The interference of tumor necrosis factor-α (TNF) signaling processes with the acquisition of tumor resistance to TNF was investigated using the TNF-sensitive human breast carcinoma MCF7 cell line and its established TNF-resistant variant (R-A1). The resistance of R-A1 cells to TNF correlated with a low level of p55 TNF receptor expression and an absence of TNF signaling through TNF receptors. Stable transfection of wild-type p55 receptor in R-A1 resulted in enhancement of p55 expression and in partial restoration of TNF signaling, including nuclear factor-κB (NF-κB) activation. However, the transfected cells remained resistant to TNF-induced apoptosis. Northern blot analysis revealed a comparable induction of manganous superoxide dismutase and A20 mRNA expression in p55-transfected cells and in sensitive MCF7 cells, making it unlikely that these genes are involved in the resistance to TNF-mediated cytotoxicity. While TNF significantly stimulated both neutral and acidic sphingomyelinases (SMases) activities with concomitant sphingomyelin (SM) hydrolysis and ceramide generation in MCF7, it failed to trigger these events in TNF-resistant p55-transfected cells. In addition, the basal SM content was significantly higher in sensitive MCF7 as compared to the resistant counterparts. Furthermore, the TNF-resistant cells tested could be induced to undergo cell death after exposure to exogenous SMase or cell-permeable C6-ceramide. This study also shows that TNF failed to induce arachidonic acid release in p55-transfected resistant cells, suggesting that an alteration of phospholipase A₂ activation may be associated with MCF7 cell resistance to TNF. Our findings strongly suggest a role of ceramide in the mechanism of cell resistance to TNF-mediated cell death and may be relevant in elucidating the biochemical nature of intracellular messengers leading to such resistance.

Tumor necrosis factor-α (TNF), originally described for its antitumor activity, is now recognized as one of the most pleiotropic cytokine to act as a host defense factor in immunological and inflammatory responses (1–3). Like other biological activities of TNF, its antitumor activity is exerted through binding to two distinct but structurally related cell surface receptors, p55 (TNF-R1) and p75 (TNF-R2). Gene knockout experiments and the use of receptor-specific agonistic antibodies confirmed that the two TNF receptors generate nonoverlapping signals (4–7). Although there is now considerable evidence that p55 is the TNF receptor that directly mediates cytotoxicity in a wide variety of cell types (8), recent studies have indicated that p75-mediated signals may cooperate with p55 to facilitate cell death in some cell types (9). Structure function analysis of p55 TNF-R signaling demonstrated that an 80-amino acid region within the cytoplasmic domain is required for initiation of apoptosis and NF-κB activation (10).

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. Cell surface expression of TNF receptors is necessary but not sufficient to induce a biological response, and post-receptor mechanisms are important in controlling the susceptibility to the cytotoxic action of TNF. The elucidation of the TNF signaling transduction pathway is particularly challenging because of the extremely wide variety of TNF responses. It is established that TNF regulates the transcription of several genes, many of which are regulated by NF-κB (11). It is also clear that the activation of this transcription factor is a pivotal and integral event for the transfer of the TNF signal to the nucleus. Several mechanisms have been reported to contribute to cellular resistance to TNF-induced cell killing, including the constitutive expression of several protective proteins in resistant tumor cells, such as MnSOD, endogenous TNF, major heat shock protein hsp70, A20 zinc finger protein (12–15). However, these proteins confer only partial protection against TNF cytotoxicity, suggesting that additional resistance mechanisms exist.

Recently, ceramide was reported to be an important lipid messenger in various pathways of TNF action (16, 17). Ceramide can be generated from sphingomyelin (SM) hydrolysis by two types of early TNF-responsive sphingomyelinases (SMases), a membrane-associated neutral (N-)SMase and an endosomai acidic (A-)SMase (18, 19). Ceramide targets may

TNF receptor; NF-κB, nuclear factor-κB; MnSOD, mitochondrial manganous superoxide dismutase; SM, sphingomyelin; SMase, sphingomyelinase; PLA₂, phospholipase A₂; AA, arachidonic acid; FCS, fetal calf serum; PBS, phosphate-buffered saline; A- and N-SMase, acidic and neutral SMases, respectively; DAPI, 4,6-diamidino-2-phenylindole.

6918 This paper is available on line at http://www-jbc.stanford.edu/jbc/
include a membrane-associated ceramide-activated protein kinase (20), a cytosolic ceramide-activated protein phosphatase (21), the mitogen-activated protein kinase cascade (22), and the stress-activated protein kinases (23). Recent studies established that ceramide generated by N-SMase directed the activation of proline-directed serine/threonine protein kinases (21). Therefore, the potential role of ceramide in mediating the cytotoxic effect of TNF, we examined its possible involvement in cell resistance to TNF. Our findings indicate that stable transfection of p55 TNF receptor restores TNF signaling including NF-xB activation in TNF-resistant MCF7 variant R-A1, but does not restore the susceptibility of these cells to TNF cytotoxicity. The data presented in this study further support the notion that the apoptotic effect of TNF is probably dissociated from NF-xB activation, and suggest that an alteration of sphingomyelinase activation and subsequent ceramide generation may represent an important additional mechanism by which human tumor cells may escape TNF-mediated apoptosis.

**EXPERIMENTAL PROTOCOLS**

Cytokines, Monoclonal Antibodies, and Reagents—Highly purified (>99%) recombinant TNF-a (TNF, specific activity 6.33 x 10^6 units/mg protein) was kindly provided by A. G. Knoll (BASF, Ludwigshafen, Germany). Monoclonal antibodies htr-9 and utr-1 directed against TNF receptors p55 and p75, respectively, were generously provided by Dr. M. Brokhaus (Hoffman-La Roche, Ltd., Basel, Switzerland). Sphingomyelinase (Staphylococcus aureus) and phospholipase D (cabbage) were obtained from Sigma. N-Hexanoyl-d-sphingosine (C6-ceramide) and C6 dihydorceramide were purchased from Matreya (Pleasant Gap, PA).

Cell Cultures and Stable Transfection of p55 TNF-R cDNA—TNF-resistant R-A1 cells were derived from a TNF-sensitive human breast carcinoma MCF7 cell line after continuous exposure to increasing doses of recombinant TNF-a (25). The wild-type human p55 TNF-R cDNA cloned in a mammalian expression vector pMPSVEH (26) was used to transfect R-A1 cells by the calcium phosphate precipitation method (27). Briefly, 1000 cells/10-cm tissue culture plate were plated. After 10–15 days of selection in growth medium containing 200 μg/ml G418 (Sigma), 4–5 resistant colonies were isolated from each plate and examined for human p55 TNF-R expression by fluorescence-activated cell sorting. The positive clones were subsequently maintained in medium with 100 μg/ml G418 for more than 2 months. The sensitivity of clones to TNF was tested every 2 weeks during culture. All cell lines were routinely cultured in RPMI 1640 medium containing 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% l-glutamine at 37°C in a humidified atmosphere with 5% CO2.

Determination of Cell Viability and DNA Fragmentation—Cell viability was determined using the crystal violet staining method as described previously (25). Absorbance (A), which was proportional to cell viability, was measured at 540 nm. Cell viability (%) = 100 x (A/TxA0), cell lysis (%) = 1 – cell viability (%), where A0 and Ax were the absorbance obtained from treated and untreated cells, respectively. The mean value of quadruplicate was used for analysis. Quantitative DNA fragmentation was determined as described previously (28). TNF- or ceramide-treated and untreated cells (1 x 10^6) were pelleted and washed in PBS. Cells were then resuspended in lysis buffer (0.5% triton X-100, 20 mM EDTA and 5 mM Tris-Cl, pH 8.0) and centrifuged at 27,000 x g for 20 min to separate the chromatin pellet from fragmented DNA. Both the pellet (resuspended in 1 mM EDTA and 10 mM Tris-Cl, pH 8.0) and supernatant were assayed to determine DNA by the spectrophotometric DAPI procedure (29).

Flow Cytometric Analysis—Indirect immunofluorescence was performed by incubating 1 x 10^6 cells with TNF receptor antibodies (htr-9 and utr-1) for 1 h on ice in PBS, 1% FCS. Cells were then washed and stained with 1:50 dilution of biotinylated goat anti-mouse IgG for 30 min. After three washes with PBS, 1% FCS, cells were incubated with 50 μl of streptavidin-phycocerythrin solution for 30 min. After additional washing with PBS, stained cells were analyzed using an EPICS profile II Couter (Coultronic, Margency, France). Fluorescence data were collected on 5 x 10^6 viable cells, as determined by forward light scatter intensity. Background fluorescence was deter-
chloroform/methanol (2:1) and quantitated by scintillation counting (36).

Measurement of [3H]Arachidonic Acid Release—According to the method of Mutch et al. (37), cells \((2 \times 10^5)\) were plated in 35-mm dishes containing 1 ml of culture medium to attach overnight and were then incubated with 0.5 \(\mu\)Ci/ml [3H]arachidonic acid \((5,6,8,9,11,12,14,15\text{-}[3H] \text{ (209 Ci/mmol, NEN du Pont, France)}\) at 37°C for 18 h. Unincorporated [3H]arachidonic acid was removed and the cells were washed twice with PBS supplemented with 0.1% BSA. Radiolabeled cells were incubated with medium or TNF. The medium was then removed and centrifuged at 2000 \(\times g\) for 5 min to separate cell debris. 0.1 ml of the supernatant fluids was mixed with 3 ml of scintillation fluid and counted. The amount of [H] release was determined in triplicate. The relative release of [H] was calculated as the percentage of arachidonic acid released in the supernatant with respect of total [H]arachidonic acid content of cells.

RESULTS

Lack of Signaling through TNF Receptors in the TNF-resistant Variant of MCF7—In an attempt to examine the mechanism of TNF-resistance acquisition by tumor cells, we established a TNF-resistant variant R-A1, derived from MCF7 cells (25). As shown in Fig. 1A, R-A1 cells were resistant to TNF compared to the parental MCF7 cells. Flow cytometry analysis (Fig. 1B) indicates that while p55 TNF-R was highly expressed in parental MCF7 cells (80%), a lower level of p55 expression (30%) was observed in R-A1. Both cell lines displayed marginal expression of the p75 receptor (10%). Data of binding experiments (Fig. 1C) using 125I-radiolabeled TNF show that receptor-bound TNF was rapidly internalized by TNF-sensitive MCF7 cells. In contrast, very little TNF binding and no TNF internalization were detected in R-A1 cells. Electrophoretic mobility shift assays for NF-\(\kappa\)B activation were performed to further evaluate the response of these cells to TNF. Treatment with TNF induced NF-\(\kappa\)B translocation in MCF7 but had no effect in R-A1 cells (Fig. 1D). The data of binding and internalization experiments are consistent with the failure of TNF to induce NF-\(\kappa\)B activation in R-A1. The hypothesis that resistance to TNF exhibited by R-A1 cells may be due to altered signaling through TNF receptors was examined next.

p55 TNF-R Expression in R-A1 Cells by Gene Transfection Restored NF-\(\kappa\)B Activation but Not TNF-induced Cell Lysis—Based on the above observations and as the p55 TNF receptor

FIG. 1. Lack of TF signaling in TNF-resistant MCF7-derived R-A1 cells. A, effect of TNF on the viability of parental MCF7 (●) and R-A1 (■) cells. Cells \((7.5 \times 10^3)\) cells/well were incubated for 72 h with the indicated doses of recombinant TNF-\(\alpha\). Cell viability was measured using the crystal violet assay as described under “Experimental Procedures.” Data presented are the means ± S.D. of quadruplicate. B, flow cytometric analysis of TNF receptor expression on MCF7 and R-A1 cells. \((1 \times 10^6)\) cells were detached from the culture and stained with the htr-9 and utr-1 monoclonal antibodies directed against the p55 and p75 TNF receptors, respectively, as described under “Experimental Procedures.” C, internalization and degradation of 125I-TNF bound to MCF7 and R-A1 cells. 125I-TNF \((50 \text{ pm})\) was allowed to bind to cells for 2 h at 4°C. Thereafter, cells were washed free of TNF and shifted to 37°C for indicated incubation times. Surface-bound (○), internalized (●), and degraded (△) 125I-TNF were determined, as described under “Experimental Procedures.” Data presented are representative of one of three independent experiments. D, effect of TNF on NF-\(\kappa\)B activation in MCF7 and R-A1 cells. Cells \((10 \times 10^3)\) were incubated for 90 min in the presence or absence of TNF (50 ng/ml). Nuclear proteins \((15 \mu\text{g})\) extracted from untreated cells (lane 1) or TNF-treated cells (lane 2) were submitted to electrophoretic mobility shift assay as described under “Experimental Procedures.” TNF-treated extracts were competed with 100-fold excess of unlabeled oligonucleotide (lane 3).
was reported to be responsible for TNF cytotoxicity signaling in most cellular models, we attempted to correct TNF signaling by transfecting R-A1 cells with a wild-type p55 expression vector, pMPSVEH-hup55 TNF-R. Following screening by fluorescence-activated cell sorting analysis using anti-TNF-R-p55 monoclonal antibody (htr-9), the stable transfected cells expressing a high level of cell surface p55 receptor (more than 70%) were selected for further study. Gel shift experiments were first performed to examine early response to TNF, i.e., NF-κB activation, in the transfected cells. As shown in Fig. 2A, exposure of three representative R-A1 p55-transfected clones (clones 1001, 2101, and 3024) to TNF (50 ng/ml) resulted in the activation of NF-κB, indicating that the TNF signaling pathway leading to NF-κB activation was functional. Interestingly, despite NF-κB activation, these p55-transfected clones remained resistant to TNF, even when a high concentration of TNF (200 ng/ml) was used (Fig. 2B). These data clearly indicate that wild-type p55 receptor expression and the resulting NF-κB activation in R-A1 cells are not sufficient to trigger TNF cytotoxic activity.

The TNF Resistance of p55-transfected R-A1 Cells Is Not Associated with MnSOD and A20 Gene Expression—Strong evidence has been provided about the role of MnSOD and A20 in cell protection against the cytotoxic action of TNF (12, 38). We therefore investigated the mRNA expression of these genes in the p55-transfected R-A1 cells to determine whether they were involved in the resistance to TNF cytotoxicity observed in these cells. Northern blot analysis showed that TNF treatment induced a similar increase in MnSOD and A20 mRNA expression in sensitive MCF7 and in p55-transfected R-A1 cells (Fig. 3). As expected, no increase in the mRNA expression level of either gene was detectable in control R-A1 cells due to the absence of TNF signaling. These data suggest that the resistance of these transfected cells was not directly related to MnSOD and A20 gene expression and further emphasize TNF signaling efficiency in these p55-transfected cells.

Fig. 2. A, effect of TNF on NF-κB activation in vector-transfected control R-A1 cells and pMPSVEH-hup55-transfected R-A1 clones (clones 1001, 2101, and 3024). Cells (10 × 10⁶) were incubated for 90 min in the presence or absence of TNF (50 ng/ml). Nuclear proteins (15 μg) were submitted to electrophoretic mobility shift assay as described under "Experimental Procedures." NF-κB activation by TNF was undetectable in control vector-transfected R-A1 cells. B, effect of TNF on the viability of parental MCF7 and transfected R-A1 cells. Cells (7.5 × 10³/well) were incubated for 72 h with the indicated doses of recombinant TNF-α. Cell viability was measured using the crystal violet assay as described under "Experimental Procedures." Data presented are the means of quadruplicate.

FIG. 3. Northern blot analysis of MnSOD and A20 gene expression following TNF treatment. Cells (10 × 10⁶) were incubated with medium (−) or 50 ng/ml TNF (+) for 6 h at 37 °C. Total RNA (15 μg/lane) were electrophoresed in a 1.2% agarose gel and hybridized with ³²P-labeled MnSOD and A20 specific cDNA probes. β-Actin probe was used to confirm comparable loading of RNA sample.

Fig. 4. A, concomitant increase in intracellular ceramide (−2.4-fold increase) was detected in MCF7 at 10−20 min of TNF incubation, preceded by a rapid SM hydrolysis (>50% decrease in SM content) that reached the maximum level within 5−10 min after TNF treatment. The measurement of neutral and acidic SMases indicates that the SM breakdown and ceramide generation in MCF7 cells was correlated with a significant induction of these two SMase activities (20% and 40% of control for neutral and acidic SMases, respectively) after 5−15 min of TNF incubation (Fig. 4B). In contrast, TNF did not induce neither ceramide generation nor SM hydrolysis in R-A1 and p55-transfected cells (clones 1001 and 3024) (Fig. 4A). This is consistent with the failure of TNF to stimulate both SMase activities in these cells (Fig. 4B). However, the defect of the SMase activation was not related to a decrease in basal SMase activities in the resistant cells, since the basal SMase activities were not significantly different in the three cell lines tested (e.g. N-SMase activities were 231, 385, and 258 pmol/h/mg proteins for MCF7, R-A1, and clone 1001 cells, respectively).

Cellular SM Content in TNF-sensitive and -resistant MCF7 Cells—In order to compare the basal level of SMase substrate in TNF-sensitive and resistant cells, the analysis of cellular SM content was performed. As shown in Table I, the percentage of SM (as compared to total phospholipids) was significantly higher in parental MCF7 cells (28−30% of increase) than in
R-A1 and clone 1001 cells. Moreover, the basal SM content was 2-fold higher in MCF7 than in the two resistant counterparts (20.9 nmol for MCF7 versus 10.7 nmol and 12.4 nmol for R-A1 and clone 1001 cells, respectively). This result suggests that the different responses induced by TNF in these cells may be dependent on the cellular SM content.

**Exogenous SMase and Ceramide Trigger Cell Death in TNF-resistant Cells**—To determine whether the activation of SMase and the production of ceramide could overcome the resistance of transfected R-A1 cells, we examined the susceptibility of these cells to exogenous bacterial-derived SMase and synthetic cell-permeable ceramide. The addition of SMase (Fig. 5A) or \(C_{\alpha}\)-ceramide (Fig. 5B) was able to induce the killing of p55-transfected cells as well as that of control R-A1 and parental MCF7 cells in a dose-dependent manner. The cytolytic effect of SMase and \(C_{\alpha}\)-ceramide on these cells was specific, since the addition of phospholipase D (data not shown) or \(C_{\alpha}\)-dihydroceramide (Fig. 5B) at equivalent concentrations failed to induce cell killing. Furthermore, DNA fragmentation analysis indicates that C6-ceramide killed both TNF-sensitive and TNF-resistant MCF7 cells through an apoptotic pathway (Fig. 6). After 24 or 48 h of treatment of TNF-sensitive MCF7 cells, more apoptotic cells were observed upon C6-ceramide treatment than that upon TNF treatment, indicating that ceramide triggers apoptosis of these cells in a more direct manner than TNF.

**TNF Induces the Release of Arachidonic Acid in Sensitive MCF7 but Not in p55-transfected R-A1 Cells**—TNF has been described to be capable of activating PLA2 and inducing the release of arachidonic acid (AA) from membrane phospholipids in several sensitive target cells (41, 42). Initial experiments using dexamethasone, an inhibitor of PLA2, indicated that the addition of this component at a subtoxic concentration (100 ng/ml) efficiently inhibited (4-fold) the killing of parental MCF7 cells by TNF (Fig. 7A). Therefore, the involvement of PLA2 in TNF signaling in MCF7 and transfected R-A1 cells was assessed by measuring the release of AA. As shown in Fig. 7B, TNF induced a significant release of \(^3\)H-labeled AA metabolites (165% of control) in sensitive MCF7 cells after 18 h of incubation. No stimulation of AA release was observed during short term (0–6 h) incubations (data not shown). In contrast, the increase in the release of \(^3\)H-labeled AA in R-A1- and p55-transfected clones was not detected at any of times tested (Fig. 7B), suggesting that the

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**Table I**

*Cellular SM content in TNF-sensitive and -resistant MCF7 cells*

| Cells     | SM contents | SM (compared to PL) |
|-----------|-------------|---------------------|
|           | nmol IP/mg protein | %                 |
| MCF7      | 20.9 ± 0.3   | 14.96 ± 0.20        |
| R-A1      | 10.7 ± 1.3   | 11.46 ± 1.60\(^a\)  |
| Clone 1001| 12.4 ± 0.3   | 11.66 ± 0.50\(^b\)  |

\(^a\) Decreased vs. % of MCF7 SM (p < 0.02).

\(^b\) Decreased vs. % of MCF7 SM (p < 0.001).

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**Fig. 4.** Induction of sphingomyelin metabolism in the parental TNF-sensitive MCF7 and in its resistant counterparts. A, ceramide and SM levels were evaluated in cells (3 x 10^6) prelabeled with [9,10-\(^3\)H]palmitic acid for 48 h. Cells were then treated with 50 ng/ml TNF for the indicated time intervals. Aliquots were then collected to prepare lipid extracts. Labeled ceramide and SM were resolved by TLC as described under “Experimental Procedures.” Results are expressed as a percentage of untreated controls. Control ceramide counts were 11,076 ± 230 and 10,190 ± 540 cpm for MCF7 and R-A1, respectively. For MCF7, ceramide data are the mean ± S.E. of three independent experiments. *, p < 0.045; **, p < 0.002, relative to time zero. SM data are from one representative of three independent experiments. For R-A1- and p55-transfected cells (clones 1001, and 3024), data are from one representative of three independent experiments. B, for neutral (N-) and acidic (A-) SMase activities, aliquots were collected after TNF treatment (50 ng/ml) and enzyme assays were performed as described under “Experimental Procedures.” Data are from one representative of two independent experiments, and they are expressed as a percentage of controls. The results for A-SMase are the mean ± S.D. of duplicate measurements. The data (not shown) for R-A1 and clone 3024 are similar to that of clone 1001.
resistance of these cells to TNF may also be related to altered AA release.

DISCUSSION

In contrast to the rapid progress that has been made in defining gene products capable of regulating TNF-induced cell death, the knowledge of the molecular components involved in cell resistance to TNF remains limited. We attempted to delineate the functional role of some second messengers in the acquisition of tumor resistance to TNF by comparing a TNF-sensitive human breast cancer cell line MCF7 with its R-A1 variant selected for resistance to TNF. This TNF-resistant variant derived from MCF7 was found to be susceptible to anti-Fas-induced cell lysis as well as to the chemotherapeutic drug adriamycin (data not shown), compelling evidence that the intracellular cell death pathway is functional in these cells. As compared to the parental MCF7 cells, the resistance of R-A1 cells to TNF correlated with a low level of p55 TNF receptor expression and an absence of TNF signaling through TNF-Rs. Although functional wild-type p55 receptor expression was re-established in R-A1 cells by gene transfer as well as subsequent NF-κB activation in response to TNF, these cells remained totally resistant to the cytotoxic action of TNF. It should be noted that transfection of rodent cells using the same vector efficiently triggered the cytotoxic effect of TNF (26). Our observations suggest that NF-κB is not sufficient to induce cell killing, and confirm that the activation of this nuclear factor and apoptosis are coincidental but that these two activities are separable. This is also in agreement with the report of Dbaibo et al. (43), indicating that the growth inhibitory effect of TNF is dissociated from the activation of NF-κB in Jurkat lymphoblastic leukemia cells.

Cell resistance to the cytotoxic action of TNF is thought to be an active process requiring the synthesis of TNF-inducible proteins, since this resistance can be overcome by protein synthesis inhibitors in some experimental systems (44). Overexpression of several TNF-inducible early response genes, such as MnSOD and A20, has been reported to protect cells against TNF cytotoxicity (12, 15). MnSOD acts as a superoxide radical scavenger, and its presence correlates with cellular protection against TNF cytotoxicity. A20 zinc finger protein is the product of a cytokine-induced primary response gene, and its overexpression inhibits TNF-induced activation of PLA₂ and TNF-mediated apoptosis (38). Although the involvement of MnSOD and A20 in cellular protection has been established, our data indicate that both genes can be induced by TNF in p55-transfected resistant cells at similar level as compared to that in TNF-sensitive MCF7 cells, suggesting that the resistance of these cells is not related to overexpression of these two genes.

FIG. 5. Effects of exogenous bacterial SMase and ceramide on parental TNF-sensitive MCF7 and its resistant counterparts. Cells were treated (7500 cells/well) with bacterial SMase (A) or synthetic cell-permeable C₆-ceramide (closed circles) or C₆-dihydroceramide (open circles) (B) at indicated concentrations. Cell viability was measured after 48 h of treatments using the crystal violet assay as described under “Experimental Procedures.” Data presented are the means of quadruplicate determinations. Experiments were repeated twice with similar results.
Ceramide has emerged as a potent second messenger in TNF signaling, and a substantial amount of evidence has been accumulated in favor of ceramide functioning as a selective mediator of the cytotoxic/cytostatic effect of TNF (16, 17). Ceramide generated by the activation of sphingomyelinase has been reported to mediate TNF-induced apoptosis in the human monocyte-like U937, human leukemic HL-60, and murine fibrosarcoma cell lines (39, 40). This study shows that TNF can activate both neutral and acidic SMases in human breast tumor MCF7 cells. A defect in the activation of these enzymes is apparently sufficient to confer resistance to TNF in R-A1 cells. Indeed, when this defect was overcome by adding exogenous SMase or ceramide, the susceptibility of R-A1 cells to apoptosis was restored. This suggests that the stimulation of ceramide-activated enzymes may constitute an important step in the regulation of programmed cell death. It is worthy to note that in our study N-SMase was more rapidly activated than A-SMase, confirming that the activation requirements of A-SMase differ from that of N-SMase. This is also in agreement with the report by Wiegmann et al. (24), which suggests that N- and A-SMase activations may be triggered by distinct pathways. When specific inhibitors of two SMases become available, the determination of the nature of SMase involved in TNF-induced apoptotic cell death would be of major interest. It is tempting to speculate that the failure of TNF to induce DNA fragmentation and apoptosis in resistant cells may be related to structural membrane organization of SM, which may constitute a limiting step for the generation of this second messenger. Our data also demonstrate that the basal level of total SM content was higher in sensitive parental MCF7 cells, as compared to the resistant counterparts (R-A1 and clone 1001 cells). On the other hand, both sensitive and resistant cells showed a similar basal level of SMase activity. Thus, the lower level of basal SM content and the absence of SMase activation in resistant cells may represent a double blockage for ceramide generation. These results confirm the hypothesis from our previous report indicating that the failure of TNF to induce either SM hydrolysis or apoptosis in resistant myeloid leukemia KG1a cells was correlated with the SM pool used for TNF signaling, which is predominantly located in the inner leaflet of the plasma membrane (45). One could speculate that the lower TNF-hydrolyzable SM pool in p55-transfected R-A1 cells and the absence of SMase activation would explain the failure of TNF signaling to induce SM hydrolysis and ceramide generation in these cells. Although the sphingomyelin/ceramide pathway was reported to be capable of signaling NF-κB translocation in HL-60 cells (46, 24), we showed here that TNF induced NF-κB translocation in p55-transfected resistant cells in the absence of ceramide generation. This is in agreement with other reports demonstrating that exogenous addition of a short chain ceramide to Jurkat cells or the inhibition of ceramide...
pathway had no effect on NF-kB activation (47, 48), and further confirming that NF-kB activation by TNF can be independent of endogenous cellular ceramide generation.

Alternatively, non-induction of apoptosis in the p55-transfected cells could be due to an abnormal expression of other genes such as the proto-oncogene bcl2, involved in the regulation of apoptosis (49, 50). However, the involvement of bcl2 in the resistance of R-A1 cells can be excluded, since the parental TNF-sensitive MCF7 cells displayed a higher bcl2 expression level than the resistant R-A1 cells (data not shown), suggesting that there is no correlation between bcl2 expression and the magnitude of TNF-induced apoptosis in these cells. In addition, we and others have reported that bcl2 acts downstream of ceramide preventing ceramide-induced cell death but not ceramide accumulation in at least two models of chemotherapy-induced cell death (51). Our demonstration that TNF failed to generate ceramide in p55-transfected R-A1 clones overrides the implication of bcl2 in this phenomenon.

A complex pattern of integrated signals may be generated in response to an elevation of cellular ceramide content. Indeed, recent studies reveal the view that ceramide produced by N-SMase triggers the mitogen-activated protein kinase cascade via ceramide-activated protein kinase, which presumably results in the activation of PLA2 (24, 52, 53). This is consistent with several lines of evidence suggesting the involvement of PLA2 in the cytocidal pathway of TNF (37, 41, 54). In addition, the AA generated by the activation of PLA2 was reported to activate sphingomyelin hydrolysis in HL60 cells (55). Data obtained in our studies indicate that the release of arachidonic acid is altered in p55-transfected TNF-resistant cells. This might be a consequence of the defect in ceramide generation. Although ceramide generation occurred rapidly (10–20 min) following TNF treatment in the sensitive cells, the activation of PLA2 and the AA release induced by TNF could not be detected in the first hours (0–6 h) after TNF treatment. The AA release probably does not precede TNF-stimulated SM hydrolysis in our system, but is involved as a later biochemical event in the signal transduction pathway. Whether ceramide and AA function independently or in coordination to transduce TNF cytocidal effect requires further investigation. Taken together, our data suggest that a selective defect in TNF signaling associated with an alteration in sphingomyelinase activity can, at least in part, confer resistance to TNF-induced cell death.

An insight into signal transduction by p55 TNF-R1 has resulted from the identification of the TNF-R1-associated protein TRADD, which interacts with the death domain of p55 and signals both cell death and NF-kB activation (56). Following TNF treatment, the association of TRADD and TNF-R1 occurs rapidly in U937 cells (57). In addition, TRADD interacts with TRAF2 (58) and FADD/MORT1 (59, 60) leading, respectively, to NF-kB activation and apoptosis induction in the overexpression systems. The importance of the signaling complex assembly is also demonstrated by the fact that dominant-negative derivatives of FADD/MORT1 abrogated CD95(Fas)-induced apoptosis and ceramide generation in a B lymphoma cell line (61). One can suggest that in the case of TNF, ceramide generation may also be a downstream event, e.g. post-TRADD and/or post-FADD/MORT1 activation. We have obtained data indicating a comparable TRADD protein expression level in parental MCF7 as well as in resistant R-A1 and clone 1001 cells, and that transient TRADD-transfection induced apoptosis and NF-kB activation in all these cells. However, no effect of TRADD overexpression on ceramide accumulation could be detected (data not shown). It seems unlikely that TRADD alone triggers cell death signaling through ceramide pathway. Whether TRADD/FADD complex formation occurs and interacts with the SM/ceramide pathway under physiological conditions remains to be determined. It would be of major interest to decipher the possible cross-talk between diverse TNF-R associated signaling molecules, such as FAN protein (62), and the sphingolipid messengers implicated in the TNF cytotoxic signaling pathway. Understanding of the molecular and biochemical mechanisms of tumor cell resistance to the cytotoxic effect of TNF may ultimately provide new approaches to enhance the therapeutic efficacy of TNF against human malignancies.

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