Overexpression of Acid Ceramidase Protects from Tumor Necrosis Factor–induced Cell Death

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Abstract

Tumor necrosis factor (TNF) signals cell death and simultaneously induces generation of ceramide. To evaluate the contribution of ceramide to TNF-dependent cell death, we generated clones of the TNF-sensitive cell line L929 that constitutively overexpress human acid ceramidase (AC). Ceramidase, in concert with sphingosine kinase, metabolizes ceramide to sphingosine-1-phosphate (SPP), an inducer of proliferation. In response to TNF, parental L929 cells display a significant increase in intracellular ceramide correlated with an "atypical apoptosis" characterized by membrane blebbing, DNA fragmentation and degradation of poly(ADP-ribose) polymerase despite a lack of caspase activity. These features are strongly reduced or absent in AC-overexpressing cells. Pharmacological suppression of AC with *N*-oleoylethanolamine restored the accumulation of intracellular ceramide as well as the sensitivity of the transfectants to TNF, implying that an enhanced metabolization of intracellular ceramide by AC shifts the balance between intracellular ceramide and SPP levels towards cell survival. Correspondingly, inhibition of ceramide production by acid sphingomyelinase also increased survival of TNF-treated L929 cells.

Key words: ceramidase • L929 cell • tumor necrosis factor • cell death • ceramide

Introduction

The sphingolipid ceramide has been described as an important bioeffector molecule involved in cellular stress responses as well as in programmed cell death (for review see references 1, 2), even though its relevance for apoptosis is a controversial subject for discussion (3–7). Stress stimuli like TNF, Fas ligand, oxidative stress, growth factor withdrawal, anticancer drugs, ionizing radiation, heat shock, or ultraviolet light induce an elevation in the endogenous cellular levels of ceramide (1, 8). A function of endogenously generated ceramide as a cofactor in the actions of stress stimuli is supported by the ability of exogenous ceramide analogues to mimic these biological responses in specific cell types (1). Ceramide is generated from the major membrane sphingolipid sphingomyelin by acid or neutral sphingomyelinases (A- or N-SMases)[1], enzymes that are activated in response to TNF and other cytokines (for review see reference 9). The catabolic pathway for ceramide involves deacylation by ceramidases to generate sphingosine, which is phosphorylated by sphingosine kinase to form sphingosine-1-phosphate (SPP). SPP in turn acts as a second messenger in cellular proliferation and survival induced by platelet-derived growth factor (PDGF) or serum (10). Previously, a model has been proposed in which the dynamic balance between the intracellular levels of ceramide and SPP (the "ceramide/SPP rheostat") is an important factor that determines whether a cell survives or dies (10).

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[1]*Abbreviations used in this paper:* 7-AAD, 7-amino-actinomycin D; AC, acid ceramidase; A-SMase, acid sphingomyelinase; C_{16}-ceramide, *N*-palmitoylsphingosine; DAPI, 4′,6-diamidino-2-phenylindole; NOE, *N*-oleoylethanolamine; N-SMase, neutral sphingomyelinase; PARP, poly (ADP-ribose) polymerase; SPP, sphingosine-1-phosphate; zDEVD-afc, benzyloxycarbonyl-Asp-Glu-Val-Asp-aminotrifluoromethylcoumarin; zIETD-afc, benzyloxycarbonyl-Ile-Glu-Thr-Asp-aminotrifluoromethylcoumarin; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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According to this model, stress stimuli like TNF activate SMases, leading to increased intracellular ceramide levels and thus to increased cell death, whereas PDGF and other growth factors stimulate ceramidase and sphingosine kinase and elevate SPP levels, resulting in cellular survival and proliferation (for review see reference 11).

Human acid ceramidase (AC) has recently been purified and cloned (12, 13). Because ceramide degradation is the only catabolic source of intracellular sphingosine (14), AC activity may be the rate-limiting step in determining the intracellular levels of sphingosine, and subsequently, SPP, playing a crucial role for the status of the ceramide/SPP rheostat and, in consequence, for cell survival or death in response to external stimuli.

Here, we demonstrate that overexpression of AC in murine fibrosarcoma L929 cells protects against TNF-induced cell death. Our results suggest that the enhanced expression of AC results in a faster removal of intracellular ceramide and therefore in a shift of the ceramide/SPP rheostat in the direction of cell survival. Thus, in L929 cells, intracellular ceramide may be a major inducer of TNF-dependent cell death.

Materials and Methods

Plasmids and Reagents. Highly purified human and murine recombinant TNF was provided by Dr. G. Adolf (Bender Research Institute, Vienna, Austria). The expression vector pSV•SPORT1-AC, containing the full-length human AC cDNA as well as the AC-specific rabbit polyclonal antiserum have been described previously (12, 13). The L929 transfectant clone Cl430 overexpressing Fas/APO-1 was provided by Dr. M. Peter (Deutsches Krebsforschungszentrum [DKFZ], Heidelberg, Germany). Collagen A was from Biochrom. *N*-lauroylsphingosine, *N*-oleoylethanolamine (NOE), and 4′,6-diamidino-2-phenylindole (DAPI) and desipramine were purchased from Sigma-Aldrich. Benzyloxycarbonyl-Asp-Glu-Val-Asp-aminotrifluoromethylcoumarin (zDEVD-afc) and benzyloxycarbonyl-Ile-Glu-Thr-Asp-aminotrifluoromethylcoumarin (zIETD-afc) were ordered from Calbiochem. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was obtained from Bachem, and *N*-palmitoylsphingosine (C_{16}-ceramide) from Biomol. D609 was purchased from MoBiTec and fumonisin B1 from Alexis Biochemicals. The mAb CH-11 specific for human Fas/APO-1 was purchased from Coulter/Immunotech. The monoclonal anti–poly(ADP-ribose) polymerase (PARP) antibody C2-10, the polyclonal Rho-GDI/D4-GDI antibody, and 7-amino-actinomycin D (7-AAD) were purchased from PharMingen. Antibodies against caspase-3 (H-277) and lamin B (M-20) were from Santa Cruz Biotechnology, Inc.

Cell Culture and Transfections. L929 cells were originally obtained from the American Type Culture Collection. Cells were maintained in a mixture of Click's/RPMI 1640 1:1 vol/vol) supplemented with 10% vol/vol calf serum, 10 mM glutamine, and 50 μg/ml each of streptomycin and penicillin in a humidified incubator containing 5% wt/vol CO_2. Stably transfected L929 cells expressing the full length AC cDNA were obtained by cotransfection of pSV•SPORT1-AC with BMGNeo (15) using electroporation at 960 μF/280 V and subsequent selection with 1,000 μg/ml Geneticin (Life Technologies). Transfectants expressing vector alone without insert were generated by cotransfecting pSV•SPORT1 with BMGNeo. For the L929 clone Cl430, expression of Fas/APO-1 on the cell surface was verified in flow cytometry analyses.

Assays for Enzymatic Activity of AC. *N*-[1-^{14}C]lauroylsphingosine was synthesized from [1-^{14}C]lauric acid (CFA106; Amersham Pharmacia Biotech; specific activity 2.0 GBq/mmol) and sphingosine as previously described (16). 10^6 cells were washed with cold PBS, resuspended in 200 μl of cold lysis buffer (0.2% vol/vol Triton X-100), left for 10 min on ice and homogenized by repeated passing through a 25-gauge needle. After centrifugation, 100 μg of protein from the supernatant was added in duplicates to a standard reaction mixture containing 150 μM *N*-lauroylsphingosine, 2 μM *N*-[1-^{14}C]lauroylsphingosine, Triton X-100 (0.05% wt/vol), Tween 20 (0.02% wt/vol), NP-40 (0.04% wt/vol), sodium cholate (0.08% wt/vol), and EDTA (5 μM) in sodium acetate buffer (250 mM, pH 4.5) in a total volume of 100 μl. The mixture was incubated for 1 h at 37°C, and the reaction was stopped by the addition of 250 μl of H_2O and 750 μl of chloroform/methanol (2:1 vol/vol). After centrifugation, the chloroform phase was evaporated under nitrogen. The extracted lipids were separated by thin layer chromatography in chloroform/methanol/acetic acid (94:1:5 vol/vol/vol), visualized by autoradiography and quantified in a PhosphorImager (Fuji). The amount of protein added to the reaction mixtures was chosen so that the reaction was linear within the time frame and the amount of N-[1-^{14}C]lauroylsphingosine hydrolyzed did not exceed 10% of the total amount of radioactive substrate added.

Immunoblots and Generation of Cytosolic Cell Extracts. Adherent and detached cells were collected and lysed in TNE buffer (50 mM Tris, pH 8.0, 1% vol/vol NP-40, and 2 mM EDTA) containing 10 μg/ml pepstatin/aprotinin/leupeptin, 1 mM sodium orthovanadate, and 5 mM NaF. For detection of AC, 25 μg of cell protein per lane were resolved by electrophoresis on 12.5% wt/vol SDS-PAGE. After electrophoretic transfer to nitrocellulose, reactive proteins were detected using AC-specific polyclonal antibodies and the ECL detection kit (Amersham Pharmacia Biotech). For detection of PARP, lamin B, D4-GDI, and caspase-3, 25 μg of protein was separated by SDS-PAGE on 8% (PARP) or 12.5% gels and analyzed using the corresponding antibodies. To generate cytosolic cell extracts (positive controls), cells were lysed in a buffer containing 10 mM Hepes, pH 7.4, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% vol/vol NP-40, 1 mM dithiothreitol, and 1 mM PMSF. The cell lysates were equilibrated for 1 h at 30°C after the addition of 1 mM dATP and 10 μM cytochrome c to permit activation of caspases and subsequent cleavage of substrate proteins as previously described (17).

Cytotoxicity Assays. 10^4 cells were seeded in flat-bottomed 96-well plates in medium containing serial dilutions of human (h) or murine (m)TNF. After 48 h, cells were washed twice with PBS and incubated for 10 min at 37°C in 50 μl of staining solution (crystal violet 0.5% wt/vol, formaldehyde 4% wt/vol, ethanol 30% vol/vol, and NaCl 0.17% wt/vol). The staining solution was washed away with tap water and the cells were dried for 1 h at 50°C. The stained cells were dissolved in acetic acid (33% vol/vol) and the intensity of the staining was colorimetrically determined at 570 nm in a microplate reader (Dynex).

DAPI-Staining of Cells. DAPI was dissolved in water at a concentration of 10 mg/ml and stored at 4°C in the dark. 2×10^4 cells per well were grown on collagen A–coated chamber slides (Nunc) and treated with either hTNF (100 ng/ml, 20 h) or Fas/APO-1 antibody CH-11 (250 ng/ml, 10 h), or else left untreated. After removal of the culture medium, cells were washed once in staining solution (500 ng/ml DAPI in methanol) and incubated in staining solution for 15 min at 37°C. The cells were

washed once in methanol, dried, and photographed under a fluorescence microscope (ZEISS Axioskop).

Hypodiploid Cell Assessment in Cell Cycle Analyses. Adherent and detached cells were collected and washed twice with cold PBS/5 mM EDTA and resuspended in 1 ml PBS/5 mM EDTA. Cells were fixed by dropwise addition of 1 ml ethanol and incubation at room temperature for 30 min, harvested, and resuspended in 0.5 ml PBS/5 mM EDTA. After digestion with 20 μl RNAse A (1 mg/ml) for 30 min at room temperature, cells were incubated for 1 h in 0.5 ml of staining solution (500 μg/ml propidium iodide in PBS/5 mM EDTA). Cell cycle analysis was performed by flow cytometry using a FACSCalibur™ (Becton Dickinson).

Agarose Gel Electrophoresis for DNA Fragmentation. 10^6 cells were seeded into 10-cm dishes and grown for 48 h, while 100 ng/ml hTNF was added for 0, 20, and 48 h. Adherent and detached cells were collected and resuspended in 20 μl of lysis buffer (10 mM EDTA, 50 mM Tris, pH 8.0, 0.5% wt/vol sodium lauryl sarcosinate, 0.5 mg/ml proteinase K), incubated for 2 h at 50°C after addition of 5 μl RNAse A (1 mg/ml), and heated to 65°C for 5 min before 10 μl of a mixture of gel-loading buffer (25% wt/vol Ficoll 400 [Amersham Pharmacia Biotech], 25 mM EDTA, and 0.5% wt/vol bromphenol blue, pH 8.0) and 1% low melting point agarose (1:1 vol/vol, heated to 65°C) were added. The heated samples were quickly transferred into dry wells of a low melting point gel (2% wt/vol NuSieve GTG agarose), separated electrophoretically, and visualized under UV light after staining with ethidium bromide.

Fluorogenic Substrate Assay for Caspase Activity. Cytosolic extracts from cells treated with hTNF or supplemented with dATP and cytochrome c to activate caspases (positive control) were prepared as described above. To measure caspase activity, 100 μl of caspase buffer (20 mM Pipes, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% wt/vol CHAPS, and 10% wt/vol sucrose, pH 7.2) containing 100 μM zDEVD-afc or zIETD-afc were added to 5 μl of cytosolic extract (50 μg protein) and incubated at 37°C. The release of afc was measured as emission at 510 nm upon excitation at 405 nm using a Labsystems Fluoroskan II fluorimeter equipped with a thermostated plate reader.

Flow Cytometric Analysis of Membrane Integrity and Phosphatidylserine Externalization. 10^6 adherent and detached cells were collected, washed with cold PBS, and resuspended in 100 μl of a buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 5 mM $CaCl_2$) containing 2% vol/vol of Annexin V–FLUOS labeling reagent (Boehringer Mannheim) and 20% vol/vol 7-AAD reagent (PharMingen). The cells were incubated for 15 min in the dark at room temperature and analyzed by flow cytometry using a FACSCalibur™ (Becton Dickinson). For permeabilization of cell membranes with ethanol, 10^6 cells were collected, washed with cold PBS/5 mM EDTA, and resuspended in 1 ml PBS/5 mM EDTA. Cells were fixed by dropwise addition of 1 ml ethanol and incubation at room temperature for 30 min, then were collected and stained as described above.

Ceramide Quantitation. Ceramide was quantitated by the diacylglycerol kinase assay essentially as described by Dressler and Kolesnick (18), following the recommendations of Perry and Hannun (5).

Measurement of Uptake of Exogenous Ceramide. 37 kBq (36.4 μM) of ^{14}C-labeled C_{16}-ceramide (ARC-831; BIOTREND Chemikalien GmbH; specific activity 2.03 GBq/mmol) were added to 10^5 cells followed by incubation for the indicated times at 37°C. Next, the cells were washed two times with PBS and lysed in 0.1% SDS. The incorporated radioactivity was determined by scintillation counting.

Results

Generation of Stable Transfectants Overexpressing AC. Parental L929 cells were stably transfected with pSV•SPORT1-AC and Geneticin-resistant colonies were isolated. In addition, several transfectants were selected that contain the expression vector pSV•SPORT1 without an insert. Of those, clones pSV2 and pSV4, together with parental L929 cells, served as negative controls in the subsequent experiments. Out of eight Geneticin-resistant clones displaying an elevated level of AC activity, four (designated AC23, AC28, AC33, and AC52) were chosen for further analysis. The basal AC activities of clones AC23 to AC52 ranged from 5- to 20-fold of those determined in parental L929 cells or in the control clones pSV2 and pSV4 (Fig. 1 A). Western blot analyses using AC-specific antiserum confirmed corresponding increases at the level of AC expression (Fig. 1 B).

Reduced Cytotoxicity of TNF in AC-Overexpressing Cells. In the subsequent cytotoxicity assays, protein biosynthesis inhibitors were omitted to avoid nonspecific effects. After treatment with hTNF, which binds to the 55- but not to the 75-kD receptor of murine L929 cells, untransfected cells and control clones pSV2 and pSV4 were efficiently killed at concentrations of 10–100 ng/ml hTNF (Fig. 2 A). At the same concentrations, all AC-transfected clones displayed a considerably higher viability (Fig. 2 B).

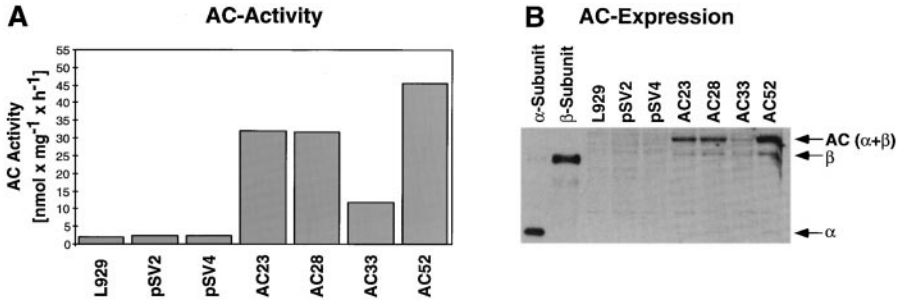

Figure 1. Activity and expression of AC in stably transfected L929 clones. (A) Enzymatic activity of AC in untransfected L929 cells, in L929 cells transfected with pSV•SPORT1 without insert (pSV2, pSV4), and in L929 cells transfected with pSV•SPORT1-AC (AC23 to AC52). The values shown represent the means from duplicate determinations. Similar results were obtained in multiple independent experiments (n = 6–9 for each AC transfectant). (B) Western blot analysis of AC expression in L929 transfectants. Purified α and β subunits of AC as well as protein extracts from parental L929 cells, pSV2 and pSV4 cells, and clones AC23 to AC52 were immunoblotted using AC antiserum. The arrows indicate the predicted sizes for mature AC (complex of α and β subunits) as well as for the individual subunits (α, 13 kD; β, 40 kD).

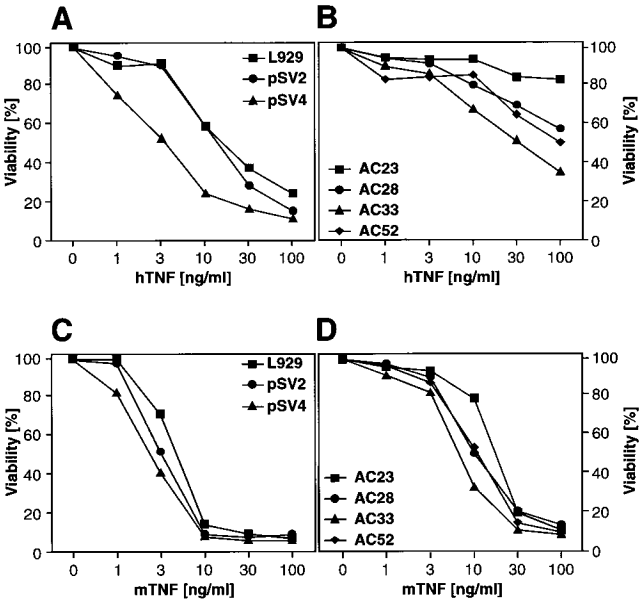

Figure 2. AC-overexpressing L929 clones are protected from TNF-induced cell death. Parental L929 cells and cells stably transfected with AC (AC23 to AC52) or vector without insert (pSV2, pSV4) were treated for 48 h with serial dilutions of hTNF (A and B) or mTNF (C and D) as indicated. Cellular viability was determined by staining with crystal violet. The values shown represent the means from six parallel determinations. SD did not exceed 15% of the means.

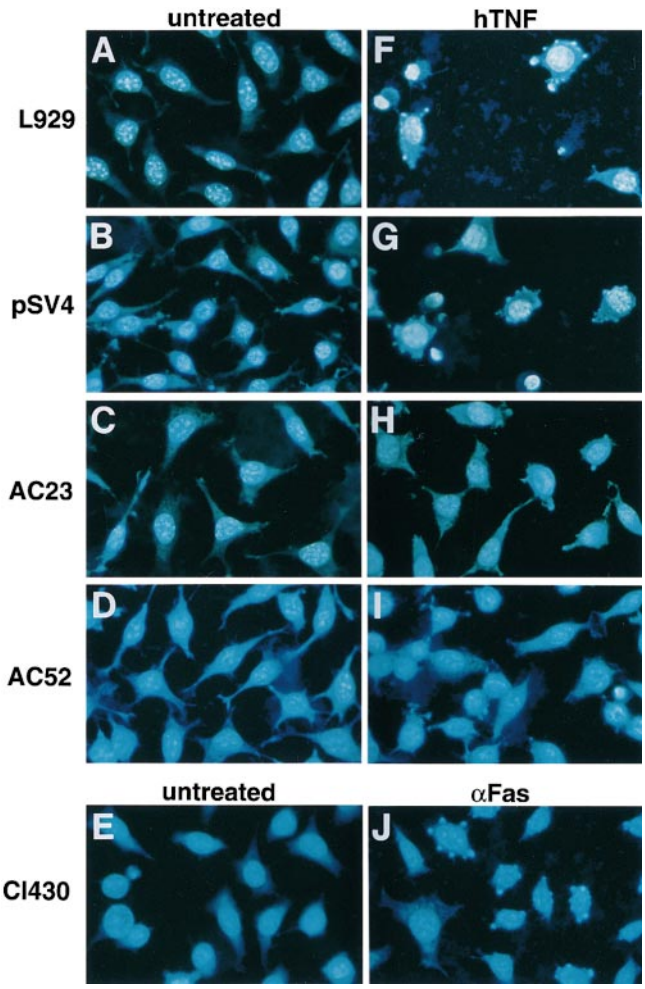

Figure 3. Morphological changes in dying parental and stably transfected L929 cells. Parental L929 cells, pSV4 cells, and clones AC23 and AC52 were either left untreated (A–D) or treated with hTNF (100 ng/ml, 20 h; F–I). In addition, clone Cl430 stably expressing human Fas/APO-1 was left untreated (E) or stimulated with the monoclonal antibody CH-11 specific for human Fas/APO-1 (250 ng/ml, 10 h; J). Cells were stained with the DNA-specific dye DAPI and photographed at an original magnification of 400.

Treatment of the transfectants with mTNF, which engages both the 55- and 75-kD TNF receptors (TR55 and TR75), showed a similar protection of clones AC23 to AC52 at concentrations of 3–30 ng/ml when compared with parental L929 cells or clones pSV2 and pSV4 (Fig. 2, C and D). However, treatment with mTNF enhanced the overall level of cell death in all cell lines. As stimulation of TR75 does not lead to activation of SMases and thus does not lead to the production of ceramide (19), it appears likely that TR75 elicits additional cytotoxic signals independent from SMases or ceramide which in consequence cannot be compensated by an enhanced enzymatic activity of AC.

TNF-induced Apoptosis in L929 Cells Shows Atypical Features and Is Strongly Reduced in AC-transfected Cells. We compared changes in nuclear and cellular morphology of untransfected and AC-overexpressing L929 cells by staining with the DNA-specific dye DAPI. Untreated cells uniformly displayed an intact nuclear morphology with localized patches of nuclear DNA (Fig. 3, A–E). Treatment with hTNF led to a blebbing of the cell membranes culminating in the formation of chromatin-containing small vesicles indicative for apoptotic bodies in both untransfected L929 cells and in pSV4 cells expressing empty vector (Fig. 3, F and G). Identical morphological changes were detectable in L929 cells stably transfected with human Fas/APO-1 (clone Cl430) that had been treated with Fas/APO-1 antibody to induce apoptotic cell death (Fig. 3 J). In contrast, only minor changes were visible in hTNF-treated clones AC23 and AC52 (Fig. 3, H and I). Treatment with mTNF elicited a greater number of dying cells in all cell lines when compared with hTNF. Nevertheless, clones AC23 and AC52 again were largely protected (data not shown). Of note, we did not observe swelling of cells and rupture of cell membranes that have been described as hallmarks of necrotic cell death (20) in any of the above experiments. However, as the morphological assessment of cells always depends on the subjective interpretation of the viewer, other, more objective parameters of cell death were analyzed subsequently.

In cell cycle analyses determining the degree of cellular DNA fragmentation, a large fraction of hypodiploid L929 and pSV4 cells was detected after treatment with hTNF (Fig. 4 A). In contrast, only a reduced number of hypodiploid cells was present in clones AC23 to AC52, indicating that overexpression of AC also prevents DNA fragmenta-

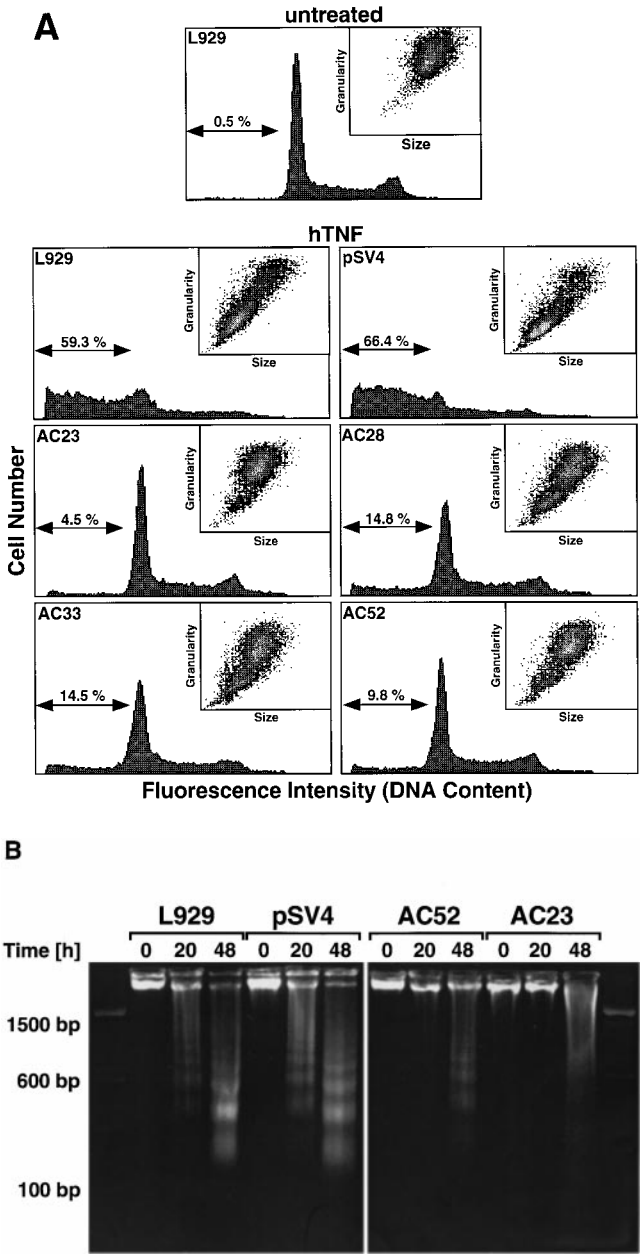

Figure 4. DNA fragmentation in parental and AC-overexpressing L929 cells. (A) Untreated L929 cells (top) or cells treated with 100 ng/ml hTNF for 48 h (bottom) were stained for cell cycle analysis. The percentage of hypodiploid apoptotic cells is indicated. The corresponding density plots of gated cells showing changes in granularity and cell size are indicated to provide a second measure for cell death. The fraction of hypodiploid cells observed in untreated clones was <0.8% (data not shown). Similar results were obtained in four independent experiments. (B) Agarose gel electrophoresis of genomic DNA from parental L929 cells, pSV4 cells, and clones AC23 and AC52 treated with 100 ng/ml hTNF for the indicated times.

tion in response to hTNF. In parallel, cellular collapse was monitored by determining changes in granularity and size of the cells (Fig. 4 A, insets). Although a certain fraction of collapsed cells could be observed in the AC-overexpressing cell lines, it was dramatically less pronounced than the fraction present in L929 or pSV4 cells treated with hTNF, indicating that elevated AC activity does protect against the cytotoxic signals of TR55, although not completely. These results were confirmed in experiments analyzing DNA fragmentation by agarose gel electrophoresis. As shown in Fig. 4 B, treatment of L929 or pSV4 cells with hTNF induced a readily visible oligonucleosomal fragmentation of nuclear DNA indicative for apoptosis, whereas in clones AC23 and AC52, the degree of DNA fragmentation was much less pronounced.

During apoptosis, PARP, a DNA-associating nuclear protein, is inactivated by processing of the mature 118-kD protein to an 85-kD cleavage product (21). When L929 or pSV4 cells were analyzed for PARP cleavage in Western blots, the intensity of the uncleaved 118-kD band started to decrease after 10 h of treatment with hTNF, resulting in the disappearance of the uncleaved protein between 15 and 20 h (Fig. 5 A). Strikingly, the 85-kD band indicative for cleavage by caspase-3 could not be detected, suggesting that a protease different from caspase-3 is responsible for the degradation of PARP in L929 cells. In contrast, no degradation of PARP was evident in clones AC23 and AC52 even after 20 h of hTNF treatment (Fig. 5 A).

When we examined cleavage of other caspase-substrate proteins, neither lamin B, D4-GDI, nor caspase-3 itself were cleaved or degraded after hTNF treatment for 20 h in parental L929 cells (Fig. 5 B) or in clones AC23 and AC52 (data not shown). This argues that TNF does not activate initiator caspases like caspase-8, -9, or -10 that induce cleavage of the above substrate proteins in L929 cells. However, by adding dATP and cytochrome c to the cell lysates we could demonstrate cleavage of all substrate proteins, confirming that the above caspases are present and can be activated in L929 cells (Fig. 5 B, Co). The fact that lamin B, D4-GDI, and caspase-3 were neither cleaved nor degraded after treatment with hTNF indicates that the observed degradation of PARP is not the result of a general destruction of cellular proteins in the course of cell death. Experiments determining cleavage of the fluorogenic caspase-3 and -8 substrates zDEVD-afc and zIETD-afc confirmed directly that hTNF induces neither caspase-3 nor -8 activity in L929 cells (Fig. 5 C).

The assumption that caspase-3 is not responsible for PARP cleavage in L929 cells gained further support from the observation that application of the broad-spectrum caspase-inhibitor zVAD-fmk in combination with hTNF did not inhibit PARP cleavage. On the contrary, zVAD-fmk reduced the PARP degradation time dramatically to <6 h (Fig. 5 D).

We monitored the externalization of phosphatidylserine and changes in plasma membrane integrity as further parameters of cell death. As a positive control for apoptotic cell death, Jurkat cells were treated with Fas/APO-1 antibody, double-stained with annexin V and 7-AAD, and analyzed by flow cytometry. As shown in Fig. 6 (top), the cells displayed distinct populations of annexin $V^+/7\text{-}AAD^-$ (early apoptotic; lower right quadrant) and double-positive cells (late apoptotic/necrotic; upper right quadrant). In

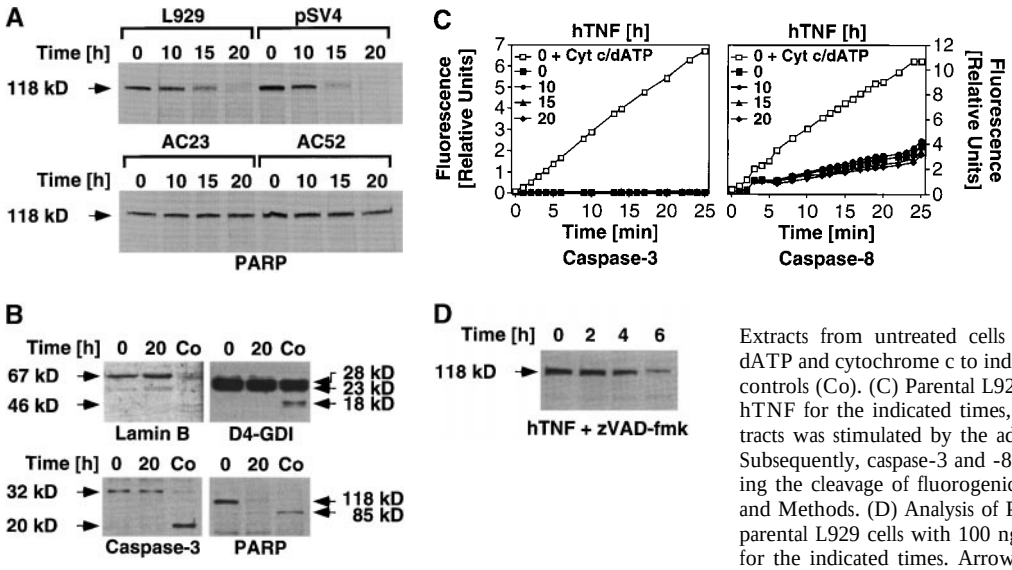

Figure 5. Caspase-3–independent degradation of PARP in L929 cells is prevented by overexpression of AC. (A) Parental and transfected L929 cells were treated with 100 ng/ml hTNF for the indicated times and analyzed for degradation of PARP. (B) Parental L929 cells were left untreated or treated with 100 ng/ml hTNF for 20 h and analyzed for cleavage of lamin B, D4-GDI, caspase-3, and PARP. Extracts from untreated cells that had been supplemented with dATP and cytochrome c to induce caspase activity served as positive controls (Co). (C) Parental L929 cells were treated with 100 ng/ml hTNF for the indicated times, or caspase activity in untreated extracts was stimulated by the addition of dATP and cytochrome c. Subsequently, caspase-3 and -8 activity was determined by measuring the cleavage of fluorogenic substrates as described in Materials and Methods. (D) Analysis of PARP degradation after treatment of parental L929 cells with 100 ng/ml hTNF and 20 μM zVAD-fmk for the indicated times. Arrowheads mark the sizes of the mature proteins and their cleavage products.

contrast, parental L929 and pSV4 cells treated with hTNF (middle) stained 7-AAD$^+$/annexin V$^-$ as early as 10 h (upper left quadrant of respective panels). However, no positive staining for annexin V was observed even after 48 h. Only when L929 cells were actively permeabilized by treatment with ethanol, a positive stain for both 7-AAD and annexin V was detectable (bottom), arguing that phosphatidylserine is present but not externalized in hTNF-treated L929 cells. Moreover, the positive staining with 7-AAD is not confirmatory for a necrotic loss of membrane integrity since annexin V should also be able to enter the cells. The AC-overexpressing clones showed a distinctly reduced population of 7-AAD$^+$/annexin V$^-$ cells (AC23, 29%; AC52, 37% vs. L929, 64%; pSV4, 78%), confirming protection against the cytotoxic effects of TNF (upper left quadrant of respective panels).

In summary, the above results demonstrate that L929 cells show features of an atypical apoptosis in response to TNF and that all of these features are strongly reduced or prevented by overexpression of AC.

Overexpression of AC Prevents Generation of Intracellular Ceramide in Response to TNF. In analyses for changes in intracellular ceramide levels, hTNF induced a marked increase in both untransfected L929 cells and in the control clone pSV4. In contrast, the intracellular ceramide content of hTNF-treated clones AC23 and AC52 was essentially unaltered (Fig. 7). Apparently, the overexpression of AC does not lead to a decrease of the basal intracellular ceramide levels. As we detected most of the enzymatic activity of overexpressed AC within the lysosomal fraction of the cells (data not shown), it is plausible to assume that only ceramide generated within the endolysosome (e.g., by endolysosomal acid sphingomyelinase) is accessible to the overexpressed AC. These results clearly demonstrate an enhanced turnover of newly generated ceramide in AC-overexpressing cells, thereby explaining their enhanced protection against TNF-induced cell death.

Exogenously Added C_{16}-Ceramide but Not SMase Abrogates Protection by AC. To determine whether the protective effect of AC overexpression could be overcome by exogenous ceramide analogues, parental and transfected L929 cells were treated with C_2-ceramide (*N*-acetylsphingosine), C_6-ceramide (*N*-hexanoylsphingosine), or C_{16}-ceramide. C_2-ceramide caused cell cycle arrest rather than cell death (22), whereas C_6- and C_{16}-ceramide induced a dose-dependent cell-death response without notable differences between parental and AC-overexpressing cells (Fig. 8 A and data not shown). When we measured the uptake of exogenous ^{14}C-labeled C_{16}-ceramide (Fig. 8 B), the amount incorporated within 2.5 h was more than 100-fold higher than the amount of intracellular ceramide that is generated in response to TNF (Fig. 7), indicating that even the enhanced capacity of the AC-overexpressing clones for ceramide degradation is overwhelmed by the massive influx of exogenous C_{16}-ceramide. When exogenous SMase from *Staphylococcus aureus* (500 mU/ml) was added to parental and transfected L929 cells, no signs of death or DNA fragmentation were detectable after 36 h, although the activity of the enzyme was verified by demonstrating phosphorylation of p42/p44 extracellular-regulated kinase within 15 min (data not shown). These results are in agreement with a recent study showing that exogenous SMase is not generally sufficient to induce cell death and that the generation of ceramide intracellularly is distinct from generation of ceramide at the outer leaflet of the plasma membrane with regard to cellular signaling (23).

Pharmacological Inhibition of AC Reduces Protection against TNF-mediated Cell Death by Restoring Accumulation of Intracellular Ceramide, Whereas Inhibition of A-SMase Increases Protection. To extend the analysis of the mechanism by which AC overexpression protects from cell death, parental and transfected L929 cells were treated with NOE, a potent in vitro inhibitor of ceramidase (24). In parental L929 or pSV4 cells, hTNF elicited a cytotoxic response

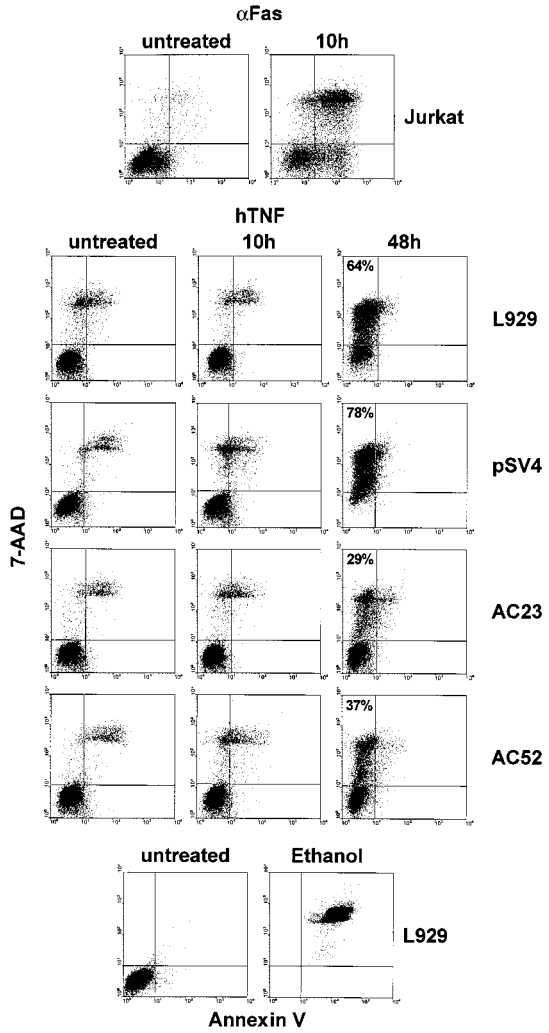

Figure 6. Flow cytometric analysis of parental and transfected L929 cells double stained with 7-AAD and annexin V after treatment with or without 100 ng/ml of hTNF for the indicated times (middle). The percentage of 7-AAD$^+$/annexin V$^-$ cells after 48 h of TNF treatment is indicated. Jurkat cells were treated with Fas/APO-1 antibody (500 ng/ml) as a positive control for apoptosis (top). In addition, parental L929 cells were actively permeabilized by treatment with ethanol to verify the presence of phosphatidylserine at the inner cell membrane (bottom).

which was not enhanced further by addition of NOE. Clones AC23 and AC52 showed protection against hTNF-induced cell death which was clearly decreased when hTNF was applied in combination with NOE (Fig. 9 A). When we measured the effect of NOE treatment on intracellular ceramide levels of AC-overexpressing L929 cells, a clear increase was detected when clones AC23 and AC52 were treated with NOE in combination with hTNF (Fig. 9 B), explaining their enhanced sensitivity seen in the corresponding cytotoxicity assays.

Parental L929 cells were treated with hTNF in the presence of desipramine or of tricyclodecan-9-yl (D609). Desipramine causes a rapid and irreversible reduction of A-SMase activity by inducing proteolytic degradation of the enzyme (25), whereas D609 prevents the TNF-dependent

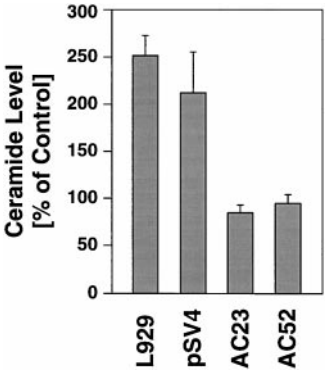

Figure 7. TNF-induced changes of intracellular ceramide in parental and AC-overexpressing L929 cells. Parental and transfected L929 cells were treated with 100 ng/ml hTNF for 20 h before intracellular ceramide was determined. Ceramide levels are expressed relative to untreated cells, in which basal ceramide was between 50 and 180 pmol per 10^7 cells. The values shown represent the means from triplicate determinations. Error bars indicate the respective SDs. Similar results were obtained in five independent experiments. Analysis of variance for repeated measurements showed that ceramide levels in AC-overexpressing cells were significantly different from those in parental or pSV4 cells ($P < 0.001$).

activation of A-SMase (19). Compared with cells treated with hTNF alone, both desipramine and D609 clearly increased cell survival. In contrast, the ceramide synthase inhibitor fumonisin B1 had no protective effect, arguing that TNF-induced accumulation of intracellular ceramide is mediated by A-SMase rather than by ceramide synthase (Fig. 9 C). Furthermore, demonstration of comparable TNF-dependent decreases in cellular sphingomyelin content as well as detection of essentially identical basal levels of A-SMase in parental and transfected L929 cells showed that overexpressed AC protects clones AC23 and AC52 by removing newly generated ceramide but not by interfering with ceramide generation through A-SMase (data not shown).

Discussion

Role of Ceramide in Cell Death. In this paper, the consequences of AC overexpression and therefore of enhanced metabolization of intracellular ceramide were investigated with respect to TNF-induced cell death. Several lines of evidence suggest that ceramide does not merely arise as a consequence of apoptosis but rather contributes to cell death itself (for review see references 1, 9). Lymphoblasts from patients with Niemann-Pick disease, an inherited deficiency of A-SMase, exhibit defects in their apoptotic response to ionizing radiation which can be restored by retroviral transfer of human A-SMase cDNA. Similarly, A-SMase knockout mice fail to generate ceramide and to develop typical apoptotic lesions in the lung, but not in the thymus, suggesting a tissue-restricted involvement of ceramide in radiation-induced apoptosis (26). Moreover, resistance to radiation-induced apoptosis in Burkitt's lymphoma cells is associated with defective ceramide signaling (27). Further support for a role of ceramide in apoptosis comes from the indirect manipulation of endogenous ceramide levels. Addition of either bacterial SMase or of inhibitors of ceramide metabolism results in increased intracellular ceramide levels and also in apoptosis (for review see reference 1). Finally, numerous experiments have used synthetic short-chain ceramide analogues. However, these experiments should be interpreted with caution. First, exogenous addition of cer-

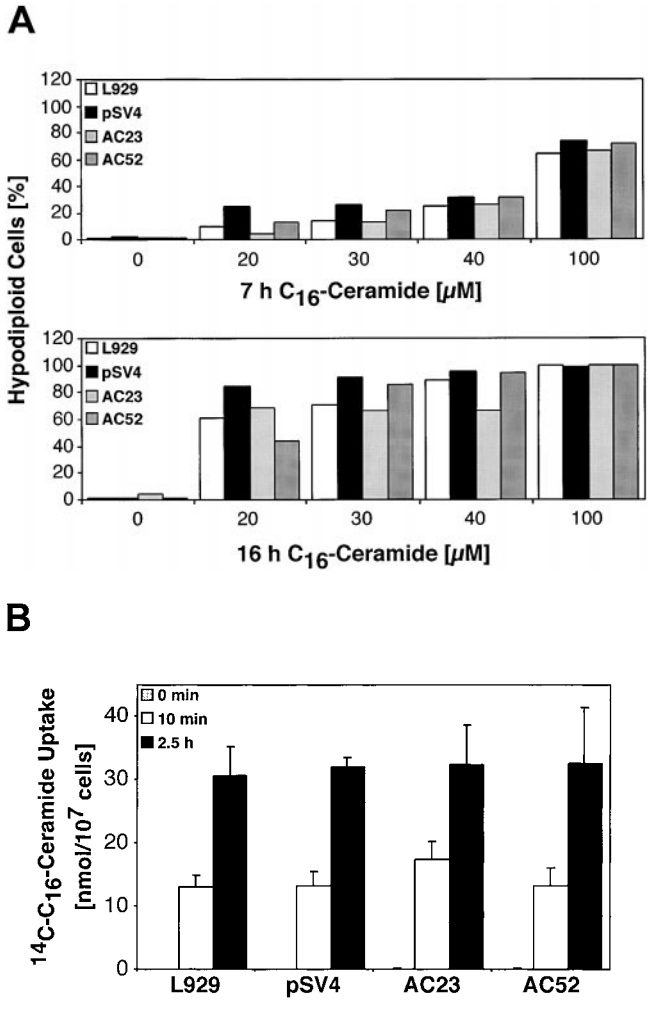

Figure 8. Protection of AC-overexpressing cells is overcome by exogenously added C_{16}-ceramide. (A) Parental and transfected L929 cells were incubated with the indicated concentrations of C_{16}-ceramide for 7 h (top) or 16 h (bottom) and stained for cell cycle analysis. The percentage of hypodiploid apoptotic cells is indicated. (B) Parental and transfected L929 cells were incubated with ^{14}C-labeled C_{16}-ceramide for the indicated times and uptake of exogenous C_{16}-ceramide was measured. The values shown represent the means from triplicate determinations. Error bars indicate the respective SDs.

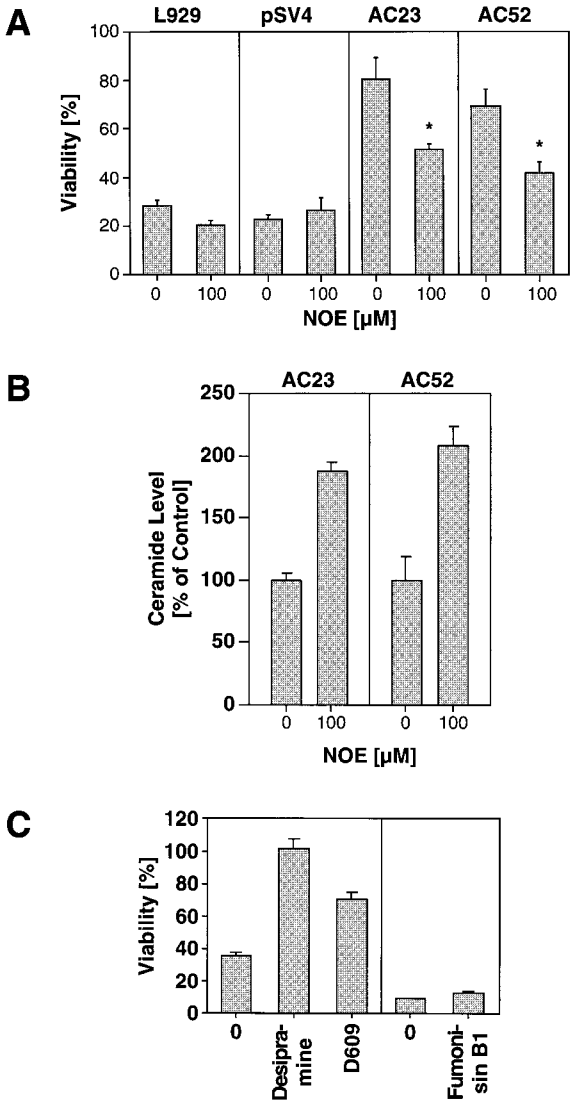

Figure 9. Pharmacological inhibition of AC diminishes protection from TNF-induced cell death, whereas inhibition of A-SMase enhances protection. (A) Parental and transfected L929 cells were incubated with or without 100 μM NOE in the presence of 100 ng/ml hTNF for 48 h. (B) Clones AC23 and AC52 were treated with 100 ng/ml hTNF with or without 100 μM NOE for 40 h before intracellular ceramide was determined. (C) Parental L929 cells were treated with or without 25 μM desipramine or 25 μg/ml D609 in the presence of 100 ng/ml hTNF for 48 h. Separately, L929 cells were incubated with 100 ng/ml hTNF for 48 h with or without addition of 50 μM fumonisin B1. Cell viability is expressed relative to untreated controls. Ceramide levels are expressed relative to untreated cells, in which basal ceramide was between 100 and 120 pmol per 10^7 cells. Values shown represent the means from three or six parallel determinations (ceramide levels or cell viability), and error bars indicate the respective SDs. *$P < 0.002$ versus cells not treated with NOE (determined by Student's *t* test). One out of three independent experiments with similar results is shown.

amide analogues may not accurately mimic the transient physiologic changes of ceramide levels within a cell. Second, synthetic ceramide analogues may be metabolized to other bioactive sphingolipids when added to intact cells. Our own results demonstrate that in L929 cells short-chain exogenous C_2-ceramide analogues or SMase do not accurately mimic the effects of intracellular ceramide elicited by TNF. In contrast to synthetic ceramide analogues, inhibitors, or bacterial SMase, the overexpression of AC used in this study permits a direct evaluation of the effects of an enhanced ceramide metabolism. The results obtained by this approach strongly suggest that in L929 cells the enhanced expression of AC directly leads to a faster removal of intracellular ceramide generated in response to TNF and thereby to the observed protection from TNF-induced cell death, further supporting a role for ceramide in cell death.

Atypical Apoptosis in L929 Cells. The murine fibrosarcoma cell line L929 represents one of the few systems in which TNF induces cell death in the absence of inhibitors

of protein biosynthesis. Therefore, L929 cells have been widely used as a model system to study TNF-dependent cell death. However, the current literature is inconsistent with regard to the exact mode of death induced by TNF in this cell line. Several studies describe necrotic death of L929 cells treated with TNF (28, 29), whereas other groups have observed apoptotic cell death (22, 30, 31). A possible solution to these divergent results may come from the explanation that most studies describing necrotic death of L929 cells have been performed with a subclone of L929 cells, L929sA (Vandenabeele, P., personal communication). L929sA has been selected for high TNF-sensitivity (32) and therefore may show responses different from those of parental L929 cells when undergoing cell death.

Consistent with our own results, Fady et al. reported both chromatin condensation and DNA laddering in TNF-treated L929 cells, which indicates apoptosis. Moreover, cell shrinkage characteristic for apoptosis was observed in both our experiments (Fig. 4 A) and those of Fady et al., whereas balloon-type degeneration of TNF-treated L929 cells typical for necrosis was not detected (30). Additionally, Hayakawa et al. describe membrane blebbing in TNF-treated L929 cells, similar to our own data shown in Fig. 3 (22).

However, there are also observations suggesting that apoptosis takes an "atypical" course in TNF-treated L929 cells. First, in agreement with reports of a delayed appearance of DNA fragmentation (30), we could detect only minor amounts of hypodiploid L929 cells at $<$20 h of hTNF treatment in cell cycle analyses (our unpublished data). Second, we and others have observed an early increase of cells staining positive for DNA-specific dyes like 7-AAD or propidium iodide after treatment with TNF (see Fig. 6 and references 30, 33). Although this increase has been interpreted as an early loss of plasma membrane integrity and therefore necrosis (33), our own data show that annexin V is unable to gain entry unless the cells are actively permeabilized, arguing that the increased staining for propidium iodide and 7-AAD does not reflect a general leakiness of the cell membrane typical for necrotic cell death but may perhaps represent a process of selective uptake. In addition, our data demonstrate that although phosphatidylserine is present at the inner membrane of L929 cells, it is not externalized after treatment with TNF, a response untypical for apoptosis (see Fig. 6). Third, Fadeel et al. have recently shown that externalization of phosphatidylserine occurs in a cell type-specific manner and is furthermore dependent on the activity of caspases (34). In agreement with this requirement, we were unable to detect activation of caspase-3 or -8 in response to hTNF, suggesting that neither initiator caspases nor executor caspases are activated by hTNF in L929 cells. Nevertheless, the capability of L929 cells to activate caspase-3 and -8 could very well be demonstrated by the addition of dATP and cytochrome c to cell extracts. These data are in line with observations by Vercammen et al., who similarly did not find an increase of caspase-3– and -1–like activities after TNF-treatment of L929 cells (Fig. 5 and reference 28). Our results indicate that unlike cell systems such as 293 human embryonal kidney cells, where liberation of ceramide is regulated by caspases (35), the generation of ceramide by TNF occurs altogether independently from caspases in L929 cells. In consequence, the observed degradation of PARP may be carried out by a protease different from the above caspases. The fact that activation of this protease by hTNF was prevented in L929 cells overexpressing AC argues that this protease is directly or indirectly activated by ceramide. Furthermore, the fact that treatment of L929 cells with the caspase-inhibitor zVAD-fmk did not prevent but rather accelerated PARP-degradation strongly suggests that caspase activity is not crucial or may not be required at all for apoptosis in L929 cells. This absence of caspase activation in response to TNF makes L929 cells a tool especially suited to study the contribution of ceramide to TNF-induced apoptosis. L929 cells may represent a biological system in which a nontypical form of apoptosis occurs in response to TNF, leading to classic morphological alterations like membrane blebbing and disintegration into apoptotic vesicles but also comprising atypical symptoms like delayed DNA fragmentation and lack of caspase activation.

Ceramide As a Potential Mediator of Caspase-Independent Apoptosis. Caspase-independent apoptosis has also been described for PML-induced death of rat embryo fibroblasts, death of activated T cells through CD2 and staurosporine, and for the Fas-mediated death of Jurkat cells in the presence of peptide inhibitors for caspase-3–like enzymes (36–38). In the latter case, cell death was characterized by cell shrinkage and surface blebbing, but lacked features like chromatin condensation and DNA fragmentation. In contrast, DNA fragmentation occurs in L929 cells, although delayed, suggesting multiple forms of apoptosis, either caspase-dependent or –independent with cell type–dependent fluent transitions between the individual forms. This may provide the cells of an organism with more flexibility, e.g., in combating infections by viruses that inhibit caspase-dependent apoptosis.

One mechanism by which caspase-independent apoptosis occurs may involve perturbations of mitochondria (37). As mitochondria represent a target for intracellular ceramide, this sphingolipid may be part of an alternative, potentially ancient cellular mechanism by which a cell can react to infections or other insults. The relevance of ceramide in this process is supported by the observation that overexpression of Bcl-2, which blocks the effects of ceramide on mitochondria, also protects L929 cells from death after treatment with TNF (39). Furthermore, Jayadev et al. have demonstrated that in L929 cells, TNF induces a 2.5-fold increase in intracellular ceramide within 24 h (corresponding to our own data shown in Fig. 7) with a concomitant activation of SMases preceding the onset of cell death, whereas a TNF-resistant L929 subline was deficient in ceramide generation (40). Correspondingly, pharmacological inhibition of A-SMase by two different inhibitors acting through independent mechanisms clearly increased the survival of parental L929 cells after treatment with TNF (see Fig. 9 C). Analogous to our own results obtained with AC-transfected cells, overexpression of glucosylceramide synthase, an enzyme that converts ceramide to glucosylcer-

amide, also conferred resistance to cell death induced by ceramide or adriamycin (41).

In summary, the results obtained by stably overexpressing AC in L929 cells indicate an important role of ceramide in the initiation of cell death. In some cell types, ceramide may act as a cofactor contributing to caspase-dependent cell death. In other cell types, ceramide may be entirely replaced by caspases. In yet other cell types, e.g., in L929 cells, ceramide may constitute an important inducer of cell death. The proapoptotic function of ceramide can apparently be regulated or attenuated at multiple checkpoints along the apoptotic signaling pathway. The data presented in this study strongly suggest that AC represents one of these checkpoints. In support of this hypothesis, intracellular ceramide levels can be controlled by regulating the activity of ceramidases such as AC. In primary rat hepatocytes, IL-1β stimulates ceramidases at low concentrations (<4 ng/ml) and inhibits them at higher concentrations. In this system, regulation of ceramidase activity may provide a "switch" controlling sphingolipid levels and thus multiple intracellular responses (42). Furthermore, nitric oxide inhibits ceramidases in renal mesangial cells while simultaneously stimulating SMases. This results in a chronic upregulation of intracellular ceramide preceding apoptosis and may be physiologically important in the resolution of glomerulonephritis (43). It is highly likely that checkpoints for ceramide accumulation like AC work in a cell type–specific manner. Therefore, although the generation of ceramide contributes to cell death, the decision whether a specific cell dies or not is probably made at the level of activity of control mechanisms such as AC.

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