Phospholipase A2 Is Necessary for Tumor Necrosis Factor α-induced Ceramide Generation in L929 Cells*

(Received for publication, January 21, 1997, and in revised form, March 21, 1997)

The role of cytosolic phospholipase A₂ (cPLA₂) in the regulation of ceramide formation was examined in a cell line (L929) responsive to the cytotoxic action of tumor necrosis factor α (TNFα). In L929 cells, the addition of TNFα resulted in the release of arachidonate, which was followed by a prolonged accumulation of ceramide occurring over 5–12 h and reaching 250% over base line. The formation of ceramide was accompanied by the hydrolysis of sphingomyelin and the activation of three distinct sphingomyelinases (neutral Mg²⁺-dependent, neutral Mg²⁺-independent, and acidic enzymes). The variant cell line C12, which lacks cPLA₂, is resistant to the cytotoxic action of TNFα. TNFα was able to activate nuclear factor κB in both the wild-type L929 cells and the C12 cells. However, TNFα was unable to cause the release of arachidonate or the accumulation of ceramide in C12 cells. C₁₀-ceramide overcame the resistance to TNFα and caused cell death in C12 cells to a level similar to that in L929 cells. The introduction of the cPLA₂ gene into C12 cells resulted in partial restoration of TNFα-induced arachidonate release, ceramide accumulation, and cytotoxicity. This study suggests that cPLA₂ is a necessary component in the pathways leading to ceramide accumulation and cell death.

The sphingomyelin (SM) cycle, first described by Okazaki et al. (1), has gained recognition over the past few years as a key mechanism for regulating anti-mitogenic signals. Activation of this cycle through the regulation of a signal-induced sphingomyelinase (SMase) results in generation of the lipid second messenger ceramide. Ceramide then modulates a number of biological fates, including growth inhibition (1–3), differentiation (2), apoptosis (4–6), and cell cycle arrest (7). Although recent studies have begun to catalogue inducers such as TNFα, interleukin-1β, nerve growth factor, and Fas that are capable of signaling through the SM cycle (see Refs. 5, 6, and 8 for reviews), the mechanisms by which these inducers stimulate SMase activity remain poorly understood.

TNFα, through interaction with either a 55- or 75-kDa TNF receptor (9, 10), impacts upon a myriad of intracellular signal cascades, including protein phosphorylation cascades, transcription factors, and lipid messengers (11). Two classes of lipid mediators have been implicated in TNFα signaling, glycerophospholipid metabolites and sphingolipid metabolites (11, 12), and recent evidence suggests that these two classes of lipids may interact (13). In HL-60 cells, a linear correlation was established among TNFα stimulation, AA generation, and SM cycle activation: TNFα-stimulated AA liberation preceded ceramide generation, and AA reproduced the effects of TNFα on the SM cycle (13). Although these studies suggested that AA and/or its metabolites may be involved in activation of SMase, the physiologic role of the PLA₂/AA pathway in regulating SMase activity has not been determined.

In this study, we examined the role of PLA₂ in SMase activation in the L929 murine fibroblast cell line. In L929 cells, TNFα treatment is known to produce potent cytotoxic effects (14). TNFα-resistant L929 cells have also been generated via clonal selection from resistant populations of L929 mouse fibroblasts grown in the presence of TNFα (14, 15). One of these cell lines, C12, differed from the original L929 cells by the absence of cPLA₂ and by the lack of inducibility of AA in response to TNFα. However, in all parameters of TNFα receptor binding and internalization, this resistant cell line was found to be analogous to the parental L929 line.

Using these two cell lines, L929 and C12, we investigated the necessity of cPLA₂ activation and AA generation for TNFα-induced ceramide generation. In the L929 model system, we found that the kinetics of cytokine-induced lipid mobilization occurred much later than previously documented in HL-60 (13, 16) and U937 (17) cells. Although the kinetics of activation were protracted in the L929 system, we found that, similar to HL-60 and U937 cells, the mechanism of ceramide generation was still through the activation of SMase and the subsequent hydrolysis of SM. Furthermore, we found that the generation of ceramide in response to TNFα did not occur in the resistant line, which was incapable of liberating AA following cytokine treatment. Finally, we found that TNFα-induced AA generation, ceramide generation, and cytotoxicity could be partly re-established in a C12 variant containing a cPLA₂ expression vector. This study implicates cPLA₂ activation and AA generation as necessary precursors to TNFα-induced activation of the SM cycle. The implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Materials
The L929 cell line and its C12 variant have been previously described (14, 15). Dulbecco’s modified Eagle’s medium and kanamycin sulfate were purchased from Life Technologies, Inc. Heat-inactivated fetal calf serum was purchased from Summit Biotechnologies (Fort Collins, CO). Arachidonic acid was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). [³H]Choline chloride and [³H]arachidonic acid were purchased from DuPont NEN. TNFα was a gift from

*This work was supported in part by National Institutes of Health Grant GM-49825. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: SM, sphingomyelin; SMase, sphingomyelinase; TNFα, tumor necrosis factor α; AA, arachidonic acid; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; PBS, phosphate-buffered saline; NF-κB, nuclear factor κB.
Hoffmann-La Roche (Basel, Switzerland). All other reagents were obtained from Sigma.

Methods

Cell Culture—All cells were maintained for up to 20 passages at 37 °C in a 5% CO2 incubator. For general maintenance, L929 and C12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 60 mg/liter kanamycin sulfate. L929/neo and CPLA2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 0.8 mg/ml G418.

For study, cells were plated at 5 × 105 cells in 12-well plates, at 5 × 104 cells in 6-well plates, or at 1 × 105 cells in 10-cm Petri plates. Cells were allowed to grow to 50–70% confluence and then washed, refed, and treated for 4 h, and treated as indicated. Time-matched controls were always run concurrently.

Arachidonic Acid Release—Cells seeded in 6- or 12-well plates were grown for 2 days and then labeled with 1 μCi/ml [3H]arachidonic acid for 24 h. Post-labeling, cells were washed, refed, and rested for 4 h. Cells were then treated as indicated, and 1.0–1.2 ml of culture medium was harvested from each treatment well. Non-adherent cells were pelleted out of the harvested medium, and the lipids were extracted via the method of Bligh and Dyer (18).

In instances where cell floaters were analyzed, the treatment media and the PBS wash were pooled and spun down. The resultant pellet was then considered the "floater" population, and lipids were harvested from these cells. Extracted lipids were dried, resuspended in chloroform, and aliquoted for phosphates (19) and diacylglycerol kinase (15) analyses as described previously. Ceramide and diacylglycerol levels were quantitated using external standards, and the resultant values were normalized against total lipid phosphate.

SM Quantitation—Cells grown in 10-cm Petri plates were grown for 2–4 days, refed, rested, and treated. Following the indicated times of treatment, the media were removed from the plates, and adherent cells were washed with PBS. Cells were then scraped, and the lipids were extracted via the method of Bligh and Dyer (18). In instances where cell floaters were analyzed, the treatment media and the PBS wash were pooled and spun down. The resultant pellet was then considered the "floater" population, and lipids were harvested from these cells. Extracted lipids were dried, resuspended in chloroform, and aliquoted for phosphates (19) and diacylglycerol kinase (15) analyses as described previously. Ceramide and diacylglycerol levels were quantitated using external standards, and the resultant values were normalized against total lipid phosphate.

SMase Isolation and Assay—Cells were seeded at 3 × 105/30-cm Petri plate in 30 ml of regular growth medium. Cells were allowed to grow for 3–4 days and then washed, refed, and treated. Following the indicated treatment times, cells were scraped into a minimal volume of serum-free medium and pelleted. Retrieved cells were resuspended in cold lysis buffer (20) and lysed via three cycles of freeze-thawing (one cycle involving freeze in a methanol/dry ice bath, 3 min at room temperature, and vortexing). By this protocol, >95% of the cells were lysed. Cells were spun at 2100 rpm (1000 × g) for 10 min to remove nuclei and the few unlysed cells. The resulting homogenate was assayed for SMase activity as described previously (13). Assay conditions for the three different sphingomyelinases were as follows: 1) neutral Mg2+-dependent; 10 nmol of SM (2 × 105 cpm), 0.1% Triton X-100, 0.1% Triton-HCl, pH 7.4, and 5 mM MgCl2; 2) neutral Mg2+-independent: 10 nmol of SM (2 × 105 cpm), 0.1% Triton X-100, and 0.1% Triton-HCl, pH 7.4, and 3) acidic: 10 nmol of SM (2 × 105 cpm), 0.1% Triton X-100, and 0.1 x sodium acetate, pH 5.0.

Thymidine Incorporation—Cells were grown in 6-well plates for 2 days and then washed, refed, and treated. Four hours prior to harvest, 1 μCi/ml [3H]thymidine was added to each well. Following the indicated treatment times, cells were harvested via a modification of a previously described method (21). Briefly, the medium was removed, and cells were washed twice with cold PBS. Cells remaining in wells were washed twice with 5% trichloroacetic acid and solubilized in 0.5 ml of 0.25 N NaOH, and 0.3 ml was collected and counted.

Cytotoxicity—Cells were grown in 6- or 12-well plates for 2 days and then washed, refed, rested, and treated. Following treatment, the medium was removed, and cells were washed with PBS. The amount of cells remaining adhered to the plate was assessed via crystal violet staining as described previously (22).

NF-κB Gel Shift—Cells were grown to 70–80% confluence and then treated in the presence of regular culture medium. Following the indicated treatment times, cells were harvested via trypan analysis, and the pellets were washed one time with cold PBS. Nuclear extractions and electrophoretic mobility shift assays were run via a modification of methods previously described (23). Briefly, the cell pellets were quick-frozen using an ethanol/dry ice bath, and the pellets were then resuspended in 50–100 μl of hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, and 1 mM dithiothreitol). This hypotonic lysis yields ~100% lysis of cells. The lysed cells were then spun, and the nuclear pellet was recovered and resuspended in 15 μl of hypotonic buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1.5 mM MgCl2, 3% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Extraction of the nuclear protein was achieved by gentle mixing of this mixture with cycloheximide. The debris was then spun down, and the resultant supernatant was diluted with 20–70 μl of dilution buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Approximately 2-μl aliquots were used for the Bio-Rad protein assay, and the remaining portion was quick-frozen and stored at −80 °C until gel shift assays were run. Protein-DNA reactions were performed in a 20-μl volume and contained 8–10 μg of nuclear extract, 1 μg of poly(dI-dC), 1 μg of poly(dI-N), 10 μg of bovine serum albumin, 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, and 10,000–50,000 cpm radiolabeled oligonucleotide probe (see Ref. 23 for sequences used). Reactions were allowed to proceed for 20 min and then terminated by the addition of 6 μl of 15% Ficoll. Nondenaturing polyacrylamide gels (5%) were then run at 200 V for 1.5 h. Gels were then dried and exposed to film. Shown (Fig. 10) is a representation of an autoradiogram obtained in this manner.

RESULTS

TNFα Is Cytotoxic to L929 Cells—L929 murine fibroblasts are acutely sensitive to the cytotoxic effects of TNFα. Treatment with 0.1–30 ng TNFα induced significant cell death within 24 h as detected via cell counts, crystal violet assays, and [3H]thymidine uptake measurements (Fig. 1). Interestingly, cytotoxicity in these cells was accompanied by the loss of adhesion to the culture plate at later time points. By 12 h of treatment, a floater population of cells was starting to appear. Following 24 h of treatment, 97% of the cells were found in this floater population, of which almost 99% were dead and only 1% remained viable (Fig. 1B). Of the cells remaining adhered to the culture plate, 50–80% of the cells remained viable as determined by trypan blue exclusion. With increased periods of TNFα treatment, fewer cells remained in the viable/adherent population, and more cells were found in the dead/floater population. Thus, loss of adhesion appears to correlate with terminal stages of the L929 death process induced by TNFα.

TNFα Stimulates Ceramide Production in L929 Cells—TNFα treatment of L929 cells also led to maximal increases in ceramide levels within 24 h (Fig. 2A). As early as 5–6 h following treatment with 5 ng TNFα, increases in ceramide of 150% were observed (Fig. 2A, inset). This delayed TNFα-induced ceramide generation differed from the previously reported effects in which cytokine-stimulated ceramide production within seconds or minutes of treatment. In fact, L929 cells showed no fluctuation in ceramide over a period of 1–60 min (data not shown), a time frame in which ceramide levels have been shown to peak and subsequently return to basal levels in HL-60 cells treated with TNFα (13). Furthermore, L929 cells demonstrated prolonged elevations of ceramide levels. Instead of attaining a peak level and quickly returning to basal levels, L929 cells were found to maintain almost an 180% increase in ceramide as long as 48 h following TNFα treatment.

More important, ceramide elevations in response to TNFα preceded the onset of death. In this delayed TNFα stimulation, which showed minimal cell death, maximal ceramide elevations were observed. Also, ceramide elevations were observed as early as 5 h following treatment; in contrast, the presence of floaters was not seen until 12 h (data not shown). Floater populations maintained high levels of ceramide, suggesting that basal levels could not be reattained prior to the onset of death (Fig. 2B).
Prolonged Elevations of Ceramide Are Accompanied by SM Hydrolysis—Since there was such a difference in the kinetics of TNFα-induced ceramide elevation in L929 cells compared with previously studied cytokine-stimulated systems, it became important to ascertain whether sphingomyelin was the source of ceramide. We therefore determined whether SM levels decreased in response to TNFα stimulation. Indeed, we found that, as early as 2 h following treatment, SM levels had decreased by ~20%, and by 14 h, when ceramide levels had increased to maximal levels, SM levels dropped by 40% as assessed by loss of label (Fig. 3).

We then investigated whether the difference in the temporal relation between TNFα induction and ceramide elevation could be explained by a difference in the type of SMase activated. In HL-60 cells, it has been shown that the rapid elevation of ceramide is the consequence of activation of a neutral cytosolic SMase activity. In contrast, stimulation of L929 cells with TNFα was found to increase the activity of not only the two neutral SMase activities (both magnesium-independent and magnesium-dependent), but also the acidic SMase activity (Fig. 4). Although the elevation of all three activities appeared to be analogous (~2-fold) in the adherent population of cells, there were some differences among the three activities in the floater population (Fig. 4). Floater/dead cells appeared to have the greatest increase in neutral magnesium-independent SMase activity (~3-fold). The neutral magnesium-dependent SMase was the first to show increased activity in response to TNFα, and its activity was increased ~2–2.5-fold at 4–6 h following treatment (data not shown).

AA Generation Temporally Precedes Ceramide Generation—As with other cell systems, TNFα stimulation of L929 cells led to an increase in AA release (Fig. 5). AA release in L929 cells demonstrated delayed kinetics, with the earliest increases observed following only 3–4 h of treatment (Fig. 5B) (14). Following 5–60 min of TNFα treatment, times in which...
HL-60 cells have been found to elevate AA levels, almost no change in AA release was found (data not shown). Furthermore, over the time period in which AA release was observed in L929 cells, the maximal level of AA elevation superseded the levels observed in other cell systems. Following 4–8 h of TNFα treatment, an almost 2-fold increase in AA release was observed.

Similar to HL-60 cells, cytokine-stimulated AA release temporally preceded ceramide elevations (Fig. 5B versus Fig. 2A, inset). Whereas ceramide increases could not be seen until 5 h following treatment with 5 nM TNFα, AA release was seen 1–2 h earlier. Temporally, therefore, the link between AA generation and ceramide generation still holds even with the prolonged kinetics observed in L929 cells.

**AA Generation Is Defective in the C12 Cell Line**—Although temporal correlations establish AA as a precedent to ceramide generation, the necessity for PLA2 activity/AA generation to modulate TNFα-induced ceramide elevation required the use of other tools. The L929 variant line C12 is known to be defective in cPLA2 as evaluated by Northern blot analysis, activity measurements (14), and Western blot analysis (data not shown). In L929 cells, 30 nM TNFα caused a 60% decrease in cell numbers as early as 16 h following treatment; in contrast, cytokine treatment of C12 cells resulted in very little cytotoxicity even following extended treatment (Fig. 6). At best, in the C12 cell line, a maximal cytotoxic effect of ~35% decrease in proliferation was observed after prolonged (48 h) stimulation with 30 nM TNFα.

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Similar to previous findings (15), we found that the resistance of C12 cells to TNFα accompanied the inability to generate AA. Whereas the parental L929 line showed a 2-fold increase in AA release, observed was prolonged (48 h) stimulation with 30 nM TNFα. The parental L929 line showed a 2-fold increase in AA release, observed was prolonged (48 h) stimulation with 30 nM TNFα.

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SM hydrolysis and ceramide generation in the resistant cells. We found that, similar to AA release, ceramide generation was perturbed in C12 cells. In L929 cells, TNFα was able to induce a 2-fold elevation of ceramide levels within 14 h of treatment with 30 nM TNFα. In contrast, C12 cells exhibited no change in ceramide levels during the same time period (Fig. 7). Neither extended (24 h) nor short-term treatment with cytokine (data not shown) elicited any change in ceramide in these resistant cells. Furthermore, we found that TNFα treatment did not lead to a decrease in SM levels (Fig. 3) or to an elevation of any of the three SMase activities that have been linked with signaling, the neutral magnesium-independent, neutral magnesium-dependent, and acidic activities (Fig. 8). The basal specific activities of all three enzymes (neutral magnesium-independent, 1000 cpm/mg/h; neutral magnesium-dependent, 2000 cpm/mg/h; and acidic, 20,000 cpm/mg/h) were equivalent in L929 and C12 cells. Indeed, treatment with TNFα in C12 cells caused a decrease in the activity of the SMases, possibly a result of activation of anti-apoptotic pathways in C12 cells (such as NF-κB) that remain responsive in this cell line (see below).

Ceramide Does Not Induce AA Generation, but Does Induce Cytotoxicity—The above results suggest that the generation of AA is coupled to ceramide generation. To establish that perturbation of ceramide generation is not the reason for the aberrant response of C12 cells in generating AA, we determined whether exogenous ceramide addition affected AA levels. In L929 cells, we found that treatment with 10 μM C6-ceramide had no stimulatory effect on AA release (Fig. 9A). In contrast, treatment of L929 cells with equivalent concentrations of C6-ceramide caused death (Fig. 9B). Concentrations as low as 1 μM C6-ceramide induced a 30% decrease in proliferation within 24 h, and concentrations of 10–40 μM caused as much as a 50–90% decrease in growth within 24 h. Thus, ceramide is able to elicit the same biological end point as TNFα without stimulating AA production, demonstrating that ceramide generation is not upstream of AA generation.

Ceramide Overrides the Resistance of C12 Cells—C12 cells,

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**TABLE I**

| Fatty acid | L929 (pmol/nmol Pi) | C12 (pmol/nmol Pi) |
|------------|---------------------|-------------------|
| 14:0       | 56                  | 59                |
| 16:0       | 700                 | 581               |
| 16:1       | 47                  | 50                |
| 18:0       | 1.122               | 961               |
| 18:1       | 761                 | 663               |
| 18:2       | 26                  | 23                |
| 18:3       | 12                  | 9                 |
| 18:1       | 17                  | 17                |
| 18:0       | 13                  | 9                 |
| 20:4       | 62                  | 50                |
| 22:0       | 6                   | 4                 |
| 22:4       | 14                  | 14                |
| 22:6       | 27                  |                   |
| 24:0       | 5                   | 4                 |
ceramide liberation, began to decline markedly by 12 h. More since disruption of PLA2 activated Signaling Cascades— as described under "Experimental Procedures." The data shown are the representative of three separate experiments. B, comparison of TNFα-induced NF-κB activation between L929 and C12 cells. Cells were treated with 0.5 nM TNFα for 15 min.

important, C12 cells, similar to L929 cells, showed nuclear translocated NF-κB within 15 min of treatment with 0.5 nM TNFα (Fig. 10B). The level of NF-κB activation seen in the TNFα-sensitive versus-resistant fibroblasts was nearly identical. Thus, NF-κB stimulation, a TNFα-responsive signaling event, appears to be intact even in cells incapable of signaling through PLA2 and AA. These results establish a specificity to the perturbation of ceramide generation in resistant cells.

**cPLA2 Activity Is Necessary for Ceramide Generation**—C12 cells, which had been found to lack TNFα-responsive cPLA2 activity, were transfected with an expression plasmid containing cPLA2 to generate the CPL4 cell line (15). These cells express cPLA2 activity (14) and cPLA2 protein as evaluated by Western blot analysis (data not shown). These CPL4 cells were utilized to assess the necessity of cPLA2 activity for ceramide generation. When treated with TNFα, CPL4 cells responded similarly to both the parental L929 and control vector-transfected L929/neo cell lines; however, CPL4 cells displayed delayed kinetics and decreased responsiveness to TNFα. Concentrations of 5 nM TNFα stimulated only 60% death within 24 h (versus 90% death in L929/neo cells) in CPL4 cells (Fig. 11A). CPL4 cells, like L929 cells, also responded to TNFα by stimulating AA release. At 1 h of TNFα treatment, when the vector (positive) controls were beginning to show a response (138%), CPL4 cells showed lower than basal levels of AA release (90%). However, by 4 h of treatment with 5 nM TNFα, CPL4 cells showed levels of AA release (150%) comparable to those seen in L929/neo cells (180%) (Fig. 11B). CPL4 cells also responded to exogenous ceramide treatment much like the other lines, with doses of 1–40 μM having potent effects within 12–24 h (Fig. 11C). Finally, CPL4 cells were able to respond to TNFα treatment with generation of ceramide. Ceramide generation corresponded to the other changes in CPL4; thus, elevations occurred later than in L929/neo cells and never reached the full extent of response attained in the vector controls. Following 5 h of treatment with 5 nM TNFα, L929/neo cells showed a response of an almost 125% ceramide increase; in contrast, CPL4 cells exhibited basal levels of ceramide at this time. By 24 h, CPL4 cells still showed decreased responsiveness compared with L929/neo cells; however, they did produce significantly more (180%) ceramide than control untreated cells (Fig. 11D). Since C12 cells did not show any level of ceramide responsiveness to cytokine, the increase in ceramide generation can be attributed to the expression of cPLA2. The attenuation in the responses of CPL4 cells may be related to the incomplete res-

**FIG. 10. Effects of TNFα on NF-κB.** Nuclear extracts were prepared from TNFα-treated cells as described under "Experimental Procedures." The amount of NF-κB activation was assessed via gel shift analysis as described. The upper band of the doublet is indicative of nuclear translocated NF-κB. The gels shown are representative of multiple experiments. A, time course with 0.2 nM TNFα treatment of L929 cells; B, comparison of TNFα-induced NF-κB activation between L929 and C12 cells. Cells were treated with 0.5 nM TNFα for 15 min.

**FIG. 9. Effects of ceramide on AA release and proliferation.** A, cells were prelabeled with [3H]AA for 24 h and then treated with vehicle, 1 nM TNFα, or 20 μM C6-ceramide. Following 24 h of treatment, label release was quantitated as described under "Experimental Procedures." The results shown are the means of duplicate samples and are representative of three separate experiments. B, cells grown in 12-well plates were treated with the indicated concentrations of ceramide for 24 h. Quantitation of proliferation was done via the crystal violet assay as described under "Experimental Procedures." The data shown are the means ± S.D.

A

**B**

similar to L929 cells, did not release AA in response to 10 μM C6-ceramide (Fig. 9A). However, concentrations of 1–40 μM C6-ceramide were able to elicit cytotoxicity in both of these cells (Fig. 9B). Notably, C6-ceramide was more cytotoxic to C12 cells than to L929 cells. Thus, the defect in C12 cells could be rectified via exogenous treatment with ceramide, suggesting that ceramide functions downstream of PLA2 activation and AA generation.

**Lack of AA Generation Does Not Perturb Other TNFα-induced Signaling Cascades**—Since disruption of PLA2 activity/A generation should exclusively affect downstream events regulated by AA, other signaling events not associated with AA should remain unperturbed. We therefore determined whether TNFα signaling to NF-κB remained intact. TNFα-induced NF-κB activation in L929 cells exhibited very different kinetics from the mobilization of AA or ceramide. Nuclear translocation of NF-κB was observed via gel shift analysis as early as 5 min following stimulation with 0.2 nM TNFα (Fig. 10A). Treatment of L929 cells over a period of hours showed that nuclear translocation of NF-κB was maintained over 4 h, but unlike AA and ceramide liberation, began to decline markedly by 12 h. More
toration of PLA₂ levels in this cell line as seen by activity measurements (14) and by Western blot analysis (data not shown). Since CPL4 cells showed significant enhancement of cytokine-induced lipid generation and growth inhibition compared with the precursor C12 line, which differs only in the expression of cPLA₂, these results establish the necessity of cPLA₂ activity/AA generation for ceramide generation and growth inhibition.

DISCUSSION

This study demonstrates the importance of PLA₂ to ceramide generation and cytotoxicity. Previous studies in the HL-60 cell system suggested a link between AA and the SM cycle in TNFα signaling (13); however, the L929 system, used here, has allowed the further development of these initial studies. L929 cells vary markedly from the HL-60 model in two critical respects. First, the temporal correlation between receptor activation and lipid mobilization (both AA release and ceramide release) is greatly attenuated in L929 cells, taking hours as opposed to minutes. Second, the magnitude of change in ceramide elicited by TNFα stimulation of L929 cells (2–4-fold changes) exceeds the levels attainable upon cytokine stimulation of HL-60 cells (at best, 1.5–1.8-fold changes) (13, 16). Despite these differences, both systems show TNFα-induced PLA₂ activation/AA generation to occur prior to SM hydrolysis and ceramide generation. Thus, the L929 model demonstrates that AA-mediated signaling to ceramide is not restricted to one cell system, but is indeed a cascade that may have greater implications.

Furthermore, the L929 model has been used here to extend our studies from the correlative level to establish the necessity of AA liberation for ceramide generation. These studies were possible because of the availability of L929 clones resistant to
the cytotoxic effects of TNFα that are defective in cPLA2. We found that, whereas L929 cells responded to TNFα stimulation by elevating AA levels within 2–4 h, C12 cells showed little if any elevation of AA release. Likewise, we found that, unlike the parental L929 line, this resistant line was incapable of responding to TNFα through the production of ceramide. In contrast, TNFα-stimulated NF-κB activation appeared to be intact, suggesting that only AA-dependent cascades were affected in the resistant cells. Thus, initial observations with the resistant lines illustrated the specificity of the interplay between AA and ceramide. We further established the necessity for AA generation through utilization of a variant strain of C12, the CPL4 line, which differed from C12 only with respect to the presence of an expression plasmid containing the murine cPLA2 gene. We found that, through recapitulating PLA2 activity in the CPL4 lines, we were able to re-establish 1) cytokine-induced AA generation, 2) TNFα-induced ceramide generation, and 3) TNFα-induced growth inhibition. Although the CPL4 system did not fully restore cytokine responsiveness, it did serve to demonstrate the interconnection among AA, ceramide, and growth.

A number of key points emerge from this study. First, it becomes apparent that the kinetics of ceramide generation are dependent upon the type of SMase activated. Whereas rapid ceramide mobilization has been attributed to the activation of one enzyme (depending on the system, a neutral, cytosolic, or acidic SMase), more prolonged ceramide generation appears to be the consequence of multiple activities perhaps acting together: a neutral Mg2+-dependent SMase, a neutral Mg2+-independent SMase, and an acidic SMase. Whether all three of these enzyme activities can be regulated by AA remains to be determined. Also, since the long-term accumulation of ceramide involves multiple enzymes, multiple pools of SM could be implicated. At a first level of examination, however, the ceramide generated in both the L929 and HL-60 systems appears to be similar (as determined by TLC) (data not shown). Whether all three SMase activities found to be stimulated by TNFα in L929 cells can act on the same pool of SM, however, remains unknown.

Second, these results raise the question of how PLA2/AA couples to SMase. The exact links between PLA2 activation/AA generation and ceramide generation remain elusive. Furthermore, other components that may be involved in regulating the signaling cascade between TNF receptor stimulation and SMase activation remain to be determined. It is important to note in this context that Fas ligand-induced death, in contrast to TNF, may not require cPLA2 (27).

Finally, the prolonged kinetics of ceramide generation raise a possibility that ceramide may function as a long-term regulator of cell growth/viability. Indeed, most studies examining the changes in ceramide associated with cell death or growth suppression disclose similar long-term changes in ceramide. These include serum withdrawal, fas stimulation, and dexamethasone-induced apoptosis (7, 24, 25). These studies have raised the question whether ceramide generation is a precedent to cell death and differentiation or whether ceramide elevations are a consequence of the biological fate and serve more as a marker of phenotype. The kinetics established in this study clearly show that both SMase activation and ceramide elevation precede the appearance of a dead/floater population of cells. The observed increase in ceramide in the adherent population of cells demonstrates cytokine-responsive SM hydrolysis prior to the onset of death, suggesting that ceramide precedes cell death. Ongoing studies also support a role for ceramide prior to the onset of actual cell death. In cells over-expressing the anti-apoptotic protein Bcl2, it appears that ceramide generation in response to chemotherapeutic agents is not perturbed, although cell death is greatly reduced (26). Such studies continue to expand our understanding of where AA and ceramide fit in the overall scheme between cell stimulation and onset of response, whether it be death, differentiation, or cell cycle arrest.

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