Temporal relationships between ceramide production, caspase activation and mitochondrial dysfunction in cell lines with varying sensitivity to anti-Fas-induced apoptosis

Claire RODRIGUEZ-LAFRASSE[1], Gersende ALPHONSE, Pierre BROQUET, Marie-Thérèse ALOY, Pierre LOUISOT and Robert ROUSSON
INSERM U189, Department of Biochemistry, Lyon-Sud Medical School, BP12, 69921 Oullins Cedex, France

To clarify the chronology of events leading to anti-Fas-induced apoptosis, and the mechanisms of resistance to this death effector, we compared the response kinetics of three tumour cell lines that display varying sensitivity to anti-Fas (based on levels of apoptosis), in terms of ceramide release, mitochondrial function and the caspase-activation pathway. In the highly sensitive Jurkat cell line, early caspase-8 activation, observed from 2 h after treatment, was chronologically associated with an acute depletion of glutathione and the cleavage of caspase-3 and poly-ADP ribosyl polymerase (PARP), followed by a progressive fall in the mitochondrial transmembrane potential ($\Delta\psi_m$), between 4 and 48 h after treatment. Ceramide levels began to increase 2 h after the addition of anti-Fas (with no increase during the first hour), and increased continuously to 640 % of control cells at 48 h. In the moderately sensitive SCC61 adherent cells, comparable results were observed, though with lower levels of ceramide and a delay in the response kinetics, with apoptotic cells becoming flotant. Finally, despite early cleavage of caspase-8 at 2 h, and a sustained level of activation until 48 h, no apoptotic response was observed in anti-Fas-resistant SQ20B cells. This was confirmed by a lack of ceramide generation and mitochondrial changes, and by the absence of any detectable cleavage of caspase-3 or PARP. Inhibition of caspase processing, and amplification of endogenous ceramide signalling by pharmacological agents, allowed us to establish the order of cellular events, locating ceramide release after caspase-8 activation and before caspase-3 activation, and demonstrating a direct involvement for ceramide release in mitochondrial dysfunction. Furthermore, these experiments provide strong arguments for the role of endogenous ceramide as a key executor of apoptosis, rather than as a consequence of membrane alterations.

Key words: Jurkat cells, SCC61 cells, sphingolipid metabolism, SQ20B cells.

INTRODUCTION

In the current paradigm for apoptotic cell death, the stimulation of the death receptor CD95 (apo1/Fas) by anti-Fas ligation initiates several distinct cellular events, involving protein or lipid mediators. The ligand–receptor interaction results in oligomerization of the intracellular death domain, and leads to the recruitment of two key signalling proteins, the adapter protein FADD (Fas-associated death domain, also called MORT-1) and caspase-8 [1], which form a death-inducing signalling complex (DISC) [2]. Downstream from caspase-8 activation by proteolytic cleavage, caspase-3 seems to be the most important effector caspase in this apoptotic pathway [3]. More recently, mitochondria have been identified as a central control point for apoptosis (for review, see [4]). Early loss of mitochondrial transmembrane potential ($\Delta\psi_m$) can be measured in response to anti-Fas together with the formation of permeability transition pores. At the same time, cytochrome *c* is inactivated and then released into the cytoplasm [5]. Cytoplasmic cytochrome *c* forms an essential part of the 'apoptosome', together with the protease-activating factor-1 (Apaf-1) and pro-caspase-9, which leads finally to caspase-3 activation [6]. From these observations, Scaffidi et al. [5] distinguished two different cell types (type I and type II) that use distinct CD95 apoptosis signalling pathways. Type-I cells use a mitochondrion-independent pathway, accompanied by high levels of caspase-8 activation by the DISC, whereas in type-II cells (such as Jurkat cells), DISC formation is strongly reduced and activation of caspase-8 and caspase-3 occurs after the loss of $\Delta\psi_m$.

Other research groups have focused on the signalling pathway involving the lipid mediator, ceramide, also implicated in death-receptor-mediated apoptosis (for review, see [7]). For instance, it has been demonstrated that the anti-Fas response involves delayed ceramide formation [8] and cytochrome *c* release [9] in Jurkat cells, although the authors have excluded a role for ceramide in the induction of caspase activation, or in conveying the apoptotic signal to the mitochondria.

The mechanism by which ceramide mediates apoptosis has not yet been fully addressed, although mitochondria are known to be ceramide targets. The addition of short-chain ceramides to isolated mitochondria or cell cultures has been reported to induce direct inhibition of complex III of the mitochondrial respiratory chain [10], the generation of reactive oxygen species [11,12], cytochrome *c* release and a decrease in transmembrane potential [13]. However, it is not clear whether short-chain analogues can mimic the behaviour of naturally occurring long-chain ceramide species exactly.

On the other hand, anti-Fas-resistance has been observed in different cell lines. Recently, anti-Fas-resistance in specific clones of melanoma cells has been attributed to a defect upstream from mitochondrial cytochrome *c* [14]. Moreover, in 11 variant clones of this cell line, susceptibility to anti-Fas and to short-chain

Abbreviations used: $\Delta\psi_m$, mitochondrial transmembrane potential; DISC, death-inducing signalling complex; DL-PDMP, D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl; FADD, Fas-associated death domain; JC1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzylimidazolylcarbocyanine iodide; PARP, poly-ADP ribosyl polymerase; TBS, Tris-buffered saline; Z-VAD-fmk, Z-Val-Ala-D,L-Asp-fluoromethylketone; Z-DEVD-cmk, Z-Asp-Glu-Val-Asp-chloromethylketone.

[1] To whom correspondence should be addressed (e-mail rodriguez@lyon-sud.univ-lyon1.fr).

ceramides was clearly correlated. However, the lack of quantification of intracellular ceramide levels does not permit an assessment of its participation in cellular resistance to anti-Fas.

To clarify the role of ceramide and mitochondrial changes in the chronology of events leading to anti-Fas-induced apoptosis or resistance, we here compare the response kinetics of three tumour cell lines (Jurkat, SCC61 and SQ20B cells), which display a gradient of sensitivity to anti-Fas (based on the percentage of apoptotic cells), in terms of ceramide release, mitochondrial function and the caspase-activation pathway.

MATERIALS AND METHODS

Chemical and biological reagents

Ceramide (type III) from bovine brain sphingomyelin, sphingomyelin, sphingosine, sphinganine and other sphingolipid derivatives were purchased from Sigma (St Louis, MO, U.S.A.), as was *o*-phthalaldehyde. Eicosasphinganine, used as internal standard, was the generous gift of Dr A. Merrill (Emory University School of Medicine, Atlanta, GA, U.S.A.). Anti-CD-95 monoclonal antibody (7C11 clone) was supplied by Immunotech (Marseille, France). Anti-caspase-8 monoclonal antibody was supplied by Euromedex (Mundolsheim, France), anti-caspase-3 polyclonal antibody by R&D Systems (Abingdon, Oxon, U.K.), and anti-(poly-ADP ribosyl polymerase) (PARP) monoclonal antibody by Pharmingen (Hamburg, Germany). The associated secondary antibodies were, respectively, horseradish peroxidase-conjugated goat anti-mouse IgG antibody, from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), horseradish peroxidase-conjugated rabbit anti-goat IgG, from Zymed (San Francisco, CA, U.S.A.) and horseradish peroxidase-conjugated goat anti-mouse IgG, from Jackson Immuno Research (West Grove, PA, U.S.A.). Z-DEVD-cmk (Z-Asp-Glu-Val-Asp-chloromethylketone) and Z-VAD-fmk (Z-Val-Ala-D,L-Asp-fluoromethylketone) were purchased from Bachem (Voisins-le-Bretonnneux, France) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzylimidazolylcarbocyanine iodide (JC1) from Molecular Probes (Leiden, The Netherlands). D,L-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl (DL-PDMP) was supplied by Biomol (Plymouth Meeting, PA, U.S.A.), and imipramine by Sigma. All other chemical reagents were of analytical grade.

Cell-culture conditions

The Jurkat E6.1 cell line was derived from human T-acute lymphoblastic leukaemia cells (European Collection of Cell Cultures, Porton Down, Salisbury, Wilts., U.K.). SCC61 and SQ20B human cell lines were derived from head and neck squamous cell carcinomas, respectively, and were kindly provided by Dr J. Bourhis (Institut Gustave Roussy, Villejuif, France) and Dr V. Favaudon (Institut Curie, Orsay, France), respectively. Adherent cells were maintained at 37 °C under 6% CO_2 in Dulbecco's modified Eagle's medium and Glutamax® (0.86 mg/ml; Life Technologies, Paisley, Scotland, U.K.) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% fungizone and 0.4 mg/ml hydrocortisone. Jurkat cells were cultured under the same conditions, but without hydrocortisone.

Experimental procedures

Anti-Fas antibody was added to culture flasks, at a concentration of 100 ng/ml in fresh medium, before further incubation at 37 °C for varying times. Incubation of SCC61 and SQ20B cells was terminated by cell scraping for the ceramide assay, by cell lysis for Western-blot analysis, and by trypsinization for cell-cycle analysis, measurement of $\Delta\psi_m$ and quantification of glutathione. Jurkat cells were collected by low-speed centrifugation, and subsequently frozen, lysed or fixed.

Caspase inhibitors (50 μM Z-VAD-fmk, 100 μM Z-DEVD-cmk) were added 2 h before the addition of anti-Fas, and maintained in the medium until the cells were harvested.

Ceramide assay

Ceramide was quantified by HPLC with fluorimetric detection using a protocol described previously [15], to which further steps have been added in the extraction procedure. A total lipid extract prepared from the cell homogenate (2×10^6 cells), and supplemented with eicosasphinganine (used as an internal standard), was divided into three aliquots. Two aliquots were devoted to the analysis of total and free sphingoid base content of cells. After evaporation of the chloroform/methanol phase, the deacylation of ceramide derivatives to sphingoid bases was achieved on the first aliquot by incubation for 1 h in 1 ml of 1 M KOH, at 100 °C. To determine the free sphingoid base content, saponification of the acylglycerolipids was performed on the second aliquot, by incubation in 1 ml of 0.1 M KOH in methanol/chloroform (2:1 v/v), for 1.5 h at 37 °C. The subsequent steps, common to both analyses, included phase partition after the addition of 1 ml of chloroform and 0.5 ml of distilled water, and a wash with 1 ml of the theoretical upper phase. After evaporation of the organic phase, long-chain bases were dissolved in 50 μl of methanol, and derivatized by the addition of 50 μl of *o*-phthalaldehyde [16]. HPLC analysis was performed as described by Rodriguez-Lafrasse et al. [15]. Samples were quantified by comparison with a standard curve constructed from known amounts of ceramide type III or free sphingosine, sphinganine or glucosylsphingosine, processed concurrently. The third aliquot of the lipid extract was used to quantify lipidic phosphorus. Finally, results were expressed as pmol/nmol of lipidic phosphorus.

Western blotting of caspase-8, caspase-3 and PARP

Cells (6×10^6) were lysed in 50 mM Tris/HCl buffer (pH 8.0), 150 mM NaCl, 1% Triton X-100 and protease inhibitors (anti-proteases complete, Boehringer Mannheim) for 1 h at 4 °C. Lysates were centrifuged for 20 min at 15000 ***g***, and supernatants diluted in reducing SDS sample buffer.

For the caspase-8 immunoblot, total proteins were separated by SDS/PAGE on 12% gels, and transferred on to nitrocellulose membranes. Blots were blocked with PBS/3% non-fat dried milk for 1 h, and probed overnight at 4 °C with anti-caspase-8 antibody at a dilution of 1:1000 in PBS/3% Tween 20/3% non-fat dried milk. After three washes with PBS/0.05% Tween 20, antibodies retained on the membrane were detected using peroxidase-conjugated secondary antibody diluted at 1:7500 with PBS/0.05% Tween 20/3% non-fat dried milk. Following three washes in PBS/0.05% Tween 20, and two washes in PBS, bands were visualized with enhanced chemiluminescence reagent (Pierce, Rockford, IL, U.S.A.), and subsequent exposure to Hyperfilm (Sigma).

For analysis of caspase-3, proteins were separated on 8–16% denaturing gels (Bio-Rad, Ivry-sur-Seine, France), and transferred on to nitrocellulose membranes. Blots were blocked with 10 mM Tris/HCl buffer (pH 8.0)/150 mM NaCl (Tris-buffered saline, TBS) with 5% non-fat dried milk for 1 h, and probed with anti-caspase-3 antibody at a dilution of 1:2000 in TBS containing 0.05% Tween 20 and 5% non-fat dried milk. After three washes with TBS/0.05% Tween 20, the membranes were

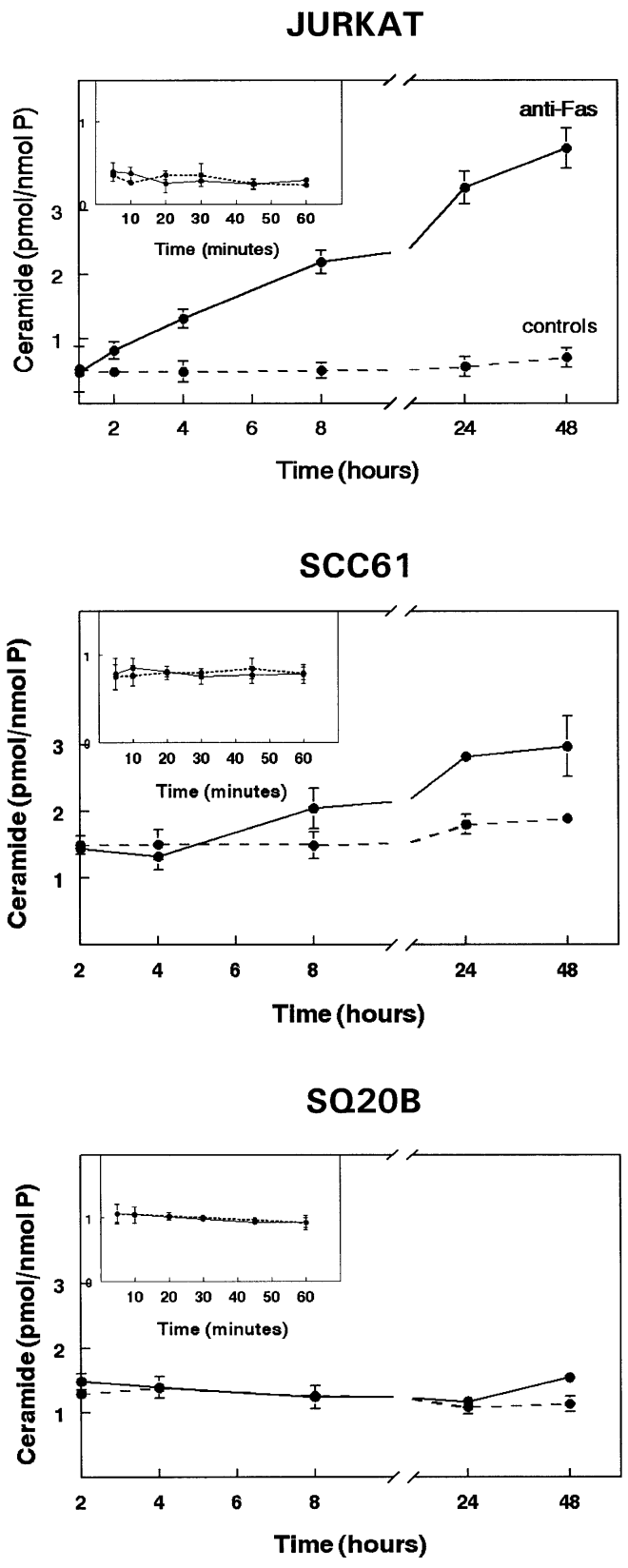

Figure 1 Kinetics of ceramide release in Jurkat, SCC61 and SQ20B cells after the addition of 100 ng/ml anti-Fas

For each time point, matched controls were assessed (dashed lines). Ceramide was quantified in cellular extracts by HPLC, with fluorimetric detection. Each value represents the mean ± S.D. from triplicate determinations. The results obtained during the first hour after treatment are shown in the insets.

incubated with secondary antibody, diluted at 1:2000 in TBS containing 0.05 % Tween 20 and 5 % non-fat dried milk. Three washes in TBS/0.05 % Tween 20, and two washes in TBS were performed before visualization.

PARP analysis was performed as for caspase-8, except that proteins were separated on 10 % reducing gels, and membranes were blocked with PBS/0.1 % Tween 20/5 % non-fat dried milk. Incubations with primary (1:2000) and secondary (1:20000) antibodies were performed in the same mixture. The subsequent washes were done with PBS/0.1 % Tween 20.

Detection of sub-G_1 peak, using propidium iodide labelling and flow cytometry

Cells were pelleted by centrifugation at 300 ***g*** (after harvest by trypsinization, for adherent cells), and washed once in PBS at 4 °C. Cells were then fixed in 2 ml of ice-cold 70 % ethanol, and stored at −20 °C for at least 24 h, until use. After washing with PBS, and subsequent centrifugation, cells were resuspended in a solution containing 0.25 ml of PBS, 0.25 ml of RNase (type I-A, Sigma; 1 mg/ml) and 0.5 ml of propidium iodide (500 μg/ml), and incubated for 15 min in the dark, at room temperature, before flow-cytometric analysis on a Coulter Epics XL-MLC.

Analysis of $\Delta\psi_m$

To measure $\Delta\psi_m$, (0.4–0.6) × 10^6 anti-Fas-treated or control cells were incubated with 5 μg/ml JC1 in the dark for 20 min, before flow-cytometric analysis. A total of 10000 events were collected for each analysis. The increase in green fluorescence (FL1), corresponding to monomer formation in the dye, was taken as a measure of the decrease in $\Delta\psi_m$.

Total glutathione analysis

Preparation of cellular extracts was carried out as outlined previously [17]. Separation was achieved by isocratic elution over a 20 cm Spherisorb 5 μm ODS2 C_{18} column, with a mobile phase composed of methanol and 0.15 M acetate buffer (3/37), pH 7, at a flow rate of 1.5 ml/min. Quantification of the fluorescent glutathione-*o*-phthalaldehyde derivatives was performed as described previously [17], at an excitation wavelength of 340 nm and an emission wavelength of 420 nm.

RESULTS

Time course for ceramide changes in Jurkat cells, and in adherent SCC61 and SQ20B cell lines, following incubation with 100 ng/ml anti-Fas antibody (7C11 clone)

The kinetics of ceramide release in response to 100 ng/ml anti-Fas were compared between the non-adherent Jurkat cell line, selected for its high sensitivity to anti-Fas [18], and two adherent squamous carcinoma cell lines, SCC61 and SQ20B, with moderate and no sensitivity to anti-Fas, respectively. Fas-receptor mRNA expression was verified in the three cell lines. The doubling times of these cells were comparable, and were found to be 18 h for SCC61, 22 h for SQ20B and 26 h for Jurkat cells. Ceramide levels, expressed as pmol/nmol of lipidic phosphorus, were measured between 5 min and 48 h after treatment to allow detection of any acute or delayed response. Each point was compared with a time-matched control (Figure 1). Ceramide was quantified by a highly sensitive HPLC method adapted from Rodriguez-Lafrasse et al. [15], which, in contrast with the diacyl-

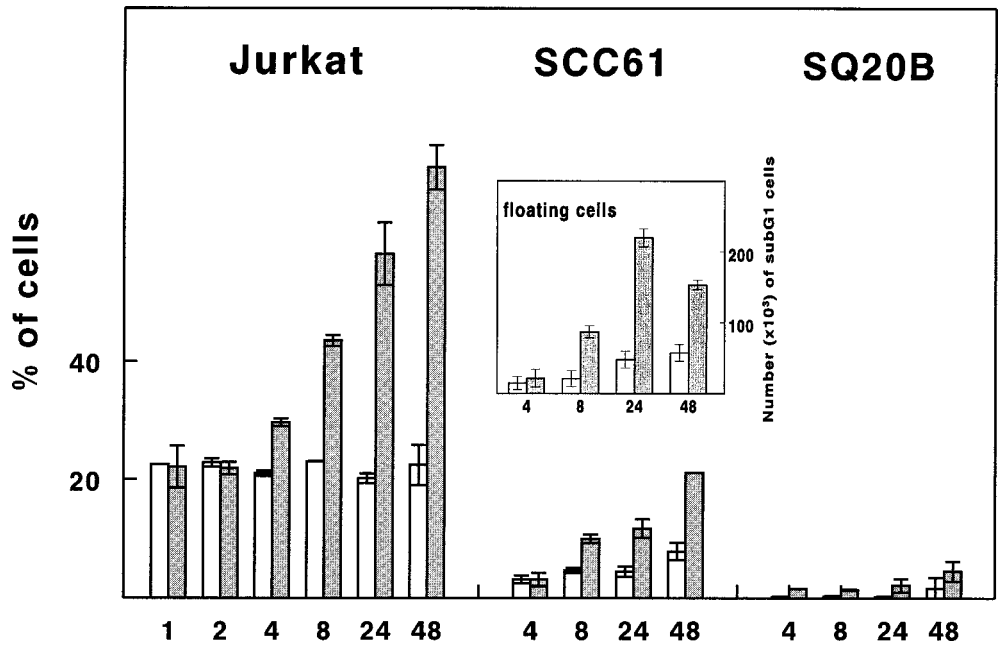

Figure 2 Percentage of Jurkat, SCC61 and SQ20B cells in the sub-G_1 phase, from 1 to 48 h after the addition of 100 ng/ml anti-Fas

Analysis of the cell cycle was performed using flow cytometry after propidium iodide labelling. For SCC61 floating cells, the absolute number of cells in the sub-G_1 phase ($\times 10^3$) is represented in the inset, since nearly all of these cells were in the sub-G_1 phase. Grey bars, incubation in the presence of 100 ng/ml anti-Fas; white bars, matched control cells.

glycerol kinase assay, allows separation and quantification of the glycosylated and reduced forms of ceramide and sphingosine.

As expected in the non-adherent Jurkat cells [8], addition of 100 ng/ml anti-Fas induced a time-dependent increase in ceramide levels, which became significant at 2 h post-stimulation, reaching 640 % of basal levels at 48 h after treatment (Figure 1, top panel). The time-matched controls showed very constant basal levels of ceramide, as indicated in the inset. No early (< 1 h) significant formation of ceramide was observed, in support of Tepper's report [8]. In SCC61 cells (Figure 1, middle panel), the profile of ceramide release showed similarities to that of Jurkat cells, but with a lesser response (190 % increase over control levels at 48 h), and a delay in the kinetics of release (a significant increase was apparent from 8 h after anti-Fas treatment). As shown in the inset, no early response could be detected in this cell line. SQ20B cells (Figure 1, bottom panel) showed a distinct response, with no variation in ceramide levels measured from 5 min to 48 h after anti-Fas treatment.

No significant variation in sphinganine or dihydroceramide levels was detected in response to anti-Fas in the three cell lines (results not shown).

In terms of ceramide release, SQ20B, SCC61 and Jurkat cells showed a gradient of sensitivity to anti-Fas, of which we took advantage to study the subsequent steps of the apoptotic process.

Relationship of ceramide formation to apoptotic commitment in Fas-sensitive and -resistant cells

To evaluate the apoptotic state of each cell line in response to anti-Fas, the percentage of hypodiploid (apoptotic) cells, which appear in the cell-cycle distribution as cells with a DNA content less than that at G_1, was assessed by cell-cycle analysis.

As expected in Jurkat cells, a significant number of sub-G_1-phase cells appeared from 4 h after the addition of anti-Fas, and increased to 73 % of cells at 48 h (Figure 2), whereas spontaneous apoptosis occurred in about 20 % of time-matched control cells. An increasing number of sub-G_1-phase cells was detected from 8 h among adherent SCC61 cells (Figure 2). Only 21 % of the remaining attached cells had a fractional DNA content 48 h after treatment, because most of the apoptotic cells became detached. The absolute number of floating cells in the sub-G_1 phase is represented in the inset of Figure 2, as nearly all the detached cells were in the sub-G_1 phase. Finally, less than 5 % of adherent SQ20B cells were in the sub-G_1 phase in response to anti-Fas (Figure 2), while only a very small proportion of cells became detached, making analysis of the cell cycle in this fraction impossible. The addition of anti-Fas at concentrations up to 400 ng/ml did not induce significant apoptosis in these cells (results not shown).

The kinetics and extent of nuclear fragmentation closely parallel ceramide formation in the three cell lines tested, which also exhibited corresponding graduated levels of apoptosis.

Processing of caspase-8, caspase-3 and PARP in anti-Fas-sensitive and -resistant cells

The kinetics of proteolytic cleavage of pro-caspase-8, pro-caspase-3 and PARP were followed by immunoblotting between 1 and 48 h after anti-Fas treatment.

In Jurkat cells (Figure 3, top panel), the expression of the p43 and p41 cleaved forms of caspase-8 was maximal 2 h after the addition of anti-Fas, and was maintained at a high level until 8 h post-treatment. Between 24 and 48 h after treatment, the expression of pro-caspase-8 and active caspase-8 decreased significantly. The p17 active form of caspase-3 likewise appeared 2 h after treatment, and was maintained at a detectable level until 24 h. Finally, the 85 kDa cleaved form of PARP was observed from 2 to 48 h after treatment with anti-Fas. In SCC61 cells (Figure 3, middle panel), anti-Fas treatment induced a cell death cascade, assessed by an increasing number of detached

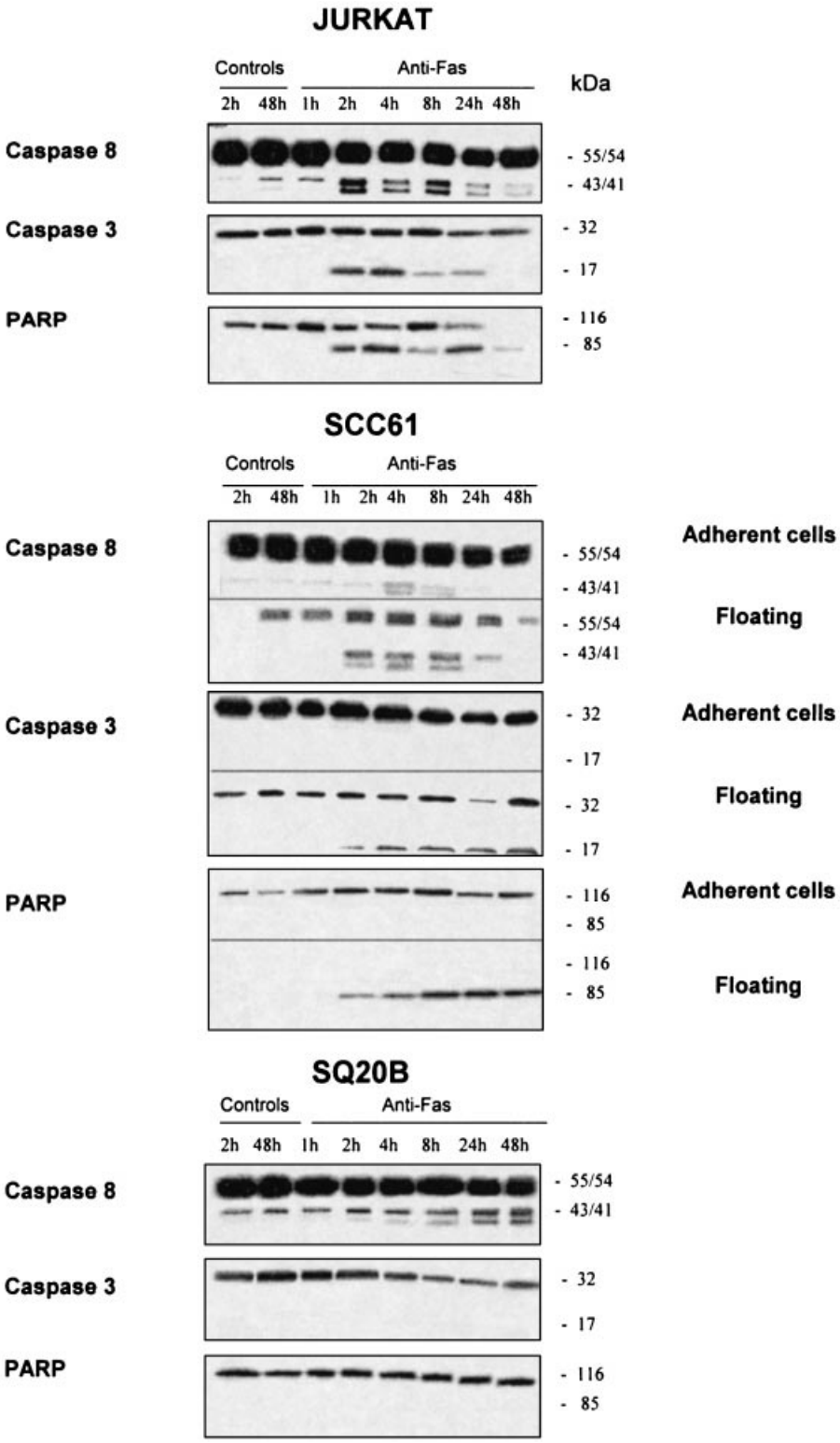

Figure 3 Kinetics of caspase-8 and caspase-3/PARP activation in Jurkat, SCC61 and SQ20B cells after incubation with 100 ng/ml anti-Fas

Cells were harvested after the indicated incubation periods. Total cellular protein (15 μg) was loaded on to the SDS/polyacrylamide gel for analysis of caspase-8 and caspase-3, and 40 μg for PARP analysis. Processing of caspase-8, caspase-3 and PARP was monitored by immunoblotting with specific antibodies as detailed in the Materials and methods section. The native forms of caspase-8, caspase-3 and PARP were 55 kDa, 32 kDa and 116 kDa, respectively. Their cleavage products were 43/41 kDa, 17 kDa and 85 kDa, respectively. The adherent and floating populations of SCC61 and SQ20B cells were collected separately.

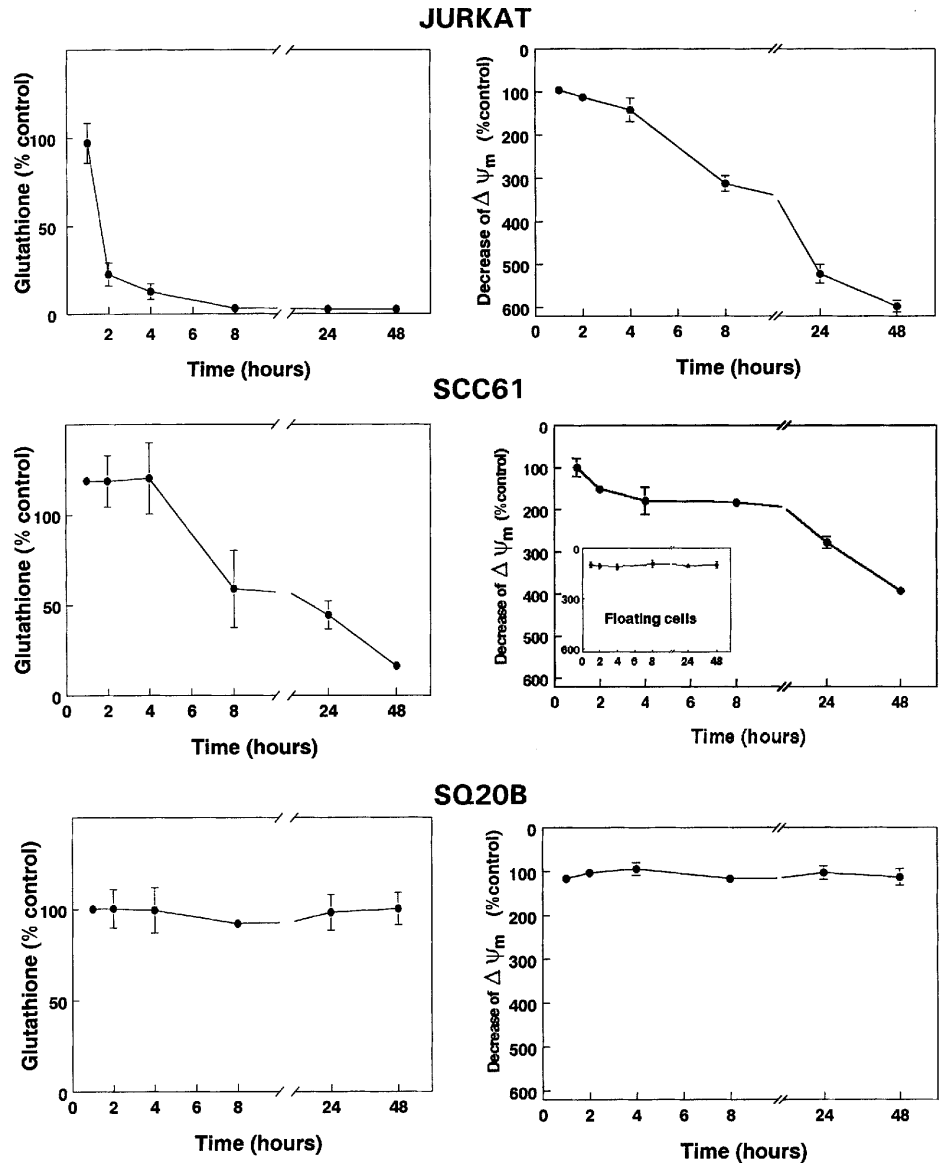

Figure 4 Kinetics of total glutathione depletion and $\Delta\psi_m$ decrease, measured in Jurkat, SCC61 and SQ20B cells, after incubation with 100 ng/ml anti-Fas

Glutathione levels were quantified by HPLC with fluorimetric detection, and $\Delta\psi_m$ was determined after JC1 labelling and flow-cytometric analysis, as described in the Materials and methods section. All results are expressed as a percentage of control cell values.

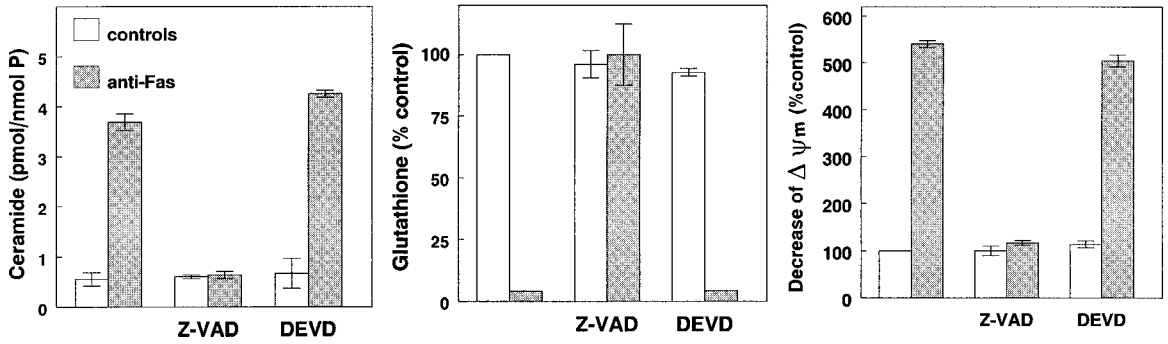

Figure 5 Effects of the caspase inhibitors Z-VAD-fmk and Z-DEVD-cmk on anti-Fas-antibody-mediated apoptosis

Jurkat cells were preincubated for 2 h with 50 μM Z-VAD-fmk or 100 μM Z-DEVD-cmk, and then treated with or without 100 ng/ml anti-Fas. After 24 h incubation, ceramide and total glutathione contents were determined, in parallel with measurement of $\Delta\psi_m$. Data are presented as means $\pm$ S.D. from triplicate samples.

Table 1 Increasing endogenous ceramide levels by inhibitors of its metabolism induces mitochondrial injury and caspase-dependent apoptosis

Jurkat cells were incubated for 24 h in the presence or absence of 20 μM DL-PDMP and imipramine. Ceramide levels, total glutathione content and changes in $\Delta\psi_m$ were assessed, processing of caspase-8, caspase-3 and PARP was analysed by Western blotting, and cell-cycle analysis was performed as described in the Materials and methods section.

				Protein cleavage			
	Ceramide (pmol/nmol of P)	Glutathione (nmol/mg of protein)	Decrease in $\Delta\psi_m$ (%)	Caspase-8	Caspase-3	PARP	Cells in sub-G_1 phase (%)
Controls	0.62 ± 0.08	19.8 ± 0.14	15.85 ± 1.84	−	−	−	14.0 ± 1
20 μM DL-PDMP/imipramine	1.56 ± 0.12	10.9 ± 0.99	37.85 ± 0.57	+	+	+	29 ± 2

cells, which led us to analyse the two populations of cells separately. Caspase-8 activation occurred from 2 h post-treatment in the floating population, and 4 h in the adherent one. The cleaved forms of caspase-8 were undetectable after 24 h. The active p17 form of caspase-3 was only detectable in the floating population, and increased from 4 to 48 h post-treatment. This process was closely associated with ongoing cell death, as the same amount of total cellular proteins was applied to each lane. Likewise, only the native form of PARP was detectable in adherent cells, but a significant decrease in the band intensity was observed at 24 and 48 h after treatment (Figure 3, middle panel). The p85 inactive form of PARP appeared in the floating cells from 2 to 24 h after treatment. A small percentage of SCC61 control cells spontaneously detached from the flask, and were also shown to express the 85 kDa form.

In the SQ20B cells, the cleaved form of caspase-8 was detectable from 2 h, and reached maximal expression at 24 and 48 h after treatment. However, cleavage of pro-caspase-3 and PARP was not observed at any time in response to anti-Fas (Figure 3, bottom panel), even when incubation with anti-Fas was prolonged to 72 h (results not shown). Interestingly, low levels of PARP were expressed in this cell line. Moreover, the percentage of floating cells was too small to permit Western-blot analysis.

Our experimental results indicate that in Jurkat and SCC61 cells the response to anti-Fas implies a common process involving ceramide generation, and activation of the caspase-8, caspase-3/PARP signalling cascade. In the SQ20B cell line, a blockage seems to occur between caspase-8 activation and the caspase-3/ceramide pathway. Furthermore, the absence of any sub-G_1-phase cells, which was demonstrated previously, confirms the absence of a caspase-3-independent apoptotic pathway in SQ20B cells.

Quantification of total glutathione and determination of $\Delta\psi_m$ in anti-Fas-sensitive and -resistant cells

Given the crucial role played by mitochondria in the apoptotic pathway, and their early participation in the chronology of events, we focused on the fall in total glutathione levels and the decrease in $\Delta\psi_m$.

As shown in Figure 4, addition of anti-Fas induced a drastic decrease in total glutathione levels in Jurkat cells. A 77 % fall was measured as early as 2 h after treatment, with levels reduced to less than 1 % of control cells 8 h after treatment. Very low levels of oxidized glutathione were detected in this cell line, and no significant variation occurred after anti-Fas stress (results not shown), confirming the results of van den Dobbelsteen et al. [19]. In SCC61 cells, the decrease in total glutathione appeared significant between 8 and 48 h post-treatment, but was less severe than in Jurkat cells. In SQ20B cells, anti-Fas treatment did not influence glutathione levels. Interestingly, the basal level of glutathione was 2–3-fold higher in SQ20B cells than in Jurkat or SCC61 cells, suggesting that the former cells are better protected against oxidative stress.

Experiments to estimate $\Delta\psi_m$ were performed in parallel with those in which glutathione was quantified. In Figure 4 decreases in $\Delta\psi_m$ are expressed as a percentage of control values. As already noted in the previous experiments (caspase processing and sub-G_1 peak detection), the Jurkat and SCC61 controls contained a significant number of stressed or apoptotic cells, as 15 % of these cells showed changes in $\Delta\psi_m$. The kinetics of $\Delta\psi_m$ decrease closely parallel the kinetics of ceramide release in Jurkat and SCC61 cells (see Figure 1), confirming the hypothesis that the two processes are linked [13]. The decrease in $\Delta\psi_m$ was more gradual and delayed, compared with the depletion of glutathione. As previously observed, SCC61 cells displayed changes in $\Delta\psi_m$ that were intermediate between those of the other two cell lines. In anti-Fas-resistant cells, less than 3 % of control or treated cells showed any decrease in $\Delta\psi_m$.

These experiments indicate a marked glutathione depletion and a progressive decrease in $\Delta\psi_m$ in Fas-sensitive cell lines. Furthermore, they also suggest that resistance to anti-Fas lies between caspase-8 activation and the mitochondria.

Ordering of cellular events on the basis of caspase-inhibitor effects and the modulation of intracellular ceramide levels

As shown in Figure 5, in Jurkat cells Z-VAD-fmk efficiently blocked ceramide formation, glutathione depletion and the fall in $\Delta\psi_m$ induced by the addition of anti-Fas. Z-VAD-fmk is known to be an irreversible broad-specificity caspase inhibitor that efficiently inhibits caspase-8 activation ($K_i = 2.5$ nM) [20]. Our data also confirm that caspase-8 activation acts before, and participates in, ceramide formation, glutathione release and changes in mitochondrial function. In our hands, Z-DEVD-cmk efficiently blocked caspase-3 processing, but did not prevent ceramide release or mitochondrial changes, suggesting that the cascade of reactions induced by caspase-3 occurs independently of, and/or downstream from, ceramide formation and the mitochondria. To elucidate whether ceramide increase is the cause or the consequence of mitochondrial changes, we attempted to amplify the endogenous ceramide signal by preventing its conversion into other metabolites. The addition of short-chain ceramide analogues to cell cultures has been demonstrated to trigger mitochondrial dysfunction (see the Introduction), but they do not mimic the behaviour of naturally occurring long-chain ceramide species exactly. DL-PDMP (20 μM), an inhibitor of the conversion of ceramide into glucosylceramide [21], was added to Jurkat cells, combined with 20 μM imipramine (a lysosomotropic agent inhibiting hydrolases such as acid sphingomyelinase [22]) to prolong and to amplify the endogenous ceramide signal. After 24 h incubation, intracellular ceramide increased to 200 % (Table 1) in response to both agents, whereas the addition of DL-PDMP or imipramine separately did not

induce significant variation in ceramide levels (results not shown). The inhibition of glucosylceramidesynthase proved sufficient, as 20 μM DL-PDMP induced a significant fall in the glucosylceramide pool at 24 h (0.08 versus 0.33 pmol/nmol of phosphorus in control cells). The 2-fold increase in ceramide levels was associated with a 45 % fall in glutathione levels, a 240 % decrease in $\Delta\psi_m$ and a 200 % increase in cells in the sub-G_1 phase (Table 1). These results indicate that increasing endogenous ceramide above physiological levels can induce mitochondrial dysfunction and apoptosis, strongly suggesting that endogenous ceramide can trigger apoptosis. These results also lend support to the argument that endogenous ceramide acts as an active factor in the apoptotic execution phase.

DISCUSSION

This paper has addressed two important and controversial issues that have arisen in the course of recent studies into the biochemistry of cell death, namely the temporal relationship between ceramide production, the activation of caspases and mitochondrial dysfunction, and their involvement in the course of anti-Fas-resistance.

First, our results point to a relationship between the sensitivity of three tumour cell lines to anti-Fas, and the degree of concomitant ceramide production and mitochondrial changes in these cells. Ceramide increases of a similar magnitude have recently been reported [9] in Jurkat cells exposed to anti-Fas, but to our knowledge no prior study has been made of the SCC61 and SQ20B adherent cell lines. In the present work, we demonstrate that ceramide production parallels the sensitivity of cells to anti-Fas, based on levels of apoptosis. Consistent with our results, the sensitivity to anti-Fas of various clones of melanoma cells was related directly to that for *N*-acetylsphingosine [14]. Interestingly, it has been demonstrated in another field of investigation that the sensitivity of human tumour cells to irradiation also correlates with the accumulation of ceramide [23]. Furthermore, a direct relationship has been observed between resistance to radiation-induced apoptosis and defective ceramide signalling [24]. A progressive increase in ceramide levels was measured from 2 to 48 h after treatment of Jurkat cells with 100 ng/ml anti-Fas, confirming the late kinetics of accumulation described previously in this cell line [7,25]. We did not detect any significant increase of ceramide during the first hour of apoptotic induction, in agreement with another finding of Tepper's group [8]. Nevertheless, our results contradict those of Cifone et al. [26], who found a transient elevation of ceramide levels in the first hour after anti-Fas addition to U937 cells. This discrepancy may be caused by the method of ceramide quantification, the diacylglycerol kinase assay used by the latter having been proved to give possible artefacts [27]. The fact that the kinetics of apoptosis and ceramide formation parallel each other suggests an involvement of ceramide in the apoptotic process. The addition of exogenous short-chain ceramides to cell culture clearly triggers apoptosis [28], but it is not clear whether this is equivalent in action to naturally generated ceramide. Furthermore, ceramide has been reported to be a 'gauge' or a 'sensor', rather than an 'executor' of apoptosis [29]. We observed significant increases in ceramide only at later times, when cell death had increased markedly, because this toxic compound is initially metabolized rapidly through the action of ceramidase or glucosyltransferase. A moderate increase in ceramide levels was detected in the SCC61 cell line, but monolayers and supernatants were pooled for the experiments, possibly attenuating the ceramide peak in apoptotic cells mainly present in the supernatant. In this context, our results show that increasing intracellular ceramide levels, via inhibitors of its metabolism, specifically induce apoptosis, supporting a significant role for ceramide in triggering apoptosis. Therefore, we disagree with two studies [30,31] that exclude the role of ceramide in Fas-induced apoptosis in HL60 cells and T-cells, in experiments based on the addition of exogenous ceramide and the inhibition of ceramide biosynthesis. We have also shown that the inhibition of glucosylceramide synthase by DL-PDMP induces the release of an endogenous ceramide pool, which can trigger apoptosis. This is contrary to Tepper's conclusions, which suggest that glucosylceramide synthase does not protect cells from the possible detrimental effects of ceramide accumulation [32].

The metabolic origin of ceramide release remains to be addressed [33,34]. We offer two different arguments to support the hypothesis that *de novo* synthesis does not contribute significantly to the delayed generation of ceramide in response to anti-Fas: (i) the absence of significant variation in the levels of sphinganine or dihydroceramide, intermediates in the synthesis of ceramide that were detected during the chromatographic run; and (ii) the lack of significant variation in the production of ceramide in cells pretreated with fumonisin B1 (results not shown), before the addition of anti-Fas. In spite of the large number of studies, the respective importance of acid and neutral sphingomyelinases in stress-induced apoptosis is still controversial. Both enzymes have been shown to be activated by anti-Fas [25,26,35,36]. Whether cells genetically deficient in acid sphingomyelinase manifest resistance to anti-Fas-induced apoptosis is also still a matter of debate [26,33,37,38]. As demonstrated in [33], a neutral sphingomyelinase is most probably responsible for the slow ceramide production in Jurkat cells upon anti-Fas ligation. Our results indicate no major contribution of acid sphingomyelinase to the process of sphingomyelin hydrolysis, because 24 h pre-incubation of cells with imipramine did not prevent ceramide increase induced by anti-Fas. However, as the inhibition of enzymic activity is not total, we cannot draw any definitive conclusions.

Previous works have suggested that Fas-mediated apoptosis is modulated by intracellular glutathione levels in human T-lymphocytes [39], or that activation of apoptosis-executing caspases may be under the control of GSH [40]. Furthermore, anti-Fas addition to Jurkat cells has been shown to induce a rapid fall in GSH, caused by an accelerated efflux from the cells rather than by an intracellular oxidation of the tripeptide [19]. We found an early and acute decrease in glutathione levels in Jurkat and SCC61 cells, preceding the more gradual decrease in $\Delta\psi_m$. In the absence of significant variation in oxidized glutathione levels, we can speculate from van den Dobbelsteen's experiments [19] that glutathione depletion may be associated with its efflux from the cells. Interestingly, it has been reported that GSH can inhibit neutral magnesium-dependent sphingomyelinase, but not acid sphingomyelinase, in some cell types, implying that depletion of cellular GSH can result in the hydrolysis of sphingomyelin and the additional generation of ceramide [41]. The increase in ceramide production seen in our experiments, from 8 h incubation with anti-Fas, could be attributable to a similar process. The molecular mechanism linking ceramide production to mitochondrial injury has not yet been clarified. Nonetheless, Decaudin et al. [42] have shown that exogenous ceramide may signal a permeability transition, a release of apoptogenic factors and the generation of reactive oxygen species in cytoplasts, indicating that these events are transcriptionally independent. Our results demonstrate that an increase in endogenous ceramide production may be responsible for mitochondrial injury. Furthermore, these experiments provide strong support for the role of endogenous ceramide as a key

executor of apoptosis, rather than as a consequence of membrane alterations.

Secondly, we found that the anti-Fas response in Jurkat and SCC61 cells implies both a mitochondrion- and a caspase-dependent pathway, as early caspase-8 activation is associated at the same time with pro-caspase-3 cleavage and glutathione depletion. These results corroborate the hypothesis that anti-Fas signalling can diverge at the caspase-8 level, with one branch of the pathway leading directly to effector caspase activation, and the other branch communicating with the mitochondria [18]. Our data are at variance with the work of Scaffidi and co-workers [5], who found that caspase-8 activation in Jurkat cells is insufficient to activate pro-caspase-3. We also contest the hypothesis that caspase-8 acts downstream from the mitochondria, because Z-VAD-fmk completely blocks mitochondrial injury. However, our results are consistent with those of Bossy-Wetzel and Green [43] and Watson et al. [44], who found that caspase-8 activation precedes mitochondrial disruption in cells undergoing apoptosis, and that mitochondrially derived factors subsequently amplify the processing of downstream caspases. We also agree with recent work of Kolesnick and co-workers [45] who conclude that ceramide generation is dependent on the caspase-8 initiator, and occurs prior to commitment to the effector phase of apoptosis. Our data support the notion that two cell lines with different histological patterns, adenocarcinoma-derived SCC61 adherent cells and lymphoblastic leukaemia-derived floating Jurkat cells, may share a common signalling pathway.

The present work confirms the involvement of the caspase-3/PARP pathway in the cascade of cellular responses to anti-Fas. Experiments performed with Z-DEVD-cmk, which efficiently blocks caspase-3 processing, did not prevent ceramide release or mitochondrial changes, suggesting that the cascade of reactions induced by caspase-3 occurs independently of, and/or downstream from, ceramide formation and the mitochondria. Nevertheless, the activation of this pathway did not occur in resistant SQ20B cells, which surprisingly displayed very low levels of PARP, compared with the other cell lines. Caspase-independent apoptosis [46] does not seem to occur in this cell line, since the sub-G_1 peak could not be detected.

Finally, we have demonstrated that SQ20B cells have a functional defect in apoptosis signalling from caspase-8 activation. Anti-Fas resistance implies an early block in the cascade of events after the activation of caspase-8 and before ceramide production, glutathione depletion and pro-caspase-3 activation. We have advanced a further step in the localization of this defect, on the work of Raisova et al. [14], who identified the block as occurring between the activation of caspase-8 and the release of cytochrome *c*. In a recent work, Juo et al. [47] suggested that FADD and caspase-8 mediate the Fas $\rightarrow$ ceramide signal, and are required for the generation of ceramide in response to Fas ligation. However, the results obtained here in SQ20B cells suggest that another activating factor intervenes between caspase-8 and ceramide release.

Our overall results indicate that a susceptibility to anti-Fas is related to the intensity of ceramide production and mitochondrial changes (glutathione depletion and $\Delta\psi_m$ decrease), and the propagation of the signal by active caspases. Although this study does not address the molecular link between caspase-8, ceramide and the mitochondria, it places ceramide in a proximal position in anti-Fas signalling. Further experiments are in progress to identify the causal defect in SQ20B cells, which should be helpful in gaining insight into chemico- and radio-resistance in tumours, since variant clones of Jurkat cells selected for resistance to CD95-induced apoptosis were also cross-resistant to etoposide and γ-radiation [18].

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