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Tumor Necrosis Factor Induces Ceramide Oscillations and Negatively Controls Sphingolipid Synthases by Caspases in Apoptotic Kym-1 Cells*

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The role, origin, and mode of action of the lipid messenger ceramide in programmed cell death and its linkage to receptor-associated apoptotic signal proteins is still unresolved. We show here in Kym-1 rhabdomyosarcoma cells that tumor necrosis factor (TNF)-induced apoptosis is preceded by a multiphasic increase in intracellular ceramide levels. Distinct enzymes were found to contribute to three waves of ceramide, neutral sphingomyelinase, ceramide synthase, and acid sphingomyelinase, with peak activities at 1–2, 40, and around 200 min, respectively, the latter coinciding with progression to irreversible damage. In parallel with ceramide generation, TNF-mediated inhibition of glucosylceramide and sphingomyelin (SM) synthase prevents the immediate metabolization of this lipid mediator. In the presence of benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) or benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethyl ketone (Z-DEVD-cmk), a broad spectrum and a caspase 3-selective inhibitor, respectively, glucosylceramide and SM synthase activity remains unaffected by TNF, and intracellular ceramide accumulation is not observed. Our results show that several lipid enzymes contribute to generation of ceramide in response to TNF and identify glucosylceramide and SM synthase as important regulators of the kinetics and magnitude of intracellular ceramide accumulation. As glucosylceramide and SM synthase activity is caspase-sensitive, our data suggest a novel functional link between caspase(s) and ceramide during apoptotic processes.

Proteases of the caspase family are regarded as important physiological signal transducers and executioners of apoptosis induced by a variety of exogeneous stimuli (1). Apoptotic signals emanating from TNF[1] receptor type 1 (p55–60) are linked via receptor-associated, death domain-containing adaptor proteins to Flice/Mach (caspase 8) as the receptor proximal protease and presumed initiator of a caspase cascade. TNF-mediated cellular activation also encompasses the generation of lipid second messengers, one of which, ceramide, potentially acts as lipid anchor and has been implicated as an important mediator of cell cycle arrest and apoptosis (2–5). A functional linkage between these two pathways at multiple levels is emerging. Thus, a role of ceramide in the activation of caspase 3 (CPP32) has been proposed (1, 6). More recently, ceramide generation has been placed downstream of Crm-A and Z-VAD-fmk-sensitive caspases, suggestive of a caspase-dependent regulation of intracellular ceramide levels (7, 8). The potential relationship between caspases and the various enzymes involved in ceramide generation or metabolism and the respective role of the latter during TNF-induced apoptosis is presently unknown. This is in part related to the fact that, with the exception of acidic and neutral sphingomyelinases (aSMase and nSMase, respectively) (9, 10), the cellular enzymes regulating ceramide levels are not yet molecularly defined. Moreover, they are located in different cellular compartments (11–15). This may not only influence their accessibility by upstream regulators, *e.g.* caspases, but also has implications for potential immediate targets of locally produced, compartmentalized ceramide. Indeed, the existence of distinct signal-transducing pools of sphingomyelin was recently suggested (15).

Here, we have investigated the contribution of different ceramide-generating and -metabolizing enzymes to intracellular ceramide levels, their kinetics of activation, and caspase dependence in a cellular model of TNF-induced apoptosis that is independent of metabolic inhibitors such as cycloheximide or actinomycin D. The cell line studied, Kym-1, is an anchorage-dependent human rhabdomyosarcoma cell line highly sensitive to TNF but resistant to Fas/Apo-1-induced apoptosis (16, 17). We present evidence that two important enzymes regulating intracellular concentrations of ceramide, glucosylceramide (GlcCer) and sphingomyelin (SM) synthase, are under control of TNF-induced caspase(s), thus representing a merging point where different proapoptotic signals emanating from TNF receptor type 1, the lipid messenger ceramide, and TNF-induced caspases, converge.

MATERIALS AND METHODS

Cell Culture and Biological Reagents—The human rhabdomyosarcoma cell line KYM-1 was generously supplied by Dr. A. Meager (Hertfordshire, United Kingdom). Kym-1 cells were cultured in Clicks RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (PAA Laboratories, Austria, Germany) at 37 °C and 5% CO_2. For all experiments Kym-1 cells were grown to 90% confluency and incubated for 15 h in 0.5% fetal calf serum and for additional 2 h in 10% fetal calf serum medium. Z-VAD-fmk and Z-DEVD-cmk were from Bachem. [γ-^{32}P]ATP, [^{14}C]sphingomyelin, and

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[1] The abbreviations used are: TNF, tumor necrosis factor; GlcCer, glucosylceramide; SM, sphingomyelin; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; Z-DEVD-cmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-chloromethyl ketone; NBD, 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); SMase, sphingomyelinases; aSMase, acidic SMase; nSMase, neutral SMase; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase.

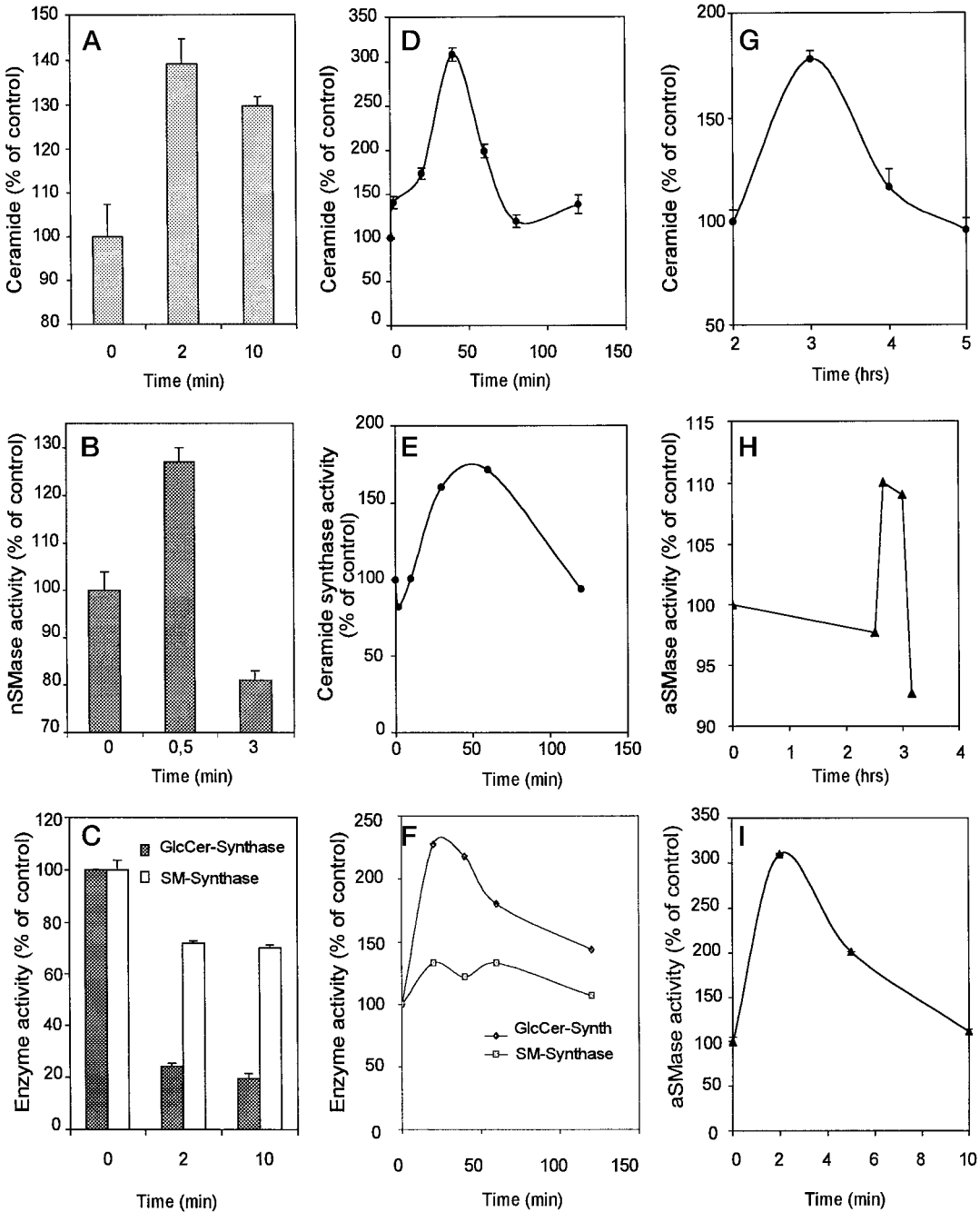

FIG. 1. *A*, kinetics of TNF-induced ceramide formation in Kym-1 cells during 10 min. 5×10^6 Kym-1 cells/dish and value were stimulated with 10 ng of TNF/ml, and ceramide levels were determined as described (28) with the diacylglycerol kinase assay. Lipids were extracted and separated by TLC (31), and [^{32}P]-labeled ceramide-1-phosphate spots were quantified by a PhosphoImager (Molecular Dynamics). *B*, activation of nSMase by TNF. 2×10^6 Kym-1 cells were stimulated with 10 ng of TNF/ml. After extraction at neutral pH, SMase activity was determined by hydrolyis of SM labeled with [*N*-methyl-^{14}C]choline. *C*, inhibition of glucosylceramide and sphingomyelin synthase by TNF. 12×10^6 Kym-1 cells were incubated with TNF (10 ng/ml), and the glucosylceramide and sphingomyelin synthase assays were performed with microsomal membranes (12). The NBD-labeled lipids were extracted, separated by TLC (31), and scanned and quantified by a PhosphoImager. *D*, kinetics of TNF-induced ceramide formation in Kym-1 cells during 120 min (see *panel A* and "Materials and Methods"). *E*, stimulation of ceramide synthase by TNF. 12×10^6 Kym-1 cells were incubated with TNF (10 ng/ml), and the ceramide synthase assay was performed with [^{3}H]sphingosine and microsomal membranes (11, 22, 31). Lipids were extracted and separated by TLC (31), which was treated with Enhance spray and autoradiographed for 5 days. Shown is the densitometric quantification of the [^{3}H]ceramide spots. *F*, activation of SM and GlcCer synthase activity by TNF (see panel *C* and "Materials and Methods" and Jeckel *et al.* (12)). *G*, ceramide formation after 3 h of TNF stimulation (same experimental design as in panels *A* and *D*; the value at 2 h has been set as 100%). *H*, activation of acidic SMase after 3 h of TNF stimulation. 2×10^6 cells/data point were treated with TNF (10 ng/ml) for the indicated times, and cell-free extracts were prepared at acidic pH. Cellular SMase activity was determined by hydrolysis of SM labeled with [*N*-methyl-^{14}C]choline (29). *I*, activation of acidic SMase of Kym-1 cells in suspension. Before TNF stimulation, cells were brought into suspension by treatment with 0.02% EDTA, washed, and equilibrated for 10 min in culture medium, and extracts and assays were performed as described in *panel H*. The results shown in panels *A–D* and *F–I* are the mean of ±S.D. of triplicate determinations. Similar results were obtained for each assay in three independent experiments.

the diacylglycerol kinase assay were from Amersham Pharmacia Biotech, [^{3}H]sphingosine was from Du Pont NEN. Semisynthetic C_6- and C_{16}-ceramide and fumonisin B1 were purchased from Biomol. NBD-C_6-ceramide and NBD-C_6-sphingomyelin were from Molecular Probes.

Determination of Intracellular Ceramide Levels—Intracellular ceramide was determined as described by Preiss *et al.* (28) with the

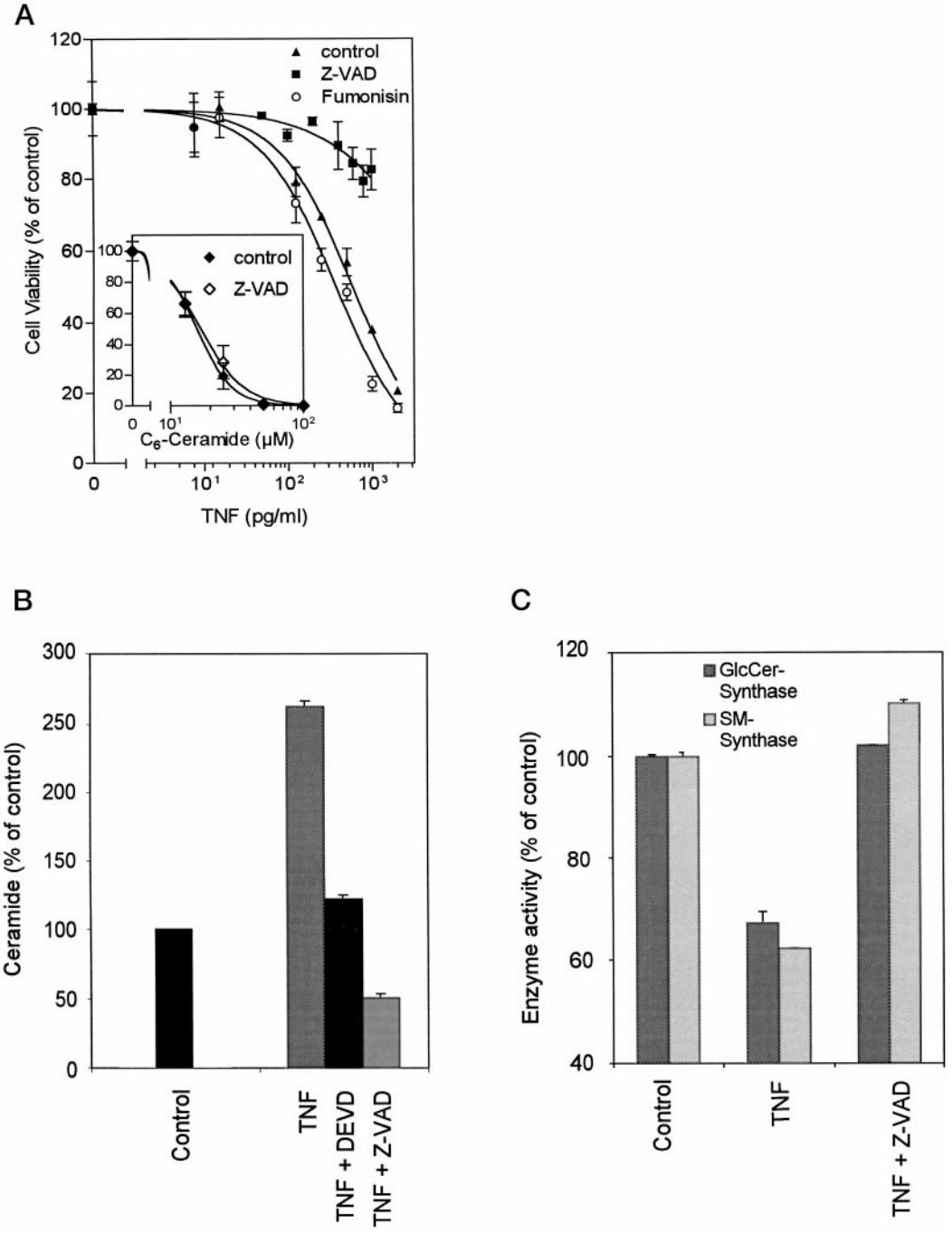

FIG. 2. *A*, inhibition of TNF-induced cytotoxicity by caspase inhibitor Z-VAD-fmk but not by fumonisin B1. Kym-1 cells were treated for 12 h with various concentrations of TNF in the absence (*filled triangles*) or presence of Z-VAD-fmk (20 μM) (Bachem) added 2 h after TNF (*filled squares*). *Inset*, apoptosis induced by C6-ceramide (Biomol) in the absence or presence of Z-VAD-fmk (20 μM). Viable cells were determined as described under "Materials and Methods." *B*, depletion of the TNF-induced third ceramide peak by Z-VAD-fmk and Z-DEVD-cmk. 5×10^6 Kym-1 cells/cell and value were stimulated with TNF (10 ng/ml) for 3 h in the presence or absence of Z-VAD-fmk (20 μM) or Z-DEVD-cmk (100 μM). To evaluate the intracellular ceramide levels, lipid extracts (31) were assayed as described in Preiss *et al.* (28), and ^{32}P-labeled lipids separated on TLC plates were quantified by a PhosphoImager. *C*, influence of Z-VAD-fmk on GlcCer and SM synthase. 1.2×10^7 cells/cell and value were stimulated with TNF (10 ng/ml) for 3 h, and Z-VAD-fmk (20 μM) was added 2 h after TNF stimulation. Microsomal membranes were prepared, and the sphingomyelin synthase assay was performed as described in Liu and Anderson (11) and Jeckel *et al.* (12). After lipid extraction and TLC, the separated lipids were quantified by a PhosphoImager. The data shown in Fig. 2, *A–C*, are the mean ±S.D. of triplicate determinations of one of three independent experiments, with similar results for each assay.

diacylglycerol kinase assay from Amersham using C_{16}-ceramide as standard.

Assays for neutral and acidic SMase were performed as described by Schütze *et al.* (29) with some modifications. Kym-1 cells grown in Petri dishes were stimulated with 10 ng of TNF/ml. At indicated times the treatment was stopped by placing the dishes into ice water, washed twice with PBS at 0 °C, and scraped from the plates with a rubber policeman in 200 μl of SMase extraction buffer.

Cytotoxicity Assay—This assay was performed with the crystal violet method. After stimulation, cells cultivated in 96-well plates were washed with 200 μl of PBS and incubated for 15 min with crystal violet (0.5% in 20% methanol), washed with distilled water, and then dried. Optical densities were read at 550 nm in an enzyme-linked immunosorbent assay reader.

Isolation of microsomal membranes was performed according to Liu *et al.* (11). 12×10^6 Kym-1 cells were placed on ice water, washed with ice-cold PBS, and scraped into 1 ml of ice-cold homogenization buffer (for the sphingomyelin and glucosylceramide synthase assay: 10 mM

HEPES, pH 7.4, 10 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin; for the ceramide synthase assay: 25 mM HEPES, pH 7.4, 5 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After homogenization in a 5-ml Dounce homogenizer (25 strokes), cell debris was removed by low speed centrifugation, and the supernatant was centrifuged at 80 000 × *g* for 30 min at 4 °C. The pellet was resuspended in 200 μl of homogenization buffer.

Ceramide Synthase Assay—This assay was performed similar to methods previously described (25, 30). 150–250 μg of microsomal proteins were added to a buffer containing 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 μM [^{3}H]sphingosine, and the reaction was started by the addition of 200 μM palmitoyl-CoA followed by incubation at 37 °C for 15 min with gentle shaking. Unspecific reactions were determined by incubation without palmitoyl-CoA. The reaction was stopped by lipid extraction with chloroform methanol 1:2 (v/v).

Sphingomyelin and Glucosylceramide Synthase Assays—These assays were performed according to a method of Jeckel *et al.* (12) with the following modifications. As substrate for glucosylceramide and sphingomyelin synthase, NBD-C_6-ceramide was inserted to phosphatidylcholine vesicles; 26 nmol of NBD-C_6-ceramide and 100 μg of phosphatidylcholine in ethanol were dried and resuspended in 50 μl of buffer containing 10 mM HEPES, pH 7.4, 10 mM EDTA, and 100 mM NaCl by ultrasonic treatment in the dark. The enzyme assays in a volume of 0.15 ml contained 100–200 μg of microsomal protein, 50 μl of NBD-C6-ceramide vesicles (13 nmol), and 50 μl of UDP-glucose (1.5 mM), in the case of standards diluted with UDP-[^{14}C]glucose (Amersham). After incubation for 15 min at 37 °C in the dark with gentle shaking, the reaction was stopped by lipid extraction with 500 μl of chloroform: methanol 1:2 (v/v).

Lipid Extraction and Thin Layer Chromatography—Lipid extraction was performed according to the method of Bligh and Dyer (31). TLC was performed on high performance TLC plates (Merck), and the samples were separated with chloroform:methanol:acetic acid (65:15:5) (v/v/v) for the diacylglycerol kinase assay and with chloroform, methanol, 3.5 N ammonium hydroxide (85:15:1) (v/v/v) for analysis of glucosylceramide and chloroform, with methanol, water, 25% ammonium hydroxide (44:44:10:2) (v/v/v/v for sphingomyelin).

Determination of PARP Cleavage—Tissue culture plates with 4 × 10^6 Kym-1 cells after stimulation with TNF (10 ng/ml) or 50 μM C_6-ceramide were placed on ice, washed, scraped from the plates, and resuspended in a buffer containing 50 mM HEPES, 2.5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM nitrophenyl phosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin. After 30 min on ice Nonidet P-40 was added to a final concentration of 0.5%, and lysates were shaken vigorously for 10 min and centrifuged for 5 min at 10,000 rpm. Equal amounts of protein were separated on SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The membrane was blocked with 3% bovine serum albumin, 3% milk, and 0.1% Tween 20 in PBS. PARP was detected by an antiserum of Upstate Biotechnology and alkaline phosphatase staining.

Labeling of Cells with NBD-C_6-ceramide and Analogues—All steps are performed in the dark. Cells grown on glass coverslips were incubated for 1 min at room temperature with 5 μM NBD-C_6-ceramide or 1-*O*-methyl-NBD-C_6-ceramide preincubated for 1 h in Click's/RPMI containing 1% bovine serum albumin (fatty acid free). Then cells were washed with 1% bovine serum albumin (fatty acid free) in Click's/RPMI and with PBS, fixed with 3.5% paraformaldehyde, covered with mowiol, and photographed with a fluorescence microscope (Zeiss, Scotch T 640 film). In long time experiments for investigation of ceramide metabolization, cells were incubated for the indicated times with 5 or 50 μM NBD-C_6-ceramide at 37 °C and washed with 1% bovine serum albumin (fatty acid free) before lipid extraction. For quantification of intracellular NBD-ceramide and metabolites, a calibration curve has been established.

RESULTS AND DISCUSSION

When Kym-1 cells were treated with TNF, a multiphasic increase in intracellular ceramide levels was discerned. Two min after onset of TNF stimulation, a first increase in ceramide (140% of control) (Fig. 1*A*) was due to rapid but transient (<60 s) activation of neutral sphingomyelinase (Fig. 1*B*). In contrast to hematopoietic cells (14), no aSMase activity could be detected in Kym-1 cells growing attached in monolayers within 2 h of TNF treatment. Interestingly, the rapid rise in ceramide levels (Fig. 1*A*) was paralleled by a simultaneous inhibition of GlcCer and SM synthase (Fig. 1*C*), determined

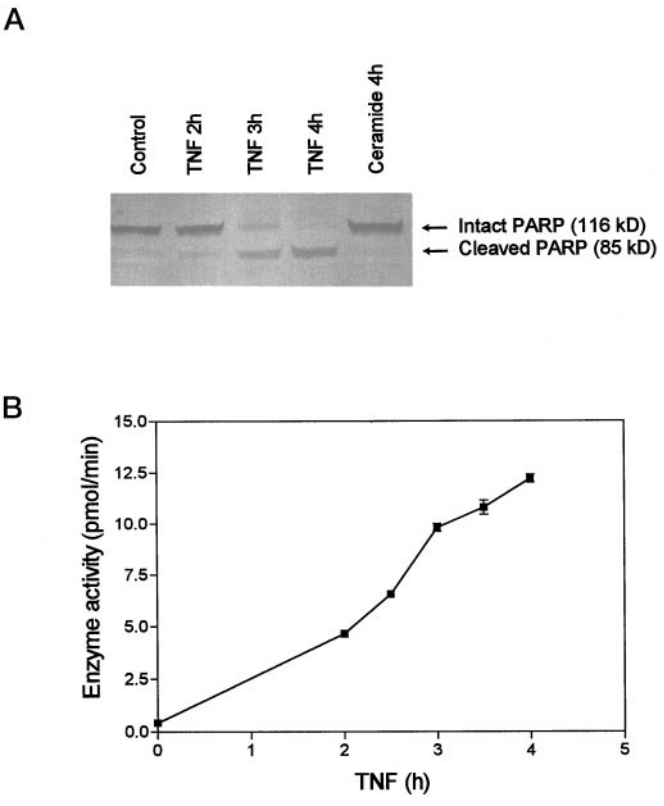

FIG. 3. **Activation of caspase 3 by TNF detected by PARP and peptide cleavage.** *A*, 4 × 10^6 Kym-1 cells were incubated with TNF (10 ng/ml) or C_6-ceramide (50 μM). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis blotted onto nitrocellulose, and PARP was immunodetected by a rabbit polyclonal antiserum (Upstate). *B*, TNF-induced caspase 3 activity was also detected by cleavage of Z-DEVD-*p*-nitroaniline using a standard kit from Biomol.

from membrane fractions of TNF-treated cells and NBD-C_6-ceramide as substrate (12). This finding contrasts to metabolic activity of untreated cells, where a rise in the intracellular ceramide concentration immediately leads to increase in GlcCer and SM synthase activity (18, 19). Thus, GlcCer and SM synthase appear to be important TNF-regulated enzymes to control the duration of the ceramide signal. The subsequent ceramide elevation of greater than 300% of control after 40 min of TNF stimulation (Fig. 1*D*) was correlated with a transient increase in ceramide synthase activity, as revealed from conversion of the precursor [^{3}H]sphingosine (Fig. 1*E*) and the absence of this ceramide peak in the presence of the ceramide synthase inhibitor fumonisin. In parallel to the second ceramide wave, GlcCer and SM synthase activity transiently increased, reaching maxima of ~230 and 130% that of control, respectively (Fig. 1*F*). The substantial increase in intracellular ceramide for at least 1 h due to concomitant *de novo* synthesis suggests that ceramide could exert a rather persistent second messenger function during this period. After 3 h of TNF stimulation, a third, again transient peak of ceramide generation was observed in Kym-1 cells (Fig. 1*G*). This late ceramide peak was caused by aSMase activation (Fig. 1*H*). Neither nSMase nor ceramide synthase was found to be activated at this time point (data not shown). It is noteworthy that the onset of the third ceramide peak in Kym-1 cells correlated with beginning loss of focal contacts, rounding, and sequential detachment of the cells, as revealed by microscopic examination. Indeed, after loosing focal contacts, detached cells clearly induce acidic SMase; *in vitro* TNF stimulation of Kym-1 cells experimentally detached by EDTA before TNF treatment resulted in rapid activation of aSMase with peak activity of 300% control (Fig.

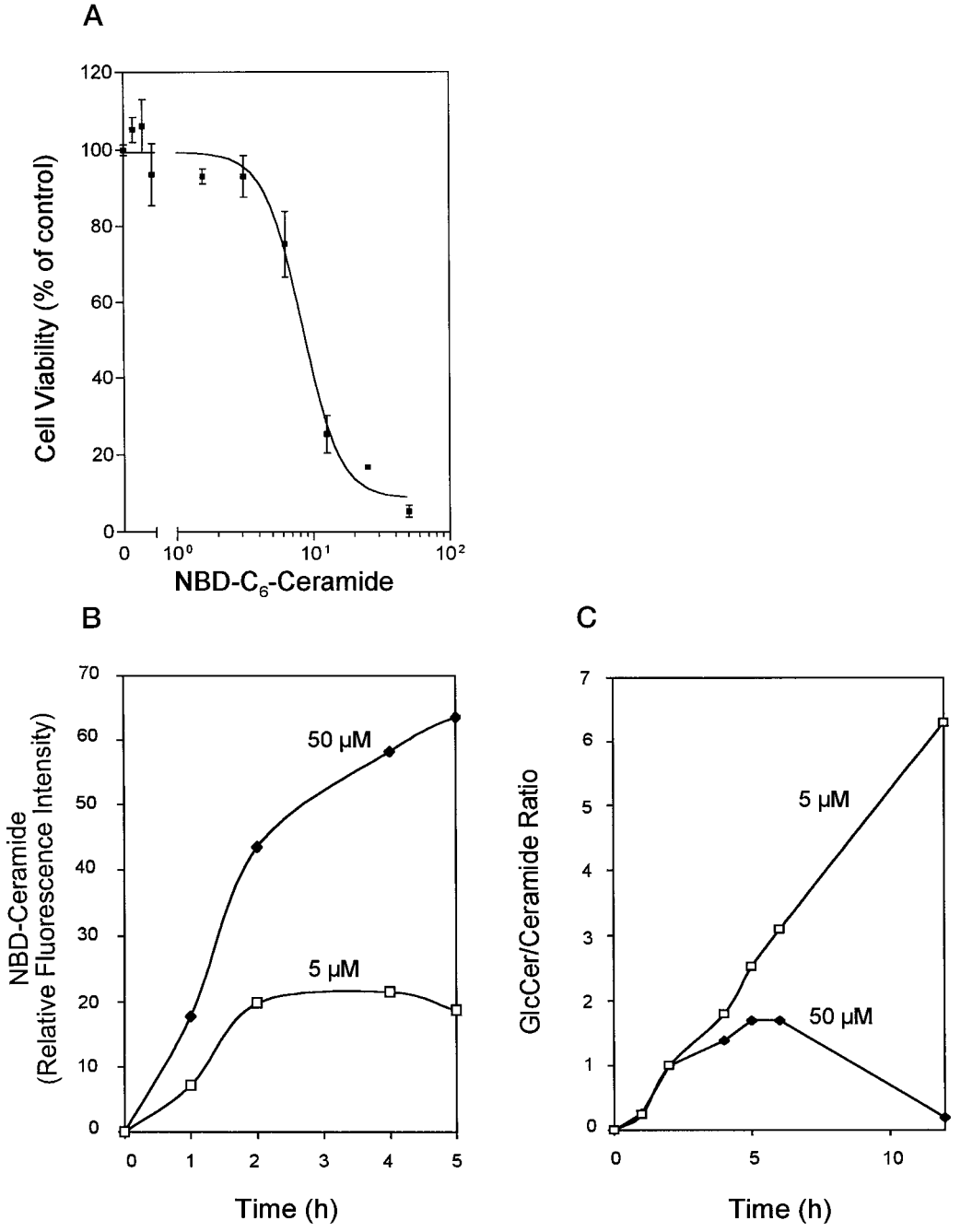

FIG. 4. *A*, titration of NBD-C_6-ceramide-induced cytotoxicity. The cells were treated for 12 h with the indicated concentrations of NBD-C_6-ceramide (Molecular Probes), and cell viability was determined as described in Bligh and Dyer (31). *B*, kinetics of ceramide accumulation with 5 or 50 μM NBD-C_6-ceramide. Cells were incubated with NBD-C_6-ceramide, washed as described under "Materials and Methods," and incubated for the indicated time. Then lipids were extracted and separated on TLC, and NBD- C_6-glucosylceramide as well as NBD-C_6-ceramide concentrations were analyzed by a PhosphoImager. Shown are the levels of NBD-C_6-ceramide. *C*, glucosylceramide/ceramide ratio after incubation with 5 or 50 μM NBD-C_6-ceramide, calculated from the lipid concentrations analyzed in *panel B*.

1*I*). Loss of cell-cell contacts appears an important prerequisite for induction of apoptosis in tissues, a mechanism that has been termed anoikis (20).

For cells undergoing apoptosis, caspases have been proposed as the upstream regulators of ceramide generation (7, 8), but the lipid enzymes potentially targeted by caspases have not been identified. In Kym-1 cells, the addition of either Z-VAD-fmk, a broad spectrum inhibitor of caspases, or Z-DEVD -cmk, a selective caspase 3 inhibitor, both of which block TNF-induced apoptosis (Fig. 2*A* and data not shown), completely abolished the TNF-induced third increase in intracellular ceramide (Fig. 2*B*). However, apoptosis of Kym-1 cells induced by exogeneous ceramide could not be blocked by Z-VAD-fmk (Fig. 2*A*, *inset*), indicating an action of ceramide downstream of Z-VAD-fmk-sensitive caspases. This is in contrast to a recent study with a T cell line, where ceramide-induced apoptosis apparently was an indirect, Fas/FasL-mediated event sensitive to the caspase inhibitor Z-VAD-fmk (21). An immediate role of ceramide in the apoptotic process of Kym-1 cells rather than an induction of the Fas/FasL pathway is strengthened by previous data showing that these cells are fully resistant to Fas-induced apoptosis (17). This suggests that ceramide may serve multiple functions in apoptotic pathways that, depending on the cellular context, can be indirect (21) or direct, as shown here for Kym-1 cells.

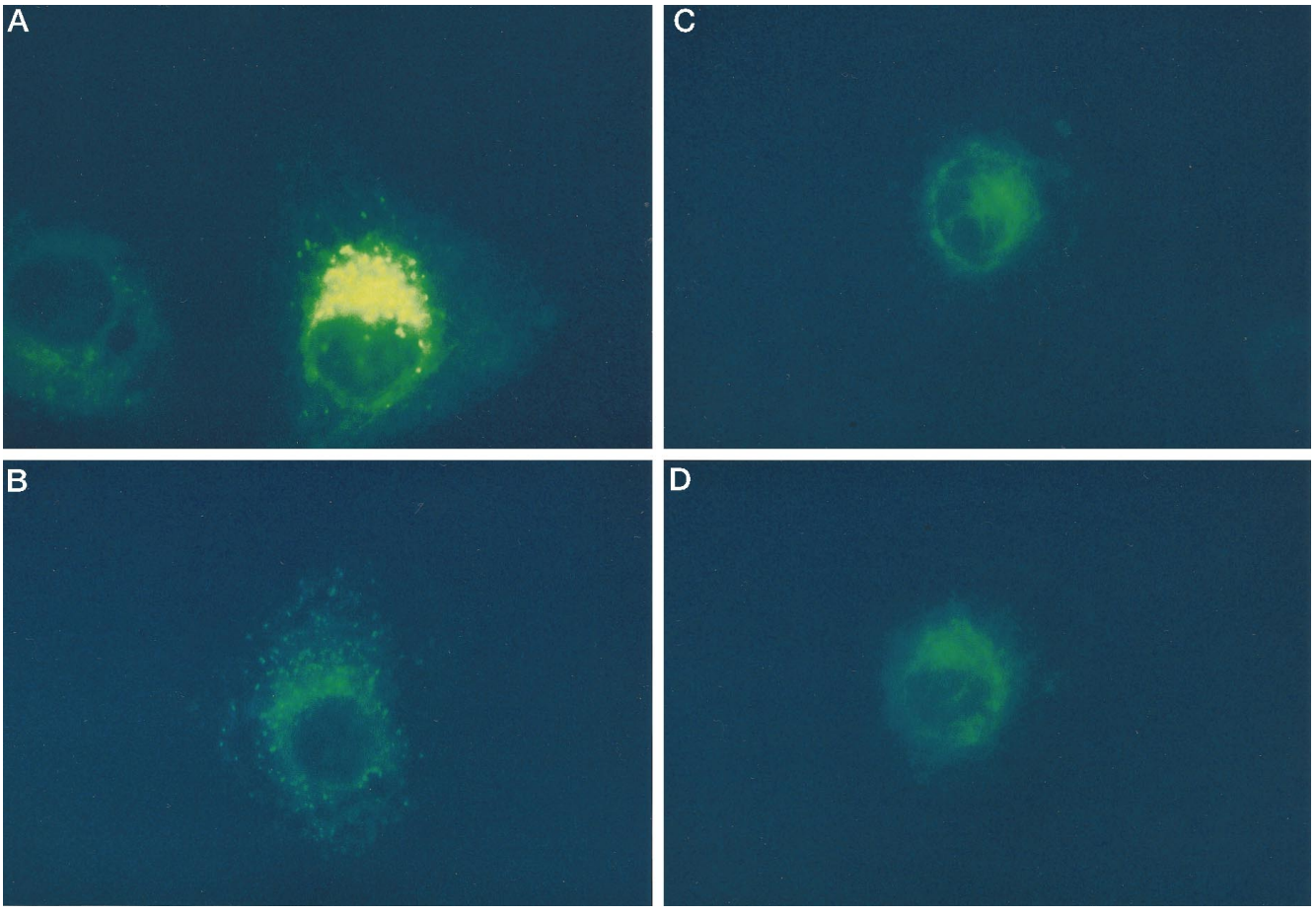

FIG. 5. **Intracellular localization of NBD-C_6-ceramide and 1-*O*-methyl-NBD-C_6-ceramide.** 5×10^4 Kym-1 cells were incubated with 5 μM NBD-C_6-ceramide (*A* and *B*) or 5 μM 1-*O*-methyl-NBD-C_6-ceramide (*C* and *D*) for 1 min (*A* and *C*) at room temperature, washed twice, and immediately fixed with 3.5% paraformaldehyde or were further incubated after washing for additional 2 h and then fixed (*B* and *D*).

With respect to the contribution of ceramide generated at different times by different enzymes to the apoptotic process, our data suggest that the very early nSMase-dependent and the ceramide synthase-dependent intermediate ceramide accumulation are likely not to be involved in processes of the apoptotic program, causing irreversible damage. This reasoning is based on the findings that i) Z-VAD-fmk still inhibited TNF-induced apoptosis even when added up to 2 h after TNF (Fig. 2*A*), just before the beginning detachment of cells and the third rise in ceramide; 2 h later, chromatin condensation was apparent by 4′-6-diamidino-2-phenylindole dihydrochloride staining in greater than 80% of the cells, and Z-VAD-fmk was ineffective at this time point (data not shown); ii) fumonisin effectively inhibited TNF-induced ceramide synthase activity, responsible for the second ceramide peak, but even at high concentrations (25 μM) failed to inhibit TNF-induced apoptosis (Fig. 2*A*); this transient ceramide synthase activity in Kym-1 cells thus apparently plays a functionally different role compared with continuous ceramide synthase stimulation, which, in a different experimental setting, was previously reported to lead to apoptosis (22, 23); iii) overexpression of the TNF receptor-associated adaptor protein and presumed activator of nSMase, FAN, does not influence apoptosis in either its active or inactive form (24).

It was noted in TNF-treated Kym-1 cells that the rapid accumulation of ceramide was paralleled by an inhibition of GlcCer and SM synthase activity (Fig. 1*C*), followed by an increased activity of the enzymes for approximately 1 h (Fig. 1*F*). Cells treated for 3 h with TNF again showed a reduced GlcCer and SM synthase activity compared with untreated cells (Fig. 2*C*). Interestingly, at this time point, the caspase

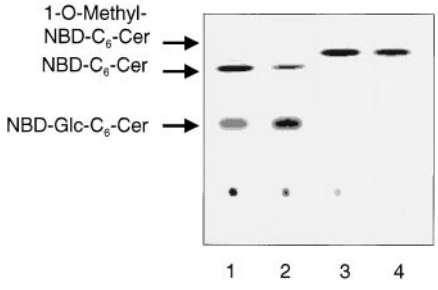

FIG. 6. **Metabolism of NBD-C_6-ceramide in Kym-1 cells.** Cells were incubated for 1 min and 2 h with NBD-C_6-ceramide (*lanes 1* and *2*) or for 1 min or 2 h with the nonmetabolizable analog 1-*O*-methyl-NBD-C_6-ceramide (*lanes 3* and *4*) and immediately washed as described under "Materials and Methods," then lipids were extracted (31), separated on TLC, and analyzed by a PhosphoImager.

inhibitor Z-VAD-fmk rendered TNF-treated cells fully resistant to down-regulation of GlcCer and SM synthase activity (Fig. 2*C*). This suggests that GlcCer and SM synthase are targets in TNF-induced apoptotic pathways that are either directly or indirectly controlled by Z-VAD-fmk-sensitive caspase(s).

To further investigate which of the caspases might be involved in GlcCer and SM synthase activity control, the activation of caspase 3, previously suggested to be a direct target of ceramide (1, 6), was determined. As expected, TNF treatment of Kym-1 cells resulted in caspase 3 activation and PARP cleavage (Fig. 3*A*). The kinetics of activation (Fig. 3*B*) paralleled the late accumulation of ceramide, with a peak of the latter approximately 3 h after the onset of TNF treatment.

Z-DEVD-cmk, a selective inhibitor of caspase 3, abolished the TNF-induced third ceramide peak (Fig. 2*B*). Exogenous ceramide had no influence on PARP cleavage (Fig. 3*A*) and did not activate caspase 3 during 4 h, although the nuclear contents of more than 60% of the cells were already encapsulated into "apoptotic bodies" after 4 h, as revealed by 4′-6-diamidino-2-phenylindole dihydrochloride staining (data not shown). These results indicate that ceramide is not an activating messenger of caspase 3.

The above data indicate a critical role of GlcCer and SM synthase as negative regulators of endogenous ceramide levels (Fig. 1*C* and 2*C*). A recent study further suggested a similar role of SM synthase for exogeneous ceramide (25). As the vast majority of exogeneous ceramide is converted into glucosylceramide rather than sphingomyelin (Ref. 26 and data not shown), we investigated the fate of apoptotic (50 μM) and non-apoptotic (5 μM) doses of NBD-C_6-ceramide by determining its intracellular concentrations and conversion to glucosylceramide in Kym-1 cells. As with C_6-ceramide, the fluorescently labeled derivative NBD-C_6-ceramide induced apoptosis at an exogeneous concentration of 50 μM, whereas 5 μM NBD-C_6-ceramide had no effect (Fig. 4*A*). In the presence of 5 μM NBD-C_6-ceramide, a steady state intracellular level of 30 pmol/10^6 Kym-1 cells was reached after 2 h (Fig. 4*B*). More relevant, the glucosylceramide/ceramide ratio was steadily increasing, indicating that GlcCer synthase is active and can fully compensate this stimulus (Fig. 4*C*). In the presence of 50 μM exogeneous ceramide, the intracellular ceramide level was continuously increasing, reaching about 90 pmol/10^6 cells after 4 h (Fig. 4*B*). Of note is that the glucosylceramide/ceramide ratio drops drastically after 6 h (Fig. 4*C*), indicative of a pronounced reduction of GlcCer synthase activity, probably by substrate inhibition. Such a mechanism cannot be overcome by caspase inhibitors, explaining why apoptosis induced by high concentrations of exogeneous ceramide proceeds in the presence of Z-VAD-fmk (Fig. 2*A*).

The intracellular location and metabolism of GlcCer and SM was monitored by labeling cells with the precursor NBD-C_6-ceramide, known as a specific marker for the Golgi compartment (27). Only recently it has been demonstrated that labeling of the Golgi by short chain fluorescent ceramide analogues requires their metabolism to GlcCer or SM. The fluorescent NBD-C_6-ceramide, but not its nonmetabolizable analogue 1-*O*-methyl-NBD-C_6-ceramide, is metabolically converted to GlcCer and to a minor extent to SM (26). Kym-1 cells show a very fast uptake of NBD-C_6-ceramide. Within minutes, NBD-C_6-ceramide was accumulated in a perinuclear space and in Golgi structures (Fig. 5*A*), in contrast to 1-*O*-methyl-NBD-C_6-ceramide, which was only diffusely distributed in the cell (Fig. 5*C*), although both compounds were taken up in equal amounts (Fig. 6). Upon further incubation for 2 h, only NBD-C_6-ceramide was distributed in vesicular structures (Fig. 5*B*), at which time it was to a large extent metabolized to glucosylceramide (Fig. 6). 1-*O*-Methyl-NBD-C_6-ceramide remained diffusely distributed in the cells (Fig. 5*D*), indicating that metabolization to glucosylceramide is a prerequisite for localization of the label in Golgi and vesicular structures, thus localizing metabolic conversion of ceramide to intracellular vesicular, trans-Golgi and/or endosomal compartments.

By comparing endogeneous, TNF-induced ceramide levels and those arising intracellularly from exogeneously added ceramide with its different physiological outputs, it becomes apparent that not the ceramide concentration *per se*, but additional parameters, are important factors in determining the specific role of ceramide in the apoptotic process. These parameters include time point of appearance and duration of local intracellular concentrations of ceramide. For example, the ceramide level transiently reached upon TNF activation of ceramide synthase is similar to the concentrations accumulating upon incubation with 50 μM exogeneous ceramide, yet only the latter induces apoptosis, probably because of sustained inhibition of GlcCer and SM synthase. Compartmentation of the ceramide-generating enzymes could be of similar importance. Indeed, evidence for the existence of distinct intracellular pools of the ceramide precursor sphingomyelin and their differential involvement in apoptosis has recently been obtained (15). Together, these data support our hypothesis that intracellular location and time point of accumulation largely determine the apoptotic signal/effector capacity of ceramide.

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