Programmed Cell Death during Regression of the MCF-7 Human Breast Cancer following Estrogen Ablation

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ABSTRACT

To study the mechanism of regression of human mammary cancer following estrogen ablation, estrogen-responsive MCF-7 human mammary adenocarcinoma cells were inoculated into ovariectomized female nude mice supplemented with exogenous 17β-estradiol (E2) via an E2 implant. Implants were then removed when MCF-7 tumors were 400 mm3 in size. Removal of the E2 implants resulted in a 50% tumor regression by 2 weeks following E2 ablation. Associated with this regression is a rapid (i.e., within 1 day following E2 ablation) enhanced expression of the transforming growth factor β1 and TRPM-2 genes, two genes the expression of which has been previously demonstrated to be enhanced in a variety of cell types induced to undergo programmed cell death (i.e., apoptosis). The enhanced expression of transforming growth factor β1 and TRPM-2 is not a nonspecific response since the expression of other genes, such as p53, c-fos, c-ha-ras, and p52, decrease following E2 ablation. Fragmentation of tumor DNA into nucleosomal oligomers and histological appearance of apoptotic bodies are characteristic early events that precede the dramatic reduction in tumor volume following E2 ablation. These results demonstrate that the regression of MCF-7 human mammary cancers in nude mice following estrogen ablation is due to a sequence of biochemical and morphological changes that result in both the cessation of cell proliferation and activation of programmed death or apoptosis of these MCF-7 cancer cells. Clarification of the biochemical pathway involved in the activation of this programmed cell death should identify new targets of therapy for even estrogen-independent human mammary cancer cells.

INTRODUCTION

Approximately one-third of women with metastatic breast cancer respond positively to estrogen ablation (1). This positive response often involves regression of at least a portion of the cancer within individual patients. Tumor regression occurs only when the rate of tumor cell death becomes greater than tumor cell proliferation. This could occur due to a decrease in the rate of cell proliferation (i.e., cytostatic effect) and/or an increase in the rate of cell death (i.e., cytotoxic effects) induced by estrogen ablation. It remains controversial as to whether estrogen ablation is simply cytostatic or whether it can also be cytotoxic to human breast cancer cells. To resolve this issue a variety of in vivo and in vitro studies have been performed using the MCF-7 human breast cancer cell line as a model (2–9).

In the present study, MCF-7 cells were inoculated into ovariectomized female nude mice supplemented with exogenous E2 to produce continuously growing tumors. The response of these MCF-7 cancers to removal or ablation of the E2 was then tested. Since these tumors regressed rapidly following E2 ablation, biochemical studies were performed to determine whether this regression was due simply to a decrease in cell proliferation (i.e., cytostatic effect) or whether this was coupled to an increase in cell death (i.e., cytotoxic effect). In addition, if an increase in cell death occurred following E2 ablation, this raised the issue of how this cell death occurred.

Previous studies have demonstrated that human prostate cancer cells, like normal prostatic glandular cells (10), can retain the ability for androgen ablation to induce a series of discrete biochemical events that result in the cessation of cell proliferation and activation of the programmed death of these androgen-dependent cancer cells (11). The death of these cancer cells induced by androgen ablation occurs as an active energy-dependent process which involves a cascade of biochemical changes, collectively referred to as programmed cell death (10–14). Associated with programmed cell death is the enhanced expression of a series of genes including TGFβ1 and the TRPM-2 gene (11, 14–16). This type of programmed cell death initially involves fragmentation of genomic DNA. This fragmentation of genomic DNA is a result of activation of a Ca2+-Mg2+-dependent endonuclease present within the nucleus induced by elevation of intracellular free Ca2+ (12, 17). This Ca2+-Mg2+-dependent nuclease selectively hydrolyzes DNA at sites located between nucleosomal units, thus resulting in the stereotypic ladder of nucleosomal oligomer DNA fragments (i.e., multiples of a 180-nucleotide base pair subunit lacking intranucleosomal breaks in the DNA) (10–12). This DNA fragmentation is subsequently followed by irreversible morphological changes, histologically defined as apoptosis, which characteristically involve chromatic condensation, nuclear disintegration, cell surface blebbing, and eventually cellular fragmentation into a cluster of membrane-bound apoptotic bodies (18, 19).

Thus, the question arose as to whether a similar programmed cell death was induced in MCF-7 cells following E2 ablation. The parameters used to determine if the MCF-7 cancer cells underwent programmed cell death following estrogen ablation included: (a) histological appearance of mitotic versus apoptotic cells; (b) pattern of DNA fragmentation and its temporal relationship with regression of the MCF-7 tumor volume following E2 ablation; and (c) level of expression of the TGF-β1 and TRPM-2 genes, which increase in a variety of cell types undergoing programmed cell death (11, 14, 16).

MATERIALS AND METHODS

MCF-7 Cell Culture. MCF-7 cells were generously obtained from Dr. Charles McGrath of the Michigan Cancer Foundation. These cells are routinely maintained in Dulbecco's minimally essential media (Gibco, Grand Island, NY) containing 10% fetal calf serum (HyClone, Logan, UT), streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C in 5% CO2.
Animals. Fifty 5-6-week-old ovariectomized female athymic Ncr-nu mice (National Cancer Institute) were anesthetized with Metofane (Pittman-Moore, Washington Crossing, NJ) and then implanted s.c. in the flank with a 0.25-cm Silastic capsule packed with E2 (Sigma Chemical Co., St. Louis, MO). The Silastic capsules were constructed from Model 602-305 (i.e., 0.078 inch inner diameter and 0.125 inch outer diameter) Silastic medical grade tubing (Dow Corning, Midland, MI) as described previously (20). This size E2 implant will maintain the serum 17β-estradiol levels between 200 to 400 pg/ml for up to 3 months as determined by radioimmunoassays as described previously (21). The E2-implanted ovariectomized mice were then given s.c. injections of 2 × 10⁶ viable MCF-7 cells. Preliminary studies demonstrated that no tumor became palpable within the first 3 months post-tumor inoculation if the animals were not implanted with such an E2 capsule. In contrast, in E2-implanted ovariectomized hosts, >90% of all MCF-7-as determined by radioimmunoassays as described previously (21). The serum 17β-estradiol levels between 200 to 400 pg/ml for up to 3 months.

The tumor volume was determined for each of these animals at various times using methods described previously (22). When the tumors reached a volume of ~400 mm³, five of the animals were killed and their MCF-7 tumors were harvested. E2 implants were removed from 40 tumor-bearing animals under anesthesia, and an additional group of 5 animals were allowed to go untreated to evaluate the tumor growth behavior. At 1, 2, 3, 7, and 14 days following removal of the E2 capsules, 5 of these castrated mice were killed and their MCF-7 tumors removed and used for assays described later. Five of the mice from which E2 capsules were removed were allowed to go untreated for 3 weeks following removal of the estrogen implants and the tumor volume was determined weekly during the interval period. Ten of the mice from which implants were removed were allowed to go untreated for 1 week, and then 5 received a new E2 capsule and 5 received a testosterone capsule implanted s.c. in the flank of each animal under anesthesia. The testosterone capsules were 1 cm long and were constructed of Model 602-305 Silastic tubing. These capsules maintained the serum testosterone level between 6 to 8 ng/ml (11). The tumor volumes were determined for each of these animals after 1 and 2 weeks of E2 or testosterone replacement.

Quantitative Kinetic Analysis of MCF-7 Tumor. After various times of treatment, host mice were killed and MCF-7 tumors were removed rapidly, weighed, fixed with 4% paraformaldehyde and 5% glutaraldehyde, and then diluted 1:1 with 0.1 M cacodylate buffer (pH 7.3 at 4°C). After 10 min, thin slices were obtained and placed in a small quantity of fresh fixative on a sheet of dental wax. These pieces were diced into cubes (<1.5 mm³) and placed in fresh fixative. After 2 h of primary aldehyde fixation, the tissue was washed in 0.1 M cacodylate buffer and postfixified for 1 h in 1% osmium tetroxide. The tissue was then embedded in PolyBed 812 (Polysciences, Inc., Warrington, PA) and sectioned (1 μm), and the sections were stained with 0.5% toluidine blue in 0.5% aqueous sodium borate as described previously (11). The mitotic and apoptotic indexes were determined at various days before and after estrogen ablation.

The daily percentage of cells undergoing proliferation was determined at various days before and after estrogen ablation by multiplying the daily fractional cell proliferation rate (i.e., kp) for the particular time point by 100. The kp values were determined by the formula presented by Steele (23) that

\[ kp = \frac{MI}{Tm} \]

where MI is the mitotic index on the particular day and Tm is the time in days for the duration of mitosis. For these calculations, a Tm value of 0.058 day (1.4 h) was used based upon the data of Brunner et al. (9).

The daily percentage of cells undergoing apoptotic death was determined at various days before and after estrogen ablation by multiplying the daily fractional apoptotic cell death rate (i.e., kd) for the particular time point by 100. The various kd values were determined by the formula

\[ kd = \frac{AI}{Ta} \]

where AI is the apoptotic index on the particular day and Ta is the time in days for the duration of apoptosis. For these calculations, Ta = 0.125 day (3 h) was used. This Ta value was determined as follows. In the presence of E2 in the animal, the kp is 0.241 (Table 1) and tumor volume-doubling time (DT) is 14 days, as determined by analysis of data presented in Fig. 1. Using these values, the kp value in the presence of estrogen of 0.192 was calculated using the Steele formula

\[ kd = kp - \ln \frac{2}{DT} \]

Using this calculated kd value and the determined AI value in the presence of E2 of 0.024, Ta was calculated as

\[ Ta = \frac{AI}{kd}. \]

Biochemical Analysis. DNA fragmentation was analyzed as described previously (11). Polyadenylated RNA from xenografts was isolated and subjected to Northern analysis as described previously (11). Insert probes used in this study were derived from plasmids kindly provided by the following individuals: TRPM-2 (15) from Dr. Martin Tenniswood (University of Ottawa, Ottawa, Ontario, Canada); TGFβ (24) from Dr. Rik Derynck (Genentech, South San Francisco, CA); pS2 (25) from Dr. Pierre Chambon, Institute de Chimie Biologique, Strasbourg, France; human c-myc (26) (exon 3 probe cloned into pTZ18R) from Dr. Paul Celano, The Johns Hopkins Oncology Center, Baltimore, MD; prasZip-6 containing murine V-Hrai (27) from Dr. Robert Weinberg, MIT, Cambridge, MA; human β-actin (28) from Dr. Don Cleveland, The Johns Hopkins University, Baltimore, MD; and human c-fos from the American Type Tissue Collection, Rockville, MD. Autoradiographs were analyzed with a scanning densitometer (Model 1650; Bio-Rad, Richmond, CA). Results were normalized by expressing the units obtained for the transcript of interest to the units obtained for the β-actin transcript.

Statistical Analysis. Data are expressed as the group (N = 5) mean ± SEM. The significance of difference was determined by analysis of variance using the Newman-Keuls test to allow for multiple comparisons.

RESULTS

MCF-7 cells are tumorigenic when injected into ovariectomized female nude mice if the animals are supplemented with estrogen. The results of these studies are presented in Table 1.
exogenous E2 capsules. Removal of the E2 capsule from such E2-supplemented animals bearing continuously growing MCF-7 tumors results in a reduction of the tumor volume so that by 2 weeks post-E2 removal, the MCF-7 tumor volume regresses by more than 70% (Fig. 1). This regression is specifically due to estrogen ablation since E2 replacement of hosts results in complete regrowth of the tumor (Fig. 1). Morphological analysis of the regressing tumor revealed a reduction in proliferative activity as indicated by the rapid decrease in the daily percentage of MCF-7 cells undergoing apoptosis (Table 1). A 3-fold increase in the daily percentage of MCF-7 cells undergoing apoptosis is detectable as early as 2 days following castration.

The pattern of DNA fragmentation in MCF-7 mammary cancer cells was determined at various times following E2 ablation of tumor-bearing hosts. The characteristic ladder of DNA nucleosomal oligomers is evident 1 day post-E2 ablation (Fig. 2A, Lane 2). Once initiated, progressive fragmentation of the higher molecular weight nucleosomal multimers into lower molecular weight oligomers continues over time (Fig. 2B, Lanes 3, 4, and 5, respectively). If after 1 day of E2 ablation exogenous E2 is replaced, however, the fragmentation ceases (Fig. 2B, Lane 3).

Since increased expression of the TGFβ1 and TRPM-2 genes are associated with programmed cell death in a variety of systems (11, 14, 15), the expression of these two genes was studied during E2 ablation-induced regression of the MCF-7 tumor. Within the first day following E2 ablation, there is a 3-fold increase in TRPM-2 mRNA levels with a nearly 5-fold increase in expression occurring by 3 days post-hormonal ablation (Fig. 3). Seven days after E2 removal, there is a small decrease in TRPM-2 mRNA levels (Fig. 3). The 2.3-kilobase molecular weight size of the TRPM-2 mRNA transcript from the MCF-7 tumors from animals supplemented with E2, or 1, 3, 7, or 14 days after removal of E2 capsules was size fractionated and blotted onto nylon membranes. The blots were initially hybridized with the appropriate 32P-labeled cDNA probes and autoradiographs developed. In B, autoradiographs were quantitated and the blots were dehybridized and subsequently re-hybridized with a 32P-labeled β-actin cDNA probe which was quantitated and these β-actin values were used to adjust for any loading differences. The TRPM-2 and TGFβ1 results were expressed as relative values by assigning a relative value of one for the particular mRNA values from E2 intact hosts (i.e., ovariectomized mice with E2 capsule present). Since the pS2 expression decreased following E2 ablation, the pS2 results were expressed relative to the values for tumors from animals which had their E2 capsules removed 14 days earlier.

Within the first day following E2 ablation, there is also a 3-fold increase in TGFβ1 mRNA levels which persists through day 3 prior to a decrease to constitutive expression levels by 7 days after E2 capsule removal (Fig. 24). In contrast to rapid elevations in TRPM-2 and TGFβ1 mRNA expression, expression of several other growth-related genes decreases in MCF-7 tumors following E2 ablation. These include a 4-fold decrease in the expression of the E2-responsive pS2 gene (23) (Fig. 3), and a 65–70-fold decrease in proliferation-associated c-fos and c-H-ras oncogene expression (Fig. 4). Interestingly, the expression of another gene often associated with growth, the c-myc oncogene, does not decrease but instead increases 5-fold by day 3 after E2 removal. A similar transient elevation in c-myc expression is also seen during the regression of the rat ventral prostate following androgen ablation (29, 30).
PROGRAMMED DEATH OF MCF-7 CELLS

In contrast to these studies, Shafie and Grantham (3) and Osborne et al. (7) demonstrated that if E$_2$ pellets were removed from ovariectomized female mice bearing growing MCF-7 tumors, the tumor stopped its progressive growth but did not regress. In additional studies, Osborne et al. (4) and Sutherland et al. (4) demonstrated that in estrogen withdrawal from MCF-7 cells in tissue culture (i.e., produced by removal of E$_2$ from the media or by the addition of the antiestrogen tamoxifen) was only cytostatic (i.e., cells accumulate in the G$_0$-G$_1$ phase of the cell cycle), not cytotoxic to MCF-7 cells. Again, these studies were performed in the presence of phenol red which may be a confounding factor.

Combining these studies, it is apparent that while MCF-7 cells are E$_2$ responsive, not all MCF-7 cells are equivalent. Apparently some MCF-7 cell variants have lost their ability to manifest a cytotoxic response but retain the ability to demonstrate a cytostatic response to estrogen ablation, while other variants have maintained both of these abilities. In the present study, the MCF-7 cell variant used produced progressive growing tumors in ovariectomized female nude mice only when supplemented with exogenous E$_2$, and these tumors regressed rapidly following E$_2$ ablation. The regression of these MCF-7 mammary cancers following E$_2$ ablation involves an inhibition of cell proliferation coupled with an activation of programmed cell death. Elevation of TGF/β expression following ablation may be involved as a negative regulatory signal to inhibit further cell proliferation once programmed cell death is induced. This possibility is supported by the previous studies which have demonstrated the ability of TGF/β to inhibit the proliferation of certain MCF-7 cells in vitro (32). Enhanced TRPM-2 expression, fragmentation of DNA into nucleosomal oligomers, and the morphological manifestation of enhanced apoptosis following E$_2$ ablation are characteristic early events that preceded MCF-7 tumor regression. These results demonstrate that E$_2$-dependent MCF-7 human mammary cancer cells retain the ability to activate a programmed cell death cascade similar to that induced in normal rat prostatic glandular cells (10, 12, 13) and PC-82 human prostatic cancer cells following androgen ablation (11). Using DNA cleavage, morphological changes, and dye exclusion techniques, Bardon et al. (8) likewise have demonstrated that antiestrogens and antiprogestins induce the programmed death of MCF-7 cells growing in vitro in phenol red-containing medium. How such antihormones in vitro or E$_2$ ablation in vivo induce the programmed death of MCF-7 cells is unclear. It does appear, however, that endonuclease-driven fragmentation of the genomic MCF-7 DNA may be critically involved, as it is in other types of systems in which programmed cell death is actively induced (10–13, 33–40). It will be crucial, therefore, to resolve whether this is due to an activation of Ca$^{2+}$-Mg$^{2+}$-dependent or independent endonuclease activity within the MCF-7 cell nuclei.

Recent studies have demonstrated that even hormonally independent cancer cells can retain the major portion of the biochemical cascade (i.e., Ca$^{2+}$-Mg$^{2+}$-dependent endonuclease-induced fragmentation of DNA, TRPM-2 induction, etc.) involved in the programmed cell death pathway. In these hormonally independent cells, however, there is a defect in these cells such that programmed death is no longer activated by hormonal removal (36). A new therapeutic approach for hormonally independent mammary cancers therefore could be to develop some type of nonhormonal ablation method to activate this programmed cell death cascade in these cells distal to the point of the defect. In order for any realistic chance to accom-
plish this goal, a more detailed understanding of the biochemical pathway involved in the programmed death of mammary cancer cells is urgently required. This MCF-7 cell line will be a valuable model in this regard.

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REFERENCES

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