Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 73–77, January 1994
Cell Biology

Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway

(signal transduction/intracellular messengers/sphingomyelinase/ceramide/leukemia)

W. DAVID JARVIS*†, RICHARD N. KOLESNICK‡, FRANK A. FORNARI*§, REBECCA S. TRAYLOR*, DAVID A. GEWIRTZ*§, AND STEVEN GRANT*§

Departments of *Medicine and §Pharmacology/Toxicology, Medical College of Virginia, Richmond, VA 23298-0230; and ‡Program of Molecular Pharmacology, Sloan-Kettering Institute, Cornell University Medical College, New York, NY 10021

Communicated by Oscar L. Miller, Jr., September 24, 1993 (received for review May 18, 1993)

ABSTRACT **The potential involvement of ceramide-related signaling processes in the induction of apoptosis by tumor necrosis factor α was assessed by multiple biochemical strategies in the human leukemic cell lines HL-60 and U937 and the murine fibrosarcoma cell lines L929/LM and WEHI 164/13. Exposure of these cells to tumor necrosis factor α resulted in internucleosomal cleavage of genomic DNA, yielding laddered patterns of oligonucleosomal fragments characteristic of apoptosis when resolved by agarose gel electrophoresis; similar responses were observed after exposure to exogenous sphingomyelinase or synthetic ceramides. Quantitative spectrofluorophotometry demonstrated that these treatments promoted time- and concentration-dependent degradation of DNA, resulting in the formation of and eventual release of small DNA fragments (≤3.0 kb). Corresponding damage to bulk DNA was demonstrated by enhanced-fluorescence alkaline unwinding analysis. DNA fragmentation was not induced by phospholipase C or synthetic diglyceride; in fact, the effects of sphingomyelinase and ceramide were substantially reduced by coexposure to these agents, suggesting opposing roles for diglyceride- and ceramide-mediated signals in the regulation of apoptosis. Phospholipase A_2 and arachidonic acid failed to promote DNA fragmentation, as did phospholipase D. Characterization of DNA strand breaks by alkaline and neutral elution analyses confirmed that ceramide action was restricted to breakage of mature, double-stranded DNA but not of nascent DNA. The induction of DNA damage was associated with appearance of apoptotic morphology and decreased clonogenicity. These results demonstrate that the ceramide-dependent signaling system selectively induces apoptosis and raise the possibility that ceramide-activated enzymes represent important components in a signaling cascade involved in the regulation of programmed cell death.**

Programmed cell death, or *apoptosis*, is an active, energy-dependent process through which living cells participate in their own destruction and is initiated by a variety of physiological and pharmacological stimuli (1–4). A fundamental component of this response is the stereotypical degradation of genomic DNA to oligonucleosomal fragments (3, 4). The inflammatory cytokine tumor necrosis factor α (TNF-α) has been shown to initiate apoptotic cell death and DNA fragmentation in several mammalian cell lines, including the human leukemia cell lines HL-60 (5, 6) and U937 (6–8) and the murine fibrosarcoma cell lines L929 (9, 10) and WEHI (11, 12). Two subtypes of TNF receptor, a high-affinity, 75-kDa (type A) form and a low-affinity, 55-kDa (type B) form, are expressed in comparable copy numbers in U937 and HL-60 cells (13); activation of the latter species has been implicated in the induction of apoptotic DNA degradation and cell death in both U937 and HL-60 cells (5–8).

While the biochemical signaling mechanisms mediating TNF-related DNA fragmentation remain obscure, the biological actions of TNF have been associated in part with enhanced sphingomyelin turnover (14, 15). This response is mediated through positive coupling of the type B receptor to a neutral sphingomyelinase (SMase) in the plasma membrane (16). SMase catalyzes the hydrolysis of sphingomyelin, yielding phosphocholine (*P*Cho) and ceramide (15); free ceramide acts as an intracellular messenger, specifically stimulating both (*i*) a membranal ceramide-activated serine (threonine) protein kinase (17) and (*ii*) a cytosolic ceramide-activated phosphoprotein phosphatase (18).

The present studies were undertaken to assess the potential involvement of ceramide-dependent signaling processes in the induction of DNA damage in a variety of mammalian cell lines. Our findings indicate that pharmacological manipulations that increase the availability of intracellular free ceramide induce apoptotic DNA fragmentation and cell death. In a recent communication, Obeid *et al.* (19) reported that exposure of U937 cells to recombinant human TNF-α (rhTNF-α) or synthetic ceramide induces endonucleolytic DNA fragmentation in a fashion consistent with apoptosis. The studies described in the present communication extend the scope of these findings to cell lines of both hematopoietic and nonhematopoietic origin; moreover, our observations provide quantitative biochemical analyses of ceramide-related DNA strand breakage and demonstrate a close association of DNA damage with biological sequelae of apoptosis such as suppression of clonogenicity and alteration of cellular morphology.¶

MATERIALS AND METHODS

Drugs and Reagents. rhTNF-α (R & D Systems) was dissolved in sterile physiological saline. SMase (from *Staphylococcus aureus*; Sigma) was prepared and diluted in sterile 50% glycerol/250 mM phosphate buffer, pH 7.5. Phospholipase (PL) C (from *Bacillus cereus*; Sigma) was prepared and diluted in 425 mM phosphate buffer (pH 7.5); PLA_2 and PLD (Sigma) were dissolved in sterile water. Synthetic ceramides [*N*-octanoylsphingosine (C_8-Cer; Biomol, Plymouth Meeting, PA) and *N*-hexanoylsphingosine and *N*-hexanoylsphinganine (Matreya, Pleasant Gap, PA)], diglyceride [1,2-dioctanoyl-*sn*-glycerol (diC_8; Sigma)], sphingosine (Biomol),

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TNF-α, tumor necrosis factor α; rhTNF-α, recombinant human TNF-α; SMase, sphingomyelinase; C_8-Cer, *N*-octanoylsphingosine; diC_8, 1,2-dioctanoyl-*sn*-glycerol; *P*Cho, phosphocholine; PL, phospholipase.

†To whom reprint requests should be addressed.

¶Portions of this work were presented at the 75th Annual Meeting of the Endocrine Society, June 9–12, 1993, Las Vegas (abstr. 1229).

and *n*-octanoic and arachidonic acids (free acid forms; Sigma) were dissolved in ethanol; heated ethanol stocks of these lipids were diluted to final concentrations in warm serum-free medium at 37°C. Synthetic *P*Cho chloride (crystalline calcium salt form; Sigma) was dissolved directly in sterile medium.

Cell Culture and Test Incubations. The human monoblastic leukemia cell line U937 (20) was obtained from the American Type Culture Collection. The human promyelocytic leukemic cell line HL-60 (21) was obtained from R. E. Gallegher (Albert Einstein College of Medicine, Bronx, NY). U937 and HL-60 cells were grown in RPMI 1640 medium (phenol red-free formulation) supplemented with 1.0% pyruvate, nonessential amino acids, L-glutamine, and antibiotics (all from GIBCO/BRL) and 10% (vol/vol) heat-inactivated fetal bovine serum. The murine fibrosarcoma cell lines L929/LM and WEHI-164/13 were obtained from the American Type Culture Collection and grown in medium 199 (GIBCO/BRL) supplemented with 0.5% gelysate peptone (Becton Dickinson). All cultures were passed twice weekly and maintained under a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells in logarithmic-phase growth were suspended at a density of 4.5×10^5 cells and exposed to test agents for various intervals in serum-free medium as described (22). Exposures were terminated by pelleting of the cells by centrifugation at $400 \times g$ at 4°C for 10 min. Cell density was determined by using a Coulter Counter, and cell viability was assessed by using a hematocytometer and trypan blue exclusion; cell pellets were then prepared for biochemical analysis as indicated below.

Analysis of DNA Fragmentation. Quantitative analysis of DNA fragmentation was performed by agarose gel electrophoresis as described (22). Pelleted cells were lysed in 0.1% Nonidet P-40/10 mM Tris·HCl/25 mM EDTA, pH 7.4, and the lysates were treated with proteinase K (500 μg/ml) at 55°C for 16 hr and centrifuged at $30,000 \times g$ for 75 min at 4°C; the pellets were discarded and the supernatants were treated with ribonuclease A (100 μg/ml) at 37°C for 18 hr. DNA fragments in the final extracts were resolved by electrophoresis at 115 V for 90–240 min on 2.25% agarose gels impregnated with ethidium bromide and were visualized under UV light. Multiple DNA molecular weight reference preparations were included to facilitate assessment of DNA fragment size. Quantitative analysis of DNA fragmentation, permitting measurement of DNA fragments in both lysate and medium preparations, was performed by bisbenzimide spectrofluorophotometry as described (23, 24). Pelleted cells were lysed in 0.1% Triton X-100/5 mM Tris·HCl/20 mM EDTA, pH 8.0, and the lysates were centrifuged at $30,000 \times g$ at 4°C for 40 min. Aliquots of the incubation medium were adjusted to 25 mM EDTA and centrifuged at $20,000 \times g$ at 4°C for 40 min. The pellets were discarded, and the presence of nonsedimenting DNA fragments in the supernatants was determined by dilution in 3 mM NaCl/10 mM Tris·HCl/1 mM EDTA, pH 8.0 containing bisbenzimide trihydrochloride (Hoechst 33258; Sigma) at 1.0 μg/ml and monitoring net fluorescence in each sample [λ (excitation) = 365, λ (emission) = 460]. DNA values were calculated against highly purified DNA standard and are expressed as nanograms of DNA recovered or released from 10^6 cells.

Analysis of Bulk DNA Damage. Bulk DNA damage was assessed by enhanced-fluorescence alkaline unwinding analysis (30), adapted as described (22, 25). Pelleted cells were resuspended in saline and subjected to timed alkaline denaturation in 0.1 M NaOH; denaturation was terminated by neutralization in 0.1 M HCl. Cells were then lysed in 0.16% *N*-lauroylsarcosine/200 mM K_2HPO_4/50 mM EDTA and sonicated. Damage to bulk cellular DNA in the lysates was quantified by spectrofluorophotometry with Hoechst 33258 [λ (excitation) = 350, λ (emission) = 450]. Values were standardized against graded DNA strand breakage induced by scaled ^{137}Cs irradiation (30–3000 rads) and are expressed as rad-equivalents.

Characterization of DNA Strand Breaks. To characterize DNA strand breaks, cells were either (*i*) prelabeled with [^{3}H]dThd (0.1 μCi/ml; 1 Ci = 37 GBq) for 24 hr (to assess damage to mature DNA) or (*ii*) pulse-labeled with [^{3}H]dThd (1.0 μCi/ml) for 6 hr (to assess damage to nascent DNA) and exposed to test agents for appropriate intervals. Strand breakage in both mature and nascent DNA was then determined by elution as described by Kohn *et al.* (26) and adapted as fully described elsewhere (22, 27), under (*i*) alkaline conditions for single-strand breaks and (*ii*) neutral conditions for double-strand breaks.

Clonogenicity. To determine cloning efficiency, pelleted cells were resuspended in cold (4 °C) medium and seeded in 12-well culture plates (400 cells per well in a volume of 1 ml) in medium containing 20% (vol/vol) fetal calf serum, 10% 5637-CM, and 0.3% Bacto agar; cultures were maintained for 10–12 days, and formation of colonies (defined as clusters of ≥50 cells) was scored as described (20).

Cell Morphology. To assess alterations in cellular morphology, pelleted cells were applied to cytocentrifuge slides and stained with 20% Wright–Giemsa stain. The occurrence and mode of cell death in each treatment group were determined based on the expression of cytoarchitectural characteristics of either *apoptosis* (cell shrinkage, nuclear condensation, extensive formation of membrane blebs and apoptotic bodies) or *necrosis* (cell swelling, nuclear expansion, gross cytolysis); 500 cells were scored for each treatment.

RESULTS

Previous investigations have established that activation of the type B TNF receptor induces fragmentation of double-stranded DNA in the human leukemia cell lines HL-60 (5, 6) and U937 (6–8). TNF has also been demonstrated to promote sphingomyelin catabolism, as reflected by decreased sphingomyelin content and reciprocally increased availability of intracellular ceramide, in HL-60 and U937 cells (15, 19). Similarly, treatment with exogenous SMase has been shown to increase levels of intracellular free ceramide in HL-60 cells from a basal value of 90 pmol per 10^6 cells to 280 pmol per 10^6 cells within 5 min (28), an effect that persists for 2–4 hr. To assess the potential capacity of ceramide to promote DNA damage, the effects of acute exposure to rhTNF-α initially were compared with those of pharmacological manipulations that increase intracellular ceramide levels. Low molecular weight DNA was resolved on agarose gels into laddered electrophoretic profiles of oligonucleosomal DNA fragments characteristic of apoptosis after a 6-hr exposure of U937 cells to rhTNF-α (100 nM) or SMase (100 milliunits/ml) (Fig. 1). Evaluation of the relative effects of the coproducts of SMase action revealed that (*i*) exposure to synthetic ceramide analogs such as C_8-Cer (10 μM) produced laddered DNA fragment profiles, whereas (*ii*) exposure to synthetic *P*Cho (10 μM) was without effect.

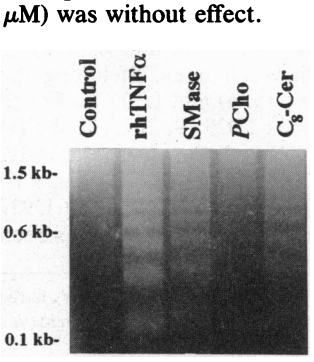

FIG. 1. Qualitative assessment of apoptotic DNA damage. U937 human monoblastic leukemia cells (5×10^5 cells per ml) were exposed to rhTNF-α (100 nM), SMase (200 milliunits/ml), *P*Cho (10 μM), or C_8-Cer (10 μM) for 6 hr. Laddered electrophoretic patterns of oligonucleosomal DNA fragments were resolved by conventional agarose gel electrophoresis.

In corresponding quantitative studies (Fig. 2), fragmentation of genomic DNA was determined by spectrofluorophotometric measurement of the formation of small (i.e., ≤3.0 kb) fragments of double-stranded DNA and their release into the incubation medium; under basal conditions, such fragments were present at intra- and extracellular levels of ≤225 and ≤45 ng per 10^6 cells, respectively. Exposure of U937 cells to rhTNF-α (100 nM) for 6 hr produced an 11-fold increase in the intracellular accumulation of DNA fragments and a 9-fold increase in the release of DNA into the medium. Similar responses were elicited by treatment with exogenous SMase (100 milliunits/ml), indicating that DNA fragmentation was associated with enhanced sphingomyelin hydrolysis in these cells. Similarly, a 6-hr exposure to C_8-Cer (10 μM) significantly increased the intra- and extracellular accumulation of DNA fragments, whereas parallel exposure to *P*Cho (10 μM) was without effect. The observed extent of DNA fragmentation was accompanied by loss of cloning efficiency: exposure to rhTNF-α, SMase, or C_8-Cer reduced clonogenicity 88%, 46%, and 32%, respectively, whereas *P*Cho was ineffective. Comparable results were obtained in HL-60 cells (data not shown).

Because induction of DNA fragmentation by TNF has also been reported in the murine fibrosarcoma cell lines L929/LM (10) and WEHI-164/13 (12), ceramide-related DNA damage was also investigated in these cells. Exposure of L929/LM cells to rhTNF-α increased ceramide levels from a basal level of 176 pmol per 10^6 cells maximally to 220 pmol per 10^6 cells within 5 min; the response was detectable within 30 sec and persisted for at least 15 min. DNA fragments were present basally in L929/LM cells at a level of 180 ± 15 ng of DNA per 10^6 cells; a 6-hr exposure to rhTNF-α (100 nM) increased levels of DNA fragments 280% to 691 ± 40 ng of DNA per 10^6 cells, whereas a parallel treatment with SMase (100 milliunits/ml) resulted in a 650% increase to 1370 ± 96 ng DNA per 10^6 cells. Ceramide also increased DNA fragmentation 311% to 560 ng of DNA per 10^6 cells, whereas *P*Cho was ineffective. Similar responses were noted in WEHI-164/13 cells (data not shown).

The time course of SMase-induced DNA fragmentation in HL-60 cells is shown in Fig. 3. The formation of DNA fragments was evident within 1 hr and became maximal within 6–9 hr; thereafter, a steady decline in the cellular DNA fragments was associated with the progressive release of DNA into the medium, an aspect of the response attributable to physical dissolution of cells undergoing apoptosis. The concentration–response characteristics of SMase action are shown in Fig. 4. Treatment of HL-60 cells for 12 hr with SMase (0.001–100 milliunits/ml) resulted in a significant, concentration-related suppression of cloning efficiency (Fig. 4*A*). The loss of clonogenicity was closely correlated in parallel studies with concentration-dependent induction of damage to HL-60 cell DNA, as demonstrated by spectrofluorophotometric measurement of the formation and release of DNA fragments (Fig. 4*B*; $r^2 = 0.981$) and by corresponding enhanced-fluorescence alkaline unwinding analysis of bulk DNA breakage (Fig. 4*C*; $r^2 = 0.965$); in both instances, DNA damage was first evident at 0.1 milliunit/ml and maximal at 10 milliunits/ml.

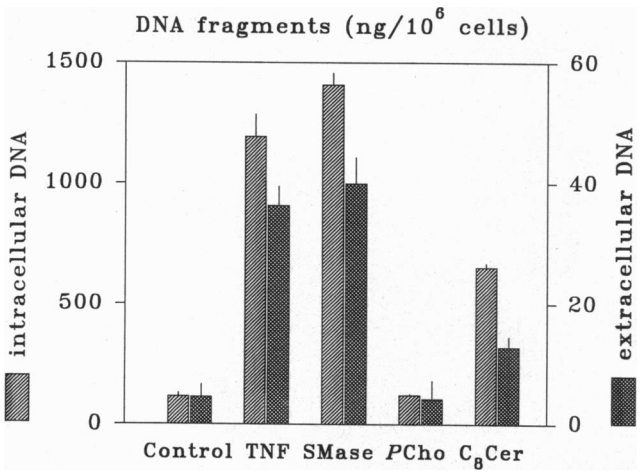

FIG. 2. Quantitative assessment of apoptotic DNA damage. U937 cells (4.5×10^5 cells per ml) were exposed to rhTNF-α (100 nM), SMase (200 milliunits/ml), *P*Cho (10 μM), or C_8-Cer (10 μM) for 6 hr. The formation (hatched bars) and release (cross-hatched bars) of double-stranded DNA fragments were determined by spectrofluorophotometry in the presence of bisbenzimide. Values reflect the mean ± SEM of quadruplicate determinations.

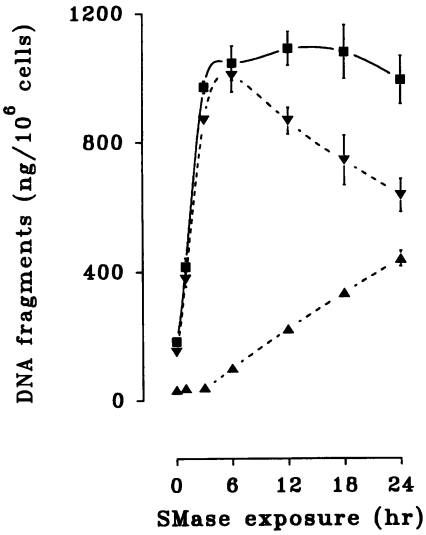

FIG. 3. Time course of SMase-induced DNA fragmentation. HL-60 human promyelocytic leukemia cells (4.5×10^5 cells per ml) were treated with SMase at 50 milliunits/ml for 0–24 hr, and accumulation of DNA fragments within both cellular (▼) and extracellular (▲) compartments was determined as before; calculated total production of DNA fragments is also shown (■). Values reflect mean ± SEM of quadruplicate determinations.

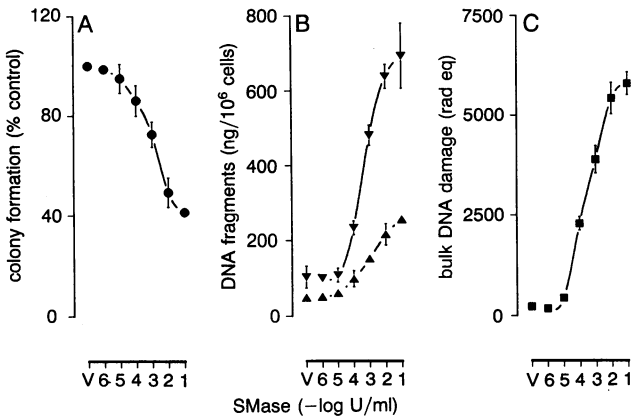

FIG. 4. Concentration–response characteristics of SMase-induced DNA damage. HL-60 cells were treated with either vehicle (V) or SMase (0.01–100 milliunits/ml) for 12 hr and prepared for evaluation of clonogenicity and DNA damage. (*A*) Reduction of cloning efficiency by assay of colony formation (●). (*B*) The formation (▼) and release (▲) of DNA fragments as determined by bisbenzimide spectrofluorophotometry. (*C*) Corresponding bulk DNA breakage (■) induced by SMase as determined by enhanced-fluorescence alkaline unwinding analysis. All values reflect the mean ± SEM of quadruplicate determinations. eq, equivalents.

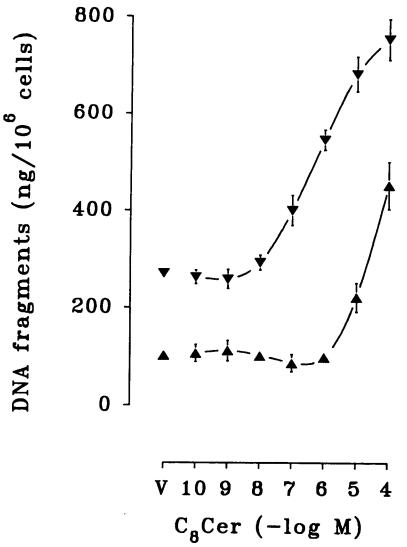

FIG. 5. Concentration–response characteristics of C_8-Cer-induced DNA damage. HL-60 cells were exposed to C_8-Cer (0.0001–100 μM) for 12 hr; the formation (▼) and release (▲) of DNA fragments were then determined by bisbenzimide spectrofluorophotometry as before. Values reflect the mean ± SEM of quadruplicate determinations.

Continual exposure of HL-60 cells to C_8-Cer for 12 hr promoted the formation and release of DNA fragments in a concentration-dependent manner (Fig. 5); the increase in intracellular DNA fragments was evident at 100 nM and maximal at 10–100 μM, while the increased presence of extracellular DNA fragments was detectable at 10 μM and maximal at 100 μM. Both sphingosine and *n*-octanoic acid were without effect, however (data not shown), indicating that the bioactivity of ceramide was not mediated by deacylation catabolites. Exposure to ceramide and dihydroceramide (*N*-hexanoylsphingosine and *N*-hexanoylsphinganine) for 4 hr resulted in comparable DNA fragmentation in HL-60 cells (data not shown).

Further characterization of DNA breakage by alkaline and neutral elution analysis of DNA from [^{3}H]dThd-prelabeled HL-60 cells confirmed that both SMase and C_8-Cer produced extensive concentration-dependent breakage of mature, double-stranded DNA (data not shown); there was no evidence of independent single-stranded DNA breakage, however, and no indication of damage to nascent DNA (data not shown).

A number of lipid messenger systems have been implicated in the mechanism of action of TNF in different cell types (29), including the generation of diglyceride by PLC and of arachidonic acid by PLA_2. Treatment of HL-60 cells with PLC (100 milliunits/ml) increased cellular diglyceride levels (5-fold within 2 min) but did not modify ceramide levels. PLC (10 milliunits/ml) failed to promote DNA fragmentation in 2- to 24-hr incubations, however, and reduced the basal level of DNA fragments (48% at 12 hr). Moreover, the formation and release of DNA fragments elicited by a 12-hr treatment with SMase (50 milliunits/ml) were substantially attenuated in the presence of equivalent amounts of PLC (54% at 12 hr). In an analogous fashion, exposure of HL-60 cells to diC_8 for 6 hr moderately reduced basal levels of DNA fragments, whereas the induction of DNA fragmentation by a 6-hr exposure to C_8-Cer (25 μM) was reduced by coexposure to equimolar concentrations of diC_8 (65% at 6 hr). In related studies, treatment of HL-60 cells with PLA_2 (100 milliunits/ml) increased levels of free arachidonic acid and lysophosphatidylcholine by 220%; nonetheless, a 12-hr treatment of these cells with PLA_2 (100 milliunits/ml) was without effect on DNA fragmentation, as was exposure to exogenous arachidonic acid (25 μM; data not shown). Treatment with PLD (50 milliunits/ml) was also ineffective (data not shown).

The induction of DNA fragmentation was invariably associated with an increased number of cells exhibiting cytoarchitectural features of apoptosis in both U937 cells (Fig. 6) and HL-60 cells (data not shown). Untreated U937 cells exhibited normal morphology (Fig. 6*A*), with spontaneous expression of apoptotic traits discernible in <2% of the cells scored. In contrast, manifestation of prominent apoptotic morphology (i.e., cell shrinkage, condensation of nucleoplasm and cytoplasm, and formation of membrane blebs and membranous apoptotic bodies) was evident in >33% of cells exposured to rhTNF-α (50 nM; Fig. 6*B*). Similarly, treatment with SMase (50 milliunits/ml; Fig. 6*C*) or exposure to C_8-Cer (10 μM; Fig. 6*E*) increased the number of apoptotic cells to 23% and 18%, respectively, whereas exposure to *P*Cho (10 μM; Fig. 6*D*) was without effect on cellular morphology. Although gradual physical dissolution of apoptotic cells was noted at longer intervals (data not shown), there was no

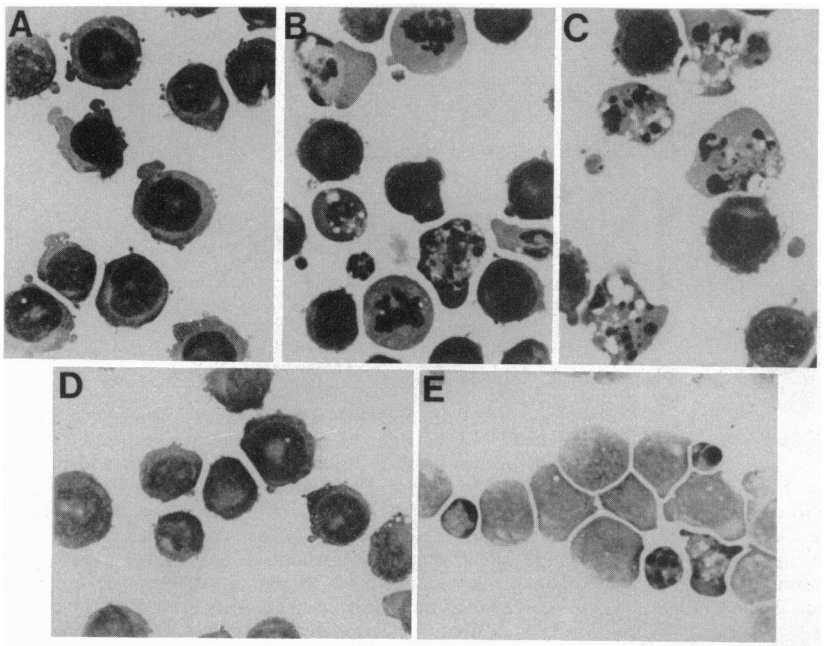

FIG. 6. Expression of morphological features of apoptosis. U937 cells were exposed to saline (*A*), rhTNF-α (50 nM; *B*), SMase (50 milliunits/ml; *C*), *P*Cho (10 μM; *D*), or C_8-Cer (10 μM; *E*) for 6 hr.

evidence of primary necrosis following exposure to any of these agents.

DISCUSSION

The induction of apoptotic cell death and DNA fragmentation by TNF has been documented in the human leukemic cell lines HL-60 (5, 6) and U937 (6, 8) and in the murine fibrosarcoma cell lines L929 (9, 10) and WEHI-164 (11, 12). Previous efforts to define a mechanism of action for TNF have demonstrated that exposure of HL-60 cells to rhTNF-α promotes the degradation of sphingomyelin to free ceramide in a time- and concentration-dependent fashion (14, 15) and that this response is mediated through activation of the type B receptor (16). The present findings confirm the ability of TNF to induce apoptosis in both hematopoietic and nonhematopoietic cell lines. In addition, these studies demonstrate apoptotic responses to experimental manipulations that increase intracellular free ceramide levels, strongly suggesting that TNF-induced apoptosis is mediated by endogenous ceramide derived from sphingomyelin hydrolysis. Biochemical characterization of apoptotic DNA damage revealed that ceramide promotes the internucleosomal degradation of DNA, resulting in the formation and eventual release of oligonucleosomal DNA fragments; this damage appears to be restricted to breakage of mature, double-stranded DNA, but not of nascent DNA, and is closely correlated with impaired clonogenicity and manifestation of apoptotic cytoarchitecture.

Apoptotic responses were also elicited by exposure to ceramide, but not to *P*Cho, demonstrating that ceramide is the principal biologically active catabolite of sphingomyelin hydrolysis mediating programmed cell death in these cells. Moreover, the induction of apoptosis was selectively associated with the action of ceramide and could not be elicited by other lipid messengers such as diglyceride, arachidonic acid, or sphingosine. In fact, the actions of SMase and ceramide were antagonized by PLC or diglyceride, suggesting opposing roles for glycerophospholipid and sphingophospholipid messenger systems. Taken together, the present results indicate that the sphingomyelin pathway (but not other lipid-dependent messenger systems) selectively mediates the induction of apoptotic fragmentation of genomic DNA by activation of the type B TNF receptor. In addition, these findings suggest an involvement of ceramide-activated protein kinase (17) and possibly of phosphoprotein phosphatase (18), rather than of protein kinase C, in the induction of apoptosis, although the extent to which these enzymes participate in the regulation of programmed cell death remains to be determined.

The expression of classically recognized morphological features of apoptosis, including nucleoplasmic and cytoplasmic condensation, formation of apoptotic bodies and membrane blebs, and loss of cell volume, was observed following rhTNF-α exposure, whereas there was no evidence of gross cytolysis or other changes indicative of necrosis. These extensive cytoarchitectural modifications, in conjunction with induction of internucleosomal DNA fragmentation, represent definitive hallmarks of apoptotic cell death, and it is therefore significant that each of these features was clearly manifested in response to SMase and various ceramide analogs such as C_8-Cer but could not be elicited by *P*Cho. The specificity of the responses to these agents is further underscored by the observations that neither PLC nor diC_8 promoted DNA damage.

In summary, pharmacological manipulations that increase the availability of free ceramide selectively induce apoptotic DNA damage and cell death in mammalian cell lines of both hematopoietic and nonhematopoietic origin, indicating that activation of ceramide-sensitive enzymes constitutes an important step in the regulation of programmed cell death. We further suggest that these findings may have potentially more generalized implications for current efforts to understand the mechanisms underlying pharmacological induction of DNA damage by other agents, including antineoplastic drugs.

This work was supported by Grants CH-523 (S.G.) and FRA-345 (R.N.K.) from the American Cancer Society, Cancer Center Support Core Grant CA-16059 from the National Cancer Institute, the Thomas F. Jeffres and Kate Miller Memorial Trust (D.A.G.), and the Medical College of Virginia Bone Marrow Transplantation Core Research Laboratory. W.D.J. is the recipient of National Research Service Award CA-09380 from the National Cancer Institute. F.A.F. is supported by Training Grant ESO-7087 from the National Institute of Environmental Health and Safety.

1. Wyllie, A. H. (1980) *Int. Rev. Cytol.* **68**, 251–306.
2. Wyllie, A. H. (1986) in *Cell Death in Biology and Pathology*, eds. Bowen, I. D. & Lockshin, R. A. (Chapman & Hall, London), pp. 9–34.
3. Gershenson, L. E. & Rotello, R. J. (1992) *FASEB J.* **6**, 2450–2455.
4. Schwartzman, R. A. & Cidlowski, J. A. (1993) *Endocrine Rev.* **14**, 133–151.
5. Elias, E., Moore, P. B. & Rose, S. M. (1988) *Biochem. Biophys. Res. Commun.* **157**, 963–969.
6. Elias, E. & Berry, C. O. A. (1991) *Leukemia* **5**, 879–885.
7. Greenblatt, M. S. & Elias, L. (1992) *Blood* **80**, 1339–1346.
8. Wright, S. C., Kumar, P., Tam, A. W., Shen, N., Varma, M. & Larrick, J. W. (1992) *J. Cell. Biochem.* **48**, 344–355.
9. Kyprianou, N., Alexander, R. B. & Isaacs, J. T. (1991) *J. Natl. Cancer Inst.* **83**, 346–350.
10. Fesel, K., Kolb-Bachofen, V. & Kolb, H. (1991) *Am. J. Pathol.* **139**, 251–254.
11. Gromkowski, S. H., Mama, K., Yagi, J., Sen, R. & Rath, S. (1990) *Int. Immunol.* **2**, 903–908.
12. Fulton, A. M. & Chong, Y. C. (1992) *Carcinogenesis* **13**, 77–81.
13. Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A. & Goeddel, D. V. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9292–9296.
14. Kim, M.-Y., Linardic, C., Obeid, L. & Hannun, Y. (1991) *J. Biol. Chem.* **266**, 484–489.
15. Dressler, K. A., Mathias, S. & Kolesnick, R. N. (1992) *Science* **255**, 1715–1718.
16. Yamaga, F. & Watson, S. (1992) *FEBS Lett.* **314**, 293–300.
17. Mathias, S., Dressler, K. A. & Kolesnick, R. N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10009–10013.
18. Dobrowsky, R. T. & Hannun, Y. (1992) *J. Biol. Chem.* **267**, 5048–5051.
19. Obeid, L. M., Linardic, C. M., Karolak, L. A. & Hannun, Y. A. (1993) *Science* **259**, 1769–1771.
20. Sundstrom, C. & Nilsson, K. (1976) *Int. J. Cancer* **17**, 565–577.
21. Collins, S. J., Gallo, R. C. & Gallegher, R. E. (1977) *Nature (London)* **270**, 347–349.
22. Grant, S., Jarvis, W. D., Swerdlow, P. S., Turner, A. J., Traylor, R. S., Wallace, H. J., Lin, P.-S., Pettit, G. R. & Gewirtz, D. A. (1992) *Cancer Res.* **52**, 6270–6278.
23. Cesarone, C., Bolognesi, C. & Santi, L. (1979) *Anal. Biochem.* **100**, 188–197.
24. Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352.
25. Ellis, A. L., Randolph, J. K., Conway, B. R. & Gewirtz, D. A. (1990) *Biochem. Pharmacol.* **39**, 1549–1556.
26. Kohn, K. W., Ewig, A. G., Erickson, L. C. & Zwellig, L. (1981) in *DNA Repair: A Laboratory Manual of Research Techniques*, eds. Friedberg, E. C. & Hannawalt, P. C. (Dekker, New York), Vol. 1, pp. 379–401.
27. Munger, C., Ellis, A., Woods, K., Randolph, J., Yanovich, S. & Gewirtz, D. A. (1988) *Cancer Res.* **48**, 2404–2411.
28. Yang, Z., Costanzo, M., Golde, D. W. & Kolesnick, R. N. (1993) *J. Biol. Chem.* **268**, 20520–20523.
29. Vilcek, J. & Lee, T. H. (1991) *J. Biol. Chem.* **266**, 7313–7316.
30. Kanter, P. M. & Schwartz, H. S. (1982) *Mol. Pharmacol.* **22**, 145–151.