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Title
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Journal
Cancer Research, 58(21)

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Publication Date
1998-11-01

Peer reviewed

Differences in Susceptibility to Tumor Necrosis Factor α-induced Apoptosis among MCF-7 Breast Cancer Cell Variants[1]

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ABSTRACT

Widespread use of MCF-7 human breast carcinoma cells as a model system for breast cancer has led to variations in these cells between different laboratories. Although several reports have addressed these differences in terms of proliferation and estrogenic response, variations in sensitivity to apoptosis have not yet been described. Tumor necrosis factor α (TNF-α) has been shown to both induce apoptosis and inhibit proliferation in MCF-7 cells. We observed that TNF-α inhibited proliferation in MCF-7 cell variants from three different laboratories (designated M, L, and N). MCF-7 M cells were resistant to TNF-α-induced apoptosis, whereas MCF-7 L cells were moderately resistant to the effect of TNF-α. A third variant, MCF-7 N, underwent apoptosis when exposed to TNF-α. Analysis of the p55 TNF-α receptor (TNFR) 1 expression revealed the greatest expression in MCF-7 N cells, whereas the MCF-7 L and M cells expressed 89 and 67% of MCF-7 N cell TNFR1 levels, respectively. Ceramide generation occurred in all three variants in response to TNF-α treatment, with MCF-7 N cells expressing the greatest increase. Cleavage of the CPP32/caspase 3 substrate poly(ADP-ribose) was observed in MCF-7 N and L cells as early as 3 and 6 h, respectively, but poly(ADP-ribose) cleavage was not observed in MCF-7 M cells. The delayed protease activation in the L variant may represent the mechanism by which these cells display delayed sensitivity to TNF-α-induced apoptosis. Expression of the Bcl-2, Mcl-1, Bcl-X, Bax, and Bak proteins was analyzed to determine whether the differences in MCF-7 cell sensitivity to apoptosis could be correlated to the differential expression of these proteins. Whereas Bak, Bcl-X, and Mcl-1 levels were identical between variants, the levels of Bcl-2 were 3.5–3.8-fold higher and the levels of Bax were 1.5–1.7-fold lower in the resistant variants (M and L) as compared with those of the sensitive variant (N). Taken together, these results suggest that differences in susceptibility to TNF-α-induced apoptosis among MCF-7 breast cancer cell variants may be explained by differences in TNFR expression, ceramide generation, differential expression of the Bcl-2 family of proteins, and protease activation.

INTRODUCTION

The MCF-7 cell line was established in 1973 from a pleural effusion of a patient with metastatic breast carcinoma who was previously treated with radiation and hormonal therapy (1). Since that time, this cell line has become a model system of ER[3]-positive breast cancers (2). Previous studies suggest that MCF-7 cell line variants possess intrinsic differences in estrogen responsiveness and proliferation rates. Osbourne *et al.* (3) reported that MCF-7 cells obtained from different laboratories varied in proliferation rates, ER and progesterone receptor levels, estrogen and antiestrogen responses, and tumorigenicity. Klotz *et al.* (4) showed that different stocks of MCF-7 cells displayed different levels of variant ER mRNAs, which correlated with their differential response to estrogen stimulation. Different MCF-7 variants tested by Villalobos *et al.* (5) exhibited different responses to 17β-estradiol-induced proliferation and expression of the estrogen-responsive genes *pS2* and *cathepsin D*. These reported variations in MCF-7 cells could potentially lead to contradictory results, depending on the origin of the variant of MCF-7 cells studied.

Apoptosis and apoptotic signaling have recently been examined in MCF-7 cells in response to a number of stimuli including okadaic acid, staurosporine, Fas, retinoic acid, vitamin D analogues, 4-hydroxy-tamoxifen, ceramide analogues, hormone withdrawal, and various chemotherapeutic drugs (6–15). TNF-α is also an effective inhibitor of proliferation and inducer of apoptosis in MCF-7 cells (7, 8, 16, 17). In other studies, MCF-7 cells reportedly responded only weakly to TNF-α (18–21). MCF-7 cells made resistant to TNF-α by continuous passaging in increasing concentrations of TNF-α express decreased levels of TNFR and do not activate SMase or phospholipase A$_2$ with TNF-α treatment (17). The reported differences in the sensitivity of MCF-7 cells to TNF-α and potentially other apoptotic-inducing agents raised the possibility that variations in MCF-7 cell strains among laboratories may account for these discrepancies.

The effects of TNF-α are mediated through two distinct but related plasma membrane receptors, p55 (TNFR1) and p75 (TNFR2). Both receptors generate distinct biological effects, with the cytotoxic effects of TNF-α being primarily mediated through TNFR1 (22, 23). Although these receptors share limited cytoplasmic homology, they activate some overlapping signaling cascades, such as nuclear factor κB, via the recruitment of specific signaling intermediates to the cytoplasmic domains (22, 23). In the case of TNFR1, TNF-α ligation results in association with TRADD (24), which then recruits TNFR-associated protein 2, receptor-interacting protein (25), and FADD/MORT1 (26), association with the receptor is followed by the association of FLICE/MACH1 with the TNFR1 complex (27, 28). Subsequent to the formation of this protein complex, the activation of several signaling cascades including phospholipase A$_2$, SMase, nuclear factor κB, stress-activated protein kinases, and apoptotic proteases occurs (22, 23). Activation of SMase, resulting in ceramide formation, represents an early event in the apoptotic signaling cascade (29, 30). MCF-7 cells have been shown to activate SMase in response to TNF-α and undergo apoptosis when exposed to water-soluble ceramide analogues (17). Additionally, studies of acidic SMase knockout mice have shown that ceramide generation may be required for apoptosis by TNF-α and other inducers in some cell types (31).

[3] The abbreviations used are: ER, estrogen receptor; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; PARP, poly(ADP-ribose) polymerase; SMase, sphingomyelinase; TRADD, TNFR1-associated death domain protein; FADD/MORT1, Fas-associated death domain; FLICE, FADD-like ICE; MACH, Mort1-associated CED-3 homologue; ICE, interleukin 1β-converting enzyme; FBS, fetal bovine serum; DAG, diacylglycerol; MTS, 3-(4,5-dimethyl-thiazol-2-yl)-5-3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
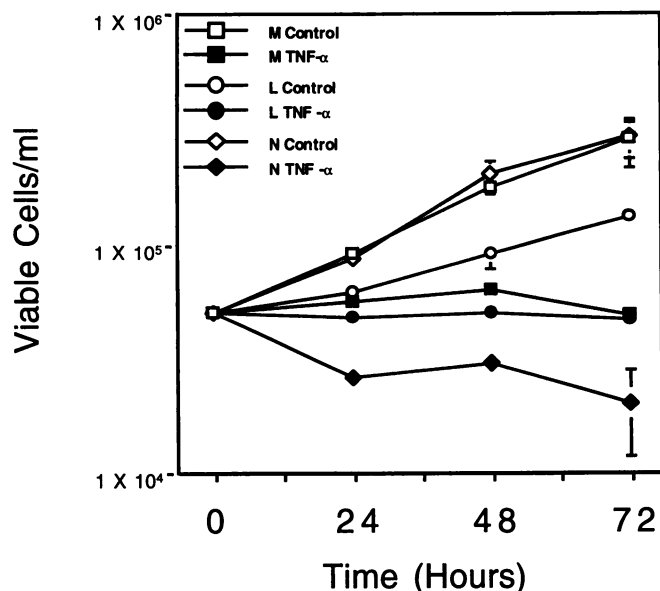
Fig. 1. Effects of TNF-α on the proliferation of MCF-7 cell variants. Each MCF-7 cell variant was plated in DMEM with 10% FBS alone or in the presence of 10 ng/ml TNF-α. Cells were harvested and counted at 24, 48, and 72 h to determine the number of viable cells/milliliter. *Error bars*, SD for duplicate experiments performed in triplicate.

Caspase activation is also thought to represent an early event in TNF-α cell death signaling. The death domain-containing protein FLICE/MACH1/caspase 8 possesses an ICE-like protease domain that becomes activated upon association with the TNFR1-TRADD-FADD complex. The activation of FLICE is thought to result in the subsequent activation of ICE/caspase 1 and CPP32/caspase 3 (32, 33). Therefore, both ceramide generation and protease activation represent potential regulatory points of TNF-α-induced apoptotic signaling.

The Bcl-2 family of proteins comprises a number of related proteins whose expression has been shown to regulate apoptosis (34, 35). This family includes antiapoptotic members (Bcl-2, Mcl-1, and Bcl-X$_L$) and proapoptotic members (Bax, Bcl-X$_S$, and Bak) whose individual expression and heterodimerization with each other is believed to regulate the sensitivity of cells to apoptosis. Although the actual biochemical function of these proteins has yet to be completely elucidated, these proteins act upstream of caspase activation through the regulation of cytochrome *c* release from the mitochondria (36, 37).

This study tests directly whether variants in the phenotype of MCF-7 cells may explain the reported differences in susceptibility to apoptosis induced by TNF-α and other agents. The molecular mechanisms for these observations are dissected by examining several events in the signal transduction cascade of TNF-α including TNFR expression and SMase and caspase activation as well as the expression of specific members of the Bcl-2 family of proteins.

MATERIALS AND METHODS

Cell Culture. MCF-7 cell variants M and L were a gift from Stephen M. Hill (Tulane University, New Orleans, LA). The MCF-7 M cell variant (passage 180) originated from the laboratory of the late William McGuire (University of Texas, San Antonio, TX.). The MCF-7 L cell variant (passage 40) originated from the laboratory of Marc Lippman (Georgetown University, Washington, DC). The MCF-7 N cell variant (passage 50) is a subclone of MCF-7 cells from the American Type Culture Collection (Rockville, MD) that was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN). All MCF-7 cells were routinely maintained and grown in DMEM supplemented with 10% FBS, BME (Basal Medium Eagle) amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Life

Technologies, Inc., Gaithersburg, MD), and 1×10^{-10} M porcine insulin (Sigma Chemical Co., St. Louis, MO) under *Mycoplasma*-free conditions.

Proliferation and Viability Assay. MCF-7 cells were plated at 5.0×10^4 cells/ml in 10-cm^2 wells. The cells were allowed to adhere for 18 h before treatment with recombinant human TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN). Cells were then counted at 24, 48, and 72 h posttreatment. The results are represented as the number of viable cells/milliliter as measured by trypan blue exclusion. Apoptosis was expressed as the percentage of trypan blue-stained cells in treated samples compared to control viability. The MTS viability assay (Promega) was performed according to the manufacturer's protocol.

DNA Fragmentation Analysis. After treatment, cells were harvested for DNA as described previously (38). Briefly, $1–2 \times 10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris-HCl, 10 mM EDTA, and 0.5% SDS (w/v; pH 7.4)] to which RNase A (100 μg/ml) was added. After incubation for 2 h at 37°C, proteinase K (0.5 mg/ml) was added, and the lysates were heated to 56°C for 1 h. NaCl was then added (final concentration, 1 M), and lysates were incubated overnight at 4°C. Lysates were centrifuged at $15,000 \times g$ for 30 min, and nucleic acids in the supernatant were precipitated in 2 volumes of ethanol with 50 mM sodium acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by ethidium bromide staining.

Western Blot Analysis. MCF-7 cells were grown for 2 days as described above, and then 5×10^6 cells were harvested in sonicating buffer [62.5 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, and 25 mg/ml aprotinin] and sonicated for 30 s. After centrifugation at $1,000 \times g$ for 20 min, 50 μg of protein were resuspended in sample loading buffer [62.5 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromphenol blue], boiled for 3 min, and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with a 0.05% PBS-Tween-5% low-fat dry milk solution at 4°C overnight. The membrane was subsequently incubated with rabbit antisera (anti-Bcl-2, 1:4,000 dilution; anti-Bax, 1:4,000 dilution; anti-Bcl-X, 1:1,500 dilution; anti-Mcl-1, 1:2,000 dilution; and anti-Bak, 1:1,000 dilution) or with mouse anti-PARP-specific monoclonal antibody (1:5,000 dilution; PharMingen, San Diego, CA) and incubated for 2 h at room temperature. Blots were washed in PBS-Tween solution and incubated with goat antirabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat antimouse antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Oxford) for 30 min at room temperature. After four washes with PBS-Tween solution, immunoreactive proteins were detected using the
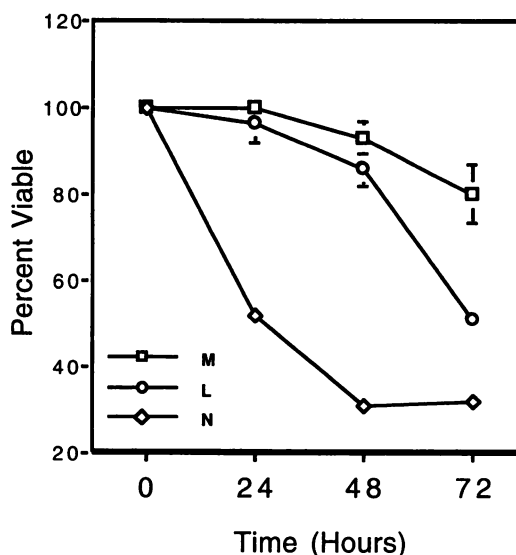

Fig. 2. Effects of TNF-α on MCF-7 cell viability. The percentage of cell death as measured by trypan blue staining at 24, 48, and 72 h of TNF-α treatment in MCF-7 cell variants M, L, and N is shown. *Error bars*, SD for three experiments. An absence of error bars represents <3% error between replicates.
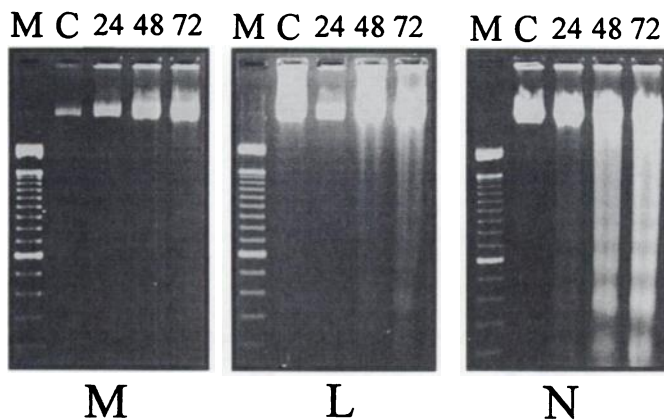
Fig. 3. TNF-α-induced DNA fragmentation of MCF-7 cells. An analysis of DNA fragmentation of MCF-7 cell variants M, L, and N by agarose gel (1.5%) electrophoresis at 24, 48, and 72 h after treatment with TNF-α (10 ng/ml) is shown.

enhanced chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad).

Flow Cytometry. Flow cytometric analysis of TNFR1 and TNFR2 was performed as described by Cai *et al.* (17). Briefly, 1×10^6 cells were harvested in PBS-EDTA and washed in 50% normal goat serum at 4°C for 15 min. Cells were washed in PBS-FBS (PBS with 1% FBS added) and incubated with 10 μg/ml mouse anti-TNF-α receptor antibodies (anti-TNFRp55 htr-9 and anti-TNFRp75 utr-1; BACHEM, Inc., King of Prussia, PA) in PBS-FBS at 4°C for 60 min. After this, the cells were washed three times in PBS-FBS and incubated with PE-conjugated goat antimouse IgG (1:40 dilution) in PBS-FBS at 4°C for 2 h. Cells were washed three times in PBS-FBS and analyzed using a Becton Dickinson FACStar flow cytometer. Excitation was at 488 nm (100 mW) using a coherent 6W argon-ion laser. For each cell, emission was measured using a photomultiplier with a 585 ± 42-nm band pass filter for phycoerythrin. Data were collected as 2,000 event list mode files and analyzed using LYSIS II (Becton Dickinson) software. Data represent duplicate counts of 2×10^3 cells, and statistical comparisons were made by Kolmogorov-Smirnov summation curves (39). Background fluorescence was determined using either unstained cells or cells stained using nonspecific mouse anti-α p65 monoclonal antibody (a kind gift of Kathleen Buckley, Department of Neuroscience, Harvard Medical School, Boston, MA).

Fluorescence Microscopy. For fluorescence microscopy, MCF-7 cells were seeded at 1×10^5 cells/ml in 10-cm² wells and treated with TNF-α (10 ng/ml) for 48 h. Samples were harvested, pelleted, and fixed in a solution of 10% formalin for 10 min and then washed with PBS and resuspended in a solution of propidium iodide in PBS (50 μg/ml). Cells were transferred to slides and visualized using a Zeiss Axioscope fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with appropriate filters.

Analysis of Ceramide. Ceramide was quantified by the DAG kinase assay as ^{32}P incorporated on the phosphorylation of ceramide to ceramide-1-phosphate by DAG kinase as described previously (40). Briefly, MCF-7 cells were treated with or without TNF-α (10 ng/ml) for the times indicated, washed in PBS, and fixed in ice-cold methanol. After extraction of the lipid, ceramide contained within the organic phase extract was resuspended in 20 μl of 7.5% α-octyl-β-glucopyranoside, 5 mM cardiolipin, and 1 mM diethylenetriamine pentaacetic acid (Sigma Chemical Co.). Thereafter, 40 μl of purified DAG kinase in enzyme buffer [20 mM Tris-HCl, 10 mM DTT, 1.5 M NaCl, 250 mM sucrose, and 15% glycerol (pH 7.4)] were added to the organic phase extract. Ten mM [γ-^{32}P]ATP (20 μl; 1000 dpm/pmol) in a buffer was added to start the reaction. After 30 min at 22°C, the reaction was stopped by the extraction of lipids with 1 ml of chloroform:methanol:hydrochloric acid (100:100:1, v/v). Buffered saline solution [170 μl; 135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES (pH 7.2)] and 30 μl of 100 mM EDTA were added. The lower organic phase was dried under N₂. Ceramide-1-phosphate was resolved by TLC using CHCl₃:CH₃OH:acetic acid (65:15:5, v/v) as a solvent and detected by autoradiography, and the incorporated ^{32}P

was quantified by a phosphorimager (Fugi BAS1000; Fugi Medical Systems). The level of ceramide was determined by comparison to a concomitantly run standard curve composed of known amounts of ceramide.

RESULTS

Using three MCF-7 cell variants (M, L, and N) from established laboratories, we compared the effect of TNF-α on proliferation and viability. Under control conditions, different basal proliferation rates were observed among cell variants with doubling times of 30.8, 45, and 28.6 h for the M, L, and N cells, respectively (Fig. 1). The addition of TNF-α (10 ng/ml) to the medium inhibited basal proliferation in all three variants in a time-dependent manner. The most striking effect was observed in MCF-7 N cells, in which the number of viable cells per milliliter decreased by 50% from that of the control by 24 h. In the TNF-α-treated samples, trypan blue staining indicated that MCF-7 M cells retained >90% viability compared to that of the control on days 1 and 2 and 80% viability on day 3, whereas the viability of the TNF-α-treated MCF-7 N cells was 52, 31, and 32% of the control on days 1, 2, and 3. MCF-7 L cells treated with TNF-α were 97, 86, and 51% viable on days 1, 2, and 3 (Fig. 2). Additionally, the MTS viability assay revealed a dose-dependent effect of TNF-α (0.1–10 ng/ml) on MCF-7 cell variant viability and proliferation (data not shown). Consistent with the literature, 10 ng/ml was the optimal dose for both the induction of cell death (MCF-7 N cells) and the inhibition of proliferation (MCF-7 M and L cells; Ref. 17). These results suggest that MCF-7 N cells are highly sensitive to TNF-α-induced cytotoxic effects. Whereas the MCF-7 M cells were resistant to the cytotoxic effect of TNF-α, they still retained their sensitivity to the antiproliferative effect of TNF-α. TNF-α treatment of MCF-7 L cells resulted in an inhibition of proliferation and a delayed cytotoxic effect.

To determine whether the rapid loss of viability in the MCF-7 N variant on TNF-α treatment was due to apoptosis, DNA fragmentation analysis was performed. As expected from their retention of viability, the MCF-7 M variant did not undergo apoptosis in response to TNF-α

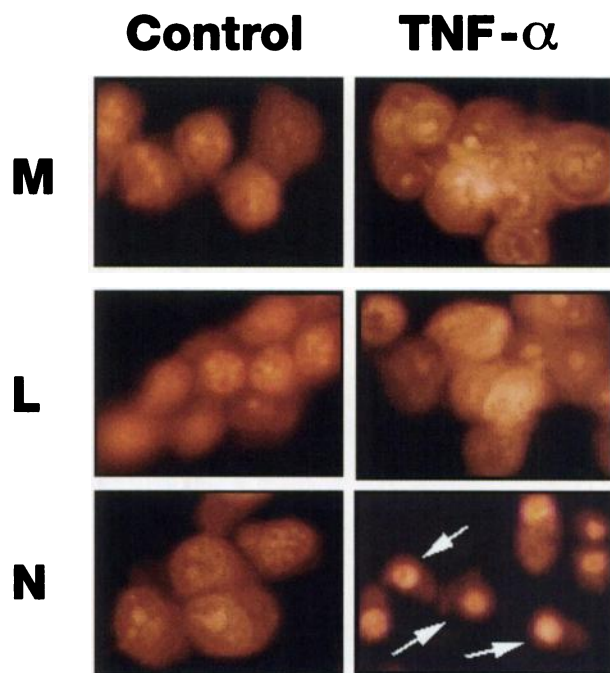Fig. 4. TNF-α induced apoptosis of MCF-7 cells. Nuclear staining with 50 μg/ml propidium iodide revealed distinct nuclear condensation of the MCF-7 N variant (*arrows*) but not the M and L variants after 48 h of treatment with 10 ng/ml TNF-α.
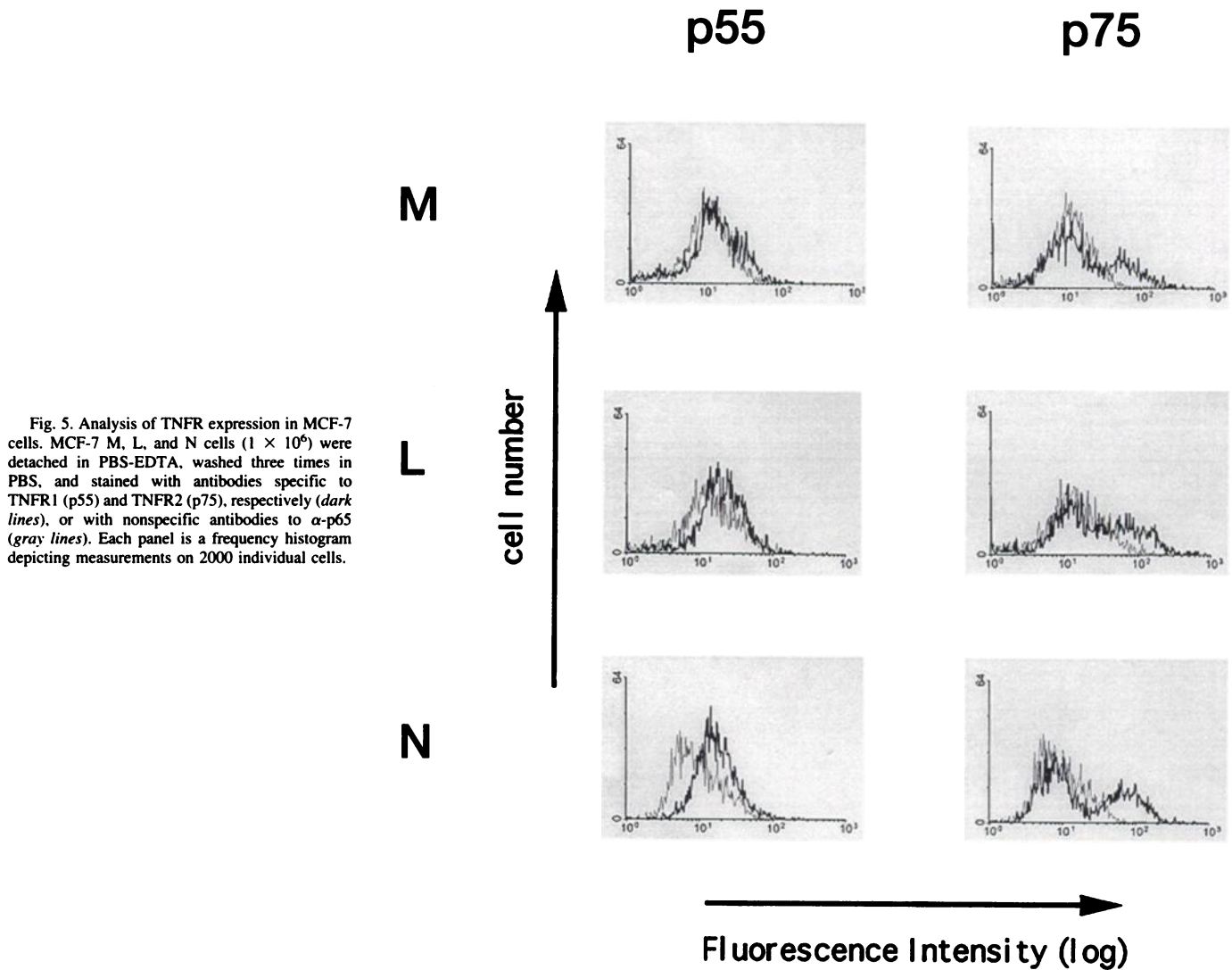
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p55   p75

Fig. 5. Analysis of TNFR expression in MCF-7 cells. MCF-7 M, L, and N cells (1×10^6) were detached in PBS-EDTA, washed three times in PBS, and stained with antibodies specific to TNFR1 (p55) and TNFR2 (p75), respectively (*dark lines*), or with nonspecific antibodies to α-p65 (*gray lines*). Each panel is a frequency histogram depicting measurements on 2000 individual cells.

treatment (Fig. 3). However, TNF-α treatment resulted in DNA fragmentation as early as 24 h in the MCF-7 N variant and in moderate DNA fragmentation in the MCF-7 L stock by 72 h. These differences were confirmed by fluorescence microscopy (Fig. 4). Condensed nuclei were observed in TNF-α-treated MCF-7 N cells at 48 h and, as expected, were absent in the MCF-7 M and L variants.

TNFR expression was analyzed by flow cytometry with antibodies specific for TNFR1 (p55) or TNFR2 (p75). In Fig. 5, each curve is a frequency histogram of measurements on 2000 individual cells, with the number of cells in each of 1024 fluorescence channels displayed on a log scale. In each panel, the histogram obtained with the specific p55 or p75 antibody (*dark lines*) is overlaid with the curve derived from a nonspecific antibody to an irrelevant protein (*gray lines*). TNFR1 expression was determined to be 89 and 67% lower in the MCF-7 L and M cells as compared with that in the N cells. This finding suggests that the resistance of MCF-7 M and L cells may be due to their decreased expression of TNFR1. All three cell variants expressed similar levels of TNFR2 (*p75*).

Ceramide generation represents an early downstream event of TNF-α-induced signaling in numerous cell lines including MCF-7 cells (17, 29, 30). Ceramide also represents a key intermediate in the transduction of apoptotic signals from TNF-α as well as Fas, chemotherapeutic drugs, and γ-radiation (29, 30). The ability of water-soluble analogues of ceramide to induce apoptosis in MCF-7 cells further implicates

ceramide as an important component in apoptotic signaling. TNF-α-induced ceramide generation was analyzed in the three MCF-7 cell variants to determine whether differences in SMase activity can account for the differential sensitivity to TNF-α-induced apoptosis. A rapid and transient increase in ceramide production was observed in the MCF-7 N variant, reaching a maximal level of 5.5 $\pm$ 0.56-fold over that of the control at 15 min with TNF-α (Fig. 6), whereas a 1.73 $\pm$ 0.37- and 1.42 $\pm$ 0.22-fold maximal increase in ceramide levels was observed in the M and L variants, respectively, at 15 min. All three cell variants possessed similar basal amounts of ceramide. Despite minimal expression of TNFR1, the MCF-7 M and L cells still responded, albeit weakly, to the ability of TNF-α to generate ceramide. Although substantial differences in TNFR1 expression exist between the MCF-7 M, L, and N cells, all cell variants express some degree of TNFR1, which suggests that other downstream events may also account for altered sensitivity to apoptosis.

The caspase family of proteases represents critical signaling intermediates and effectors of the apoptotic program (23, 32, 33). PARP is a proteolytic substrate for Asp-Glu-Val-Asp (DEVD)-specific caspases including CPP32/caspase 3 and caspase 7. Cleavage of PARP from its M_r 116,000 precursor to its M_r 29,000 and M_r 85,000 subunits is indicative of apoptosis and is a useful tool for the measurement of the time course of caspase activity (32, 33). Western blot analysis revealed caspase activity as early as 3 h in the TNF-α-
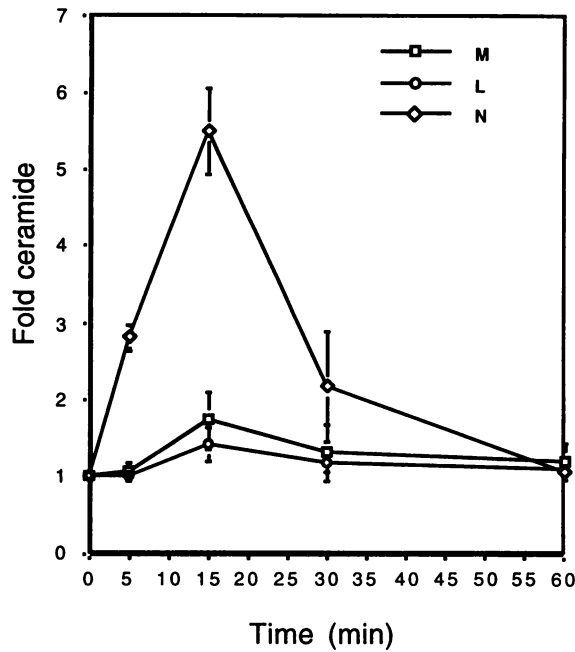
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Fig. 6. Ceramide generation in MCF-7 cell variants with TNF-α treatment. MCF-7 M, L, and N cells (4 × 10⁶) were treated with TNF-α (10 ng/ml) for the times shown above. Cells were harvested in ice-cold methanol; the lipid extraction and ceramide assay were performed as described in "Materials and Methods." The ceramide generated represents the fold change over control in nanograms of ceramide/milligrams of protein; error bars, SD of three independent experiments performed in duplicate.

sensitive N cells (Fig. 7). PARP cleavage in the moderately TNF-α-sensitive MCF-7 L cells was observed only at 6 h and was not observed in MCF-7 M cells.

bcl-2 proto-oncogene expression imparts considerable resistance to apoptosis induced by a variety of stimuli (34, 35). The relative expression of various members of the Bcl-2 family of proteins was analyzed in the three MCF-7 stocks by Western blot analysis (Fig. 8). Bcl-X, Mcl-1, and Bak protein expression was not appreciably different in the three stocks. However, striking differences were observed in the expression of Bcl-2 and Bax. Bcl-2 expression was 3.8 and 3.5 times higher in the apoptosis-resistant cell variants MCF-7 M and L, respectively, as compared to that in MCF-7 N. Bax expression was found to be 1.7- and 1.5-fold higher in the apoptosis-sensitive MCF-7 N variant as compared to that in the MCF-7 M and L variants.

DISCUSSION

Reported discrepancies exist concerning the apoptotic responses of MCF-7 cells to TNF-α and anti-Fas antibody treatment. Several studies have indicated that MCF-7 cells readily undergo apoptosis in response to TNF-α and anti-Fas (7, 8, 17). However, some reports have indicated that TNF-α and Fas only weakly induce apoptosis in MCF-7 cells (18–21). Others have shown that the cytotoxic versus the cytostatic effects of TNF-α depend on the media and serum conditions used to culture the MCF-7 cells (41). We report that under identical culture conditions, variations in apoptotic responses exist among three different MCF-7 cell strains obtained from established laboratories (M, L, and N). It was determined that proliferation of all three variants was inhibited by TNF-α, with the cell number of the MCF-7 N variant decreasing below control in parallel with a decrease in viability. The loss of viability in TNF-α-treated MCF-7 N cells was due to an induction of apoptosis observed as early as 24 h, whereas the MCF-7 L cells seemed moderately sensitive to the apoptotic effects of TNF-α only at 72 h. MCF-7 M cells were sensitive to the antiproliferative

effect of TNF-α but resistant to the cytotoxic effects of TNF-α. Examination of the TNFR expression revealed a similar expression of p75 TNFR2 among all three cell variants. p55 TNFR1 was expressed at the highest levels in MCF-7 N cells and MCF-7 L cells, whereas MCF-7 M cells expressed the lowest levels of TNFR1. The decreased expression of TNFR1 in MCF-7 M cells may account in part for their lowered sensitivity to TNF-α apoptosis as well as a lowered genera-
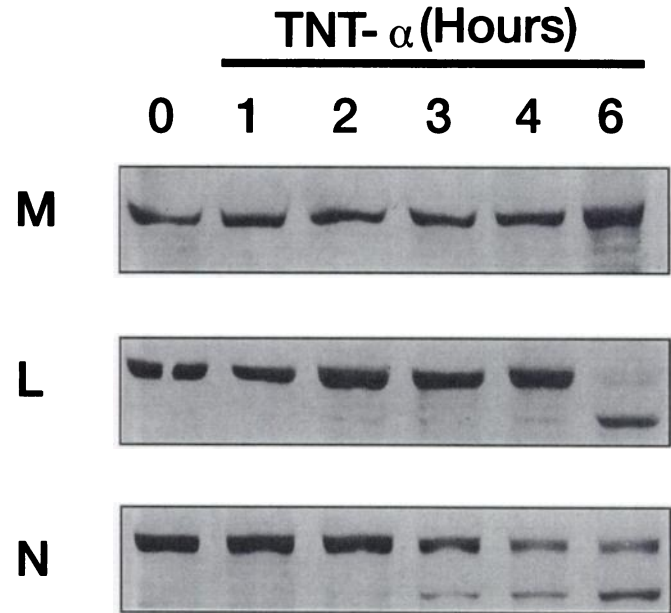

Fig. 7. Western blot analysis of PARP cleavage in MCF-7 cell variants. MCF-7 M, L, and N cells (2 × 10⁶) were treated with TNF-α (10 ng/ml) for 1, 2, 3, 4, and 6 h. Cells were then harvested in PBS-EDTA and assayed for PARP cleavage as described in "Materials and Methods."
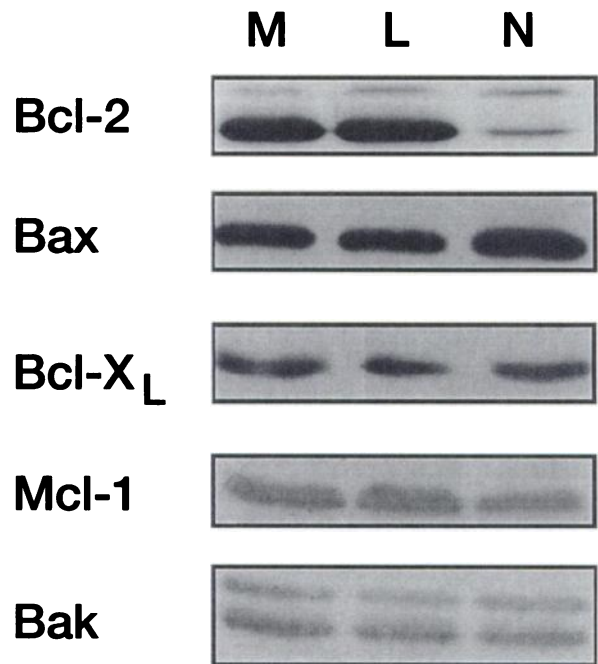

Fig. 8. Expression of Bcl-2 family proteins in MCF-7 cell variants. MCF-7 cell variants M, L, and N were grown for 2 days in normal media and harvested for Western blot analysis as described in "Materials and Methods" for the expression of Bcl-2, Bax, Bak, Bcl-X$_L$, and Mcl-1.

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tion of ceramide. It is possible that despite lowered expression, TNFR1 or even TNFR2 may provide the antiproliferative signal in these cells. Given the role of ceramide in the inhibition of proliferation, the 1.7-fold increase in ceramide in the M cells may be sufficient for the suppression of cell proliferation but insufficient to induce apoptosis. We cannot rule out the possibility that altered expression or activation of TRADD, FADD, FLICE, or other proteins in the TNF signaling cascade may account for the inability to activate apoptosis in the M cells.

Examination of several members of the Bcl-2 family of apoptosis-regulating proteins suggests that the intrinsic resistance of the M cells and the delayed apoptotic DNA laddering and protease activation in the MCF-7 L cells as compared to the N variant were correlated with a higher expression of Bcl-2 and a lower expression of Bax. Many studies confirm that an increase in the expression of Bcl-2 correlates with resistance to apoptosis induced by a number of agents (34, 35). However, contradictory reports exist as to the ability of Bcl-2 or Bcl-X$_L$ expression to inhibit TNF-α-induced apoptosis in MCF-7 cells. Vanhaesebroeck et al. (42) showed that overexpression of Bcl-2 in MCF-7 cells failed to offer a survival advantage to treatment with TNF-α. Conversely, Jaattela et al. (43) showed that overexpression of Bcl-2 and Bcl-X$_L$ was correlated with an increased resistance to TNF-α-induced apoptosis. Again, these reported differences may be due to the individual MCF-7 cell variants used by each laboratory and may potentially be due to the variations in constitutive expression of other members of the Bcl-2 family, such as Bax. Overexpression of Bax or Bcl-X$_S$ in MCF-7 cells resistant to chemotherapeutic drug treatment, serum starvation, and Fas-induced apoptosis has been shown to sensitize these cells to the induction of apoptosis (44–46). Thus, cells expressing high levels of Bax may not be as resistant to apoptosis, even when overexpressing Bcl-2. However, the Bcl-2 family of proteins may not account for all of the differences in apoptotic sensitivity reported here. Both the M and L stocks express similar levels of Bcl-2, Bax, Bak, Bcl-X, and Mcl-1; however, the L cells undergo apoptosis in response to TNF-α, whereas the M cells are resistant, suggesting that other differences within variants of MCF-7 cells will affect the antiapoptotic role of Bcl-2. Reports have indicated that Bcl-2 does not block ceramide generation but does inhibit ceramide analogue-induced apoptosis (47). Given the ability of Bcl-2 to block protease activation through the inhibition of cytochrome c release from the mitochondria (36, 37), the increased Bcl-2 expression and decreased Bax expression in MCF-7 L cells account for the delayed activation of PARP-specific caspases but not for the suppressed generation of ceramide.

Our results suggest a potential molecular basis for the differences in susceptibility to apoptosis among MCF-7 breast cancer cell variants. The increased generation of ceramide in the most apoptosis-sensitive variant cells (MCF-7 N) may account for their response to TNF-α as compared to the antiproliferative action of TNF-α in the less apoptosis-sensitive variants (MCF-7 M and L). This decreased ceramide generation may be due in part to decreased expression of TNFR1, as in the MCF-7 M cells, or possibly to an alteration in the ability of TNF-α to activate SMases, which may be the case in MCF-7 L cells. In MCF-7 cells, ceramide generation is early and transient, suggesting that its SMase activation is not a result of the apoptotic process but an early signaling intermediate. Gamen et al. (48) implicated CPP32/caspase 3 in the regulation of Fas-induced ceramide generation but not TNF-α-induced ceramide generation. Additionally, it was shown that REAPER-induced ceramide generation occurring at 1 h or later is blocked by an ICE-like protease inhibitor (49). We cannot rule out the possibility that ceramide generation may be mediated by events subsequent to FLICE/MACH1/caspase 8 or early caspase-dependent activation. In summary, our data indicate that the sensitivity of MCF-7

cells to apoptosis induced by TNF-α and other agents differs depending on the origin of the cells. Given the extensive use of MCF-7 cells as an ER-positive breast cancer model and a system for studying apoptotic signaling, the constitutive expression and regulation of apoptotic signaling molecules are therefore an important consideration.

ACKNOWLEDGMENTS

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