Programmed Cell Death in an Estrogen-independent Human Breast Cancer Cell Line, MDA-MB-468

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ABSTRACT

Previous studies have demonstrated that estrogen-responsive human breast cancer cells can be induced to undergo an energy-dependent, genetically programmed series of biochemical changes that result in the active suicide of the cells following estrogen ablation. In contrast, estrogen-independent human breast cancer cells do not activate this programmed cell death pathway following estrogen ablation. This could be due either to the absence of the machinery required for programmed cell death or simply to the inability of estrogen ablation to activate this machinery. To discriminate between these two possibilities, the MDA-MB-468 estrogen-independent human mammary adenocarcinoma cell line was used as a model system to study the mechanism of cell death following cytotoxic drug treatment. Exposure of these cells to the fluorinated pyrimidines, 5-fluoro-2'-deoxyuridine or trifluorothymidine, resulted in growth inhibition and loss of proliferative capacity within 24 h. These changes occurred while cell membrane integrity was intact as measured by either cellular morphology or trypan blue exclusion. After 48 h of drug treatment, loss of cell membrane integrity was followed by cell lysis and a rapid decline in cell number. The addition of 16 μM thymidine prior to drug treatment prevented cell death, but thymidine did not rescue these cells once drug treatment was initiated. Analysis of DNA revealed the characteristic fragmentation into nucleosomal oligomers that is a hallmark of programmed cell death. Associated with this death pathway was a 15-fold induction of transforming growth factor β1 gene expression that has been previously observed in a variety of cellular systems undergoing programmed cell death. These results indicate that MDA-MB-468 estrogen-independent human mammary carcinoma cells retain the ability to undergo programmed cell death after treatment with cytotoxic drugs that induce a "thymineless" state.

INTRODUCTION

Breast cancer is a common and often fatal disease. Estrogen frequently plays an important role in sustaining its growth, and therapies aimed at interruption of estrogen action are often useful in treating patients whose tumor contains estrogen receptor. However, many initially estrogen-responsive tumors progress to a more aggressive estrogen-independent form that no longer responds to endocrine therapies, while other tumors are intrinsically resistant at initial diagnosis (1). Therefore, the need to develop more effective therapies for estrogen-independent breast cancer is pressing. The growth of a tumor is determined by the relationship between the rates of cell proliferation and cell death. Tumor regression occurs only when the rate of cell death is greater than the rate of cell proliferation. Thus, successful treatment for estrogen-independent breast cancer can be obtained by lowering the rate of cell proliferation and/or raising the rate of cell death to a point where the number of cells dying exceeds the number of cells proliferating.

Programmed cell death, or apoptosis, is an active, energy-dependent process in which a distinct series of biochemical and molecular events leads to the death of responsive cells by specific signals (2, 3). This process of selective cellular deletion occurs widely throughout nature in a variety of organized tissue reactions such as embryonic development, morphogenesis, hormone-induced tissue involution, lymphocyte deletion in gential centers, and cell-mediated immunity (4). Additionally, it has long been known that apoptotic bodies are readily identifiable in most malignant tissues and that this may explain the high rate of spontaneous cell loss known to occur in tumors (4). This observation has led to speculation that tumors may retain the capacity for activating programmed cell death. Indeed, evidence is accumulating that apoptosis is activated in tumor tissue by a number of anticancer agents including 5-FdUrd, cisplatin, methotrexate, etoposide, and chlorambucil.

Previous in vivo studies have demonstrated that androgen-dependent human prostate cancer cells, like normal prostatic tissue (9), retain the capacity for androgen ablation to induce a series of discrete morphological, biochemical, and molecular events that result in both the cessation of cell proliferation and the activation of the programmed death of these cells (10). The cascade of events associated with this response includes the activation of a Ca²⁺-Mg²⁺-dependent endonuclease present within the nucleus and induced by elevation of intracellular free Ca²⁺ (11), the fragmentation of genomic DNA at internucleosomal sites resulting in a stereotypical ladder of nucleosomal oligomer DNA fragments (9, 10, 12), and the enhanced expression of the TGF-β1 and TRPM-2 genes (10, 12, 13). DNA fragmentation is subsequently followed by irreversible morphological changes, histologically defined as apoptosis, which characteristically involve chromatin condensation, nuclear disintegration, cell surface blebbing, and eventually cellular fragmentation into a cluster of membrane-bound apoptotic bodies (14, 15). These observations have been extended to estrogen-responsive MCF-7 human breast cancer cells where the characteristic DNA fragmentation and apoptosis described above have been documented to be induced in vivo after estrogen withdrawal (16). Additionally, in vitro studies have demonstrated that androgen-independent prostatic cancer cells can activate the biochemical cascade of programmed cell death in response to the cytotoxic drugs, 5-FdUr and TFF (5). These fluorinated pyrimidines exert their primary effect through direct inhibition of thymidylate synthetase leading to depletion of intracellular thymidine 5'-triphosphate pools and a toxic "thymineless state" (17).

The above observations led us to investigate the possibility that estrogen-independent human breast cancer cells might retain the ability to activate the programmed cell death pathway...
way. To test this possibility, in vitro response of estrogen-independent MDA-MB-468 human breast cancer cells to the cytotoxic drugs 5-FdUrd and TFT was examined. To assess whether MDA-MB-468 cancer cells undergo programmed cell death following exposure to cytotoxic drugs, the following parameters were examined: (a) the pattern of DNA fragmentation and its temporal relationship to various measurements of cellular proliferative capacity and viability; (b) the level of expression of TGF-β1, and TRPM-2 genes as well as other nuclear oncoproteins; and (c) the morphological appearance of these cells via time-lapse videomicroscopy and scanning electron microscopy.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all reagents including 5-FdUrd and TFT were purchased from Sigma Chemical Co. (St. Louis, MO). TFT and 5-FdUrd were prepared at a concentration of 10 mM in water, sterilized via filtration, aliquoted, frozen at −20°C, and thawed immediately before use. MDA-MB-468 cells were obtained from Dr. M. Lippman (Vincent T. Lombardi Cancer Center, Georgetown University, Washington, DC). TGF-β1 was purchased from R & D Systems (Minneapolis, MN), reconstituted in sterile 4 mM HCl with 1 mg/ml bovine serum albumin, aliquoted, and stored at 4°C.

Cell Culture. MDA-MB-468 cells were routinely maintained in improved minimal essential media with 5% fetal calf serum (both from Biofluids, Rockville, MD), grown at 37°C in 5% CO2, and passaged weekly.

Clonogenic Assay. Cells in logarithmic growth phase were exposed to 100 μM 5-FdUrd or TFT for 0, 6, 12, 18, 24, or 48 h. Media were removed and nonadherent cells isolated by centrifugation. Adherent cells were detached by trypsinization, pooled with nonadherent cells, rinsed with phosphate-buffered saline, counted, and plated in triplicate in media with 10% fetal calf serum. After 7 days, cells were fixed with 25% methanol and stained with crystal violet. Colonies of >50 cells were counted visually by microscopy. The clonogenic ability of untreated cells was 28.5 ± 2.3% (SEM) by this method. The results of drug treatment upon clonogenic ability were expressed as the fraction of the clonogenic percentage of treated versus untreated cells. For assessment of effect of thymidine rescue after drug treatment, cells were plated in triplicate with or without the addition of 16 μM thymidine.

Growth Curves. Cells were plated in triplicate in media containing 5% fetal calf serum. After 24 h, 5-FdUrd or TFT was added to attain a 100 μM concentration of drug. At the indicated time points, media with nonadherent cells were removed, and adherent cells were detached by trypsinization. Medium and trypsinized cells were pooled and counted by hemocytometer. For trypan blue exclusion studies, an aliquot of this cell suspension was mixed with an equal volume of 0.08% trypan blue in Hanks’ balanced salt solution (GIBCO BRL, Gaithersberg, MD). After 10 min, dye exclusion was assessed visually by microscopy. Viable cell number was calculated by multiplying total cell number by fraction of cells excluding trypan blue. For assessment of effect of concurrent treatment of cells with thymidine and drug, cells were plated as above with the addition of a second set of samples plated as in the section on DNA isolation and analysis. Total cellular RNA was isolated from pooled adherent and nonadherent cells according to the urea-lithium chloride method of Auffray and Rougeon (19). For Northern analysis, 30 μg total RNA were size-fractionated and transferred as described previously (12). DNA probes used for hybridization analysis in this study were kindly provided by the following individuals: TRPM-2 cDNA (20) from Dr. M. T.的时候wood (University of Ottawa, Ottawa, Ontario, Canada); human TGF-β1 cDNA (21) from Dr. R. Derynk (Genentech, Inc., South San Francisco, CA); human c-Jun cDNA (22) from Dr. D. Bohmann (University of California, Berkeley, Berkeley, CA); human c-myc cDNA (23) from Dr. P. Celano (Johns Hopkins Oncology Center, Baltimore, MD); human γ-actin cDNA (24) from Dr. L. Kedes (Stanford University, Palo Alto, CA); and pE7 human EGF cDNA (25) from Dr. I. Pastan (National Cancer Institute, Bethesda, MD). The human c-fos probe was obtained from the American Type Culture Collection (Rockville, MD). Autoradiographs were analyzed by scanning densitometry on a model TBX densitometer (Tobias Associates, Inc., Ithvand, PA). Equivalent loading was confirmed by photography of ethidium bromide-stained gels, and densitometry results were normalized by expressing the units obtained for a specific transcript relative to the units obtained for the γ-actin transcript.

RESULTS

Effect of Fluoropyrimidines on MDA-MB-468 Cell Proliferation and Viability. Treatment of MDA-MB-468 cells with 100 μM 5-FdUrd (Fig. 1A) or TFT (Fig. 1B) resulted in sustained inhibition of cellular proliferation followed by a decline in viable cell number after 48 h of treatment. Of note, >90% of drug-treated cells continue to exclude trypan blue at 48 h, indicating that the early effect of TFT and 5-FdUrd on these cells is not

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cell. The observation that the loss of cellular proliferative ability occurs before the loss of cellular membrane viability is a hallmark of apoptosis, or programmed cell death, as opposed to necrosis, during which loss of membrane integrity is an early event (2).

To determine whether thymidine could rescue cells after drug treatment, parallel clonogenic assay experiments were performed with the inclusion of 16 μM thymidine to cell culture media subsequent to various periods of fluoropyrimidine treatment. This thymidine treatment after drug exposure failed to rescue cells from loss of clonogenic capacity induced by 100 μM 5-FdUrd or TFT. The survival curves of cells plated in media with 16 μM thymidine after treatment with 100 μM TFT or 5-FdUrd were superimposable on the curves obtained with unsupplemented media as seen in Fig. 2. Thus, simultaneous exposure of these cells to thymidine and drug prevents drug toxicity (Fig. 1); however, this dose of thymidine cannot rescue cells after exposure to 100 μM concentrations of 5-FdUrd or TFT for 6 or more h. This observation supports the hypothesis that the toxicity of 5-FdUrd and TFT in these cells is due to the imposition of a “thymineless state” via inhibition of thymidylate synthetase and suggests that irreversible commitment to programmed cell death can occur as early as 6 h in some cells.

The effect of treatment with 100 μM 5-FdUrd on the incorporation of [3H]thymidine into logarithmically growing MDA-MB-468 cells (Fig. 3) was also examined. [3H]Thymidine incorporation progressively declined to undetectable levels over 96 h. The absence of a precipitous early decline in [3H]thymi-
dine incorporation into drug-treated cells demonstrates that cells progress into the S-phase of the cell cycle even in the presence of 5-FdUrd, although mitotic activity (see below) and cell proliferation (Fig. 1) cease very early during treatment.

Morphological Changes in MDA-MB-468 Cells after Fluoropyrimidine Treatment. Time-lapse videomicroscopy and scanning electron microscopy were used to assess morphological changes in MDA-MB-468 cells induced by fluoropyrimidine treatment. By 12 h of exposure to 100 μM 5-FdUrd, mitotic activity ceased completely (Fig. 4). This cessation of morphologically detectable mitosis correlates well with clonogenic assay results presented earlier that demonstrate >80% loss of clonogenic capacity by 12 h of treatment (Fig. 2). This is followed by a 30-h time span during which cell lysis is not detected morphologically. This “lag period” represents a time when cell membranes are intact both by direct morphological assessment and trypan blue exclusion as noted previously. Treatment with 100 μM TFT resulted in similar temporal pattern of morphological changes (data not shown).

Scanning electron microscopic analysis demonstrated characteristic differences between untreated and treated MDA-MB-468 cells (Fig. 5). Exposure to 100 μM 5-FdUrd (Fig. 5B) or 100 μM TFT (Fig. 5C) for 24 h induced a “rounding up” of cells and the development of plasma membrane projections that, by time-lapse videomicroscopy, were highly dynamic, giving the appearance of membrane “boiling.” These surface projections could release small, membrane-bound cytoplasmic buds that frequently string together (i.e., cellular fragmentation). The TFT-treated cells underwent the same membrane “boiling” and cellular fragmentation but in addition contained numerous invaginations that formed deep cavities in the plasma membrane. This cell membrane hyperactivity was episodic in nature and was noted in all drug-treated cells by 24 h of treatment. Eventually, such membrane hyperactivity resulted in the fragmentation of the cells into characteristic membrane-bound apoptotic bodies. In contrast, the untreated control cells (Fig. 5A) were larger, remained very flat, and did not contain these membrane “blebs.”

Biochemical Correlates of Programmed Cell Death in Fluoropyrimidine-treated MDA-MB-468 Cells. As programmed cell death is characterized by stereotypic DNA fragmentation, the pattern of DNA fragmentation was determined at various times following exposure to 100 μM 5-FdUrd (Fig. 6). The characteristic ladder of DNA nucleosomal oligomers, indicating cleavage of chromosomal DNA at internucleosomal loci, was noted at the earliest treatment time point examined, 6 h. Once initiated, progressive fragmentation of the higher molecular weight nucleosomal multimers into lower molecular weight oligomers continued over time. Clear evidence of DNA ladder formation was best detected in the nonadherent cells; adherent cells retained primarily high molecular weight DNA. An identical pattern of fragmentation was noted after treatment with 100 μM TFT (data not shown). Thus, as cellular DNA is progressively fragmented into lower molecular weight nucleosomal oligomers, the cells lose proliferative capacity, as measured by clonogenic assay (Fig. 2) but retain membrane integrity (Fig. 4). This discordance between DNA fragmentation and loss of cellular proliferative capacity and cell membrane viability is again indicative of programmed cell death as opposed to necrosis (4).

Since increased expression of the TGF-β1 gene is associated with programmed cell death in a variety of systems (10, 12, 16, 26), expression of TGF-β1 mRNA was studied during fluoropyrimidine treatment of MDA-MB-468 cells. Within 12 h after 100 μM 5-FdUrd treatment, a 3-fold increase in TGF-β1 mRNA levels was noted. This reached a level 15 times control by 48 h of treatment (Fig. 7). TFT treatment resulted in a similar induction of TGF-β1 (data not shown).

Finally, the expression of TRPM-2 (Fig. 8), EGFR, and the growth-related genes, c-myc, c-jun, and c-fos (data not shown), was examined. Northern analysis revealed detectable basal levels of these transcripts, but we found no significant change in mRNA expression for any of these genes after 6, 12, 18, 24, or 48 h of 5-FdUrd or TFT treatment. The 2.3-kilobase molecular weight size of the TRPM-2 mRNA transcript in MDA-MB-468 human breast cancer cells was indistinguishable from that induced in rat ventral prostate (9), although level of expression in MDA-MB-468 cells was >75-fold lower than expression in the rat ventral prostate after castration.

DISCUSSION

The present study demonstrates that MDA-MB-468 estrogen-independent human breast cancer cells are inhibited from proliferating and induced to undergo programmed cell death by treatment with the fluorinated pyrimidines, 5-FdUrd and TFT. This cytotoxic response results from the induction of a “thymineless state” due to the inhibition of thymidylate synthetase by these drugs. The demonstration that simultaneous inclusion of a sufficient level of exogenous thymidine in culture media is capable of completely protecting the MDA-MB-468 cells from the cytotoxicity of these fluoropyrimidines illustrates the critical role of the “thymineless state” in toxicity of these drugs. The fragmentation of DNA into nucleosomal oligomers, loss of cellular proliferative capacity, and enhanced TGF-β1 mRNA expression in MDA-MB-468 cells following fluoropyrimidine treatment precede the loss of cellular viability as measured by trypan blue exclusion and plasma membrane lysis. Based upon the temporal pattern of the characteristic biochemical and morphological changes, it is clear that these fluoropyrimidines activate programmed, not necrotic, death of the MDA-MB-468 cells. Thus, estrogen-independent MDA-MB-468 cells, although insensitive to estrogen withdrawal, can activate the programmed cell death pathway previously described in estrogen-dependent MCF-7 cells (16), with appropriate treatment. Elevated TGF-β1 transcripts have been shown to be associated with programmed cell death in a number of systems including the in vivo death of hormone-dependent breast and prostatic tumors by hormone withdrawal (9, 16). Additionally, exogenous
Fig. 5. Scanning electron microscopic evaluation of MDA-MB-468 cells. A, untreated MDA-MB-468 cells × 2200; B, MDA-MB-468 cells exposed to 100 μM 5-FdUrd for 24 h. Arrow, membrane-bound cytoplasmic bud. × 2200; C, MDA-MB-468 cells exposed to 100 μM TFT for 24 h. Arrow, surface craters × 2200.

Fig. 6. Electrophoretic pattern of DNA isolated from MDA-MB-468 cells after exposure to 100 μM 5-FdUrd. Letters above lanes, DNA from either adherent cells (A) or non-adherent (N) cells. Hours above lanes, duration of exposure to 5-FdUrd before DNA was isolated. Size and location of a 123-base pair (bp) molecular weight marker are shown on the right.
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TGF-β1 mRNA expression in MDA-MB-468 cells following treatment with 100 μM 5-FdUrd. A, total mRNA (30 μg/Lane) isolated after 5-FdUrd treatment for 0, 6, 12, 18, 24, or 48 h was size-fractionated and blotted onto nylon membranes. The blots were hybridized with the appropriate 32P-labeled cDNA probes and autoradiographs developed. B, autoradiographs were quantitated by densitometry, and γ-actin values were used to adjust for any loading differences. Relative expression of TGF-β1 was calculated by assigning an arbitrary value of one to the control (zero-hour) mRNA value.

Enhanced expression of the TRPM-2 gene has been associated with programmed death under conditions of hormone withdrawal in androgen-dependent normal rat prostatic glandular cells (9) as well as androgen-dependent human prostatic cancer cells (10) and estrogen-dependent human breast cancer cells (16). Other studies have demonstrated that enhanced expression of the TRPM-2 gene occurs in a variety of cell types induced to undergo programmed death by a series of additional means other than hormone withdrawal (5, 13, 33). In the present study, however, we found no induction of TRPM-2 mRNA in MDA-MB-468 cells undergoing fluoropyrimidine-induced programmed cell death, although low levels of TRPM-2 transcript, indistinguishable from that seen in rat prostatic tissue after castration, are observed. To our knowledge, programmed cell death without TRPM-2 induction has been reported in only one other model system: AT-3 rat prostatic cancer cells induced to die by the calcium ionophore, ionomycin (18). The lack of induction of TRPM-2 mRNA in these studies suggests that enhanced expression of this transcript is not an absolute requisite for DNA fragmentation. Because the increased intracellular calcium levels induced by calcium ionophore activate the Ca2+-Mg2+-dependent endonuclease (18), TRPM-2 may be involved in the calcium flux necessary for endonuclease activation, and ionomycin treatment may bypass this step in inducing programmed death of AT-3 cells. It is thus possible that MDA-MB-468 cells have acquired an alternative pathway to increase intracellular calcium and activate the endonuclease, or that a Ca2+-independent endonuclease is involved in the death process induced by fluoropyrimidines. In this regard, future studies will examine the effects of TFF and 5-FdUrd on intracellular calcium levels in MDA-MB-468 cells as well as the ability of calcium ionophore to trigger the programmed cell death cascade.

The precise mechanism by which TGF-β exerts a negative effect on the growth of MDA-MB-468 cells remains unclear. In normal rat kidney cells and a tumorigenic derivative of these cells, both of which require epidermal growth factor for anchorage-independent growth under serum-free conditions, TGF-β causes alterations in EGFR as well as altered growth of these cells. Although MDA-MB-468 cells overexpress the EGFR and are growth-inhibited by epidermal growth factor (31), we have not detected changes in the level of EGFR mRNA with fluoropyrimidine treatment at the time points studied. TGF-β has also been proposed to exert a negative proliferative effect by regulation of c-myc gene transcription in keratinocytes (32); however, we detected no changes in the level of this transcript at the time points studied. The temporal relationship of induction of the TGF-β1 gene with the programmed death of MDA-MB-468 cells in the current study suggests that this growth factor may act not only as a regulator of cell growth but of cell death as well. Further study of the effects of exogenous TGF-β will be required to address this possibility.
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