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13. ABSTRACT *(Maximum 200 words)*

To investigate the role of environmental estrogens in breast cancer cell apotosis we began by utilizing the estrogen responsive MCF-7 breast cancer cell line. Widespread use of MCF-7 human breast carcinoma cells as a model system for breast cancer has lead 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. We observed that TNF inhibited proliferation in MCF-7 cell variants from three different laboratories (designated M, L and N). MCF-7 M cells were resistant to TNF-induced apoptosis while MCF-7 L cells were moderately resistant to TNF's effect. A third variant, MCF-7 N, underwent apoptosis when exposed to TNF. Our results also suggest that differences in susceptibility to TNF-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. The most sensitive MCF-7 cell variant was used to examine the effects of estrogens and environmental estrogens on TNF-induced apoptosis. 17-β-estradiol, o,p'DDT and alachlor were shown to suppress apoptosis in MCF-7N cells and increase expression of Bcl-2.

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INTRODUCTION

The MCF-7 cell line was established in 1973 from a pleural effusion of a patient with metastatic breast carcinoma previously treated with radiation and hormonal therapy (1). Since that time this cell line has become a model system of estrogen receptor-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. reported that MCF-7 cells obtained from different laboratories varied in proliferation rates, estrogen receptor (ER) and progesterone receptor (PgR) levels, estrogen and antiestrogen responses, and tumorigenicity (3). Klotz et al. showed that different stocks of MCF-7 cells displayed different levels of variant ER mRNAs, which correlated with their differential response to estrogen stimulation (4). Different MCF-7 variants tested by Villalobos et al. exhibited different responses to 17β-estradiol-induced proliferation and expression of the estrogen responsive genes pS2 and cathepsin-D (5). 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 sphingomyelinase (SMase) or phospholipase A_2 (PLA_2) with TNF-α treatment (17). The reported differences in 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 NF-κB, via recruitment of specific signaling intermediates to the cytoplasmic domains (22,23). In the case of TNFR1, TNF-α ligation results in association of TNFR1-associated death domain protein (TRADD) (24) which then recruits TNFR-associated protein 2 (TRAF2), Receptor interacting protein (RIP)(25) and Fas-associated death domain (FADD/MORT1) (26). FADD/MORT1 association with the receptor is followed by association of FLICE/MACH1 with the TNFR1 complex (27,28). Subsequent to formation of this protein complex, activation of several signaling cascades including PLA2, SMase, NF-κB, stress activated protein kinases and apoptotic proteases occurs (22,23). Activation of SMase resulting in ceramide formation event in the apoptotic signaling cascade (29,30). MCF-7 cells have been shown to activate sphingomyelinase in response to TNF-α and undergo apoptosis when exposed to water soluble ceramide analogues (17). Additionally, studies of acidic SMase knock-out (KO) mice have shown that ceramide generation may be required for apoptosis by TNF-α and other inducers in some cell types (31). 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. Activation of FLICE is thought to then result in 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 anti-apoptotic members (Bcl-2, Mcl-1, Bcl-X_L) and pro-apoptotic members (Bax, Bcl-X_s, 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 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 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 TNF-α's signal transduction cascade including TNFR expression, sphingomyelinase and caspase activation as well as expression of specific members of the Bcl-2 family of proteins.

Evidence is accumulating that steroid hormones regulate apoptosis in hormone-responsive tissues. Both prostate and breast epithelial cells undergo apoptosis upon removal of testosterone and estrogen, respectively (50-52). This dependence upon hormone for survival and proliferation extends to neoplasms arising from these tissues. The MCF-7 breast cancer cell line has been shown to form tumors in nude ovariectomized mice only in the presence of estrogen. Upon removal of estrogen the tumor cells begin to undergo apoptosis leading to tumor regression (53). Additionally, two recent studies have shown that pretreatment of MCF-7 cells grown *in vitro* with estrogen reduces the induction of apoptosis by cytotoxic drug treatment as well as tamoxifen (54,55). These studies provide evidence that estrogens play a role in both tumorigenesis, as well as drug resistance, through reduction of apoptosis.

In addition to estrogen, a class of compounds referred to as environmental estrogens has recently been suggested to play a role in breast cancer (56,57,58). These compounds represent natural or synthetic chemicals that can mimic the activity or effects of endogenous estrogens. The potential exists that these compounds, acting through the estrogen receptor, can affect the apoptotic pathways of estrogen responsive cells. Certain organochlorine compounds have been shown to act as environmental estrogens by causing developmental defects in different animals (59). The heavy use of these compounds as herbicides and insecticides, combined with their long half-lives, make them ubiquitous contaminants in our environment (60). Several recent papers have shown that organochlorine compounds, including DDT, endosulfan and alachlor, act like estrogen and are capable of binding to the estrogen receptor (ER), causing transcription from estrogen response elements (EREs) in DNA, and causing proliferation of MCF-7 cells *in vitro* (61,62). Although these compounds may possess only weak estrogenic activity as compared to estrogen, their ability to bioaccumulate and biomagnify may allow them to elicit significant estrogenic effects in hormone responsive tissues. With mounting evidence for the role of estrogen in apoptosis, we suggest that these environmental estrogens can act like endogenous estrogen to inhibit apoptosis.

Several recent reports now show that one mechanism by which estrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis regulating proteins. Bcl-2 was discovered in 1985 as a gene product overexpressed in non-Hodgkin's B-cell lymphomas as compared to normal B cells because of the translocation breakpoint between chromosomes 14 and 18 (63). Unlike other typical oncogenes, Bcl-2 was found to possess an unusual mechanism of action, in that it is involved in the inhibition of cell death pathways (64). Since its initial discovery, Bcl-2 has been found to be expressed at high levels in many cancer types and cancer cell lines and is associated with both tumor progression and drug resistance (65,66). Clinical studies have now linked high expression of Bcl-2 to a poor prognosis in cancer treatment (66,67). The discovery of several Bcl-2 related proteins such as Bcl-x_L, Mcl-1, and Bag-1, that are also involved in the cell death process, suggests that the regulation of apoptosis is critical to normal cell and tissue function (68-70). Another group of Bcl-2 related proteins including Bax, Bcl-x_S, Bak, and Bad were discovered and found to act as pro-apoptotic factors (71-73). Although the mechanism by which these factors regulate apoptosis is not completely understood, it is known that these factors do dimerize with one another and increased levels of the anti-apoptotic members of this family protect cells against many forms of cell death.

MATERIALS & METHODS

Cell Culture. MCF-7 cell variants M and L were a gift from Stephen M. Hill (Tulane University). The MCF-7 M cell variant (passage 180) originated from the laboratory of the late William McGuire (University of Texas). The MCF-7 L cell variant (passage 40) originated from the laboratory of Marc Lippman (Georgetown University). MCF-7 N cell variant (passage 50) is a subclone of MCF-7 cells from American Type Culture Collection (Rockville, MD) generously provided by Louise Nutter (University of Minnesota). All MCF-7 cells were routinely maintained and grown in DMEM supplemented with 10% FBS, BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (GibcoBRL, Gaitherburg MD) and porcine insulin $1x10^{-10}$ M (Sigma Chemical Co., St. Louis MO).

Compounds treated with. 17β-estradiol was purchased from Amersham Corporation (Arlington Heights, IL), *o,p'* DDT [1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] was purchsed from Sigma Chemical Co. (St. Louis, MO), Alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide] was purchased from AccuStandard (New Haven CT).

Proliferation and Viability Assay. MCF-7 cells were plated at $5.0x10^4$ cells/ml in 10 cm^2 wells. The cells were allowed to adhere for 18 hours before treatment with recombinant human TNF-α (10ng/ml) (R&D systems, Minneapolis MN). Cells were then counted at 24, 48 and 72 hours post-treatment. The results are represented as the number of viable cells/ml as measured by trypan blue exclusion. MTS viability assay (Promega) was performed according to manufacturer's protocol.

DNA Fragmentation Analysis. Following treatment, cells were harvested for DNA as described previously (38). Briefly, 1-2 $x10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS(w/v) pH 7.4] to which RNAse A (100 µg/ml) was added. After incubation for two hours at 37°C, proteinase K (0.5 mg/ml) was added and the lysates were heated to 56°C for 1 hour. NaCl was then added (final concentration, 1M) and lysates were incubated overnight at 4 °C. Lysates were centrifuged at 15,000 X g for 30 minutes, and nucleic acids in the supernatant were precipitated in two volumes of ethanol with 50 mM Na acetate. Isolated DNA was then separated by electrophoresis on 1.5 % agarose gels for two hours and visualized by ethidium bromide staining.

Western Blot Analysis. MCF-7 cells were grown for two days as described above and then 5 $x10^6$ cells were harvested in sonicating buffer (62.5 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol. 1 mM phenylmethylsulfonyl fluoride (PMSF), (25 mg/ml leupeptin, 25 mg/ml aprotinin) and sonicated for 30 seconds. Following centrifugation at 1,000 x g for 20 minutes, 50 ug of protein was resuspended in sample loading buffer (62.5 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 minutes and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with PBS-Tween (0.05%) - 5% lowfat dry milk solution at 4°C overnight. The membrane was subsequently incubated with rabbit antisera (anti-Bcl-2 1:4000 dilution, anti-Bax 1:4000, anti-Bcl-X 1:1500, anti-Mcl-1 1:2000 and anti-Bak 1:1000) or with mouse anti-PARP specific monoclonal antibody 1:5000 (Pharmingen, San Diego, CA) and incubated for 2 hours at room temperature. Blots were washed in PBS-Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat anti-mouse antibodies conjugated to horseradish peroxidase (1:5000 dilution; Oxford, Oxford, MI) for 30 minutes at room temperature. Following four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL 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 (BioRad).

Flow Cytometry. Flow cytometric analysis of TNFR1 and TNFR2 was performed as described by Cai et al. (17). Briefly $1x10^6$ cells were harvested in PBS-EDTA and washed in 50% normal goat serum @ 4^0C for 15 min. Cells were washed in PBS-FBS (PBS with 1% FBS added) and incubated with mouse anti-TNF-α receptor antibodies (anti-TNFRp55 htr-9, anti-TNFRp75 utr-1: BACHEM Inc. (King of Prussia, PA)) 10 μg/ml in PBS-FBS @ 4^0C for 60 min. Following this cells were washed 3X in PBS-FCS and incubated with PE-conjugated goat anti-mouse IgG (1:40 dilution) in PBS-FBS @ 4^0C for 2 hours. Cells were washed 3X in PBS-FBS and analyzed using a Becton Dickenson FACStar flow cytometer. Excitation was at 488nm (100mW) 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 events list mode files and analyzed using LYSIS II [Becton-Dikenson, Mountain View, CA] software. Data represent duplicate counts of 2×10^3 cells and statistical comparisons were made by Kolgomorov-Smirnoff summation curves (39). Background fluorescence was determined using cells either unstained or stained using non-specific mouse anti-α p65 mAb (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 at 1×10^5 cells/ml in 10 cm^2 wells and treated with TNF-α (10 ng/ml) for 48 hours. Samples were harvested, pelleted and fixed in a solution of 10% formalin for 10 min, 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 diacylgycerol (DAG) kinase assay as ^{32}P incorporated upon 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/ 5mM cardiolipin/ 1mM diethylenetriamine pentaacetic acid (Sigma Chemical Co.). Thereafter, 40 μl of purified DAG kinase in enzyme buffer (20 mM Tris-HCL, 10 mM dithiothreitol, 1.5 M NaCl, 250 mM sucrose and 15% glycerol, pH 7.4) was added to the organic phase extract. [γ-^{32}P]ATP (20 ul 10 mM; 1000 dpm/pmol), in buffer, was added to start the reaction. After 30 min at 22 ^{0}C, the reaction was stopped by 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 $CaCl_2$, 0.5 mM $MgCl_2$, 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 N_2. Ceramide-1-phosphate was resolved by TLC using $CHCl_3$:CH_3OH:acetic acid (65:15:5, v/v) as solvent, detected by autoradiography, and the incorporated ^{32}P was quantified by phosphoimaging (Fugi BAS1000, Fugi Medical Systems, USA). 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 hours 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/ml decreased by 50% from control by 24 hours. In the TNF-α treated samples trypan blue staining indicated that MCF-7 M cells retained greater than 90 % of viability compared to control on days 1 and 2, and 80% on day 3, whereas the viability of the TNF-α treated MCF-7 N cells was 52, 31 and 32 % of 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, 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 induction of cell death (MCF-7 N) and inhibition of proliferation (MCF-7 M,L) (17). These results suggest that MCF-7 N cells are highly sensitive to TNF-α-induced cytotoxic effects. While the MCF-7 M cells were resistant to the cytotoxic effect of TNF-α they still retained their sensitivity to TNF-α's anti-proliferative effect. TNF-α treatment of MCF-7 L cells resulted in inhibition of proliferation and a delayed cytotoxic effect.

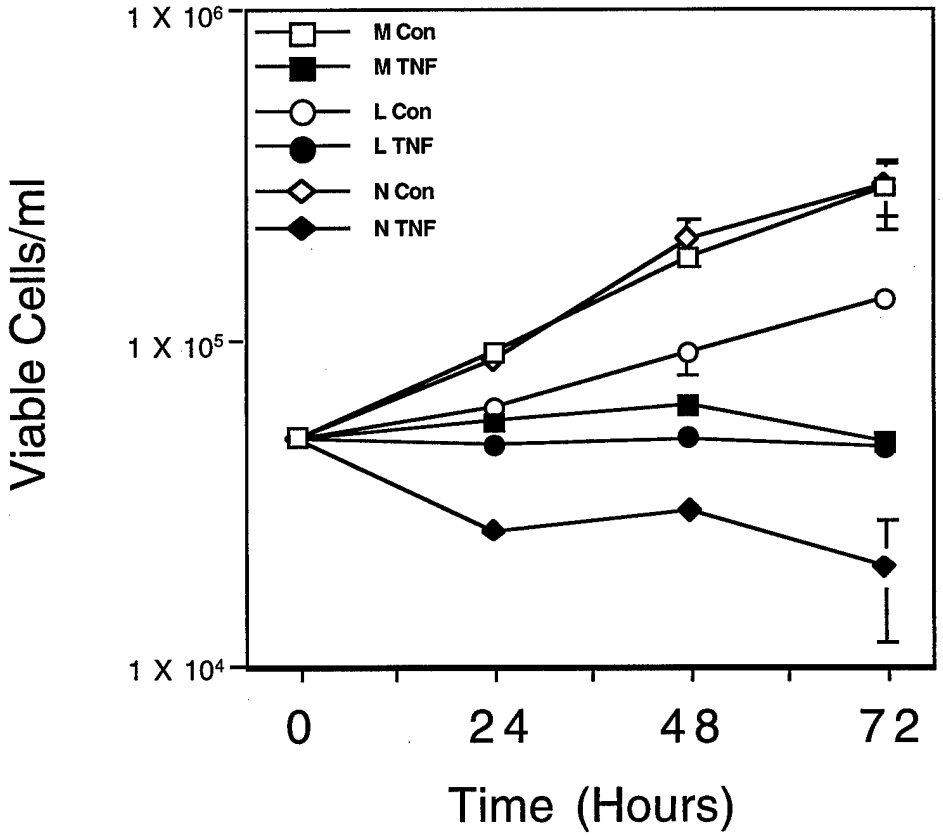

Fig 1

Effects of TNF-α on 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 hours to determine number of viable cells/ml. Error bars represent standard deviation for duplicate experiments performed in triplicate.

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

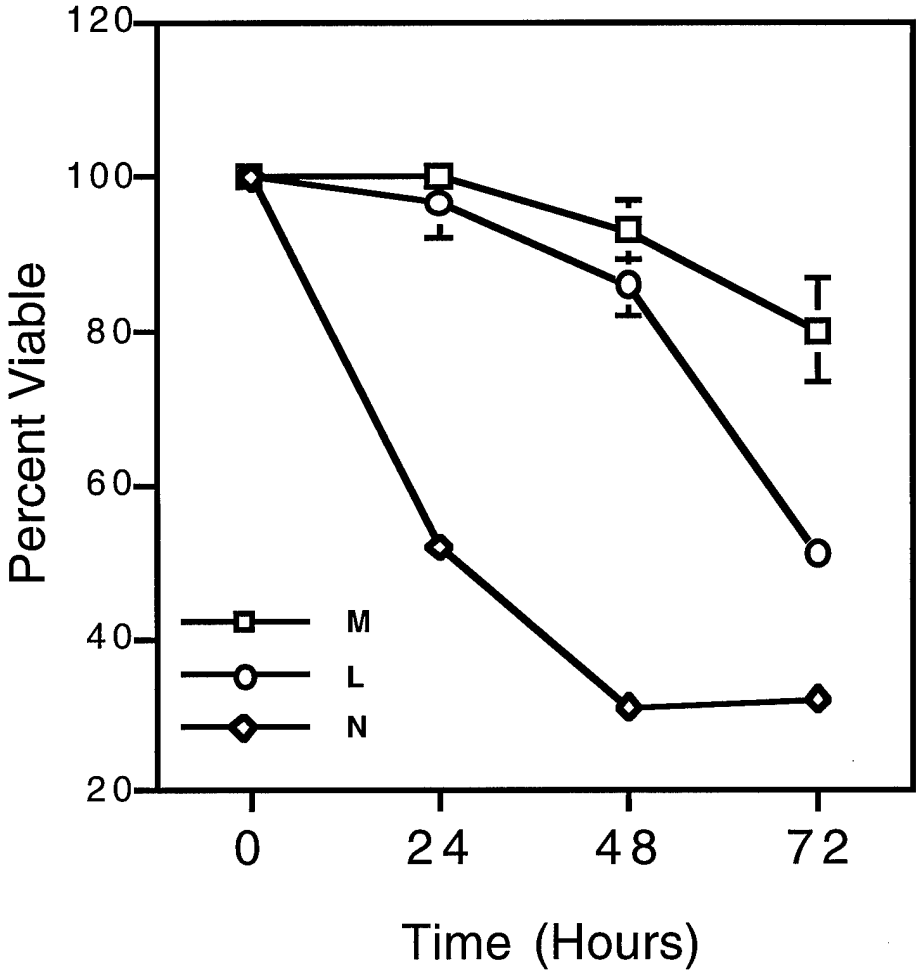

Fig 2
Effects of TNF-α on MCF-7 cell viability
Percent cell death as measured by trypan blue staining at 24, 48 and 72 hours of TNF-α treatment in MCF-7 cell variants M,L and N. Error bars represent standard deviation for three experiments. Absence of error bars represents less than 3 % error between replicates

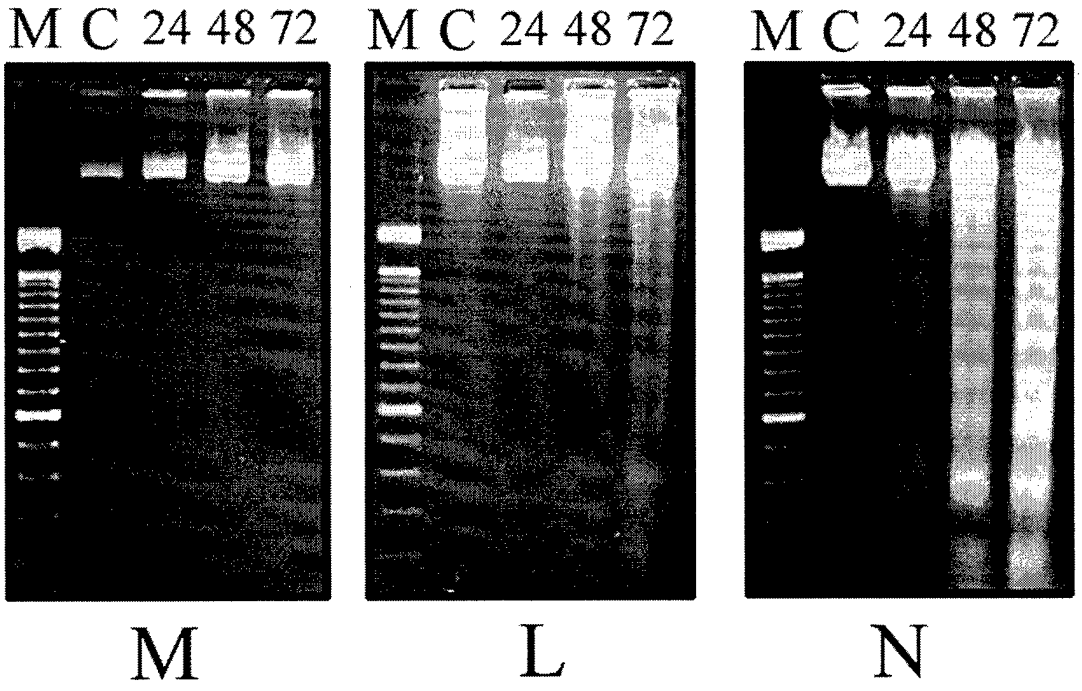

Fig 3
TNF-α induced DNA fragmentation of MCF-7 cells. Analysis of DNA fragmentation of MCF-7 cell variants M,L and N by agarose gel (1.5%) electrophoresis at 24,48, and 72 hours after treatment with TNF-α (10ng/ml).

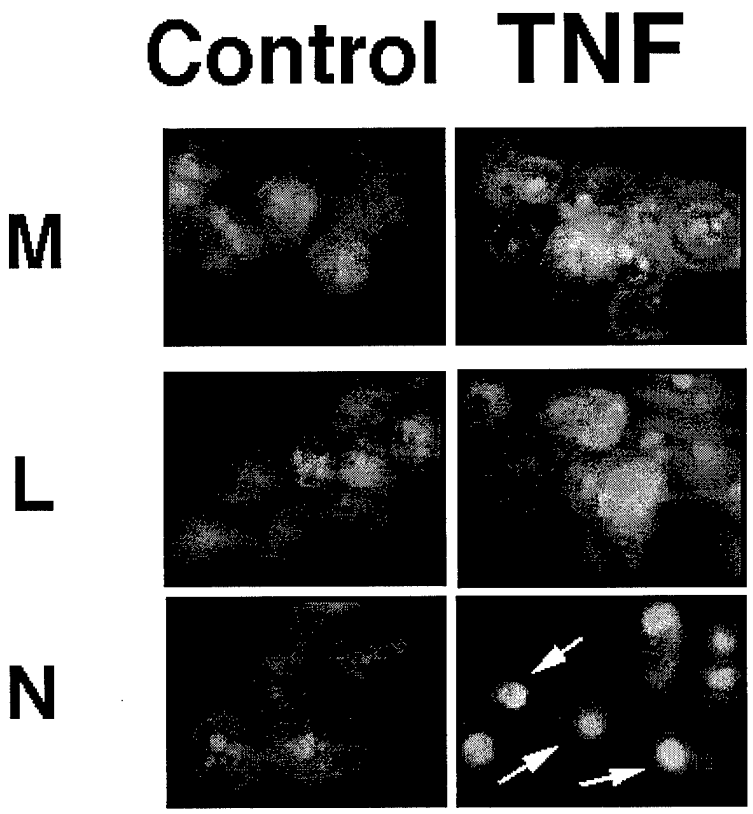

Fig 4
TNF-α induced Apoptosis of MCF-7 cells. Nuclear staining with 50 μg/ml propidium iodide revealed distinct nuclear condensation of MCF-7 N variant (arrows) but not M and L variants after 48 hours of treatment with 10ng/ml TNF-α.

TNFR expression was analysed by flow cytometry with antibodies specific for TNFR1 (p55) or TNFR2 (p75). In figure 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 non-specific 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 to the N cells. This finding suggests that the resistance of MCF-7 M cells may be due to their decreased expression of TNFR1. Similar levels of TNFR1 between the MCF-7 L and N cells suggest that other differences may account for altered sensitivity to apoptosis. All three cell variants expressed similar levels of TNFR2 (p75).

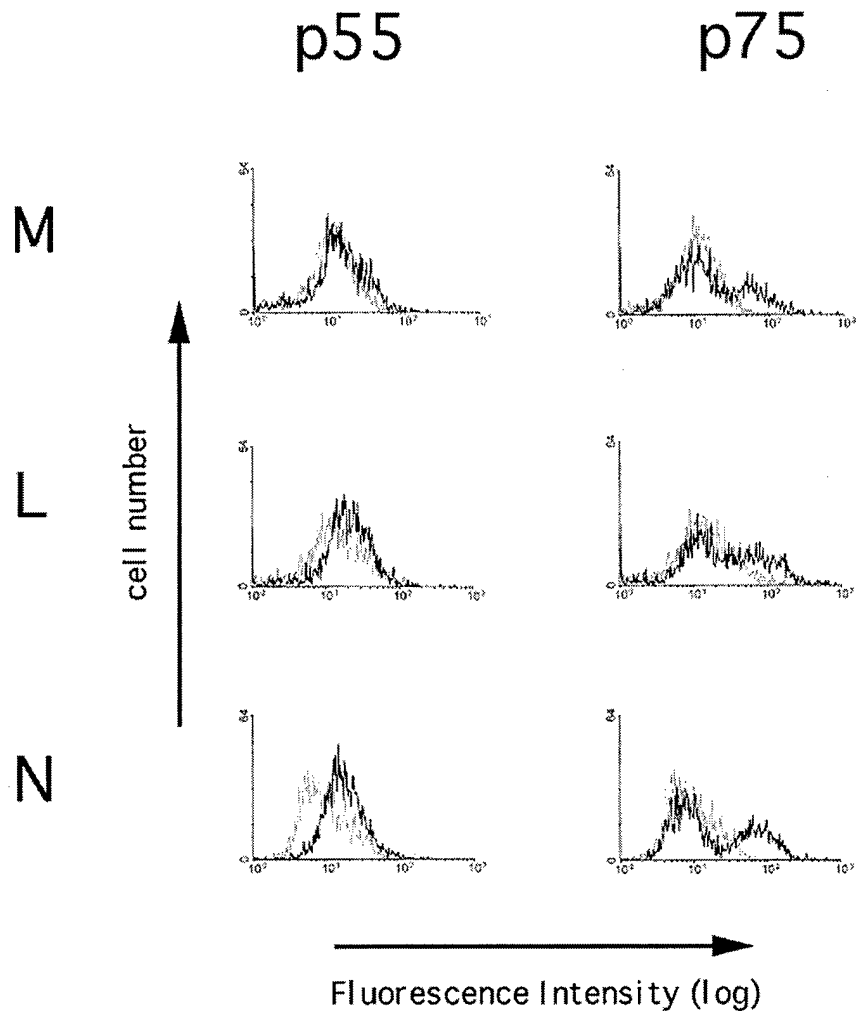

Fig 5
Analysis of TNF-α receptor expression in MCF-7 cells. MCF-7 M, L and N cells (1 x 10^6) were detached in PBS-EDTA, washed in PBS X3 and stained with antibodies specific to TNFR1 (p55) and TNFR2 (p75), respectively (dark lines) or with non-specific antibodies to α-p65 (gray lines). Each panel is a frequency histogram depicting measurements on 2,000 individual cells.

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 if differences in sphingomyelinase 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 ± 0.56 fold over control at 15 minutes with TNF-α (Fig. 6), whereas a 1.73 ± 0.37 and 1.42 ± 0.22 fold maximal increase in ceramide levels was observed in the M and L variants respectively at 15 minutes. All three cell variants possessed similar basal amounts of ceramide. Despite minimal expression of TNFR1 the MCF-7 M cells still responded, although weakly to TNF-α's ability to generate ceramide. Of interest is the finding that MCF-7 L cells which generated the lowest levels of ceramide, displayed similar levels of expression of TNFR1 as the apoptosis-sensitive MCF-7 N cells.

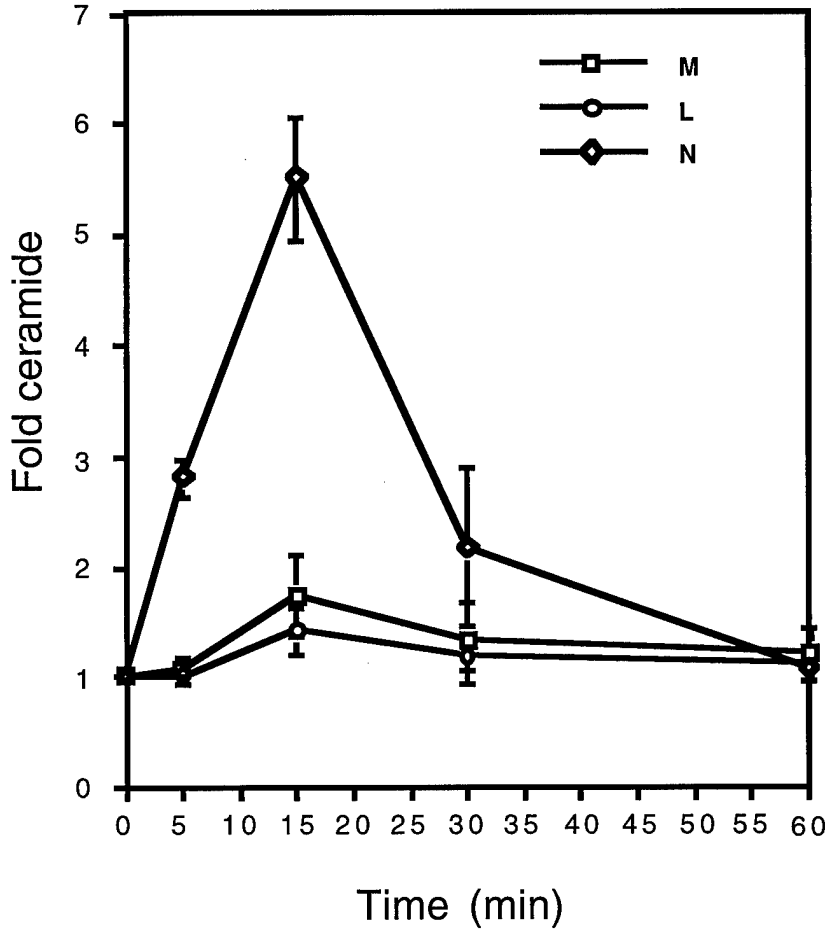

Fig 6
Ceramide generation in MCF-7 cell variants with TNF-α treatment.
4 x 10^6 MCF-7 M,L and N cells were treated with TNF-α (10 ng/ml) for time shown above. Cells were harvested in ice cold methanol; lipid extraction and ceramide assay were performed as described in "Materials and Methods". Ceramide generated represents fold change over control in ng of ceramide per mg of protein with error bars representing the standard deviation of three independent experiments performed in duplicate.

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 caspase-3/CPP32 and caspase-7/Mch3/ICE-LAP3. Cleavage of PARP from its 116 kDa precursor to its 29 and 85 kDa 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 hours in the TNF-α sensitive N cells (Fig. 7). PARP cleavage in the moderately TNF-α sensitve MCF-7 L cells was observed only at 6 hours and was not observed in MCF-7 M cells.

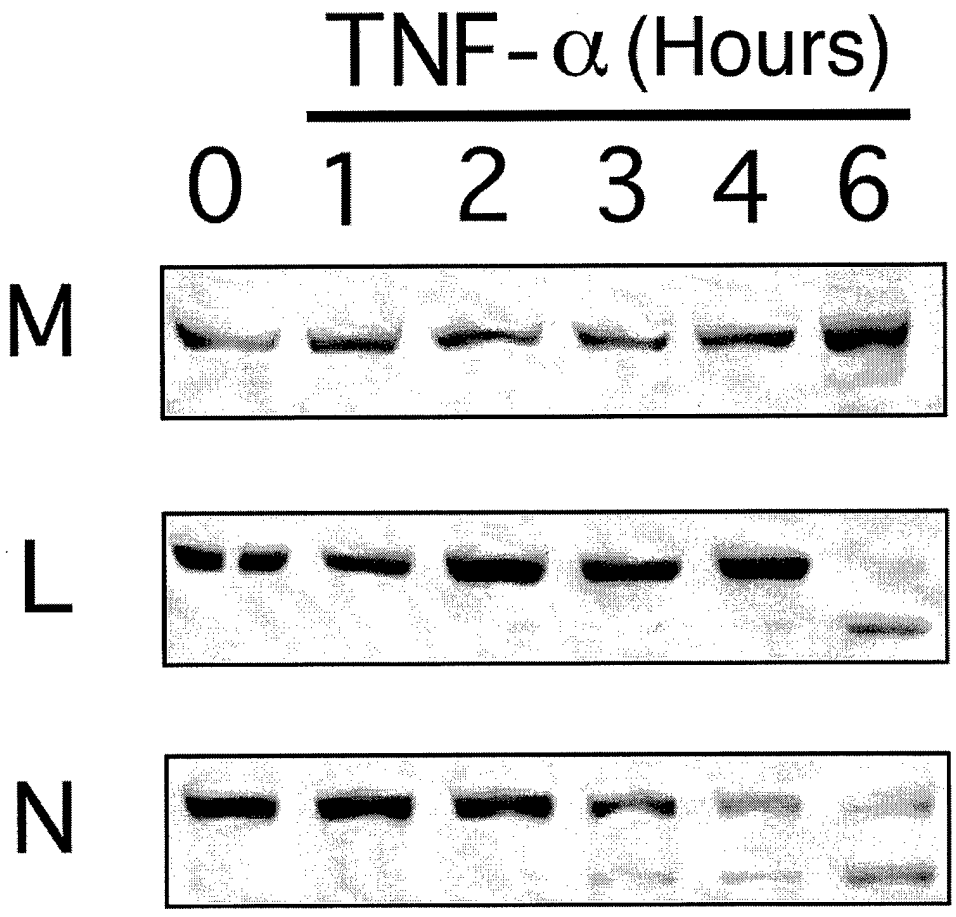

Fig 7
Western blot analysis of PARP cleavage in MCF-7 cell variants
MCF-7 M,L and N cells (2 X 10^6)were treated with TNF-α (10 ng/ml) for 1,2,3,4 and 6 hours. Cells were then harvested in PBS-EDTA and assayed for PARP cleavage as desrcibed in "Materials and Methods".

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 two apoptosis-resistant cell variants MCF-7 M and L, respectively as compared to 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 MCF-7 M and L variant.

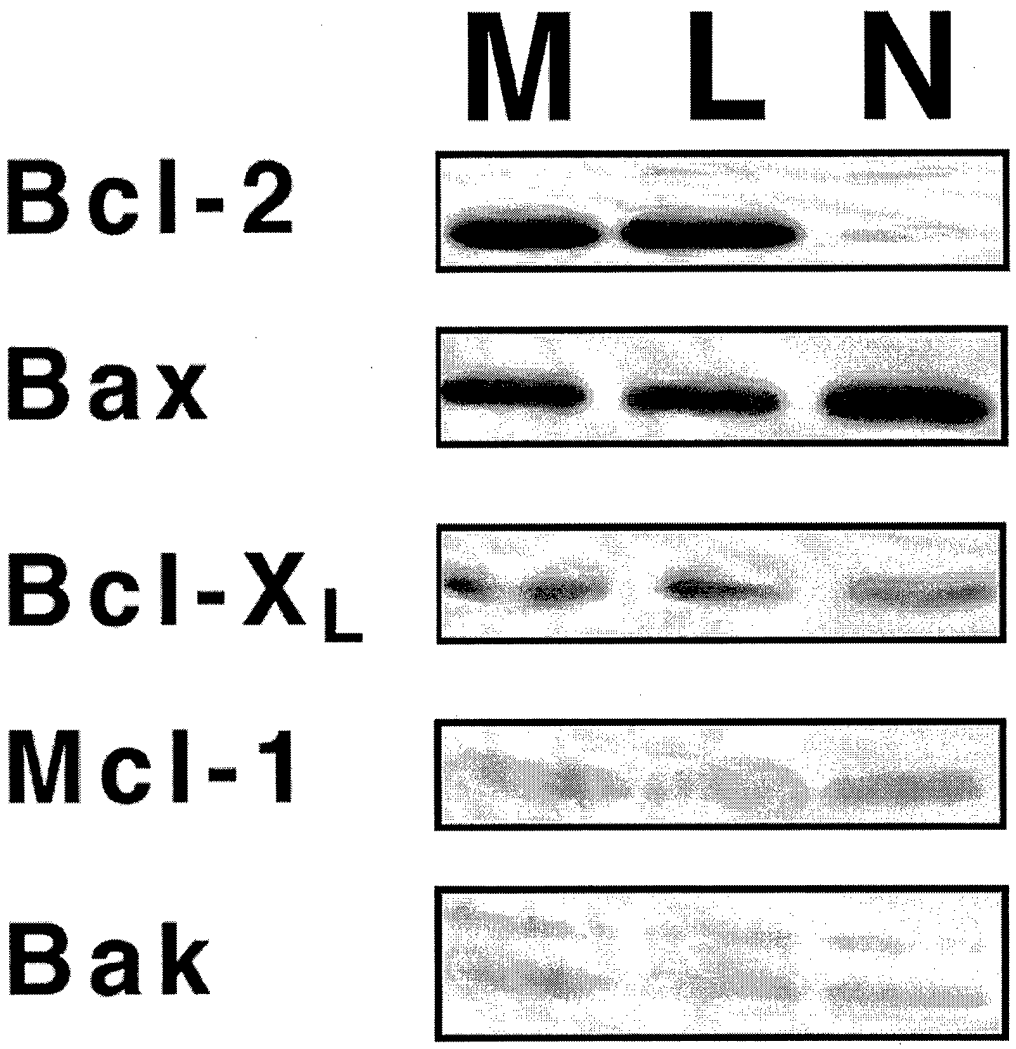

Fig 8
Expression of Bcl-2 family of proteins in MCF-7 cell variants. MCF-7 cell variants M,L and N were grown for 2 days in normal media and harvested for western blot analysis as described in "Materials and Methods" for expression of Bcl-2, Bax, Bak, Bcl-x and Mcl-1.

Inhibition of TNFα induced cell death by estrogen, *o,p'*-DDT and alachlor. Trypan blue viability assays illustrate that E_2 (1 nM), DDT (100 nM) and alachlor (1 mM) suppressed TNFα-induced apoptosis in MCF-7 cells by approximately 30%, 31%, and 23%, respectively (Fig. 9).

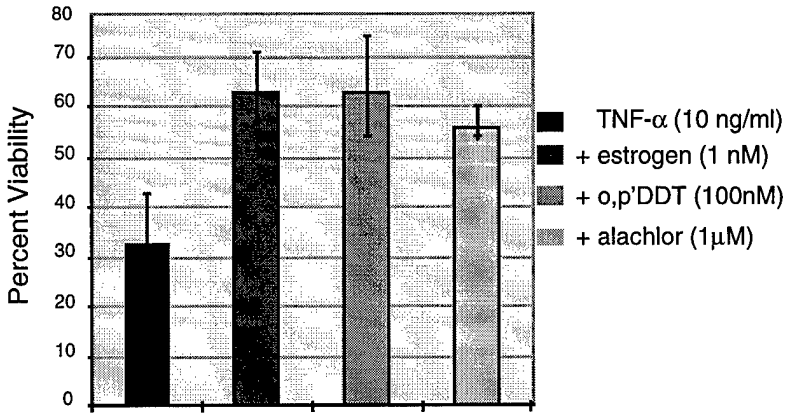

Fig. 9. MCF-7 cells previously grown for 5 days in DMEM with 5% charcoal stripped FBS, were plated out in T-25 flasks and treated with the various estrogenic compounds as shown above 24 hr prior to treatment with TNFα (10 ng/ml). Cell survival was then measured at 48 hr using trypan blue viability and represented as relative change in viability as compared to control. Each point represents the mean of 5 experiments with 500 cell counts each.

Inhibition of TNFα-induced DNA fragmentation by estrogen, *o,p'*-DDT and alachlor. As shown in Figure 6, E_2 ,DDT and alachlor were capable of decreasing TNFa-induced apoptosis as visualized by DNA ladder.

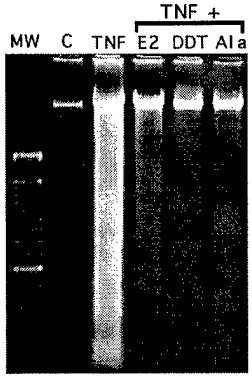

Fig. 10. Effects of environmental estrogens on TNFα-induced apoptosis. MCF-7 cells were grown in DMEM with 5% charcoal stripped FBS and treated with 1 nM β-estradiol (E_2), 100 nM *o,p'*-DDT (DDT) or 1mM alachlor (Ala) for 24 hr prior to induction of apoptosis with TNFa. Following treatment, cells were harvested for DNA isolation at 48 hours. Isolated DNA was then separated on a 1.5% agarose gel for visualization. 100 bp molecular weight marker (MW) is shown in lane 1.

Bcl-2 protein expression, which often correlates with protection from apoptosis, is shown in Figure 11. In the presence of E_2 (1 nM), Bcl-2 protein expression increased as compared to control cells. A specific ER antagonist, ICI 182,780 blocked the up-regulation of Bcl-2 induced by E_2 (1 nM). *o,p'*-DDT (100 nM) and alachlor (1 mM) increased Bcl-2 expression much like E_2 did, and the effects appear to be ER mediated, as illustrated by their inhibition by the ICI compound. These results suggest that at least two environmental chemicals, DDT and alachlor, can increase Bcl-2 in MCF-7 cells just as E_2 does, and that their effects are ER mediated.

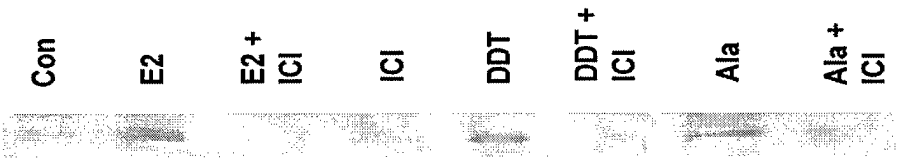

Fig. 11. Effects of environmental estrogen exposure on Bcl-2 expression in MCF-7 cells. MCF-7 cells were grown in 5% charcoal stripped media for 5 days. Cells were treated as shown above and harvested 48 hr later. The pure anti-estrogen ICI 182,780 was used as a specific inhibitor of ER. Bcl-2 protein content was determined using Western blotting and probing with Bcl-2 specific anti-sera.

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 hours, while the MCF-7 L cells appeared moderately sensitive to the apoptotic effects of TNF-α only at 72 hours. MCF-7 M cells were sensitive to the anti-proliferative effect of TNF-α but were resistant to TNF-α's cytotoxic effects. Examination of TNFR expression revealed a similar expression of p75 TNFR2 among all three cell variants. The p55 TNFR1 receptor was expressed at the highest levels in MCF-7 N cells and MCF-7 L cells, while MCF-7 M cells expressed the lowest levels of TNFR. The decreased expression of TNFR1 in MCF-7 M cells may account for their lowered sensitivity to TNF-α apoptosis as well as lowered generation of ceramide and protease activity. This however does not explain MCF-7 M cell sensitivity to the TNF-α anti-proliferative effect. It is possible that despite lowered expression, TNFR1 or even TNFR2 may provide the anti-proliferative signal in these cells. Given ceramide's role in inhibition of proliferation the 1.7 fold increase in ceramide in the M cells may be sufficient for 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 was correlated with higher expression of Bcl-2 and lower expression of Bax. Many studies confirm that an increase in expression of Bcl-2 correlates with resistance to apoptosis induced by a number of agents (34,35). Contradictory reports however 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. showed that overexpression of Bcl-2 in MCF-7 cells failed to offer a survival advantage to treatment with TNF-α (42). Conversely, Jaattella et al. showed that overexpression of Bcl-2 and Bcl-X_L was correlated with an increased resistance to TNF-α apoptosis (43). Again these reported differences may be due to the individual MCF-7 cell variants used by each laboratory and potentially 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 induction of apoptosis (44-46). Thus, cells expressing high levels of Bax may not be as resistant to apoptosis even when over-expressing Bcl-2. The Bcl-2 family of proteins may, however, 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, but 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 anti-apoptotic 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 inhibition of cytochrome c release from the mitochondria (36,37), the

increased Bcl-2 and decreased Bax expression in MCF-7 L cells accounts for the delayed activation of PARP specific caspases but not the suppressed generation of ceramide.

Our results suggest a potential molecular basis for differences in susceptibility to apoptosis among MCF-7 breast cancer cell variants. The increased generation of ceramide in the most apoptosis-sensitive variant (MCF-7 N) may account for their response to TNF-α as compared to the anti-proliferative action of TNF-α in the less apoptosis-sensitive variants (MCF-7 M and L). This decreased ceramide generation may be in part due to decreased expression of the TNFR1 as in the MCF-7 M cells or possibly to an alteration in the ability of TNF-α to activate sphingomyelinases as may be the case in MCF-7 L cells. In MCF-7 cells ceramide generation is early and transient suggesting its sphingomyelinase activation is not a result of the apoptotic process but an early signaling intermediate. Game et. al. implicated CPP32/ caspase-3 in regulation of Fas induced- but not in TNF-α-induced ceramide generation (48). Additionally it was shown that REAPER-induced ceramide generation occurring at 1 hour 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 capase-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+ breast cancer model and as a system for studying apoptotic signaling, the constitutive expression and regulation of apoptotic signaling molecules is therefore an important consideration.

CONCLUSIONS

To investigate the role of environmental estrogens in breast cancer cell apotosis we began by utilizing the estrogen responsive MCF-7 breast cancer cell line. However, discrepincies in the literature, alluded to in the introduction, lead us to believe that differences in MCF-7 cell lines from laboratory to laboratory may exist in terms of estrogenicity as well as responsiveness to apoptosis. These differences may represent a source of variability among research from lab to lab depending on the the particular variant of MCF-7 cell used. Using MCF-7 cell line variants from three well established sources we initially assesed the responsiveness of these cells to TNF-induced apotosis and then investigated the molecular differences which accounted for variations in responsiveness to apoptosis. We felt the work was important to properly establish and characterize our model system and because of the differences identified we have submitted the work shown in figures one through eight as a manuscript to Cancer Research.

Subsequent to the identification of the MCF-7N variant as an ER+, apoptotically sensitive breast cancer cell line, we assesed the role that estrogens play in suppression of TNF-induced cell death in these cells. Our results indicate that exposure of these cells to environmental estroge, and the endogenous estrogen 17-β-estradiol results in an increased expression of the anti-apototic protein Bcl-2 and concurrent suppression of TNF-induced apoptosis. These findings suggest that, consitent with previous studies, estrogenic compounds may suppress apotosis through an increase in expression of Bcl-2. We are pursuing the third specific aim of this grant by overexpressing Bcl-2 in MCF-7N cells and subsequently examining the effects on TNF- and chemotherapeutic drug-induced apoptosis.

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