Sphingosine-induced apoptosis is dependent on lysosomal proteases

Katarina KÅGEDAL, Ming ZHAO, Iréne SVENSSON and Ulf T. BRUNK[1]

Division of Pathology II, Faculty of Health Sciences, Linköping University, Linköping, Sweden

We propose a new mechanism for sphingosine-induced apoptosis, involving relocation of lysosomal hydrolases to the cytosol. Owing to its lysosomotropic properties, sphingosine, which is also a detergent, especially when protonated, accumulates by proton trapping within the acidic vacuolar apparatus, where most of its action as a detergent would be exerted. When sphingosine was added in low-to-moderate concentrations to Jurkat and J774 cells, partial lysosomal rupture occurred dose-dependently, starting within a few minutes. This phenomenon preceded caspase activation, as well as changes of mitochondrial membrane potential. High sphingosine doses rapidly caused extensive lysosomal rupture and ensuing necrosis, without antecedent apoptosis or caspase activation. The sphingosine effect was prevented by pre-treatment with another, non-toxic, lysosomotropic base, ammonium chloride, at 10 mM. The lysosomal protease inhibitors, pepstatin A and epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester ('E-64d'), inhibited markedly sphingosine-induced caspase activity to almost the same degree as the general caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone ('Z-VAD-FMK'), although they did not by themselves inhibit caspases. We conclude that cathepsin D and one or more cysteine proteases, such as cathepsins B or L, are important mediators of sphingosine-induced apoptosis, working upstream of the caspase cascade and mitochondrial membrane-potential changes.

Key words: cathepsins, lysosome, lysosomotropic detergents, mitochondrion, necrosis.

INTRODUCTION

Apoptosis is a physiologically and morphologically distinct process that eliminates cells without inducing an inflammatory response. This type of cell death occurs, for example, during embryonic development and organ remodelling. Disturbed apoptosis is involved in a variety of pathological conditions, such as cancer, AIDS and many autoimmune and degenerative diseases [1–3]. Morphological and biochemical hallmarks of apoptosis include cytoplasmic blebbing, nuclear pycnosis and fragmentation, caspase activation and mitochondrial leakage to the cytosol of cytochrome *c* and other apoptosis-activating factors [4,5].

One mechanistic pathway proposed for apoptosis involves sphingolipids such as sphingosine and different ceramides, some of which are also involved in the regulation of calcium homoeostasis and proliferation [6,7]. Sphingolipids occur in the membranes of all eukaryotic cells, and recently have been shown to mediate apoptosis following ligation of the Fas receptor and the p55 [tumour necrosis factor (TNF)] receptor (TR55), as well as when added to cells in culture [8–10]. Recent evidence indicates that TR55 ligation activates the lysosomal enzyme, acid sphingomyelinase (A-SMase) [11], allowing formation of ceramide and, after further ceramidase-dependent cleavage, of sphingosine. It also was shown recently [12] that sphingosine and ceramide bind to and activate the lysosomal pro-form of cathepsin D, which provides a link between lysosomal proteolytic enzymes and TNF-induced apoptosis.

A series of previous studies from our laboratory have shown that lysosomes have an important role in apoptosis. A partial rupture of the lysosomes occurs during the early phase of apoptosis, when this process is initiated by agonists, such as oxidative stress, selective lysosomal photo-oxidation, the artificial lysosomotropic detergent *o*-methyl serine dodecylamide hydrochloride ('MSDH'), oxidized low-density lipoprotein, growth-factor withdrawal and Fas activation [13–19]. This rupture, and the resultant leakage to the cytosol of lysosomal contents, were found to take place before other processes thought to be involved in apoptosis, such as caspase activation and mitochondrial leakage of cytochrome *c* [16–24]. These findings, together with the fact that sphingosine, with an estimated pK_a value of between

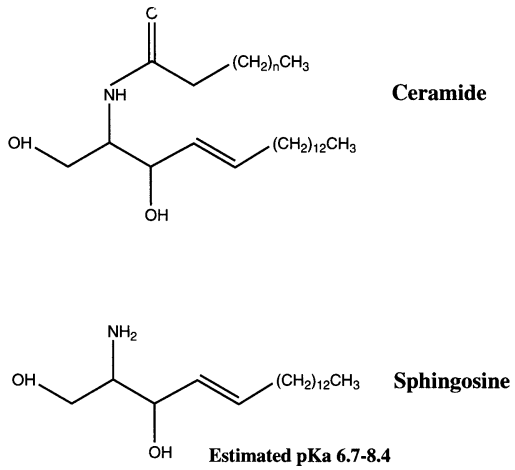

Figure 1 Structures of ceramide and sphingosine

Note the detergent-type structure of sphingosine, with a polar head and a long lipophilic tail. Also note the -NH_2 group within the polar head, conferring lysosomotropic properties (pK_a 6.7–8.4) that would aid its intralysosomal accumulation by proton-trapping.

Abbreviations used: Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin; AO, Acridine Orange; A-SMase, acid sphingomyelinase; E-64d (EST), epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester; FBS, fetal bovine serum; LEHD-AFC, Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin; LY, Lucifer Yellow; NAG, *N*-acetyl-β-glucosaminidase; Rh-123, rhodamine-123; TNF, tumour necrosis factor; TR55, p55 TNF receptor; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

[1] To whom correspondence should be addressed (e-mail ulf.brunk@pat.liu.se).

6.7 and 8.4 ([25,26] and R. A. Firestone, personal communication), has detergent as well as lysosomotropic properties owing to its long hydrophobic tail and polar head containing a proton-trapping amino group [27], led us to hypothesize that sphingosine induces apoptosis via lysosomal rupture (Figure 1). At the acidic pH (≈ 4.5) of lysosomes, the charged form of sphingosine would predominate over the uncharged form by > 100-fold and, therefore, accumulate heavily intralysosomally. Moreover, by protonation of its hydrophilic end, its capacity to act as a detergent would increase drastically, analogous to what has been shown for synthetic lysosomotropic detergents [17,28,29].

Here we provide evidence to support this hypothesis, and propose that sphingosine causes relocation of proteases from the acidic vacuolar compartment to the cytosol, which activates the apoptotic cascade, either by direct proteolytic activation of pro-caspases [30], via other cytosolic lytic pro-enzymes or pro-apoptotic proteins [18,31], or via proteolytic attack on mitochondrial membranes, with ensuing release of pro-apoptotic factors, such as cytochrome *c* and apoptosis-inducing factor ('AIF'). [20–23,32].

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) and culture media (RPMI 1640 and F-10) were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Glutamine, penicillin G and streptomycin were from Flow Laboratories (Rickmansworth, Herts., U.K.). Acridine Orange (AO) was from Gurr (Poole, Dorset, U.K.), while epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester (E-64d; also known as EST), Lucifer Yellow (LY) and rhodamine 123 (Rh-123) were from Sigma (St Louis, MO, U.S.A.). *N*-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) was from Pharmingen (San Diego, CA, U.S.A.), and Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (LEHD-AFC) was from R&D Systems (Minneapolis, MN, U.S.A.). Sphingosine, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) and pepstatin A were from Calbiochem (San Diego, CA, U.S.A.), and Annexin V-FLUOS™ and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside were from Boehringer Mannheim (Mannheim, Germany).

Cells and culture conditions

Jurkat T-lymphoma cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, Wilts., U.K.). Cells were grown in suspension in plastic flasks (Costar, Cambridge, MA, U.S.A.) at 37 °C in humidified air with 5% CO_2 in RPMI 1640 culture medium containing 10% (v/v) FBS, 10 mM Hepes, 2 mM glutamine, 50 units/ml penicillin G and 50 μg/ml streptomycin. The cells were maintained at a density of $(0.3–0.9) \times 10^6$ cells/ml medium, subcultivated three times a week, and then used for experiments 24 h after subcultivation.

J774 cells, a murine macrophage-like cell-line, were obtained from the Coriell Institute (Camden, NJ, U.S.A.), and were cultured in plastic flasks in F-10 culture media [supplemented with 10% (v/v) FBS, 2 mM glutamine, 50 units/ml penicillin G and 50 μg/ml streptomycin] at 37 °C in humidified air with 5% CO_2. The cells were subcultivated once a week, and used for experiments 24 h later at a density of 15000 cells/cm^2.

Lysosomal stability assay

Utilizing the AO uptake and relocation methods, cells were exposed to the lysosomotropic weak base AO as described previously [13–19,33,34]. Owing to proton trapping, this vital dye accumulates mainly in the acidic vacuolar apparatus, preferentially in secondary lysosomes. AO is also a metachromatic fluorophore. When excited by blue light (relocation method), it shows red fluorescence at high (lysosomal) concentrations and green fluorescence at low (nuclear and cytosolic) concentrations. If, however, green excitation light is used (uptake method), only concentrated (lysosomal) AO is demonstrated by its red or orange fluorescence. Rupture of initially AO-loaded lysosomes may be monitored as an increase in cytoplasmic diffuse green, or a decrease in granular red, fluorescence. The number of intact lysosomes (assayed as red fluorescence following excitation with green light) was monitored by both static and flow cytofluorimetry, as described previously [16–19].

For imaging, J774 cells (Jurkat cells are round and their lysosomes cannot easily be focused) were grown on coverslips and exposed to sphingosine (15–60 μM) for 30 min. The cells were then loaded with AO, and pictures were obtained using a 488 nm argon laser in a LSM410 confocal microscope (Ziess, Jena, Germany). Microscopy was performed using a red channel (590 nm barrier filter) and a $\times 63/1.25$ Plan-Fluar lens.

In some experiments, cells were pre-treated with 10 mM ammonium chloride for 30 min (to raise lysosomal pH, thereby preventing sphingosine uptake), and then exposed to sphingosine in the continued presence of ammonium chloride.

J774 cells were grown on coverslips and exposed to LY (0.25 mg/ml medium), a bright fluorochrome and a marker of endocytosis, for 30 min under otherwise standard culture conditions. The cells were kept for a further 4 h in medium lacking the marker molecule (to obtain selective labelling of secondary lysosomes) and, finally, were exposed to 30 μM sphingosine for 1 or 2 h. Images were obtained using a Nikon microphot-SA fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Hamamatsu (Bridgewater, NJ, U.S.A.) C4742-95 digital camera, and stored on a PC hard disk.

A rat liver lysosomal–mitochondrial (0.6 mg protein/ml) fraction (in 300 mM sucrose with 2 mM Hepes, pH 7.4, and 5 mM $MgCl_2$) was incubated with sphingosine (0–100 μM) at 37 °C for 0–90 min, and then centrifuged at 10000 ***g*** for 10 min. The activity of a lysosomal-marker enzyme, *N*-acetyl-β-glucosaminidase (NAG), was assayed as described previously [35] using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as a substrate, and then expressed as the percentage of activity in the supernatant over total activity of the fraction [lysosomal rupture was induced by 0.1% (w/v) Triton X-100].

Detection of cell death

Morphology and plasma membrane stability (light microscopy)

Cell death was induced by exposing the cells to various concentrations of sphingosine under otherwise standard culture conditions. Cellular membrane permeability was assayed using the Trypan Blue (0.05%) and propidium iodide (1 μg/ml) dye exclusion tests; stained cells were considered to be either post-apoptotic necrotic or primarily necrotic. The frequency of cells with apoptotic or necrotic morphology (cytoplasmic budding and pycnotic or fragmented nuclei as measured against membranous rupture and cellular/nuclear swelling respectively) was also estimated.

Phosphatidylserine exposure and plasma membrane stability (flow cytofluorometry)

Jurkat cells were stained with a combination of 2 μl of Annexin V-FLUOS™ and 2 μl of propidium iodide (1 μg/ml final con-

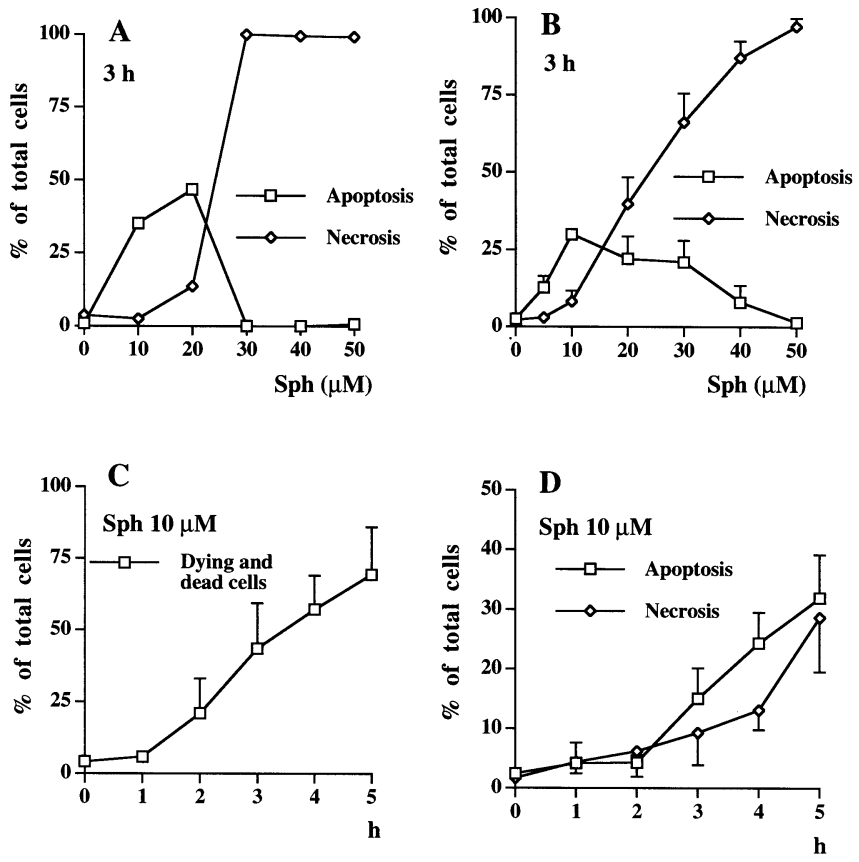

Figure 2 Analysis of apoptotic and necrotic cell death by morphology, phosphatidylserine expression, Trypan Blue and propidium iodide dye-exclusion studies

Jurkat cells were exposed to 0–50 μM sphingosine for 3 h, and the rate of apoptosis and necrosis was evaluated by (**A**) morphology in combination with Trypan Blue staining and by (**B**) flow-cytometric analysis of phosphatidylserine exposure, assaying the binding of FITC-conjugated annexin V in combination with propidium iodide exclusion. Jurkat cells also were exposed to 10 μM sphingosine for up to 5 h and, again, analysed for dying and already dead cells (apoptosis and necrosis) by (**C**) morphology and (**D**) flow cytofluorometry. For methodological details, see the Materials and methods section. Values shown are means $\pm$ S.D. ($n = 3$).

centration) in 100 μl of incubation buffer [10 mM Hepes (pH 7.4)/140 mM NaCl/5 mM $CaCl_2$] for 10 min on ice. Cells (10^5 per sample) were then analysed in a flow cytofluorometer (Becton Dickinson, Mountain View, CA, U.S.A.) using Lysys II software. Cells binding annexin but not stained by propidium iodide were considered to be apoptotic, whereas cells with an increased propidium iodide fluorescence with or without bound annexin were considered to be post-apoptotic necrotic or simply necrotic.

Activation of caspases

Caspase activation was determined using substrates that are cleaved into fluorescent reaction products by caspase-9 or -3-like proteases. For analysis of caspase-3-like activity, cells were collected and lysed in 100 μl of lysis buffer containing 10 mM Tris/HCl, pH 7.5, 130 mM NaCl, 1 % (w/v) Triton X-100 and 10 mM sodium pyrophosphate. The lysate was incubated with 20 μM Ac-DEVD-AMC in reaction buffer [20 mM Hepes/10 % (v/v) glycerol/2 mM dithiothreitol] at 37 °C for 1 h. The fluorescence intensity (expressed as arbitrary units/μg protein) of liberated AMC (7-amino-4-methyl coumarin) was measured using spectrofluorimetry (excitation wavelength 380 nm; emission wavelength 435 nm). Protein was assayed by the method of Lowry. Caspase-9-like activity was analysed using LEHD-AFC as a substrate, according to the manufacturer's instructions. The cell lysate was incubated at 37 °C for 1 h with the substrate. Fluorescence intensity of liberated AFC (7-amino-4-trifluoromethyl coumarin) was measured using spectrofluorimetry (excitation wavelength 400 nm; emission wavelength 505 nm), and values were expressed in arbitrary units/μg protein. It is recognized that overlapping with respect to substrate specificity might occur between caspases when crude cell preparations are used.

Inhibition experiments

Pepstatin A was dissolved in water-free DMSO (25 mM stock solution), and aliquots were added to the culture medium to obtain final concentrations of 1 μM, which was found to be optimal. E-64d was dissolved in DMSO (10 mM stock solution) and added to the culture medium to a final optimal concentration of 10 μM. Pre-treatment with pepstatin A and E-64d was initiated 2–16 h before ensuing sphingosine exposure; the inhibitors were also present during sphingosine exposure.

Cathepsin B- and D-like activities were determined as described by Barrett and Kirschke [36], using benzyloxycarbonyl-Arg-Arg-AMC and haemoglobin respectively as the substrate.

Both pepstatin A and E-64d were tested for their influence on active caspase-3 protease in test-tube experiments.

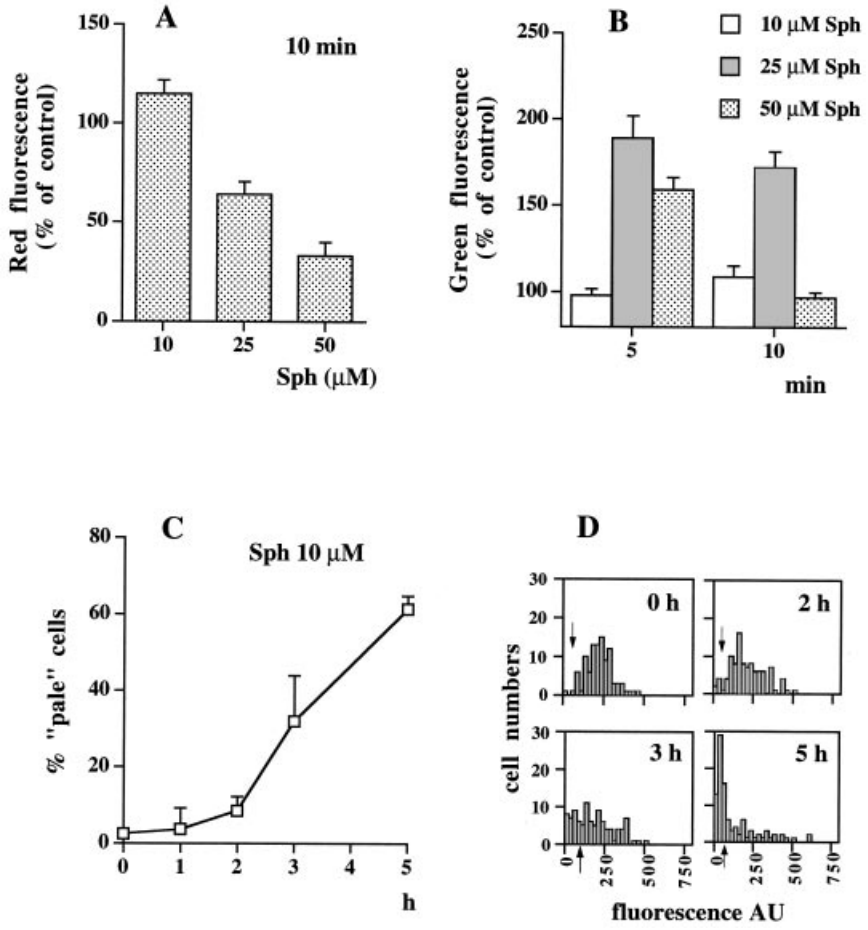

Figure 3 Analysis of lysosomal stability on whole cells

A decrease in lysosomal (red) and an increase in cytosolic (green) fluorescence of AO-stained cells reflecting lysosomal rupture. (**A**) Static cytofluorimetry of lysosomal red fluorescence in Jurkat cells, initially loaded with the lysosomotropic weak base AO and then exposed to 10–50 μM sphingosine for 10 min. The increase in red fluorescence following exposure to 10 μM sphingosine reflects expansion of the acidic vacuolar apparatus due to reparative autophagocytosis. Green light excitation was used. (**B**) Flow-cytometric analysis of cytosolic green fluorescence in Jurkat cells initially loaded with AO and then exposed to 10 μM sphingosine. Blue light excitation was used. The decline in green fluorescence at 10 min (compared with the value at 5 min) for the higher (25 and 50 μM) sphingosine concentrations is a consequence of plasma membrane damage, with resulting leak of cytosolic AO into the surrounding medium. (**C**) Uptake of AO by Jurkat cells following the addition of sphingosine. Number of 'pale' cells measured against time is given [pale cells are defined as cells with a reduced number of intact lysosomes, as assayed by estimation of AO-induced red fluorescence following excitation with blue light; the principle for their selection is shown in (**D**), cells left of the arrows]. Values shown are means $\pm$ S.D. ($n = 3$).

Mitochondrial stability assay

J-774 cells, grown on coverslips, were incubated for 20 min in 2 ml of culture medium with 10 μg/ml Rh-123 at 37 °C. The cells were then rinsed in PBS and exposed to 15 μM sphingosine for different periods of time under otherwise standard culture conditions. Rh-123 accumulates within the mitochondrial matrix as a function of the proton gradient over the inner membrane. This uptake was assayed by measuring the yellowish emission resulting from excitation with blue light. Depolarized control cells were obtained by exposure for 15 min to 1 μM valinomycin (a drug known to decrease $\Delta\Psi_m$, i.e. the mitochondrial membrane potential).

Statistical analysis

All experiments were repeated at least three times. The significance of differences between groups of cells was evaluated by the Mann–Whitney U-test. Values are given as means $\pm$ S.D. P values < 0.05 (marked with *) were considered to be significant (** $P < 0.01$; *** $P < 0.001$).

RESULTS

Sphingosine causes selective lysosomal rupture and, dose-dependently, induces either apoptosis or necrosis

To investigate the apoptosis-inducing property of sphingosine, Jurkat and J774 cells (results shown mainly for Jurkat cells, although both cell lines behaved similarly) were exposed to increasing concentrations of sphingosine for 3 h. Exposure of cells to sphingosine at low or moderate concentrations ($< 20\,\mu$M) caused a time-dependent increase in apoptosis, as judged from both morphological (Figures 2A and 2C) criteria (nuclear alterations, apoptotic body formation) and exclusion of Trypan Blue or propidium iodide in combination with plasma membrane binding of FITC-conjugated annexin V (Figures 2B and 2D). However, following exposure to sphingosine at higher concentrations ($> 20\,\mu$M), both morphology (nuclear and cellular swelling) and uptake of Trypan Blue and propidium iodide revealed necrosis without preceding apoptosis to be the predominant form of cell death (Figures 2A and 2B).

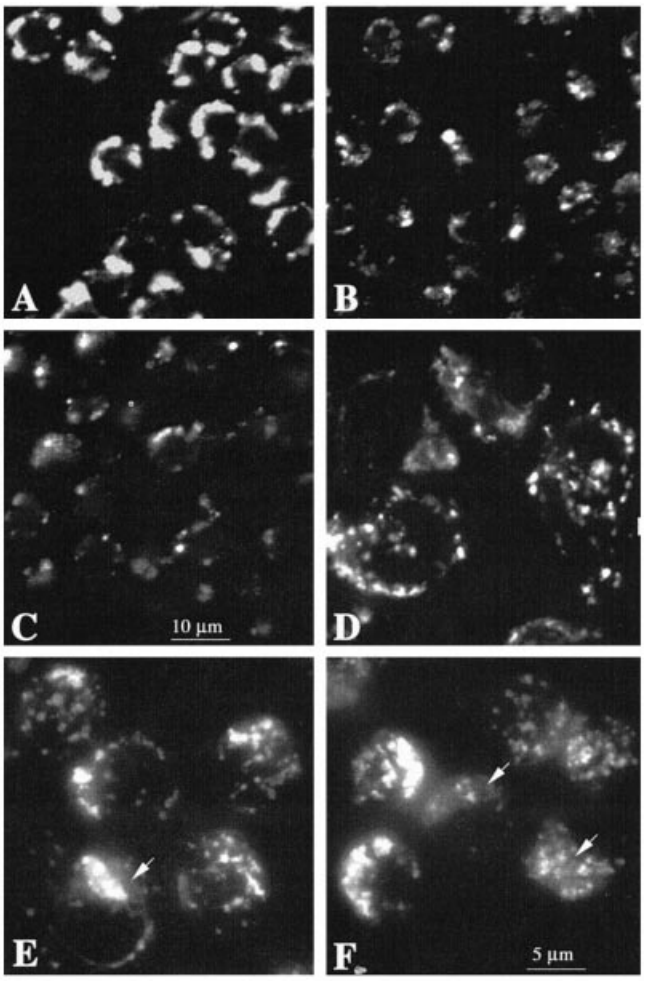

Figure 4 Visualization of lysosomal rupture in J774 cells using two different techniques (AO and LY)

Cells were initially exposed to sphingosine (0–60 μM) for 30 min, and then labelled with AO and finally imaged in a confocal microscope using a red channel (590 nm barrier filter): (**A**) for control cells; (**B**) in the presence of 30 μM sphingosine; and (**C**) in the presence of 60 μM sphingosine. Note the concentration-dependent decline in the number of fluorescent, intact lysosomes within most cells. Other cells (**D–F**) were initially exposed to LY for 30 min, kept under standard culture conditions for another 4 h, and then exposed to 30 μM sphingosine for 0–2 h and finally imaged electronically using a fluorescence microscope and a digital camera. Experimental conditions were as follows: (**D**) control cells; (**E**) in the presence of sphingosine for 1 h; (**F**) sphingosine for 2 h. Note the diminishing number of remaining intact lysosomes with time, and increasing cytosolic fluorescence, resulting from leakage of endocytosed LY from lysosomes to the cytosol (indicated by arrows in **E** and **F**).

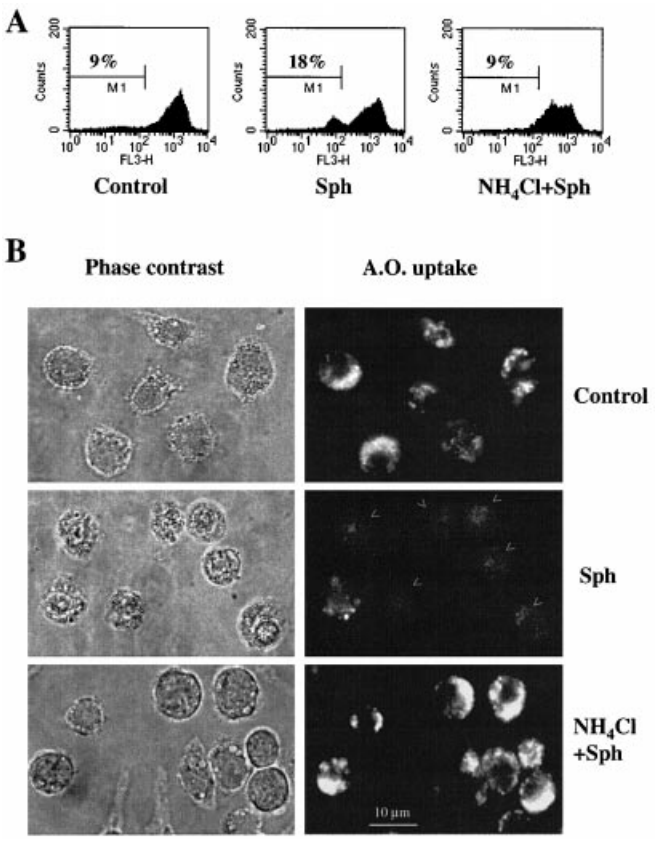

Figure 5 Sphingosine is a lysosomotropic detergent

J774 cells (3×10^5 in 35 mm dishes) were seeded and grown for 24 h under standard culture conditions. Cells were then: (i) stained with AO only; (ii) exposed to 15 μM sphingosine for 2 h and then stained with AO; or (iii) pre-exposed to 10 mM NH_4Cl for 30 min, then exposed to a combination of 15 μM sphingosine and 10 mM NH_4Cl for another 2 h and, finally, rinsed and stained with AO. Following AO staining, cells were either analysed by flow cytofluorimetry (**A**; note the decreased uptake of AO in cells exposed to NH_4Cl + sphingosine, but no increased number of 'pale' cells) or electronically imaged by light microscopy using phase and fluorescence modes (**B**). NH_4Cl protects against sphingosine-induced lysosomal rupture. Most cells exposed only to sphingosine (indicated by arrowheads) contain few intact lysosomes compared with both control cells and NH_4Cl + sphingosine-exposed cells, which are about equal.

The lysosomotropic and detergent-like structure of sphingosine (Figure 1) led us to consider whether its apoptosis-inducing effect was caused by lysosomal leakage. To determine this, utilizing the AO uptake and relocation methods [13–19], Jurkat cells were first loaded with the lysosomal marker AO and then exposed to sphingosine at different concentrations. A 10 min exposure to a low dose of sphingosine (10 μM) resulted in a small increase in green light excitation-induced red fluorescence, probably indicating either a slightly expanded lysosomal apparatus due to reparative autophagocytosis or increased osmotic pressure with ensuing hydration and swelling of the lysosomal apparatus after sphingosine uptake. However, higher doses of sphingosine (25 and 50 μM) caused a rapid and extensive decline in red fluorescence (upon green light excitation), indicating rupture of numerous lysosomes (Figure 3A), which later resulted in necrotic cell death (compare with Figures 2A and 2B). Importantly, the remaining non-ruptured lysosomes retained normal AO-induced red fluorescence (indicating an unchanged lysosomal pH) when excited with green light.

A low apoptosis-inducing dose of sphingosine (10 μM) also rapidly (within 10 min) increased green cytoplasmic AO-induced fluorescence upon blue light excitation, demonstrating the existence of partial lysosomal rupture, with release to the cytosol of some AO, already during the early phase dominated by reparative autophagocytosis. Exposure to the higher doses (25 and 50 μM) resulted in drastically increased green fluorescence (upon blue light excitation), indicative of a more extensive lysosomal rupture, after only 5 min. After 10 min, the green fluorescence had declined for the highest concentrations (25 and 50 μM) as a result of damage to plasma membranes, with a resulting diffusion of AO to the surrounding medium (Figure 3B; compare with Figure 3A), whereas the low (10 μM) apoptosis-inducing dose led to increased green fluorescence (upon blue light excitation) within intact plasma membranes. Both Jurkat cells (Figure 3C)

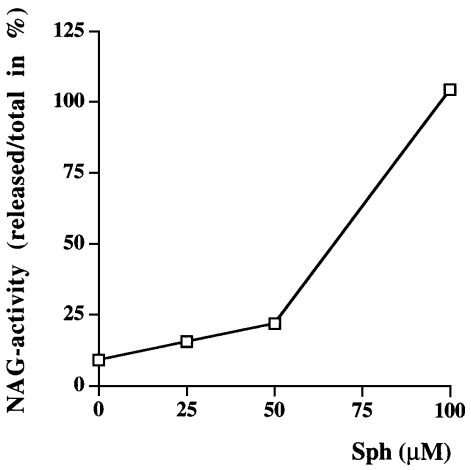

Figure 6 Analysis of lysosomal stability on a lysosomal fraction

A rat liver mitochondrial–lysosomal fraction (0.6 mg protein/ml) in buffered 300 mM sucrose was exposed for 30 min at 37 °C to sphingosine at the concentrations indicated. After centrifugation (10 000 ***g*** for 10 min), supernatant activity was expressed as a percentage of total activity (following treatment with 0.1 M Triton X-100). Note the dose-dependent increase in lysosomal rupture as a result of sphingosine exposure.

and J774 cells (results not shown) showed a time-dependent increase of 'pale' cells (cells with a reduced number of lysosomes showing normal red fluorescence, indicating lysosomal rupture) [18] after sphingosine exposure at a low dose. Figure 3(D) shows how 'pale' cells are defined (bars to the left of arrows). At 5 h, when most cells were undergoing apoptosis following exposure to 10 μM sphingosine, the majority of the cells contained only a few granules exhibiting red fluorescence after exposure to AO (intact lysosomes).

J774 cells, exposed to sphingosine for 30 min and then loaded with AO (Figures 4A–4C), showed a dose-dependent decrease in the number of AO-loaded (intact) lysosomes (compare with Figure 3A).

J774 cells exposed to LY for 30 min possessed strongly fluorescent secondary lysosomes due to the endocytotic uptake of the fluorochrome. Secondary treatment of such labelled cells with sphingosine (30 μM) caused their lysosomes to burst time-dependently, spilling LY into the cytosol (Figure 4D–4F). The two methods of lysosomal labelling are on the basis of either proton trapping or endocytotic uptake (for AO and LY respectively), and thus they are complementary, thereby confirming that secondary lysosomes do burst when cells are exposed to sphingosine.

To study further the role of sphingosine lysosomotropism, cultures were pre-treated with 10 mM ammonium chloride for 30 min before being exposed to sphingosine in the continued presence of ammonium chloride. Under these circumstances, NH_3 is formed, which diffuses into the cells and their lysosomes. In the latter compartment, the base becomes protonated and trapped as NH_4^+, substantially increasing the lysosomal pH [37] and preventing accumulation of other lysosomotropic substances, such as sphingosine. In support of the requirement for lysosomal concentration of sphingosine in causing cellular toxicity, ammonium chloride significantly protected cells against sphingosine (Figure 5).

In order to quantify further lysosomal rupture following exposure to sphingosine, a rat liver lysosomal fraction was incubated with sphingosine at different concentrations. As shown in Figure 6, there was a dose-dependent increase in sphingosine-induced lysosomal rupture, detected as an increased activity of the lysosomal enzyme NAG in the supernatant. This rupture was completed within 30 min when the highest dose (100 μM) of sphingosine was used.

These findings, using four separate techniques, indicate that both lysosomotrophic and detergent properties, as well as apoptosis-inducing activity, can be attributed to sphingosine.

The apoptosis-inducing property of sphingosine was confirmed by caspase-activation assays. Exposure of Jurkat and J774 cells to sphingosine at moderate doses resulted in the induction of both caspase-3- and -9-like activities, as is shown in Figure 7. It should be noted that this activation was preceded by moderate lysosomal rupture. However, the almost complete lysosomal rupture, with ensuing necrosis, that resulted from sphingosine exposure at high doses was not accompanied by caspase activation in either cell line (results not shown).

Inhibition of lysosomal aspartic and cysteine proteases prevents apoptosis

A series of reports on different cell types have shown that the lysosomal enzyme cathepsin D, which is an aspartic protease, and the cysteine proteases, cathepsins B and L, are involved in apoptosis induced by a variety of agonists [23,30,32,38–42]. Because the early rupture of lysosomes seems to be correlated with apoptosis, we decided to study further the involvement of lysosomal enzymes.

As shown in Figure 7(D), pre-treatment of J774 cells with the lysosomal cysteine protease inhibitor E-64d (optimal concentration 10 μM) reduced the sphingosine-induced activation of caspase-3-like proteases by approx. 40 %, whereas pre-treatment with the aspartic inhibitor pepstatin A (optimal concentration 1 μM) caused a reduction of approx. 55 %. This inhibition was comparable with the effect of the broad-spectrum caspase-inhibitor, Z-VAD-FMK. In whole-cell lysates from untreated J774 cells, E-64d inhibited cathepsin B-like activity by 55 %, while pepstatin A blocked cathepsin D-like activity by 80 %. Furthermore, neither 10 μM E-64d nor 1 μM pepstatin A inhibited 50 ng/ml active caspase-3, with 98 ± 7 % activity remaining following exposure to E-64d and 109 ± 9 % activity remaining following exposure to pepstatin A.

Homogenates of J774 cells contained active cathepsin B- and D-like proteases. The activities did not significantly change during the first few hours after exposure to sphingosine in low apoptotic doses (results not shown).

Lysosomal leakage precedes changes of mitochondrial membrane potential

To investigate further the early events of sphingosine-induced apoptosis, changes in mitochondrial membrane potential were compared with the time-frame of lysosomal leakage. The mitochondrial membrane potential (assayed as the uptake of Rh-123) remained stable in J774 cells during the first 30 min of exposure to a moderate dose (15 μM) of sphingosine. After 1 h, however, there was a slight decline (10 %), whereas after 3 h the fluorescence was only approx. 40 % of the initial value (Figure 8), although, as described above, lysosomal leakage was evident after only 10 min when cells were exposed to 10 μM sphingosine (compare with Figure 3B).

Pre-treatment of cells with E-64d and pepstatin A (10 and 1 μM respectively) in combination prevented mitochondrial membrane destabilization to a significant degree following en-

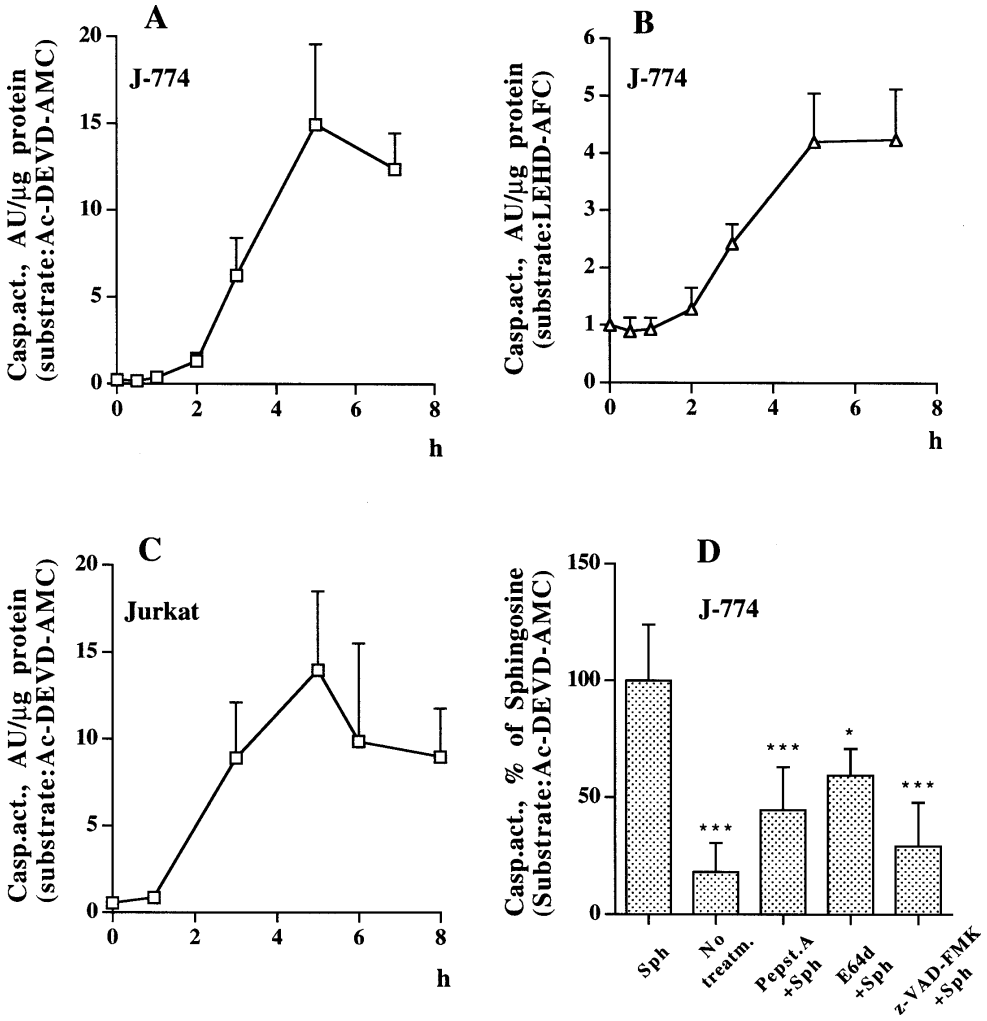

Figure 7 Determination of caspase activities

Using two different substrates, Jurkat and J774 cells were assayed for caspase activity. (**A**) Caspase activity in J774 cells, using Ac-DEVD-AMC, exposed to 15 μM sphingosine. (**B**) Caspase activity, using LEHD-AFC, of J774 cells exposed to 15 μM sphingosine. (**C**) Caspase activity of Jurkat cells, using AC-DEVD-AMC, exposed to 10 μM sphingosine. (**D**) Demonstration of caspase inhibition, using the lysosomal-protease inhibitors E-64d and pepstatin A, as well as the broad-spectrum-acting caspase inhibitor Z-VAD-FMK, following exposure of J-774 cells to 15 μM sphingosine for 4 h. Values are means $\pm$ S.D., $n = 3$–5.

suing exposure to sphingosine (Figure 8), suggesting such destabilization to be secondary to lysosomal breakage.

DISCUSSION

For some years, ceramides have been considered to be mediators of apoptosis; sphingosine has also been found to induce apoptosis in a variety of cell types [8–10,43]. On the basis of its chemical structure and pK_a value (Figure 1), sphingosine should be lysosomotropic, and, in its charged form, also a detergent. These might be important properties, in view of the fact that several studies have confirmed the involvement of the lysosomal proteases cathepsins D, B and L in apoptosis [20,23,30,32,38–40,42].

In the present report, we have investigated a putative novel mechanism of sphingosine-induced apoptosis, which focuses on its ability to act as a lysosomal detergent, thereby causing release of lysosomal contents to the cytosol. When J774 and Jurkat cells in culture, or rat liver lysosomes in suspension, were exposed to sphingosine, lysosomes ruptured in a dose- and time-dependent manner, as shown by an altered intracellular distribution of endocytosed LY, reduced granular uptake of the lysosomotropic drug AO, redistribution of AO from lysosomes to the cytosol, and release of the lysosomal-marker enzyme NAG from the sedimentable to the unsedimentable part of the lysosomal rat-liver fraction. The importance of lysosomal accumulation of sphingosine is supported by the observation that, in the presence of ammonium chloride (which elevates lysosomal pH and prevents proton trapping), there is a dramatic protection against its lysosomolytic and apoptosis-inducing effects.

Previously, lysosomal rupture, assayed by the AO uptake and relocation techniques, has been shown to coincide with the relocation of lysosomal-marker enzymes, such as cathepsin D, to the cytosol, as demonstrated by immunocytochemistry and, importantly, by the later initiation of mitochondrial destabilization and, finally, by apoptosis [15–23,44]. Cells exposed to TNF-α or anti-Fas antibodies have been reported to demonstrate increased A-SMase activity preceding the onset of apoptosis, and also increased production of ceramide in acidic organelles [45].

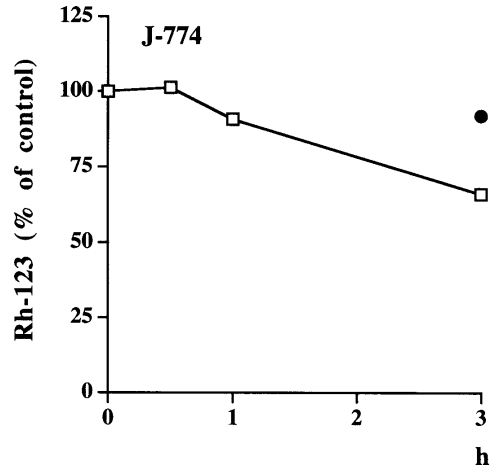

Figure 8 Mitochondrial stability of J774 cells

Cells, pre-treated (●) or not (□) with E-64d + pepstatin, were exposed to 15 μM sphingosine. Mitochondrial stability was evaluated by static cytofluorimetry of Rh-123-induced yellowish fluorescence. Note that the mitochondrial alterations occurred after lysosomal rupture (compare with Figure 3), and were partly inhibited by lysosomal-protease inhibition. For methodological details, see the Materials and methods section. Values shown are means for duplicate experiments.

However, conflicting data have been published on this topic, and it has been claimed that A-SMase, which resides in the lysosomal apparatus, is poorly positioned to be a key enzyme in signal transduction [46]. Nevertheless, the intralysosomal formation of ceramide by A-SMase, acting upon autophagocytosed membraneous material, its later splitting by lysosomal acidic ceramidase during formation of sphingosine, and a final lysosomotropic detergent effect of the latter compound might offer an explanation.

By measuring AO relocation to the cytosol from only a few ruptured lysosomes, we were able to confirm that exposure to moderate amounts of sphingosine caused early lysosomal leakage, which happened before activation of pro-caspases. The latter event occurred concomitantly with the first signs of apoptosis, reached its maximum when apoptosis was at its peak, and then declined. However, at higher concentrations of sphingosine, which cause rapid and almost complete lysosomal rupture and necrosis, there was no, or only insignificant, caspase activation. We conclude that the partial release of lysosomal hydrolytic enzymes results in moderately enhanced cytosolic proteolytic activity, resulting either in caspase activation, possibly directly, perhaps via lysosomal cysteine proteases [30,42], or indirectly by activation of pro-apoptotic proteins, e.g. Bid, or, alternatively, in mitochondrial destabilization by lysosomal or other lytic enzymes, which might become activated by released lysosomal hydrolases [18,31,32], with the ensuing release of apoptotic factors. More complete release to the cytosol of lysosomal hydrolases would result in extreme proteolytic activity, where pro-caspases and caspases alike might become substrates of various lysosomal proteases, explaining the lack of active caspases in cells exposed to sphingosine in high doses.

As was reported from our laboratory previously, lysosomal leakage might be an early phenomenon during apoptosis caused by a multitude of agonists [13–24,44]. For example, during oxidative-stress-induced apoptosis, we proposed that lysosomal membranes are exposed to increased amounts of cytosolic hydrogen peroxide. Inside the lysosomal apparatus, low-molecular-mass iron (a consequence of normal autophagocytotic degradation of metalloproteins, such as cytochromes), the acidic milieu and the existence of the reducing amino acid cysteine would promote iron reduction and Fenton-like chemistry, destabilizing the lysosomal membranes and, thereby, causing lysosomal leakage [13–14,18]. Moreover, other laboratories have also reported the involvement of both lysosomal cysteine proteases (such as cathepsins B and L) and the aspartic protease cathepsin D in the apoptotic process. Thus it has been shown that lysosomal cysteine proteases have the capacity to activate a cytosolic caspase-3-like protease, and that bile-salt-induced apoptosis can be prevented by inhibition of cathepsin B [30,47,48]. Cathepsins B and D have also been shown to process caspase zymogens and to induce nuclear apoptosis [42]. Recent findings therefore indicate that lysosomes and cathepsins are, indeed, implicated in apoptosis (reviewed in [49–51]).

Accumulating evidence thus points to the lysosome as an organelle that, perhaps in co-operation with mitochondria, might be intimately involved in apoptosis. In the present study, the importance of lysosomal proteases during apoptosis was emphasized further by the inhibition of caspase activation that was obtained using inhibitors of lysosomal cysteine proteases (E-64d) and aspartic proteases (pepstatin A). The effect was comparable with that of the broad and irreversible caspase inhibitor Z-VAD-FMK, while the inhibitors had no direct effect on caspases. Part of the effect of Z-VAD-FMK might even be mediated via its cathepsin B-inhibiting properties [52].

Apoptosis due to 'internal' mechanisms is often thought to be mediated by mitochondrial changes. However, there was no decline in the inner mitochondrial membrane potential, as measured by uptake of Rh-123, during the early events (including lysosomal leakage) of the apoptotic process. Sometime later, however, the mitochondrial membrane potential declined, although significantly less so in cells that were pre-treated with inhibitors of lysosomal cathepsins. We hypothesize that this mitochondrial alteration is an effect of released lysosomal hydrolytic enzymes activating either pro-apoptotic proteins, such as Bid, or cytosolic pro-active lytic enzymes, or acting directly on their membranes. Lysosomal and mitochondrial factors might thus sustain and augment each other as activators of various pro-caspases.

In summary, when relocation to the cytosol of lysosomal contents is of a moderate magnitude, a regulated and apoptotic type of degradation follows, while a massive release of lysosomal enzymes induces rapid and uncontrolled necrotic degradation without significant caspase activation. We suggest that partial lysosomal leakage is an early event in apoptosis, and that released lysosomal lytic enzymes might activate the apoptotic process via several direct or indirect mechanisms. Moreover, we suggest that liberated lysosomal enzymes may act in concert with caspases during the apoptotic process. Sphingosine, known to be an apoptotic agent, is suggested to be a lysosomotropic detergent, inducing apoptosis via its lysosome-rupturing action.

We thank Drs. John W. Eaton, Raymond A. Firestone, and Mark Wilson for their interest and helpful suggestions and Ms Diana Konzen for expert linguistic help. This work was supported by grants from the Swedish Cancer Foundation (grant 4296) and the Swedish Medical Research Council (grant 4481).

REFERENCES

1 Jacobson, M. D., Weil, M. and Raff, M. C. (1997) Programmed cell death in animal development. Cell **88**, 347–354
2 Wang, X. W. (1999) Role of p53 and apoptosis in carcinogenesis. Anticancer Res. **19**, 4759–4771
3 Czech, C., Tremp, G. and Pradier, L. (1999) Presenilins and Alzheimer's disease: biological functions and pathogenic mechanisms. Prog. Neurobiol. **60**, 363–384

4 Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. (1997) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science **275**, 1132–1136

5 Kroemer, G., Zamzami, N. and Susin, S. A. (1997) Mitochondrial control of apoptosis. Immunol. Today **18**, 44–51

6 Hakomori, S. and Igarashi, Y. (1993) Gangliosides and glycosphingolipids as modulators of cell growth, adhesion, and transmembrane signaling. Adv. Lipid Res. **25**, 147–162

7 Hakomori, S. and Igarashi, Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling. J. Biochem. (Tokyo) **118**, 1091–1103

8 Sweeney, E. A., Inokuchi, J. and Igarashi, Y. (1998) Inhibition of sphingolipid induced apoptosis by caspase inhibitors indicates that sphingosine acts in an earlier part of the apoptotic pathway than ceramide. FEBS Lett. **425**, 61–65

9 Sweeney, E. A., Sakakura, C., Shirahama, T., Masamune, A., Ohta, H., Hakomori, S. and Igarashi, Y. (1996) Sphingosine and its methylated derivative N,N-dimethylsphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. Int. J. Cancer **66**, 358–366

10 Hung, W., Chang, H. and Chuang, L. (1999) Activation of caspase-3-like proteases in apoptosis induced by sphingosine and other long chain bases in Hep3B hepatoma cells. Biochem. J. **338**, 161–166

11 Schutze, S., Machleidt, T., Adam, D., Schwandner, R., Wiegmann, K., Kruse, M. L., Heinrich, M., Wickel, M. and Kronke, M. (1999) Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signaling. J. Biol. Chem. **274**, 10203–10212

12 Heinrich, M. and Wickel, M. (1999) Cathepsin D targeted by acid sphingomyelinase-derived ceramide. EMBO J. **18**, 5252–5263

13 Zdolsek, J. M., Zhang, H., Roberg, K. and Brunk, U. T. (1993) H_2O_2-mediated damage to lysosomal membranes of J-774 cells. Free Radicals Res. Commun. **18**, 71–85

14 Öllinger, K. and Brunk, U. T. (1995) Cellular injury induced by oxidative stress is mediated through lysosomal damage. Free Radicals Biol. Med. **19**, 565–574

15 Brunk, U. T., Dalen, H., Roberg, K. and Hellquist, H. B. (1997) Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. Free Radicals Biol. Med. **23**, 616–626

16 Brunk, U. and Svensson, I. (1999) Oxidative stress, growth-factor starvation and Fas-activation may all cause apoptosis through lysosomal leak. Redox Report **4**, 3–11

17 Li, W., Yuan, X., Nordgren, G., Dalen, H., Dubowchik, G. M., Firestone, R. A. and Brunk, U. T. (2000) Induction of cell death by the lysosomotropic detergent MSDH. FEBS Lett. **470**, 35–39

18 Zhao, M., Eaton, J. W. and Brunk, U. T. (2000) Protection against oxidant-mediated lysosomal rupture: a new anti-apoptotic activity of bcl-2? FEBS Lett **485**, 104–108

19 Antunes, F., Cadenas, E. and Brunk, U. T. (2001) Apoptosis, induced by the exposure to a low and steady state concentration of hydrogen peroxide, is a consequence of lysosomal rupture. Biochem. J. **356**, 549–555

20 Roberg, K. and Öllinger, K. (1998) Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes. Am. J. Pathol. **152**, 1151–1156

21 Roberg, K. (2001) Relocalization of cathepsin D and cytochrome *c* early in apoptosis revealed by immunoelectron microscopy. Lab. Invest. **81**, 149–158

22 Zang, Y., Beard, R. L., Chandraratna, R. A. S. and Kang, J. X. (2001) Evidence of a lysosomal pathway for apoptosis induced by the synthetic retinoid CD437 in human leukemia HL-60 cells. Cell Death Diff. **8**, 477–485

23 Roberg, K., Johansson, U. and Öllinger, K. (1999) Lysosomal release of cathepsin D precedes relocation of cytochrome *c* and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress. Free Radicals Biol. Med. **27**, 1228–1237

24 Öllinger, K. (2000) Inhibition of cathepsin D prevents free-radical-induced apoptosis in rat cardiomyocytes. Arch. Biochem. Biophys. **373**, 346–351

25 Merrill Jr, A. H., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. J., Riley, R. T., Voss, K. A. and Wang, E. (1997) Sphingolipids–the enigmatic lipid class: biochemistry, physiology, and pathophysiology. Toxicol. Appl. Pharmacol. **142**, 208–225

26 Bottega, R., Epand, R. M. and Ball, E. H. (1989) Inhibition of protein kinase C by sphingosine correlates with the presence of positive charge. Biochem. Biophys. Res. Commun. **164**, 102–107

27 de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. and Van Hoof, F. (1974) Lysosomotropic agents (commentary). Biochem. Pharmacol. **23**, 2495–2531

28 Firestone, R. A., Pisano, J. M. and Bonney, R. J. (1979) Lysosomotropic agents. 1. Synthesis and cytotoxic action of lysosomotropic detergents. J. Med. Chem. **22**, 1130–1133

29 Wilson, P. D., Firestone, R. A. and Lenard, J. (1987) The role of lysosomal enzymes in killing of mammalian cells by the lysosomotropic detergent N-dodecylimidazole. J. Cell Biol. **104**, 1223–1229

30 Ishisaka, R., Utsumi, T., Kanno, T., Arita, K., Katunuma, N., Akiyama, J. and Utsumi, K. (1999) Participation of a cathepsin L-type protease in the activation of caspase-3. Cell Struct. Funct. **24**, 465–470

31 Stoka, V., Turk, B., Schendel, S. L., Kim, T. H., Cirman, T., Snipas, S. J., Ellerby, L. M., Bredesen, D., Freeze, H., Abrahamson, M. et al. (2001) Lysosomal protease pathways to apoptosis. Cleavage of Bid, not pro-caspases, is the most likely route. J. Biol. Chem. **276**, 3149–3157

32 Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingen, P. A., Peters, C., Kaufmann, S. H. and Gores, G. J. (2000) Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c*. J. Clin. Invest. **106**, 1127–1137

33 Rundquist, I., Olsson, M. and Brunk, U. T. (1984) Cytofluorometric quantitation of acridine orange uptake by cultured cells. Acta Path. Microbiol. Scand. A **92**, 303–309

34 Zdolsek, J. M., Olsson, G. M. and Brunk, U. T. (1990) Photooxidative damage to lysosomes of cultured macrophages by acridine orange. Photochem. Photobiol. **51**, 67–76

35 Olsson, G. M., Brunmark, A. and Brunk, U. T. (1989) Acridine orange-mediated photodamage of microsomal- and lysosomal fractions. Virchows Arch. B, Cell Pathol. Incl. Mol. Pathol. **56**, 247–257

36 Barrett, A. J. and Kirschke, H. (1977) in Proteases in Mammalian Cells and Tissue (Barrett, A. J., ed), pp. 209–248, Elsevier/North-Holland Biochemical Press, Amsterdam

37 Seglen, P. O. (1983) Inhibitors of lysosomal function. Methods Enzymol. **96**, 737–764

38 Deiss, L. P., Galinka, H., Barissi, H., Cohen, O. and Kimchi, A. (1996) Cathepsin D protease mediates programmed cell death induced by interferon-γ, Fas/APO-1 and TNF-α. EMBO J. **15**, 3861–3870

39 Wu, G. S., Saftig, P., Peters, C. and El-Deiry, W. S. (1998) Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity. Oncogene **16**, 2177–2183

40 Isahara, K., Ohsawa, Y., Kanamori, S., Shibata, M., Waguri, S., Sato, N., Gotow, T., Watanabe, T., Momoi, T., Urase, K. et al. (1999) Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. Neuroscience **91**, 233–249

41 Shibata, M., Kanamori, S., Isahara, K., Ohsawa, Y., Konishi, A., Kametaka, S., Watanabe, T., Ebisu, S., Ishido, K., Kominami, E. and Uchiyama, Y. (1998) Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation. Biochem. Biophys. Res. Commun. **251**, 199–203

42 Vancompernolle, K., Van Herreweghe, F., Pynaert, G., Van de Craen, M., De Vos, K., Totty, N., Sterling, A., Fiers, W., Vandenabeele, P. and Grooten, J. (1998) Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. FEBS Lett. **438**, 150–158

43 Ariga, T., Jarvis, W. D. and Yu, R. K. (1998) Role of sphingolipid-mediated cell death in neurodegenerative diseases. J. Lipid Res. **39**, 1–16

44 Neuzil, J., Svensson, I., Weber, T., Weber, C. and Brunk, U. T. (1999) α-Tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation. FEBS Lett. **445**, 295–300

45 Cifone, M. G., Roncaioli, P., De Maria, R., Camarda, G., Santoni, A., Ruberti, G. and Testi, R. (1995) Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. EMBO J. **14**, 5859–5868

46 Segui, B., Bezombes, C., Uro-Coste, E., Medin, J. A., Andrieu-Abadie, N., Auge, N., Brouchet, A., Laurent, G., Salvayre, R., Jaffrezou, J. P. and Levade, T. (2000) Stress-induced apoptosis is not mediated by endolysosomal ceramide. FASEB J. **14**, 36–47

47 Ishisaka, R., Utsumi, T., Yabuki, M., Kanno, T., Furuno, T., Inoue, M. and Utsumi, K. (1998) Activation of caspase-3-like protease by digitonin-treated lysosomes. FEBS Lett. **435**, 233–236

48 Jones, B., Roberts, P. J., Faubion, W. A., Kominami, E. and Gores, G. J. (1998) Cystatin A expression reduces bile salt-induced apoptosis in a rat hepatoma cell line. Am. J. Physiol. **275**, 723–730

49 Salvesen, G. S. (2001) A lysosomal protease enters the death scene. J. Clin. Invest. **107**, 21–22

50 Leis, M. and Jäättelää, M. (2001) Triggering of apoptosis by cathepsins. Cell Death Diff. **8**, 324–326

51 Brunk, U. T., Neuzil, J. and Eaton, J. W. (2001) Lysosomal involvement in apoptosis. Redox Report, **6**, 91–97

52 Schotte, P., Declercq, W., Van Huffel, S., Vandenabeele, P. and Beyaert, R. (1999) Non-specific effects of methyl ketone peptide inhibitors of caspases. FEBS Lett. **442**, 117–121

Received 9 March 2001/18 June 2001; accepted 8 August 2001