Analysis of Human Breast Adenocarcinoma MCF7 Resistance to Tumor Necrosis Factor-induced Cell Death

LACK OF CORRELATION BETWEEN JNK ACTIVATION AND CERAMIDE PATHWAY*

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Considerable progress has been made in the understanding of tumor necrosis factor (TNF) signaling; however, the molecular and biochemical basis of tumor resistance to the cytotoxic action of TNF are still not definitively identified yet. Although a role of c-Jun N-terminal kinase (JNK) pathway has been suggested as an effector in TNF signaling, its exact relative contribution and its interaction with ceramide pathway and tumor resistance to TNF remain unknown. The relationship between JNK activation and human breast adenocarcinoma MCF7 resistance acquisition to the cytotoxic action of TNF was therefore investigated. We demonstrate that TNF triggers JNK activation in both TNF-sensitive MCF7 cells and its resistant derivative, RA1/1001. In addition, when MCF7 cells were stably transfected with mitogen-activated protein kinase kinase 4 (MKK4) dominant-negative cDNA or transiently transfected with a dominant-negative c-Jun mutant (TAM 67), their susceptibility to the cytotoxic action of TNF remains comparable with control cells. We also demonstrated that JNK activation does not require ceramide generation since in MCF7 cells transfected with a dominant-negative derivative of FADD (FADD-DN), which are resistant to the cytotoxic action of TNF, TNF induced JNK activation in the absence of ceramide generation. Furthermore, our data indicate that exogenous permeable synthetic ceramide C-6 induced the killing of MCF7 cells transfected with MKK4 dominant-negative cDNA. These results provide strong evidence indicating that tumor acquisition of resistance to the cytotoxic action of TNF may occur either independently or at a level downstream of JNK activation and suggest that JNK activation is not linked to ceramide pathway in TNF-mediated apoptosis.

Tumor necrosis factor α (TNF) is a cytokine with powerful direct tumor-killing capability (1, 2). This cytokine has also been shown to play a role in tumor regression mediated by cytotoxic T cells. In fact, TNF may be released by cytotoxic T cells clones and significantly contributes to the local immune response to the tumor (3). Thus, when its secretion is confined to the area of tumor growth, TNF may fulfill its promise as an anticancer agent. It is well established that release of cytotoxic cytokines such as TNF triggered by T cell receptor engagement may be even more important to tumor destruction than direct lysis by cell-cell contact. This factor is now recognized as the most pleiotropic cytokine acting as a host defense factor in immunological responses and may contribute to tumor cell destruction (4).

Whereas expression of the TNF receptor 1 (TNFR1) alone is necessary for providing a biological response, it is not sufficient to induce the cytolytic process (5, 6). Despite the major advance in understanding the early events in TNF signaling and the identification of molecules that are recruited to TNFR1, the mechanisms of resistance to TNF observed in some tumor cells remain largely unknown. It has become clear that the initiation of intracellular signaling events through TNFR1 depends on protein intermediates that interact with specific cytoplasmic domains of this receptor. In fact, the death domain motif of TNFR1 plays a central role in the interactions between TRADD (TNFR1-associated death domain) (7, 8) and its association with FADD (Fas-associated death domain) (8–10), RIP (receptor-interacting protein) (11, 12), and TRAF2 (TNFR-associated factor 2) (8, 13). It is well established that among the early downstream effects of TNF are the activation of several kinases (4) and the transcription factors NF-κB (14) and AP-1 (15).

Evidence has been provided indicating that signaling pathways initiated by TNF include the activation of neutral or acidic sphingomyelinases (16–18) leading to the elevation of cellular ceramide that induces apoptosis in several cell types (19). The targets of ceramide are multiple (20–21), and above all, recent studies have suggested the implication of c-Jun N-terminal kinases (JNKs)/SAPK as a critical mediator in apoptosis triggered by ceramide (22–25). The prototypical JNK/SAPK pathway involves the sequential activation of mitogen-activated protein kinase kinase 1, mitogen-activated protein kinase kinase 4 (MKK4), JNK, and c-Jun. The activated JNKs translocate to the nucleus where they phosphorylate transcription factors such as c-Jun and ATF2 (26–29). JNK activation requires phosphorylation at 2 residues, Thr-183 and Tyr-185, by MKK4, a dual-specific protein kinase (30–32) that is structurally related to mitogen-activated protein kinase kinase 4. MKK4 itself is phosphorylated and activated by the up-

DAPI, 4',6-diamidino-2-phenylindole; MKK4, mitogen-activated protein kinase kinase 4.
stream mitogen-activated protein kinase kinase kinase 1 (30, 34).

The original observation that apoptosis may be linked to the activation of JNK cascade was made in PC-12 pheochromocytoma cells during nerve growth factor withdrawal (22). Moreover, introduction of constitutively active mitogen-activated protein kinase kinase kinase 1 resulted in increased apoptosis in PC-12 cells, whereas dominant interfering mutants of c-Jun, a downstream target of the JNK cascade, blocked apoptosis induced by nerve growth factor withdrawal (22). The requirement of JNK signaling for TNF-induced cell death remains controversial (22–25, 35–39), and the involvement of JNK activation and its interaction with ceramide pathway in the control of cell susceptibility to the cytotoxic action of TNF remain unknown.

The present data demonstrate that the resistance of MCF7 cells to the cytotoxic action of TNF is not associated with a defect in SAPK/JNK activation and emphasizes the absence of interaction between JNK and ceramide pathways in TNF-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Reagents—Highly purified (99.9%) recombinant TNF (specific activity 6.63 × 10^6 units/mg of protein) was kindly provided by Dr. Aplfier Isle. (Bender Nien). Hexanoyl-b-sphingosine (C6-ceramide) and C6-dihydroceramide were purchased from Matreya (Pleasant Gap, PA). [9,10-3H]Palmitic acid and [methy-14C]choline were purchased from NEN Life Science Products. DAPI (4',6-diamidino-2-phenylindole) was purchased from Sigma.

Cell Lines and Culture—TNF-resistant cells, 1001, were derived from RA-1 cells transfected by p55 TNF receptor cDNA as described (6). MCF7 FADD dominant-negative cells were kindly provided by V. Dixit (University of Michigan Medical School, Michigan). MKK4-DN-expressing cells were obtained by stable transfection of the MCF7 cells with an expression vector encoding MKK4 dominant-negative cDNA. All cell lines were routinely cultured in RPMI 1640 medium containing 5% fetal serum, 1% penicillin-streptomycin, 1% L-glutamine at 37 °C in a humidified atmosphere with 5% CO2.

Determination of Cell Viability—Cells were seeded in 96-well plates (7 × 10^4 cells/well) and treated either with human recombinant TNF, synthetic cell-permeable C6-ceramide, or with C6-dihydroceramide. After incubation for 72 h with TNF or 48 h with ceramide at 37 °C, the medium was replaced with 0.5% crystal-violet solution. Plates were incubated for 72 h with TNF or 48 h with ceramide at 37 °C, the medium was replaced with 0.5% crystal-violet solution. Plates were then incubated for 10 min at room temperature and washed, and viable crystal-violet-stained cells were lysed with 1% SDS. Absorbance (A540), proportional to cell viability, was then measured at 540 nm. Cell lysis was then stopped by the addition of the following ratiometric cDNA: 100 × (A510/A540); cell lysis (%) = 1 − cell viability (%), where A10 and A540 were the absorbance obtained from treated and untreated cells, respectively. The main value of quadruplicate was used for analysis.

Metabolic Labeling, Extraction, and Analysis of Cellular Phospholipids—Cells were incubated in RPMI medium containing 5% fetal calf serum and labeled with 0.5 μCi/ml [9,10-3H]palmitic acid (35 Ci/mmol) for ceramide analysis or 0.5 μCi/ml [methy-14C]choline for sphingomyelin analysis. After 8 h of incubation, the medium was removed, and cells were washed several times with phosphate-buffered saline. Cells (5 × 10^6) were then resuspended and treated with TNF (50 ng/ml) for various times. Lipids were extracted by the method of Bligh and Dyer (41) and separated by thin layer chromatography (TLC) as developing solvent systems for ceramide analysis chloroform/methanol/water (100:42:6) followed by a second step using hexane/diethyl ether/formic acid (55:45:1). For sphingomyelin analysis, lipids were separated using chloroform/methanol/water (70:30:5). Radioactive lipid spots were detected upon exposure to iodine vapor, scraped into scintillation fluid, and counted. The positions of ceramide on TLC plates were determined by comparing with concurrently run nonradioactive ceramide (type III) (Sigma). Statistical analysis was performed using Student’s t test.

Immunocomplex Kinase Assay—JNK activity was performed as described previously (41, 42). Briefly, cells were lysed at 4 °C in lysis buffer containing 25 mM HEPES (pH 7.5), 0.3 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were centrifuged at 25,000 × g for 15 min, and JNK was immunoprecipitated from the supernatant at 4 °C for 2 h using affinity-purified rabbit anti-JNK antibody (Santa Cruz Biotech). Immune complexes were immobilized on Sepharose-coupled protein G for 30 min at 4 °C (Amersham Pharmacia Biotech). Eluted twice in lysis buffer consisting of 12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate. After washing, the immune complexes were resuspended in buffer supplemented with 2 μg of GST-Jun (amino acids 1–79), 20 μM unlabeled ATP, and 5 μC of [γ-32P]ATP. After incubation at 30 °C for 20 min, the reaction was stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Western Blotting—Proteins were separated on 10% SDS-polyacrylamide gels and electroblotted onto hybond™ membranes (Amersham Pharmacia Biotech). After blocking, the membranes were probed with anti-JNK polyclonal antibody (Santa Cruz Biotech) as described elsewhere (43). The complexes were detected using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) and autoradiography.

Transfection and Apoptosis Assays—For transient transfection, cells were plated overnight at a density of 10^6/60-mm plate and then co-transfected with vector encoding the dominant-negative mutant of Jun pCMVTAM67 (27, 44) or the dominant-interfering pCNA3-Flag-MKK4 (Ala) mutant (27) together with the pEGFP vector (CLONTECH) expression vector encoding for the green fluorescent protein, according to the manufacturer instructions (Qiagen). After 24 h, cells were subsequently treated or not with TNF (50 ng/ml) for 16 h. For apoptosis analysis, cells were stained with DAPI at a concentration of 1 mM (Sigma) and examined with a Zeiss Axiophot fluorescent microscope. For stable transfection, MCF7 cells were transfected with the MKK4 dominant-negative vector (pCNA3-FMKK4) and selected for 350 μg/ml of G418. G418 resistance was confirmed by the PCR method at least twice. For analysis of protein expression per cell, treated cells were lysed in SDS sample buffer. Samples were separated by SDS-polyacrylamide gel electrophoresis and autoradiographed.

RESULTS

Effect of TNF on JNK Activation in TNF-sensitive (MCF7) and -resistant (RA1/1001) Cells—To further analyze the biochemical basis of cell resistance acquisition to the cytotoxic action of TNF, we first performed experiments to investigate the possible implication of the JNK/SAPK pathway in the acquisition of MCF7 resistance to the cytotoxic action of TNF. For this purpose, we have used the TNF-sensitive human breast adenocarcinoma MCF7 cell line and its well characterized TNF-resistant variant (RA1/1001) established by prolonged culture of MCF7 in the presence of increasing concentrations of TNF (6). As expected, over 70% MCF7 cells undergo apoptotic cell death in response to TNF, whereas less than 10% RA1/1001 clone exhibited such a phenotype (Table I). The resistance exhibited by these cells was not due to a lack of TNF receptor expression or TNF signaling (6, 45). To determine whether the RA1/1001 cell resistance to the cytotoxic action of TNF interferes with JNK activation, JNK activity was examined in these cells by an immunocomplex

| Table I |
|---|---|
| Effect of TNF on the viability of parental human MCF7 breast cancer cells and its resistant derivative 1001 clone |
| TNF | MCF7 | RA1/1001 |
| ng/ml | Cell Viability | % control | % control |
| 0 | 100 | 100 |
| 2.5 | 63.2 ± 13.12 | 95.47 ± 0.7 |
| 5 | 39.8 ± 12.4 | 97.33 ± 2.72 |
| 25 | 35.5 ± 9.5 | 95.54 ± 1.14 |
| 50 | 27.5 ± 3.45 | 95.53 ± 0.8 |

phenethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were centrifuged at 25,000 × g for 15 min, and JNK was immunoprecipitated from the supernatant at 4 °C for 2 h using affinity-purified rabbit anti-JNK antibody (Santa Cruz Biotech). Immune complexes were immobilized on Sepharose-coupled protein G for 30 min at 4 °C (Amersham Pharmacia Biotech). Eluted twice in lysis buffer consisting of 12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate. After washing, the immune complexes were resuspended in buffer supplemented with 2 μg of GST-Jun (amino acids 1–79), 20 μM unlabeled ATP, and 5 μC of [γ-32P]ATP. After incubation at 30 °C for 20 min, the reaction was stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.
TNF.

kinase assay using GST-Jun (1–79) as substrate. The phosphorylated
lanes 1 and 2 indicated concentrations of TNF. Cell lysates were immunoprecipitated
with anti-JNK, and immunoprecipitates were subjected to
induction of JNK and cell death in response to TNF. Therefore, we stably transfected MCF7 cells with an expressing vector
encoding a dominant-negative mutant of MKK4 (MKK4 ala),
which has a single mutation at the ATP-binding site, abrogat-
ing the kinase activity (31). Fig. 2A shows that stable expres-
sion of MKK4 ala inhibited (70%) the activation of JNK by
TNF, indicating that TNF signaling leading to JNK activation
was concomitantly observed (126%) (Figs. 3B). Interestingly, when
MCF7 cells transfected with FADD-DN or the control vector were treated with TNF, a similar JNK activation was observed
in both cells (Fig. 3, panels B and C). These data suggest that the
relationship between ceramide generation and JNK activation,
we stably transfected MCF7 cells with the dominant interfer-
ating mutant of FADD (FADD-DN) (10). The data shown in Fig. 3A clearly demonstrate that in FADD-DN-expressing cells, which are resistant to the cytotoxic action of TNF (data not shown), TNF was inefficient in inducing sphingomyelin hydrol-
ysis and ceramide generation (Fig. 3B). In contrast, in MCF7
cells transfected with the control vector, a sphingomyelin hydrol-
sis and a significant boost in intracellular ceramide were
concomitantly observed (126%) (Figs. 3B). Interestingly, when
MCF7 cells transfected with FADD-DN or the control vector were treated with TNF, a similar JNK activation was observed
in both cells (Fig. 3, C and D). These data suggest that the
abrogation of ceramide generation does not result in the alter-
ation of JNK activation, indicating that ceramide release and
JNK activation can occur independently.

Exogenous Ceramide-induced Cell Death in the Absence of JNK Activation—It has been reported that ceramide-induced apoptosis in U937 leukemia cells and bovine aorta endothelial cells was associated with induction of JNK activity (22). It is becoming increasingly clear that exogenous ceramide induced JNK activation through phosphorylation of MKK4 (48). We reasoned if ceramide induced JNK activation, which in turn leads to apoptosis, then expression of MKK4 dominant-interfering mutant would block ceramide-induced cell death. Using MCF7 transfected with MKK4 dominant-interfering mutant, we demonstrated that exogenous synthetic cell-permeable C
-ceramide-induced cell death was not altered in these cells. Data depicted in Fig. 4 show a significant cell death of MCF7
MKK4-DN cells (60% of lysis), comparable with cells trans-

FIG. 1. Lack of correlation between JNK and induction of apoptosis by TNF. A, cells (0.5 × 10^6) were treated for 15 min with the indicated concentrations of TNF. Cell lysates were immunoprecipitated with anti-JNK, and immunoprecipitates were subjected to in vitro kinase assay using GST-Jun (1–79) as substrate. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. B, kinetics of JNK activation by TNF. MCF7 cells and 1001 clone were incubated in the absence (lanes 1 and 6) or in the presence of TNF (50 ng/ml) for the indicated time. Cell lysates (85 μg) were subjected to in vitro kinase assay as described under “Experimental Procedures.” C, the level of JNK-1 expression was determined by immunoblotting with JNK-1 antibody.
The cytotoxic effect of TNF toward tumor cells can be affected by both intrinsic and acquired cell resistance. However, the current understanding of the molecular mechanisms critical for tumor resistance to TNF and for subsequent tumor progression remains limited. Overexpression of several TNF-induced early response genes such as MnSOD, A20, HSP 70, and IAPs has been reported to protect cells against TNF cytotoxicity (49–54). Recently, we provided evidence indicating that the alteration of sphingomyelinase activation and the subsequent ceramide generation may represent a potential...
additional mechanism by which human tumor cells may escape TNF-mediated apoptosis (6).

It is well established that several protein kinases are rapidly activated in response to TNF, including JNKs (55–56). Recently, much emphasis has been placed on the potential role of JNK as mediator of TNF signaling. Although JNK and its target c-Jun were suggested as critical mediators of apoptosis induced by TNF (22–25), their involvement in the control of TNF-induced cell death remains controversial. The relationship of JNK activation with respect to the acquisition of tumor resistance to TNF was particularly investigated. We used a cell model that is a valid tool to further dissect the biochemical mechanisms associated with the acquisition of tumor resistance to TNF. The present studies provide direct evidence indicating that TNF-induced JNK activation similarly occurs in

![Fig. 3. Activation of JNK in the absence of ceramide generation. Vector control (closed circles) and FADD-DN (open circles) transfected MCF7 cells were prelabeled with either [methyl-3H]choline (for sphingomyelin analysis) (A and B) or [9,10-3H]palmitic acid (for ceramide analysis) (B) for 48 h in 1% fetal calf serum. Cells (5 × 10⁶) were treated with 50 ng/ml TNF for the time intervals indicated. Labeled sphingomyelin (SM) and ceramide were resolved by analytic thin layer chromatography as described under “Experimental Procedures.” Results are expressed as the percentage of untreated controls. Results are the mean ± S.E. of three independent experiments (*, p < 0.05). C, activation of JNK by TNF in vector control and FADD-DN-transfected MCF7 cells. Cells were treated with the indicated concentrations of TNF for 15 min. Cells lysates were subjected to in vitro kinase assay, and the phosphorylated GST-Jun proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. D, kinetic analysis of JNK activation by TNF. MCF7 cells and FADD-DN-transfected MCF7 cells were incubated in the absence (lanes 1 and 6) or in the presence of TNF (50 ng/ml) for various time periods. Cell lysates were assessed for JNK activity. E, the level of JNK-1 expression was determined by immunoblotting with JNK-1 antibody.]

![Fig. 4. Dominant-negative mutant of MKK4 does not abrogate exogenous ceramide sensitivity in MCF7 breast cancer cells. pc-DNA-3 (A) and MKK4-DN-transfected cells (B) were treated with synthetic cell-permeable C₆-ceramide (closed circles) or C₆-dihydroceramide (open circles) for 48 h at indicated concentrations. Cell viability was measured using the crystal violet assay as described under “Experimental Procedures.” Data presented are the means ± S.D. of quadruplicate determinations. C, pc-DNA and MKK4 DN cells were incubated in the absence (-) or in the presence (+) of synthetic cell-permeable C₆-ceramide (25 μM) for 20 min. Cell lysates (85 μg) were subjected to in vitro kinase assay as described under “Experimental Procedures.”]
TNF-sensitive MCF7 cells and its resistant counterpart (RA-1/1001), suggesting that JNK activation was not abrogated in TNF-resistant cells. However, it should be noted that in TNF-resistant cells, more endogenous JNK protein was observed than in TNF-sensitive cells. Whether JNK plays a role in TNF resistance has to be determined. Our data are consistent with the concept that the JNK cascade does not play a role in TNF-induced cell death and are in agreement with previous reports indicating that induction of apoptosis by TNFR1 or by Fas was not hindered by disruption of the JNK cascade (e.g. by introduction of dominant-interfering TRAF2 or JNK mutants) (35). Since similar JNK activation by TNF occurs in MCF7 and its resistant derivative, it is tempting to speculate that the activation of the JNK cascade by TNFR1 is a bystander event that follows rather than leads to apoptosis, as suggested previously by others (35). In addition, the findings that CD40 ligation, which protects B cells against apoptosis, causes potent JNK activation are also in support of the view that apoptosis can be triggered in the absence of JNK activation (57–58). We also obtained data demonstrating that transient TAM 67 expression had no effect on TNF-induced apoptosis in MCF7. In contrast, Verheij et al. (23) have shown that C2-ceramide-induced apoptosis was inhibited in transiently transfected U937 and bovine aorta endothelial cells with TAM 67. It should be noted that the role of JNK cascade in stress-induced apoptosis should consider the type of cellular stress that is involved. Recently, Chauhan et al. (60) suggested that there are at least two different apoptotic pathways in multiple myeloma cell lines, one that involves activation of JNK as induced by irradiation and another that is independent of JNK as triggered by dexamethasone. In addition, evidence has been provided indicating the existence of tissue specificity differences in the role of JNK in apoptosis (59). Taken together, our data clearly indicate the existence of tissue specificity differences in the role of JNK in stress-induced apoptosis (59). Since similar JNK activation by TNF occurs in MCF7 and its resistant derivative, it is tempting to speculate that the role of JNK in stress-induced apoptosis is not a crucial mediator of TNF and ceramide-induced cell death.

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