shown to exert a wide range of biological effects, depending on the cellular model, including in particular apoptosis in many cellular models such as leukemic U937<sup>5</sup> and lymphoblastoid<sup>15</sup> and endothelial cells.<sup>10</sup> Ceramide has been described to activate stress-activated protein kinases (SAPK/c-Jun kinase), which may be closely related to ceramide-induced apoptosis in U937 and in endothelial cells.<sup>9</sup> In addition, during apoptosis, ceramide has been shown to activate transcription factors such as NF- $\kappa$ B<sup>16</sup> and AP-1.<sup>17</sup> Nevertheless, the terminal effectors of the sphingomyelin-ceramide apoptosis pathway remain elusive.

Recent studies have demonstrated that specific protease activation is a critical element of the apoptotic process. In most systems described to date, the proteases implicated are members of a cysteine protease family which share an absolute requirement for aspartic acid in the P1 position of proteolytic substrates.<sup>18–20</sup> The twelve described members of the mammalian family of these proteases, which are present as inactive precursors in resting cells, have been termed caspases (for cysteinyl aspartate-specific proteinase).<sup>21</sup> During apoptosis, these zymogenes are cleaved at Asp-X sites thus generating two subunits, which together constitute the active protease. Activation of effector caspases, such as caspase 3, which hydrolyse target proteins exhibiting a DEVD sequence, is considered as the common event initiated by the multiple stimuli that induce apoptosis.

Fas (APO-1) is a 45-kD membrane receptor which, when engaged by Fas ligand or anti-Fas antibody, triggers programmed cell death.<sup>22</sup> The death pathway initiated by Fas activation is now well documented and involves a series of death-inducing molecules. FADD (Fas-associating protein with death domain) is recruited to Fas upon its engagement,<sup>23,24</sup> leading to the activation of procaspase<sup>8,25–27</sup> followed by activation of other caspases and ending by caspase 3.<sup>28</sup>

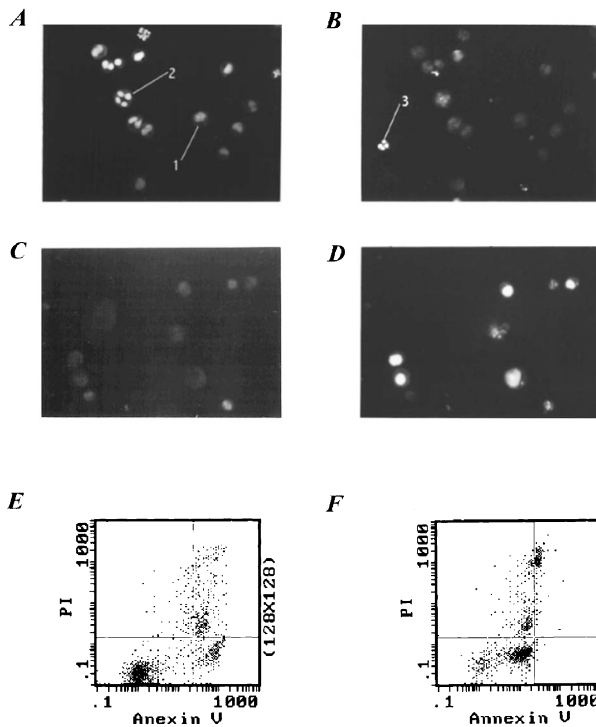
Shu-Ching *et al*, have shown that Fas-mediated and ceramide-induced death pathways are not sensitive to the same inhibitors<sup>29</sup> and conclude that ceramide is not the mediator of Fas- initiated apoptosis. However, the role of caspase as a terminal effector in the ceramide-induced pathway of apoptosis remains to be elucidated. Therefore, we investigated Fas-mediated and Cer-induced apoptosis in two human cell lines: the U937 leukemic cell line and the MCF7 caspase 3 deficient cell line.

## Results

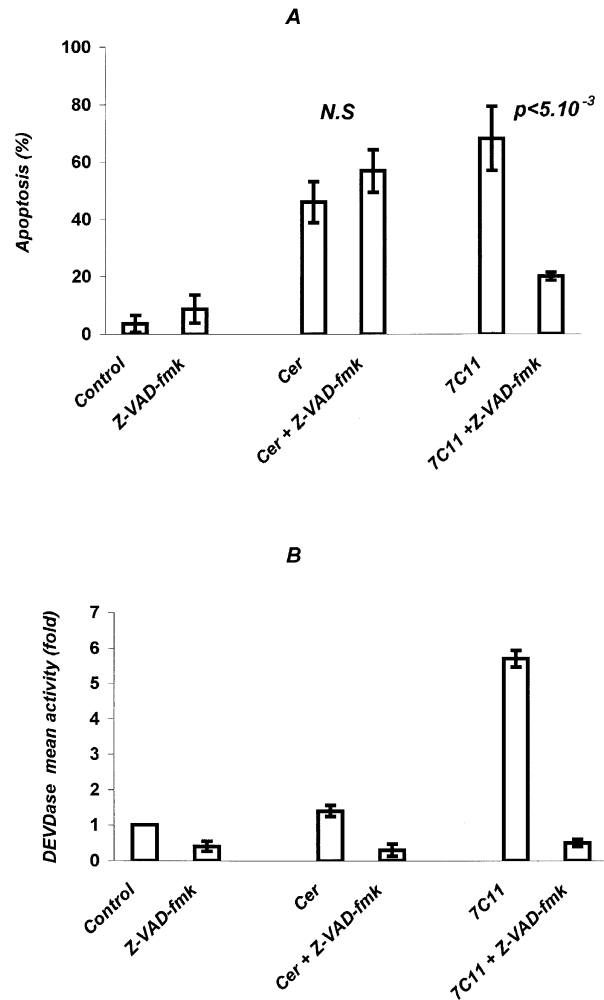
### Ceramide induces apoptosis and not necrosis in U937

Figure 1 shows the effect of Cer and H<sub>2</sub>O<sub>2</sub> on U937 cells. The cells were double stained with both the cell permeant acridine orange and the cell impermeant propidium iodide nucleic acid probes. After Cer treatment (Figure 1A and B), cells with green fluorescent nuclei (due to the binding of acridine orange to DNA), exhibiting characteristic features of apoptosis such as nuclear fragmentation and condensation were observed besides remaining intact cells (Figure 1A). The cytoplasm of non-permeabilised cells fluoresced in red due to the binding of

acridine orange on RNA (Figure 1B). Conversely, when the cells were treated by high concentrations of H<sub>2</sub>O<sub>2</sub> (Figure 1C and D), the nuclei were large, diffuse and brightly red fluorescent suggesting that these cells were permeable to propidium iodide and thus were necrotic cells (Figure 1D). Some red fluorescent nuclei were also observed in Figure 1B but they always were also fractionated nuclei and thus very late stages of apoptosis (secondary necrosis). These results were confirmed by analysis of phosphatidylserine exposure and permeability to propidium iodide by flow cytometry (Figure 1E and F). When treated with Cer, some U937 cells became brightly positive for annexin V binding but remained imperme-



**Figure 1** Ceramide induces apoptosis in U937 cells. U937 cells were treated with either 50 μM Cer (A, B, E) or 1 mM H<sub>2</sub>O<sub>2</sub> (C, D, F) for 18 h. The cells were then stained with 3 μg/ml acridine orange and 3 μg/ml propidium iodide (PI) and analyzed by fluorescence microscopy (× 100) through green (A, C) or red (B, D) emission band-pass filters. Arrow 1 points to an intact cell, arrow 2 to an apoptotic cell and arrow 3 to a cell in late apoptosis. The cells were also stained with FITC-Annexin V and PI and analyzed by flow cytometry (E, F). Green fluorescence (Annexin V) is plotted on the x axis and red fluorescence (PI) on the y axis



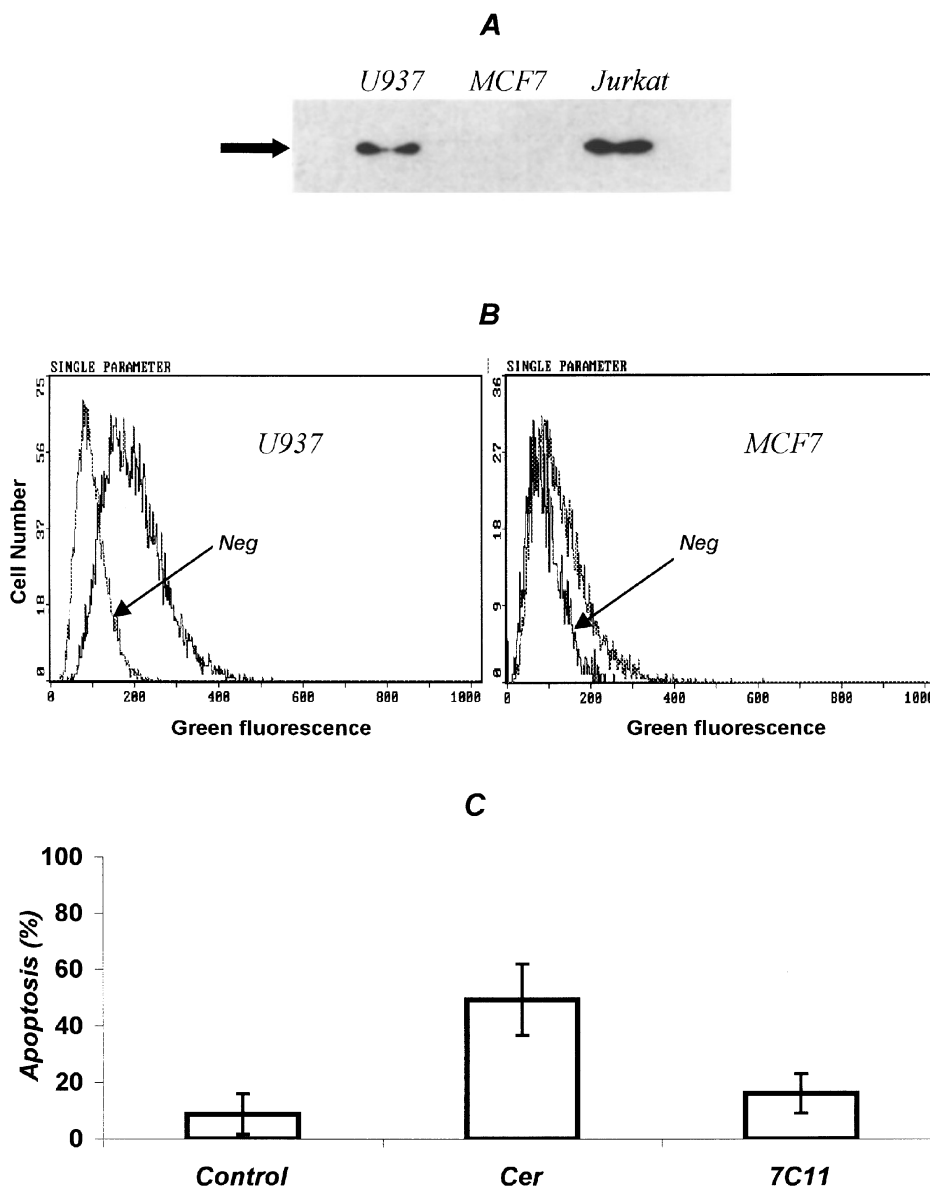
**Figure 2** Effect of caspase inhibition on the apoptotic events induced by Cer of Fas ligation. U937 cells were preincubated in the presence or the absence of Z-VAD-fmk for 1 h when indicated and either 50 μM Cer, or 0.1 μg/ml anti-Fas IgM (7C11) were added for 18 h. (A) the apoptotic cells with condensed chromatin and/or fractionated nucleus were identified by fluorescence microscopy after acridine orange staining. The percentage of apoptotic cells was plotted for each treatment group (mean ± S.D. from three separate experiments). (B) The DEVDase activity was measured as described in Materials and Methods using a fluorogenic substrate and spectrofluorimetry. A control without apoptosis inducer was processed for each experiment. The relative caspase activity (activity of the sample/activity of the untreated control) was plotted for each treatment group (mean ± S.D. from three separate experiments)

able to propidium iodide (Figure 1E), while when treated with H<sub>2</sub>O<sub>2</sub>, most U937 cells exhibited a red fluorescence concomitant with a partial accessibility of phosphatidylserines to annexin V (Figure 1F). It was thus concluded that, unlike H<sub>2</sub>O<sub>2</sub>, ceramide induced apoptosis but not necrosis in U937 cells.

### DEVD specific caspase activation is not necessary for Cer-induced apoptosis

The apoptosis potential was evaluated in U937 cells in response to either Cer or Fas ligation. Both Cer and 7C11

produced a significant amount of apoptosis cells after overnight incubation. This was found at the nucleus level by analyzing chromatin condensation and/or nucleus fragmentation (Figure 2A), and at the mitochondrial level by measuring membrane potential with DiOC6(3) and flow cytometry (data not shown). When the cells were pretreated for 1 h with Z-VAD-fmk, a wide spectrum caspase inhibitor, Fas-driven apoptosis was significantly reduced while Cer-induced apoptosis was not inhibited. This suggests that at least one VAD-inhibitable caspase necessary for Fas apoptosis is not involved in Cer apoptosis. The DEVDase activity was



**Figure 3** (A) Procaspase 3 expression in U937, MCF7 and Jurkat cells. Untreated U937, MCF7 and Jurkat cells were analyzed for their procaspase 3 content by Western blot using an anti-CPP32/Caspase-3 antibody (total protein content from a lysate of  $5 \times 10^5$  cells). The arrow indicates the 32 kD procaspase. (B) Fas expression in U937 and MCF7 cells. U937 and MCF7 cells were labelled with FITC-coupled anti-CD95 IgG and analyzed by flow cytometry. The fluorescence histograms are shown. Neg indicate histograms obtained with an isotopic unrelated FITC-coupled IgG as a negative control. (C) Effect of Cer and Fas ligation on MCF7 cell apoptosis. MCF7 cells were treated for 18 h with either 50  $\mu$ M Cer or 0.1  $\mu$ g/ml 7C11. The cells were then trypsinised, and analyzed for apoptosis at the nucleus level with acridine orange and fluorescence microscopy. The results show the percentage of apoptotic cells for each treatment group (mean  $\pm$  S.D. from three separate experiments)

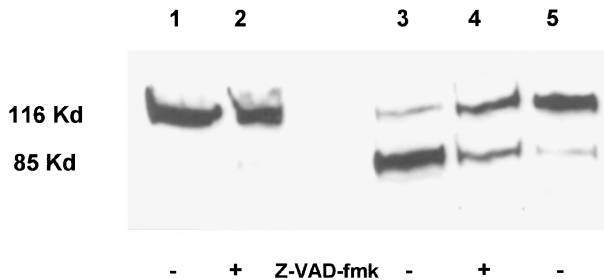
measured using a fluorogenic substrate (Figure 2B) after either Cer or 7C11 treatment. While 7C11 induced a sixfold increase in DEVDase activity, the latter was only 1.5-fold that of the control after Cer treatment. This activity was completely abolished in all the Z-VAD-fmk treated samples. Thus, no activation of DEVDase occurred in Cer-treated U937 cells, which could account for the lack of effect of Z-VAD-fmk on apoptosis. Because caspase 3 constitutes the major part of DEVDase and because it is thought to be the final caspase effector in apoptosis, we checked whether caspase 3 activity was necessary for Cer apoptosis induction.

### Caspase 3 is necessary for Fas-mediated but not for Cer-induced apoptosis

MCF7, a human breast carcinoma cell line, has been previously described as deficient in procaspase 3.<sup>30,31</sup> It was thus interesting, to verify whether MCF7 responded differently to Cer and 7C11 treatments. Figure 3A confirms that no procaspase protein could be detected in MCF7 cells while it could easily be revealed in U937 and Jurkat cells. Fas was expressed on the MCF7 membrane at a level which was comparable with its expression in U937 cells as measured by immuno-fluorescent labelling and flow cytometry analysis (Figure 3B). However, while a consistent level of apoptotic cells was observed in response to Cer treatment, 7C11 was without effect on apoptosis induction (Figure 3C). These results confirm that caspase 3 is not involved in the Cer pro-apoptotic effect but is essential for Fas-mediated apoptosis.

### Cer-induced apoptosis occurs through activation of a leupeptin-sensitive protease

Because poly(ADP-ribose) polymerase (PARP) is one of the natural *in-situ* substrates for DEVD-specific caspases, it was interesting to analyse PARP cleavage in the cells after Cer and 7C11 treatment. After 18 h, the latter resulted in an almost complete cleavage of PARP (Figure 4, lane 3). It may be noticed that Z-VAD-fmk only partly inhibited PARP cleavage after Fas ligation (Figure 4, lane 4 compared to

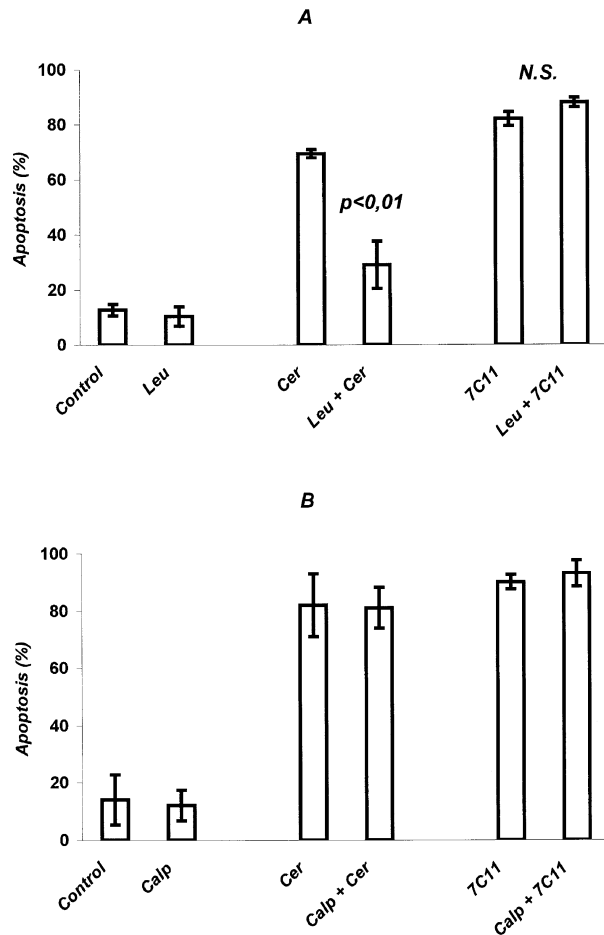


**Figure 4** Effects of Cer, Fas ligation and caspase inhibitor on the PARP cleavage. U937 cells were preincubated in the presence or the absence of Z-VAD-fmk for 1 h when indicated and either 50  $\mu$ M Cer, or 0.1  $\mu$ g/ml 7C11 were added for 18 h. The cell extracts were then processed for SDS-PAGE and Western blot using an anti-PARP monoclonal antibody as a probe. The positions of the 116 kD intact PARP and of the 85 kD cleaved PARP fragment are indicated. Lane 1, untreated control; lane 2, Z-VAD-fmk treated cells; lane 3, 7C11 treated cells; lane 4, Z-VAD-fmk and 7C11 treated cells; lane 5, Cer treated cells

lane 3) while apoptosis and DEVDase activity were completely abolished (Figure 2). When the cells were treated by Cer, a faint cleaved fragment of PARP was detected (Figure 4, lane 5), due either to the low activation of DEVD-specific caspases (Figure 2) or to another protease.

To characterise such an hypothetical protease, several protease inhibitors were assayed for their ability to inhibit Cer-induced apoptosis. One of them, leupeptin, a wide spectrum inhibitor, was found to strongly inhibit Cer-induced apoptosis, but remained without effect on Fas-mediated apoptosis (Figure 5A), thus suggesting that Cer-induced apoptosis occurs through the activation of an unknown leupeptin-inhibitible protease.

A Western-blot analysis showed that after Cer activation the PARP cleavage was not observed in MCF7 cells, which were deficient in caspase 3 (data not shown). Moreover, in U937, Cer activation induced a faint PARP cleavage which was not inhibited by leupeptin (data not shown). Therefore, the PARP cleavage observed in Cer-treated U937 cells



**Figure 5** Effect of protease inhibitors on the apoptosis induced by Cer or Fas ligation. U937 cells were pretreated for 1 h with either 20  $\mu$ M leupeptin (A) or 100  $\mu$ M calpeptin (B). Either 50  $\mu$ M Cer, or 0.1  $\mu$ g/ml 7C11 were then added for 18 h. Apoptosis was determined by fluorescence microscopy after acridine orange staining. A control without apoptosis inducer was processed for each experiment. The percentage of apoptotic cells was plotted for each treatment group (mean  $\pm$  S.D. from three separate experiments)

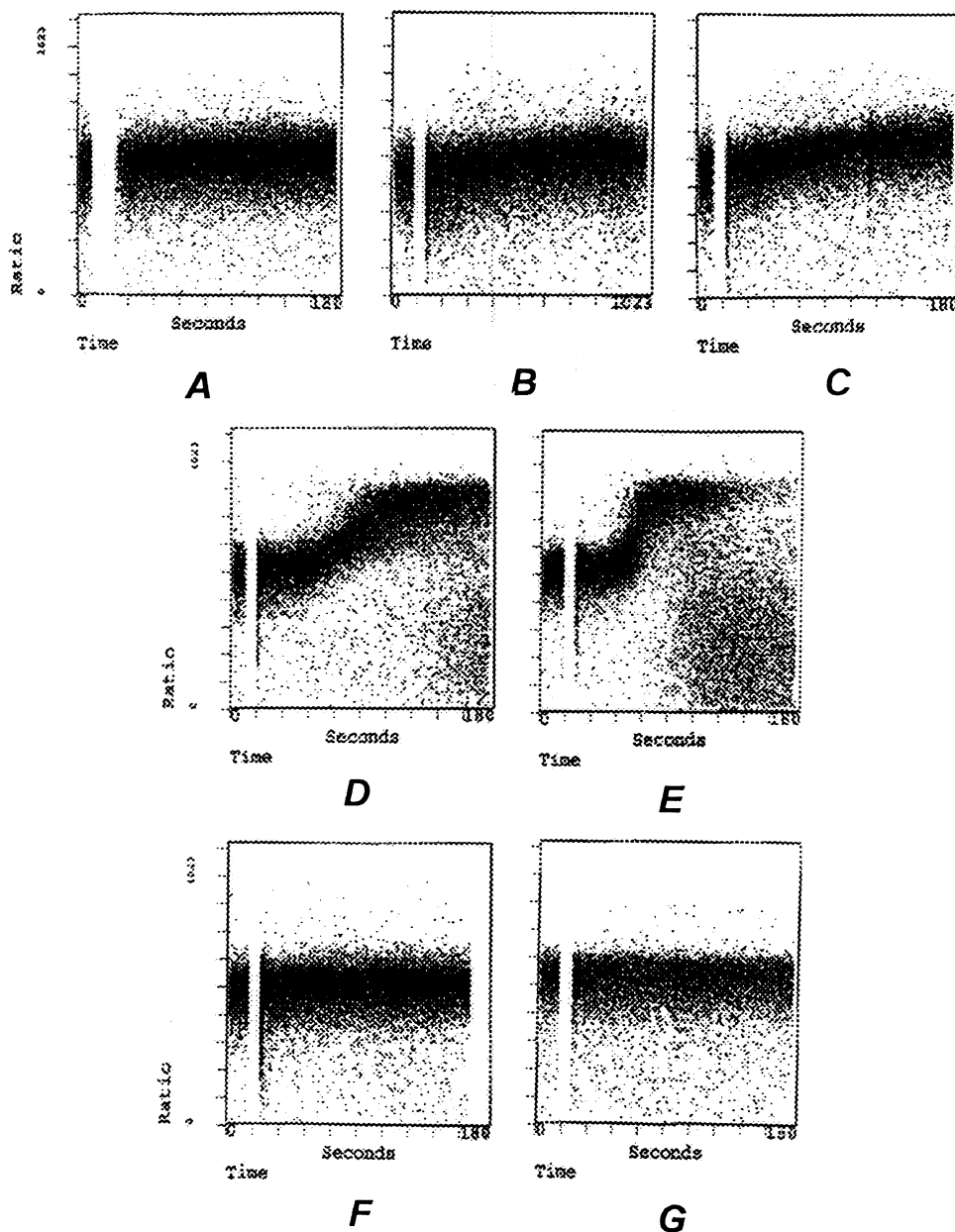
cannot be attributed to the Cer-activated-leupeptin-inhibitable protease, so the low DEVDase activation observed in Figure 2 in these conditions could be sufficient to explain the cleavage observed.

Leupeptin has been previously reported to inhibit calpain.<sup>32</sup> Thus a specific calpain inhibitor, calpeptin (calp), was also tested but was found to be without effect on both Cer and 7C11-induced apoptosis (Figure 5B). Similarly, TPCK, an inhibitor of chymotrypsin was found to have no effect, while MG-132, a specific inhibitor

of proteasome<sup>33</sup> induced apoptosis by itself (data not shown).

### Cer induces a release of cytoplasmic Ca<sup>2+</sup> while 7C11 does not

We used flow cytometry to monitor the release of Ca<sup>2+</sup> in the cytoplasm of U937 cells after Cer or 7C11 treatment. It was found (Figure 6) that Cer induced a release of Ca<sup>2+</sup> in a dose-dependent way. The higher the Cer concentration, the faster



**Figure 6** Comparative effects of Cer and Fas ligation on the cytoplasmic Ca<sup>++</sup> release in U937 cells. U937 cells were loaded with Indo 1-AM (4.5 μM) for 90 min 37°C. The indicated amount of inducer was then added in an aliquot and the fluorescence ratio 410/480 nm was measured on a cell-by-cell basis using flow cytometry. The analysis was performed at a rate of 200 events/second for 3 min. The ratio was plotted as a function of the time after injection of 0 (A), 50 (B), 75 (C), 100 (D), or 200 (E) μM Cer. Two concentrations 0.1 (F) and 1 μg/ml (G) of 7C11 were also assayed. The empty region in the kinetics corresponded to the addition of the inducer

the release. A 2–4-fold increase in Cer concentration was accompanied by a steep increase in cytoplasmic  $\text{Ca}^{2+}$ , and was followed by a cell toxicity which appeared as a low  $\text{Ca}^{2+}$ -containing population on Figure 6D and E. On the contrary, Fas ligation was not followed by a short-term change in cytoplasmic  $\text{Ca}^{2+}$  content, even when the 7C11 concentration was increased tenfold (Figure 6F and G). This experiment strongly suggests that Cer-induced and Fas-driven apoptosis occur through different cytoplasmic events which lead to similar nuclear and mitochondrial effects.

## Discussion

When caspase 3 was identified as the mammalian analogue of the *C. elegans* ced-3 gene product,<sup>34</sup> it was suggested that this protease could be a confluent effector for all the apoptosis pathways. A caspase 3 substrate was then described in mice which, after cleavage, released an active DNase responsible for the typical internucleosomal fragmentation of chromatin during apoptosis.<sup>35</sup> However, mice with an homozygous deletion in the *CASP-3* gene were able to complete a normal development of all organs except the brain,<sup>36</sup> suggesting that alternative apoptosis pathways could be effective independently of caspase 3.

We investigated in this study the role of caspases in Cer-induced and Fas-mediated apoptotic pathways in U937 cells, and confirm that both pathways result in chromatin condensation and nuclei fragmentation as observed by fluorescence microscopy. However, Z-VAD-fmk, a potent inhibitor of caspase activation, was found to be without effect on Cer-induced apoptosis, while it completely abolished Fas-mediated nuclei fragmentation. Moreover, Cer-induced apoptosis was accompanied only by a very faint activation of DEVD-specific caspases. Because caspase 3 is considered to be the main DEVD-specific caspase, it was interesting to verify if caspase 3 was essential for Cer-induced apoptosis. We addressed this question with the caspase-3 deficient MCF-7 cell line which has been described as having a functional deletion of the *CASP-3* gene.<sup>30</sup> We found that MCF-7 cells were competent to complete nuclear apoptosis in response to Cer, but were not so in response to the Fas agonist antibody 7C11 although they expressed Fas. This strongly supports the idea that caspase 3 is not necessary for Cer-induced apoptosis. It has been previously described in MCF-7 cells that caspase 3 is not essential for tumour necrosis factor- or staurosporine-induced cell death, but is required for DNA fragmentation and typical apoptotic morphological changes.<sup>30</sup> Assuming that chromatin condensation and nuclei fragmentation result from DNA cleavage,<sup>37</sup> it is probable that caspase 3 is not essential for DNA fragmentation in response to Cer treatment.

We have also shown that Cer-induced apoptosis resulted only in a minimal PARP cleavage, while almost all the PARP was cleaved after Fas ligation in U937 cells. It is possible that the very low DEVDase activation observed in Figure 2 was sufficient to partially cleave PARP (Figure 4 and data not shown). This would be sufficient to complete as much apoptosis as with Fas ligation. However, no PARP cleavage at all was observed in MCF7 cells in response to

Cer, while a significant amount of apoptotic nuclei was detected. This weakens the claim that PARP cleavage is a universal marker for apoptosis and disagrees with previous results where PARP cleavage and Z-VAD-fmk inhibition of cell death were described in MCF7 cells, in the absence of caspase 3, after tumour necrosis factor  $\alpha$ /cycloheximide or staurosporine treatment.<sup>31</sup> This further suggests that Cer-induced apoptosis uses a specific pathway independently of DEVD-specific caspase activation, which would be essential for Fas-, tumour necrosis factor  $\alpha$ - and staurosporine-mediated apoptosis.

Such an hypothesis is reinforced by our finding that Cer-induced apoptosis was inhibited by leupeptin, while Fas-mediated apoptosis was insensitive to this protease inhibitor. Since leupeptin was without effect on DEVD-specific caspase,<sup>34</sup> this suggests that Cer treatment might activate another protease or protease family which would be sufficient to complete apoptosis in the nucleus. The protease, which was not inhibited by the calpain inhibitor calpeptin, was not responsible for the partial PARP cleavage which was observed after Cer treatment in U937 cells, suggesting that activation of this protease does not necessarily exclude a partial activation of caspase 3 when possible. This ceramide-activated protease resembles the mitochondrial apoptosis inducing factor (AIF) which has been recently characterised.<sup>38</sup> However, it is not known if AIF is inhibited by leupeptin.

Finally, we show that Cer treatment is followed by a short-term, dose-dependant release of  $\text{Ca}^{2+}$ , while Fas ligation remains without effect on the cytoplasmic  $\text{Ca}^{2+}$  content whatever the dose. This result is also in agreement with the existence of different apoptotic pathways for Cer-induced and Fas-mediated apoptosis, although both resulted in a decreased mitochondrial membrane potential, a condensed chromatin and typical nuclear morphological changes.

## Material and Methods

### Cells and reagents

The experiments were performed on human monocytic U937, lymphocytic Jurkat, and MCF7 breast carcinoma cell lines. The cells were maintained in RPMI 1640 (Gibco BRL, Eragny, France) supplemented with 10% foetal calf serum, 1 mM glutamine (Gibco BRL, Eragny, France), 10 mM HEPES (Gibco BRL, Eragny, France), 100 units/ml penicillin (Gibco BRL, Eragny, France), 50  $\mu\text{g}/\text{ml}$  streptomycin (Gibco BRL, Eragny, France), in a humidified 5%  $\text{CO}_2$ , 37°C warmed incubator. Cells were incubated with several drugs. The caspase inhibitor Z-VAD-fmk (20  $\mu\text{M}$ , Calbiochem, Meudon, France) was added 1 h before inducing apoptosis with either 50  $\mu\text{M}$  Cer (C2-Ceramide, Molecular Probes, Interchim, Montluçon, France) or 0.1  $\mu\text{g}/\text{ml}$  anti-Fas IgM (7C11, Immunotech, Marseille, France). Other protease inhibitors such as leupeptin (20  $\mu\text{M}$ , Sigma, St. Quentin Fallavier, France), the calpain specific inhibitor, calpeptin (100  $\mu\text{M}$ , Calbiochem, Meudon, France), the chymotrypsin inhibitor TPCK (10  $\mu\text{M}$ , Calbiochem, Meudon, France), or the proteasome inhibitor MG-132 (30  $\mu\text{M}$ , Calbiochem, Meudon, France) were used in the same way. All experiments were done on exponentially growing cells.

## Apoptosis determination

For all cell lines, apoptosis was determined after 18 h of either Cer or 7C11 treatment by using acridine orange and DiOC<sub>6</sub>(3) assays. The acridine orange assay consisted in staining of cells in their culture medium with 3 µg/ml acridine orange and 3 µg/ml propidium iodide (Molecular Probes, Interchim, Montluçon, France) and fluorescence microscopic analysis. Cells with green fluorescent condensed chromatin and/or fractionated nucleus were scored as apoptotic.<sup>39</sup> The mitochondrial potential was assayed with DiOC<sub>6</sub>(3). Cells were incubated in culture medium for 30 min at 37°C with 100 ng/ml DiOC<sub>6</sub>(3) (Molecular Probes Interchim, Montluçon, France) and analyzed by flow cytometry as previously described.<sup>39–41</sup> Low mitochondrial potential cells were scored as apoptotic.

## Fluorogenic protease assays

DEVDase activity was assayed as previously described.<sup>39</sup> Briefly, cells ( $5 \times 10^5$ ) were washed in PBS and then suspended in 50 µl of a pH 7.4 buffer containing 72 µM Ac-DEVD aminomethylcoumarin (UBI, Euromedex, France). The cells were incubated for 10 min at 37°C and then centrifuged for 3 min at 14 000 r.p.m. The supernatant was diluted in 1 ml dH<sub>2</sub>O. Fluorescence was measured using a Jobin and Yvon spectrofluorometer ( $\lambda_{exc}=380$  nm,  $\lambda_{em}=480$  nm). A blank was performed without addition of cells in the substrate buffer and its value was subtracted from all the measurements. A control without any drug treatment was processed for each experiment. The relative enzyme activity was expressed as the ratio treated sample activity/untreated control activity.

## Western blot analysis

Western blots were performed as previously described.<sup>42</sup> PARP cleavage was analyzed in 7.5% acrylamide gel and procaspase 3 expression in a 10% acrylamide gel. Antibodies used were 1 µg/ml anti PARP (Zymed, Clinisciences, Montrouge, France) and 2 µg/ml anti CPP32/Caspase-3 (clone 19, Transduction Laboratories, Interchim, Montluçon, France).

## Fas expression

Fas expression was determined by direct immunolabelling on living U937 and MCF7 cells as previously described.<sup>42</sup> Negative control were performed with an isotypic unrelated FITC-coupled IgG. The fluorescence histograms are shown in Figure 3B.

## Intracytoplasmic calcium release

Intracytoplasmic calcium release was monitored by flow cytometry with the fluorescent calcium chelator Indo-1 acetoxymethyl ester (Indo-1 AM, Molecular Probes, Interchim, Montluçon, France) as previously described.<sup>43</sup> Briefly,  $3 \times 10^5$  U937 cells in 1 ml of culture medium were loaded with the fluorochrome by incubating with 4.5 µM Indo-1 AM for 90 min at 37°C. The cells were analyzed by flow cytometry, the blue fluorescence was split by a long pass 450 nm interferential filter and the resulting fluorescences were measured through band filters of  $410 \pm 10$  and  $480 \pm 10$  nm respectively. The ratio of 410/480 fluorescences was calculated on a cell by cell basis by the cytometer and plotted as a function of time. The vector (ethanol), Cer (50 to 200 µM) or 7C11 (0.1 and 1 µg/ml) solution were added after 15 s of analysis and the analysis was prolonged for 3 min.

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