Tumor Necrosis Factor-α (TNF-α) Signal Transduction through Ceramide

DISSOCIATION OF GROWTH INHIBITORY EFFECTS OF TNF-α FROM ACTIVATION OF NUCLEAR FACTOR-κB

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Tumor necrosis factor-α (TNF-α) exerts pleiotropic biologic effects. Although TNF-α appears to activate a number of signal transduction pathways, the role of second messengers in mediating the different effects of TNF-α are not well defined. In this study, we investigated the role of ceramide as an intracellular mediator of TNF-α action. In Jurkat T cells, TNF-α caused early activation of the sphingomyelin cycle with peak hydrolysis of sphingomyelin observed at 30 min following addition of TNF-α. In this cell line, TNF-α caused potent activation of nuclear factor-κB (NF-κB) and exerted potent cytostatic/cytotoxic activity. C2-ceramide mimicked the effects of TNF-α on cell growth in a dose-dependent manner, but C2-ceramide was unable to induce activation of NF-κB under multiple conditions investigated. C2-ceramide, however, enhanced activation of NF-κB in response to TNF-α with peak effects observed at a concentration of C2-ceramide of 5 μM. Thus, ceramide functions as a selective mediator of the cytostatic/cytotoxic effects of TNF-α and plays a positive feedback role in activation of NF-κB. TNF-α signaling, therefore, involves multiple second-messenger pathways that function independently or coordinately to transduce distinct functions of TNF-α.

Sphingolipids play important roles in the regulation of cell growth and differentiation, cell-cell contact, and oncogenesis (1–3). A role for sphingolipids as precursors for intracellular modulators and second messengers has been proposed (2) following the discovery of inhibition of protein kinase C by sphingosine (4).

Evaluation of sphingolipid turnover as a mechanism for cell regulation led to the discovery of a sphingomyelin cycle in human promyelocytic leukemia cells (5). The action of extracellular agents such as 1α,25-dihydroxyvitamin D3 (5), γ-interferon, and tumor necrosis factor-α (TNF-α) results in activation of a neutral sphingomyelinase which causes sphingomyelin hydrolysis and the generation of ceramide (6, 7). The effects of TNF-α on sphingomyelin hydrolysis in HL-60 cells have also been confirmed recently (8).

The elevation in intracellular ceramide levels has been proposed to mediate the effects of TNF-α on cell growth and differentiation (7). This was primarily based on the ability of exogenous, cell-permeable, ceramide analogs to specifically cause cell differentiation and growth inhibition. Moreover, it was shown that exogenous C2-ceramide down-regulates c-myc protooncogene RNA levels (7). The early time course and low concentrations required for these effects are consistent with a role for endogenously generated ceramide in c-myc regulation. These results suggest that sphingomyelin turnover is an important effector mechanism for transducing the effects of TNF-α on cell growth and differentiation.

TNF-α exerts multiple biologic activities in different cell systems including inhibition of cell growth, cytotoxic activity, and modulation of gene transcription (9–12). The regulation of expression of the interleukin-2 receptor gene and other genes by TNF-α involves activation of the transcription factor NF-κB (13–16). Within minutes following addition of TNF-α, cytosolic NF-κB appears to be released from its inhibitor, IκB (17), and translocates to the nucleus, where it participates in regulation of gene transcription. However, the signaling mechanisms involved in transduction of the multiple biochemical and biological effects of TNF-α remain largely undefined (see “Discussion”). Because of the potential role of ceramide in mediating the effects of TNF-α on cell differentiation and growth inhibition, we evaluated the ability of cell-permeable ceramide analogs to modulate the expression of NF-κB.

In this study, we show that TNF-α induces sphingomyelin hydrolysis which leads to ceramide production. Ceramide, in turn, mimics the effects of TNF-α on growth of Jurkat lymphoblastic leukemia cells but is unable to activate NF-κB on its own. However, C2-ceramide enhances activation of NF-κB by TNF-α. These results suggest that ceramide is sufficient to mediate the effects of TNF-α on growth inhibition, but is not sufficient to induce NF-κB. Furthermore, ceramide may have a positive feedback modulatory role in regulation of NF-κB expression. The role of multiple signaling mechanisms in transduction of TNF-α effects are discussed.

EXPERIMENTAL PROCEDURES

Materials

Jurkat cells were a kind gift from Jerry Molitor (Howard Hughes Medical Institute, Duke University). C2-ceramide was synthesized as described (6). [3H]Choline and [γ-32P]ATP were from Du Pont-New England Nuclear. Poly[d(I-C)] and pdN4 were from Pharmacia LKB Biotechnology, Inc.

17762
**Methods**

**Cell Culture—**Jurkat (acute lymphocytic T-cell leukemia) cells were grown in RPMI 1640 (GIBCO) supplemented with 10% (v/v) fetal bovine serum. Cells were incubated for 4–12 h in serum-free medium prior to treatment with C2-ceramide delivered in ethanol vehicle (final ethanol concentration was always <0.1%). Cell proliferation studies, TNF-α, C2-ceramide, dihydro-C2-ceramide, or vehicle were added to cells, and cells were counted at the indicated time points. Cell viability was evaluated by trypan blue exclusion.

**C2-ceramide Metabolism—**H-Labeled C2-ceramide was synthesized by acetylation of sphingosine with [3H] (CH&O)20 and purified by TLC (chloroform/methanol/2N NH4OH; 4:1:0.1). Specific activity of the labeled ceramide was -2.22 x 10^6 dpm/nmol and purity was ~100%. Jurkat cells at a density of 5 x 10^5 cells/ml and total volume of 50 ml were treated with 5 μM H-labeled C2-ceramide at 30 min, 4, 12, and 24 h prior to cell harvest. Lipids from cells were extracted (Bligh and Dyer method) and applied to TLC. Radioactive spots of H-labeled C2-ceramide were scraped and counted. No metabolites were evident within the limits of detection (metabolites with >5% of C2-ceramide radioactivity would have been detected).

**Nuclear Extracts—**The nuclear extraction procedure was modified from Dignam (18) and Osborn (19). Briefly, 10^6 cells were washed once in phosphate-buffered saline. The cell pellet was rapidly frozen in dry ice and isopropanol then thawed by adding 100 μl of ice-cold Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol) resulting in 100% cell lysis. The nuclei were pelleted by microcentrifugation at 3500 rpm for 10 min at 4°C. The supernatant was discarded, and the nuclei were suspended in 15 μl of Buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1.5 mM MgCl2, 25% v/v glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The suspension was mixed gently for 20 min at 4°C then microcentrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was diluted with 50-70 μl of Buffer D (20 mM Hepes, pH 7.9, 50 mM KCl, 20% v/v glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and aliquots stored at -80°C. Protein concentrations were determined using the Bio-Rad assay.

**Electrophoretic Mobility Shift Assay—**Reactions were performed in a 20-μl volume, using 8–10 μg of nuclear extract in the presence of 1 μg of poly(dI-dC)-1, 1 μg of poly(Na), and 10 μg of bovine serum albumin. Incubations were in the presence of HDKE buffer with the following final concentrations: 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, and 5 mM dithiothreitol. Radiolabeled oligonucleotide probe (10–50,000 cpm) was added to each reaction. After incubation for 10 min the reactions were terminated by adding 6 μl of 10% ficol solution containing indicator dyes. Equal amounts of the reactions were loaded on 5% nondenaturing polyacrylamide gels in 1X TBE and run at 200 V.

**Oligonucleotides—**The probe utilized was a synthetic oligonucleotide identical to the NF-κB binding site in the interleukin-2Ra promoter with the following sequence: 5'-CAACGGCAGATCTATGATTCTCTCTCCTTCTT-3'. It was end-labeled using T4 kinase and [γ-32P]ATP. Two mutant oligonucleotides were used in competition experiments: Mutant 1: 5'-CAACGGCAGATCTATGATTCTCTCTCCTT-3'; Mutant 2: 5'-CAACGGCAGATCTAGATGATTCTCTCTT-3'.

**Sphingomyelin Assay—**Cells were labeled with [3H]choline (specific activity of 1 mCi/ml) at 0.5 μCi/ml for 48–72 h in serum-free medium then washed three times with phosphate-buffered saline and resuspended in serum-free media for 2–3 h prior to treatment with either TNF-α or vehicle. Lipids were extracted, and labeled sphingomyelin was quantitated as described (6, 7).

**RESULTS**

**Induction of Sphingomyelin Turnover by TNF-α in Jurkat T Cells—**The effects of TNF-α on activation of NF-κB-B have been best studied in the Jurkat T cell line. Because the effects of TNF-α on sphingomyelin turnover have been determined primarily in HL-60 promyelocytic leukemia cells, it became important to demonstrate the ability of TNF-α to induce sphingomyelin hydrolysis in Jurkat T cells. Treatment of cells with TNF-α resulted in a time-dependent hydrolysis of sphingomyelin with initial hydrolysis detected as early as 7.5 min, and nadir sphingomyelin levels were observed at 30 min. Sphingomyelin levels then returned toward baseline by 90-120 min (Fig. 1). The effects of TNF-α on Jurkat T cells were qualitatively similar to its effects in HL-60 cells. These studies demonstrate the ability of TNF-α to induce sphingomyelin hydrolysis in Jurkat T cells.

**C2-ceramide Mimics the Effects of TNF-α on Cell Growth Inhibition but Not on Induction of NF-κB—**The above studies suggest that sphingomyelin hydrolysis may be an effector mechanism mediating some of the biologic activities of TNF-α in Jurkat T cells. In this cell line, TNF-α inhibits cell growth (Fig. 2A) and causes potent activation of NF-κB (14). To define what role sphingomyelin hydrolysis and the generated ceramide may have in mediating the diverse actions of TNF-α, the ability of the cell-permeable, C2-ceramide, to mimic the action of TNF-α was investigated. C2-ceramide caused a dose-dependent inhibition of Jurkat T cell growth with initial inhibition observed at a concentration of 1-3 μM (Fig. 2A). At a concentration of 5 μM, C2-ceramide mimicked the effects of TNF-α, while at higher concentrations C2-ceramide was toxic to cells (Fig. 2A). To determine the specificity of this growth-inhibitory effect of ceramide, cells were treated under identical conditions with dihydro-C2-ceramide, which differs from C2-ceramide by the loss of the double bond between carbons 4 and 5 (Fig. 2B). As shown in Fig. 2C, there was minimal inhibition of cell growth by dihydro-C2-ceramide when compared with C2-ceramide. Therefore, ceramide specifically mimics the effects of TNF-α on cell growth, providing further support for a role for endogenous ceramide in mediating the effects of TNF-α on growth inhibition.

To determine the fate of C2-ceramide once it enters the cells, radiolabeled C2-ceramide was used to treat Jurkat cells at different time points. Radioactivity attributable to C2-ceramide was then measured using TLC. As shown in Fig. 3, the fraction of C2-ceramide recovered between 30 min and 24 h after treatment was stable. During the time course of the experiment 20% of the radioactivity remained in the medium as unchanged C2-ceramide. This, in addition to the lack of appearance of novel radioactive products on TLC (data not shown), indicates the absence of significant metabolism of C2-ceramide in Jurkat cells.

The ability of ceramide to induce NF-κB activation was investigated next. For these studies, Jurkat T cells were treated with either ethanol vehicle, C2-ceramide, or TNF-α, and then nuclear proteins were extracted. NF-κB activation 2

2 In other studies we have demonstrated that the effects of TNF-α and ceramide on cell growth and toxicity involved programmed cell death rather than nonspecific necrotic effects (Obeid, L. M., Linardic, C. M., and Hannun, Y. (1995) Science 259, 1769–1771).
FIG. 2. Effects of TNF-α and C2-ceramide on Jurkat T cell growth. A, Jurkat T cells were treated with 10 nM TNF-α or the indicated concentrations of C2-ceramide. Control cells were treated with ethanol vehicle. Cell counts were determined by counting with a hemocytometer. B, the structures of C2-ceramide (C2) and dihydro-C2-ceramide (DHC2). C, specificity of C2-ceramide action. The closely related analog, dihydro-C2-ceramide, was used under identical conditions. Cells were counted after 72 h of treatment (represents two experiments).

was evaluated by the formation of a distinct and specific complex in a gel-shift DNA-binding assay. Treatment of Jurkat T cells with 4 nM TNF-α resulted in activation of NF-κB (Fig. 4). This gel-shift assay detected a specific band in response to TNF-α that was competed off by unlabeled (wild type) probe but not by similar probes mutated in the NF-κB binding sequence (Fig. 4A). Thus, TNF-α caused specific activation of NF-κB, consistent with previous studies.

However, C2-ceramide, under a variety of experimental conditions, failed to induce NF-κB (Fig. 4). To fully rule out any effect of ceramide, cells were treated with ceramide delivered either in a 1:1 complex with bovine serum albumin (data not shown) or in ethanol vehicle (Fig. 4, B and C). Under these conditions, C2-ceramide was unable to induce NF-κB. C2-ceramide failed to induce NF-κB in concentrations ranging from 1 to 10 μM when delivered in ethanol vehicle (Fig. 4B). Lower concentrations of C2-ceramide were also tested and found to be inactive (data not shown), while higher concentrations became toxic to cells. In a complex with bovine serum albumin, C2-ceramide also failed to induce NF-κB in concentrations ranging from 1–20 μM (data not shown).

Since TNF-α causes early (1–5 min) activation of NF-κB that lasts over minutes and hours, we evaluated a maximal concentration of C2-ceramide (as determined under identical conditions from cell growth studies; see Fig. 2) for effects on NF-κB over 0–60 min. Again, over this time range, C2-ceramide was unable to induce NF-κB whether delivered in complex with bovine serum albumin (data not shown) or in ethanol vehicle (Fig. 4C). Thus, it can be concluded from these studies that C2-ceramide is insufficient to induce activation of NF-κB.

Ceramide Enhances NF-κB Activation by TNF-α—Since ceramide is a putative second messenger involved in mediating at least part of the effects of TNF-α, ceramide may participate in positive or negative feedback regulations of TNF-α effects. Therefore, the ability of ceramide to modulate NF-κB activation by TNF-α was evaluated next. Treatment of Jurkat T cells with 4 nM TNF-α in combination with variable concentrations of C2-ceramide resulted in a dose-dependent enhancement of NF-κB activation (Fig. 5). Initial effects were observed with a concentration of C2-ceramide of 2 μM, and peak effects were observed at a concentration of C2-ceramide of 5 μM (Fig. 5). Higher concentrations of C2-ceramide were toxic (the combination of C2-ceramide and TNF-α was more toxic than either alone). Thus, while ceramide was insufficient to induce NF-κB, it played a positive modulatory role in augmenting NF-κB activation.

DISCUSSION

Little is known about the initial events of signal transduction in response to TNF-α (20). Sequence analysis of the two receptors for TNF-α shows no homology to protein kinase receptors or to receptors coupled to G proteins (12, 21) although TNF signaling has been shown to result in activation of G proteins (22–24) and in early modulation of protein phosphorylation (25, 26). TNF-α has been shown to induce translocation of protein kinase C (27, 28) suggesting activation of the diacylglycerol/protein kinase C pathway (20, 29). The functions mediated by this pathway remain to be defined, but studies suggest a possible role for protein kinase C in activation of c-Jun in response to TNF-α (27). TNF-α has
also been shown to induce mobilization of arachidonic acid (30, 31) possibly through activation of phospholipase A₂ (32, 33). Lipooxygenase metabolites of arachidonic acid have been implicated in mediating the effects of TNF-α on c-fos expression (34). Because of the existence of these multiple potential signaling mechanisms for TNF-α action, it has become important to delineate the functional roles of the respective second messengers (ceramide, diacylglycerol, arachidonate, and eicosanoids) in mediating activities of TNF-α. 

In many cell types, TNF-α has potent cytoplastic/cytodical activity which is manifest several hours to a few days following treatment with TNF-α (11, 12, 35). On the other hand, TNF-α causes very early activation of NF-κB (within 1–5 min) which in turn induces transcription of interleukin-2, and interleukin-2 receptor genes resulting in an autocrine loop that may mediate activation and growth promotion of T cells (13). The growth promoting and cytodial activities may represent two opposing effects of TNF-α with the effects on growth inhibition predominating in Jurkat T cells.

Activation of these two opposing pathways of cell growth regulation may involve distinct signaling mechanisms. In a study in L929 mouse fibrosarcoma cells, it was suggested that activation of interleukin-6 by TNF-α does not correlate with the level of activation of NF-κB (35). Results from the current study show that ceramide is capable of mimicking the effects of TNF-α on growth inhibition, while dihydro-C₂-ceramide (Fig. 2C), diacylglycerol, and arachidonate are not (data not shown). On the other hand, ceramide is unable to activate NF-κB under various conditions tested, while phorbol 12-myristate 13-acacetate and diocytanolglycerol are able to induce NF-κB activation (14, 17, 36, 37). Therefore, at this point we may begin to define divergent signaling pathways involved in mediating distinct effects of TNF-α. The sphingomyelin/ceramide pathway may be involved in the cytokastic/cytodial activities of TNF-α, whereas other pathways may be involved in the regulation of NF-κB. The nature of signaling mechanisms mediating the effects of TNF-α on NF-κB remain poorly defined. Doubt has been cast on the role of protein kinase C in NF-κB regulation by studies in which inhibitors and down-regulation of protein kinase C failed to abrogate the effects of TNF-α (36, 38). Also, activation of NF-κB has been shown to be independent of cAMP (39). Recent studies have suggested a role for oxidation-reduction in modulating the activity of NF-κB (40–42). Further studies are required to elucidate the signaling pathways involved in NF-κB regulation.

An important point raised by these studies concerns the role of ceramide in modulating NF-κB activation. While the current results clearly show that ceramide is not sufficient to activate NF-κB, they do not rule out the possibility that ceramide is necessary for NF-κB activation in response to TNF. Thus, it is conceivable that TNF-α launches multiple signals that are necessary for NF-κB activation but none of these may be sufficient.

Our studies also demonstrate the ability of ceramide to enhance NF-κB activation in response to TNF-α. Because the kinetics of ceramide generation (5–30 min) occur later than initial activation of NF-κB (1–5 min), ceramide may play an important role in maintaining NF-κB activation following TNF-α. The lack of inhibitors for spheromyelinase or ceramide action preclude examination of these possibilities at this point.

The results from this study have implications on the role of NF-κB in growth regulation and in regulation of c-myc levels. Studies in WEHI 231 lymphoma cells have shown that...
an NF-xB-like factor may interact with the 5′ regulatory region of the c-myc gene (45). Also, it has been suggested, based on sequence analysis, that NF-xB may regulate transcription elongation of c-myc (44). In HL-60 cells, the growth inhibitory effects of TNF-α are related to down-regulation of the c-myc protooncogene (45, 46), and ceramide was shown to down-regulate c-myc mRNA levels (7) and to inhibit cell growth. However, in HL-60 cells ceramide fails to induce NF-xB activation. Thus, the ability of ceramide to inhibit c-myc levels and cell growth without activating NF-xB suggests that NF-xB activation can be dissociated from c-myc regulation and growth inhibition.

In conclusion, ceramide is an important mediator of the cytostatic/cytotoxic effects of TNF-α and other agents. A complex picture of TNF-α signaling is emerging whereby different second messengers and intracellular mediators regulate different activities of TNF-α independently and/or coordinately.

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Addendum—Since submission of this manuscript, Schütze et al. (47) have shown that exogenous ceramide can induce activation of NF-xB in permeabilized but not in intactJurkat cells; the specificity of this effect (with respect to ceramide) was not investigated. These results raise the possibility that while ceramide is not sufficient to activate NF-xB in intact cells (the current study) it may act with other components (induced by permeabilization of cells) to activate NF-xB. Further studies are required to determine if ceramide is necessary for mediating the effects of TNF-α on NF-xB.

REFERENCES


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