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Tamoxifen Induces TGF-β1 Activity and Apoptosis of Human MCF-7 Breast Cancer Cells In Vitro

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Abstract We report here that the antiestrogen tamoxifen (TAM) induces cell death in human breast cancer cell line MCF-7. We assessed the type of cell death induced by TAM in this breast cancer cell line on the basis of morphological and biochemical characteristics. Dying cells showed morphological characteristics of apoptosis, such as chromatin condensation and nuclear disintegration. DNA isolated from these cells revealed a pattern of distinctive DNA bands on agarose gel. The DNA fragmentation in MCF-7 cells induced by TAM could also be detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling. Northern blot hybridization revealed a substantial increase in the amounts of TRPM-2 and TGF-β1 mRNAs in MCF-7 cells after treatment with TAM. In contrast, the mRNA level of the estrogen-induced pS2 gene was strongly suppressed. The biological activity of TGF-β was increased at least fourfold in the media from MCF-7 cells treated with TAM. The results presented in this study suggest that TAM induces apoptosis of MCF-7 cells and it may be mediated by the secretion of active TGF-β. © 1996 Wiley-Liss, Inc.

Key words: antiestrogen, human breast cancer, programmed cell death, tamoxifen, TGF-β1

Cell death can be accidental or programmed in a multicellular organism [Kerr et al., 1972]. Accidental death by the necrotic pathway results in cell lysis and rupture of the cells. Once lysed, cellular contents are spilled into surrounding tissue, triggering an inflammatory response. Programmed cell death, or apoptosis, on the other hand, is not accidental. Two of the distinct changes noted to occur during apoptosis are a variety of well-documented morphological changes [Kerr et al., 1972] and changes in DNA structure, reflecting its degradation by an endogenous endonuclease [Wyllie et al., 1986]. Morphologically, in apoptosis, the plasma membrane becomes blebbed and ruffled, and eventually cellular fragmentation into a cluster of membraned-bound apoptotic bodies occurs [Wyllie et al., 1986]. The internal components of the cells are not spilled into the surrounding tissue and therefore do not trigger an inflammatory response. The nucleus shrinks and the chromatin becomes very dense, collapsing into patches, then into crescents in tight apposition to the nuclear envelope and finally into dense spheres. One of the biochemical hallmarks of apoptosis is the appearance of DNA degradation into a ladder of regular nucleosomal subunits of 180–200 base pairs [Tomei and Cope, 1991].

Recently Kyprianou et al. [1991] determined that hormone ablation (castration) of female mice bearing human breast tumors caused the tumors to decrease in size. Through molecular techniques, it was determined that the tumor cells were regressing because apoptosis was occurring in a large proportion of the tumor. Indeed, a common feature of many endocrine tissues is that they are absolutely dependent on tropic hormone support, without which they involute: this is true, for example, in prostate, adrenal cortex, follicle, testis, and breast [Collins and Rivas, 1993]. In each of these cases, cells are actively deleted via apoptosis. This scheme is in keeping with the notion that all cells require "survival" signals, without which programmed cell death runs by default [Raff et al., 1993]. It has been shown that the growth of

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; TAM, tamoxifen; TdT, terminal deoxynucleotidyl transferase; TGF-β1, transforming growth factor-β1; TRPM-2, testosterone-repressed prostate message-2 gene; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

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hormone-dependent tumors can be arrested by increasing the apoptotic rate of cells following administration of inactive hormone analogs [Wärri et al., 1993]. Tamoxifen (TAM) is a nonsteroid antiestrogen that binds with high affinity to the estrogen receptor, thereby blocking the action of native estrogen. Subsequently it inhibits or modifies the interaction of estrogen receptors with DNA. TAM strongly counteracts estrogen effects, including secretion of several growth factors and growth controlling enzymes [Dickson and Lippman, 1987], so that a woman's own estrogen cannot stimulate growth of the tumor cells. This approach is of interest in treatment of a variety of endocrine tumors.

The aim of this study was to further examine the mechanism of growth inhibition of breast cancer cells by TAM. In particular, we sought to determine if TAM treatment induced programmed cell death in MCF-7 human breast cancer cells. Programmed cell death was evaluated by examing coordinate hallmark morphological changes, DNA fragmentation, expression of genes associated with MCF-7 cell death (TGF-β1 and TRPM-2) or normal function (pS2) before and after TAM treatment, and the production of bioactive TGFβ1.

MATERIALS AND METHODS

Cell Cultures

MCF-7 breast cancer cells were obtained from Dr. Xiao-kun Zhang, La Jolla Cancer Research Foundation, La Jolla, California. These cells are routinely maintained in Dulbecco's modified Eagle's medium (DMEM) media (Gibco, Grand Island, NY) containing 10 μg/ml bovine insulin, 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C in 5% CO_2. In TAM treatment, various amounts (1×10^{-7} M, 5×10^{-7} M, 1×10^{-6} M, 5×10^{-6} M, 1×10^{-5} M) of tamoxifen (Sigma) were added (in 0.1% ethanol) to the media and cells were treated for a period of times as indicated.

For light microscopy, cells were cultured on 8-well Lab-Tek chamber slides (Nunc Inc., Naperville, Illinois). After TAM treatment, cells were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin.

DNA Isolation and Agarose Gel Electrophoresis

MCF-7 cells were treated with TAM (1×10^{-6} M) for two weeks and floating cells were collected every 3 days. Cells were lysed in TE-buffer (5 mM Tris-HCl (pH 7.5)-5 mM EDTA-0.5% Triton X-100). Low molecular weight DNA was extracted as described [Fukuda et al., 1993a] by ethanol precipitation of the supernatant. The DNA pellets were then dissolved in TE buffer, extracted twice with phenol/chloroform, and treated with 200 μg/ml RNase A at 65°C for 1 hour, followed by proteinase K (100 μg/ml) treatment at 37°C for 2 hours. After phenol/chloroform extraction again, precipitated DNA was resuspended in TE buffer, and DNA concentration was determined from the absorbance at 260 nm. Each DNA sample (500 ng) was dephosphorylated by calf-intestinal alkaline phosphatase (Gibco). End-labeling was carried out by T4 kinase and ^{32}P-γATP for 1 hour at 37°C. Following passage through a Sephadex G-50 column to remove free ATP, equal counts of samples were electrophoresed on a 1.5% agarose gel [Huang and Plunkett, 1992]. The gel was dried on DEAE membrane and exposed to Kodak X-ray film at −70°C.

In Situ Detection of DNA Fragmentation

MCF-7 cells were grown in Lab-Tek chamber slides with/without TAM for various times/doses and then fixed in 4% buffered paraformaldehyde. Apoptotic cells were then identified by TUNEL, an acronym introduced by Gavrieli et al. [1992] for a method using terminal deoxynucleotidyl transferase (TdT) to insert biotinylated-dUTP onto free 3′-ends of DNA; biotin signal is then detected by standard streptavidin-peroxidase/AEC. The in situ detection of DNA fragmentation was performed as described by Gavrieli et al. [1992]. Protein was stripped from DNA by incubation in proteinase K (25 μg/ml, 37°C, 10 min) and cells were washed 3 times (2 min each) in distilled water. Endogenous peroxidase was blocked by incubation in 0.6% hydrogen peroxide for 15 min. Slides were rinsed in distilled water and preincubated in TdT buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), covered with 30 units of TdT and 0.5 nmol of biotinylated-dUTP in 100 μl TdT buffer, and incubated in a humid chamber (37°C, 60 min). The reaction was stopped by transferring slides to TB buffer (300 mM NaCl, 30 mM sodium citrate) for 20 min. Slides were then rinsed in distilled water and incubated in 2% BSA for 30 min. Biotinylated-dUTP incorporated into DNA was visualized with streptavidin horseradish peroxidase and AEC chromagen to produce a red color. Slides were mounted in

GVA. In positive controls, a step prior to TdT treatment was introduced in which cells received 10 μg/ml type I DNase to allow all nuclei to label with TdT. Three negative controls were performed routinely: omission of either biotinylated-dUTP, TdT, or avidin peroxidase.

Analysis of TRPM-2, TGF-β1, and pS2 Gene Expressions by mRNA Determination

RNA extraction and Northern blotting analysis were performed as previously described in our laboratory [Cook and Chiu, 1986]. Briefly, total cellular RNA was isolated from MCF-7 cells and RNA samples were electrophoretically separated on formaldehyde-agarose denaturing gels [Lehrach et al., 1977]. RNA was then transferred to nitrocellulose filters, prehybridized, and hybridized to radioactively labeled TRPM-2, TGF-β1, or pS2 cDNA [Cook and Chiu, 1986]. Plasmids containing either TRPM-2, TGF-β1, or pS2 cDNAs were radioactively labeled by the random primer method [Feinberg and Vogelstein, 1983]. TRPM-2 cDNA plasmid [Leger et al., 1987] was obtained from Dr. Dr. Martin Tenniswood, W. Alton Jones Cell Science Center, Lake Placid, NY. TGF-β1 cDNA plasmid [Kondaiah et al., 1988] was obtained from Dr. Anita B. Roberts (NCI, NIH). pS2 cDNA plasmid [Masiakowski et al., 1982] was acquired from Dr. P. Chambon (Institut De Chimie Biologique, Strasbourg, France).

TGF-β1 Bioassay

Serum-free media were conditioned for various periods of time (from 24 to 72 h) by exponentially growing MCF-7 cells in DMEM media containing 10 μg/ml bovine insulin, penicillin (100 units/ml), streptomycin (100 units/ml), in the presence or absence of TAM (1×10^{-6}). Media were collected and then treated with PMSF (0.15 μM) and leupeptin (1 mg/ml). Transient acid treatment (1 h, 20°C, pH 2) and then neu-

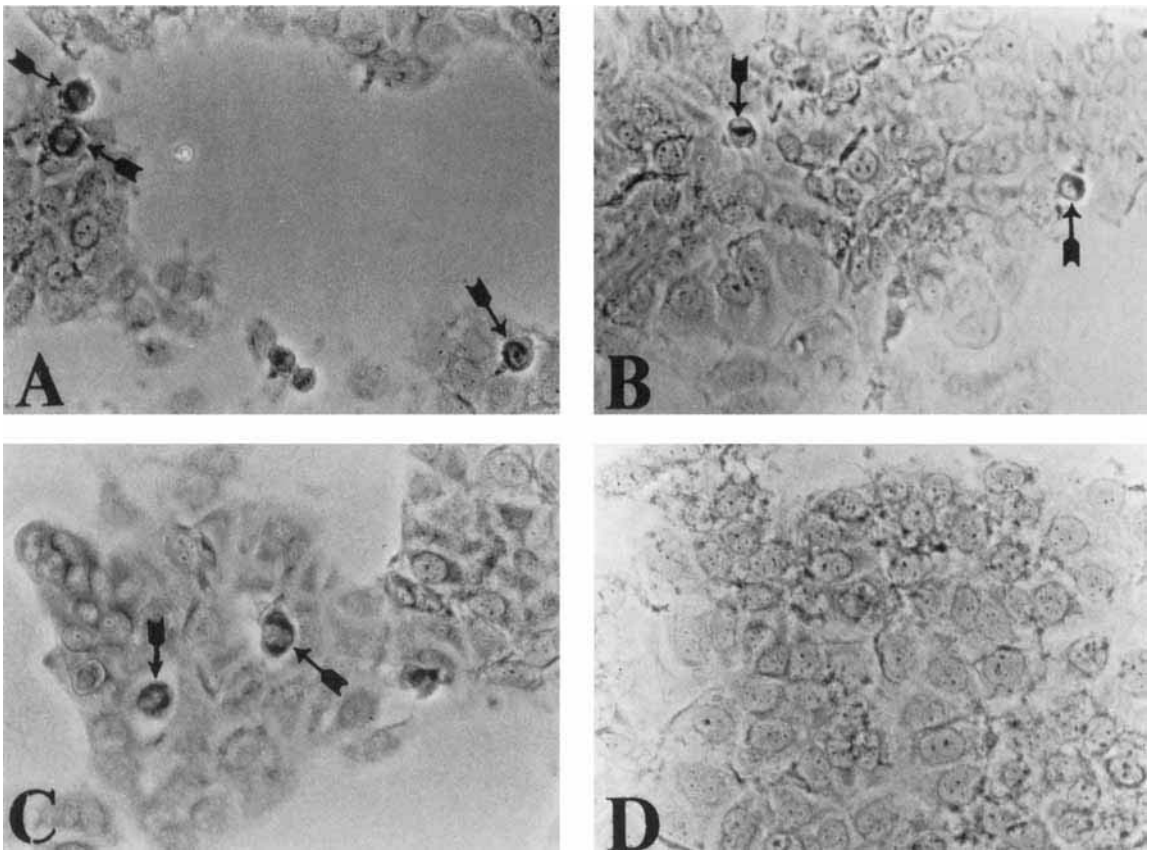

Fig. 1. H & E stain of MCF-7 cells treated with 1×10^{-6} M tamoxifen (**A–C**) or vehicle (0.1% ethanol) (**D**) for 3 days. Many scattered apoptotic cells (*arrows*) are observed in tamoxifen-treated MCF-7 cells. These apoptotic cells contain condensed nuclear fragments and irregular aggregation of chromatin. ×150.

tralization is necessary to measure total TGF-β1 activity (active + latent form). Conditioned media were adjusted to 0.5% BSA and 25 mM HEPES, and were tested for TGF-β1 activity in a Mv1Lu (mink lung epithelial cell) bioassay as previously described [Absher et al., 1991; Kelley et al., 1992]. Mv1Lu cells were obtained from ATCC (Rockville, MD) and maintained in high-glucose DMEM supplemented with 10% fetal calf serum. For assay, cells were incubated in DMEM plus 0.5% BSA and 25 mM HEPES.

RESULTS

To examine whether TAM induced apoptosis in MCF-7 breast cancer cells, MCF-7 cells were treated with either 1×10^{-6} M TAM or vehicle (0.1% ethanol) for 4 days. The morphological changes of MCF-7 cells following this treatment are shown in Figure 1A–C. Several apoptotic cells (indicated by arrows) were easily identifiable on H&E-stained samples by cell shrinkage with condensed and fragmented nuclei. For comparison, Figure 1D shows control cells without TAM treatment, where apoptotic features are absent. These data strongly suggested that increased MCF-7 cell death following treatment with TAM, as described by Bardon et al. [1987], is probably due to apoptosis.

To confirm morphologic changes suggestive of apoptosis, we determined if the nucleosomal fragmentation pattern typical of apoptotic cells also appeared in MCF-7 cells after TAM treatment. DNA was extracted from adherent and floating cells and analyzed by agarose gel electrophoresis after end-labeling with ^{32}P-γ-ATP [Huang and Plunkett, 1992]. Data is shown in Figure 2. Lanes 1 and 2 are the DNA samples isolated from MCF-7 cells treated with vehicle or TAM, respectively. DNA fragmentation did occur in MCF-7 cells treated with TAM but not in the cells treated with vehicle. However, the DNA is not fragmented into the typical nucleosomal size of 180–200 bp; rather, a distinctive pattern of larger size fragments was observed (Fig. 2).

The above procedures involve genomic DNA extraction and analysis on agarose gels, and do not allow distinction between individual cells. The method of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) [Gavrieli et al., 1992] was applied to in situ detection of DNA fragmentation in MCF-7 cells treated with 1×10^{-6} M TAM for 5 days. The controls were MCF-7 cells incubated without TAM for 5 days. At the end of the incubation period, cell samples were fixed and processed for TUNEL. Results shown in Figure 3 confirmed that substantial cell death take places in MCF-7 cells treated with TAM. Figure 3A shows a positive control in which MCF-7 cells without treatment of TAM were briefly treated with DNase I before the TUNEL assay; Figure 3B is a negative control in which terminal deoxynucleotidyl

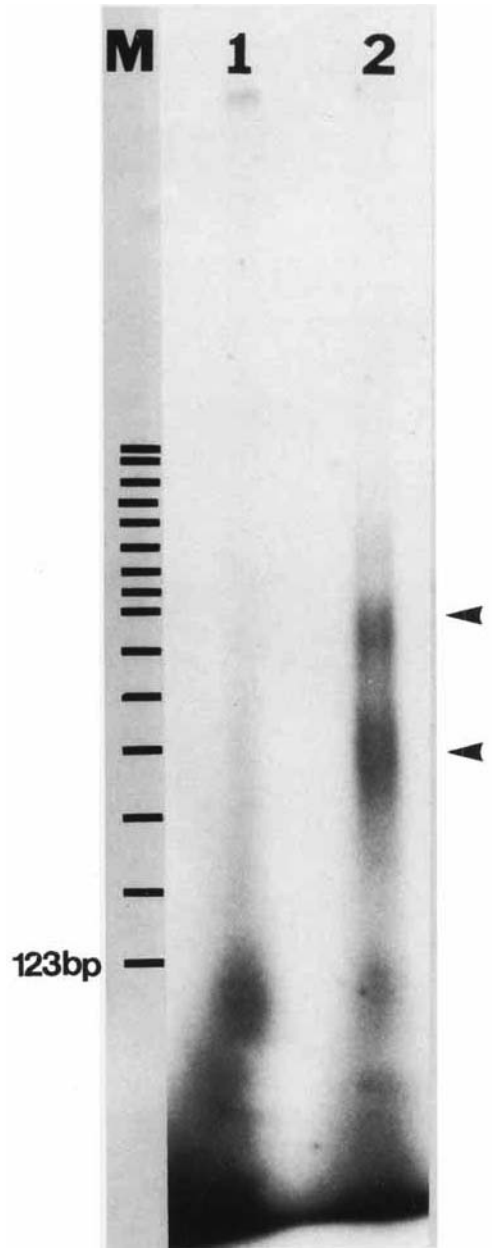

Fig. 2. Electrophoretic analysis of DNA isolated from breast cancer cells treated with (2) or without (1) 1×10^{-6} M tamoxifen for 12 days. *Lane M* contains 123 bp DNA molecular weight markers. *Arrows* indicate fragmented DNA.

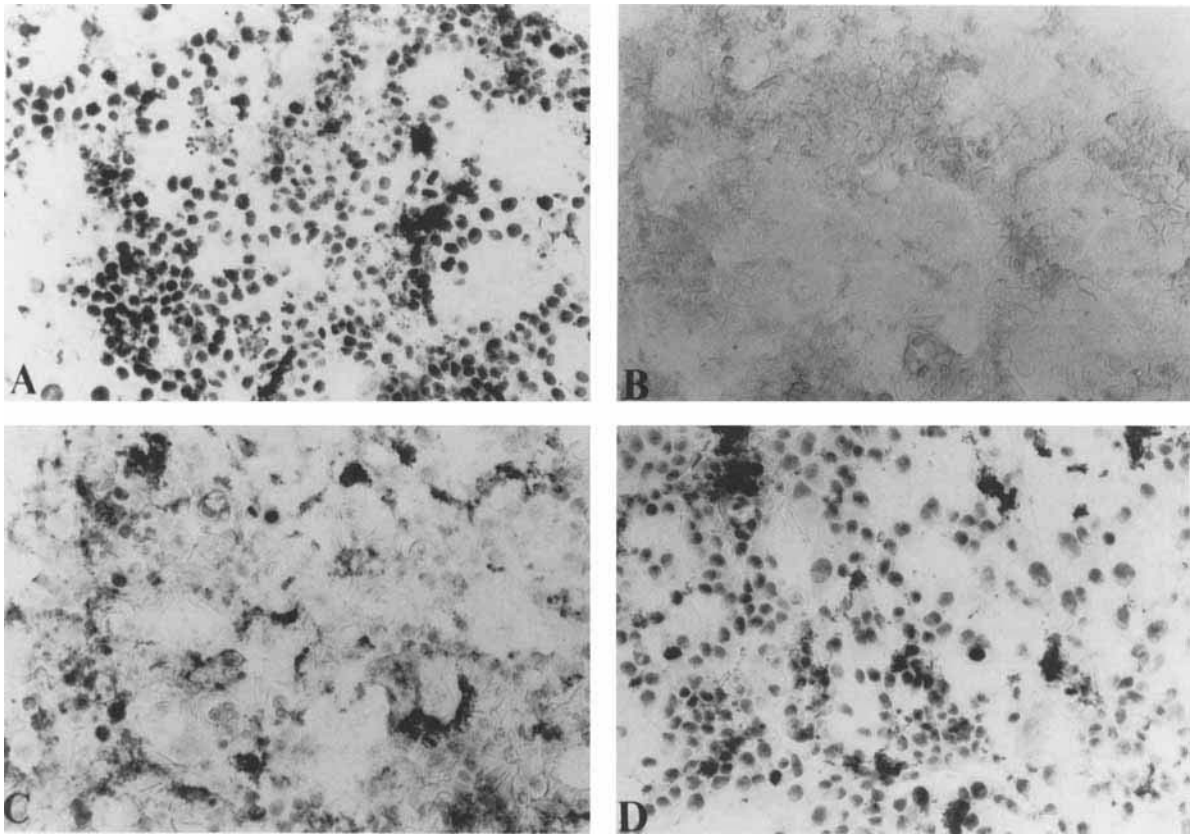

Fig. 3. In situ detection of DNA fragmentation in MCF-7 breast cancer cells by TUNEL. MCF-7 cells were cultured either with (**D**) or without (**A, B** and **C**) 1×10^{-6} M tamoxifen for 5 days. Panel A is a positive control in which MCF-7 cells were briefly treated with DNase I before TUNEL assay. B is a negative control in which terminal deoxynucleotidyl transferase was omitted. C: MCF-7 cells were treated with 0.1% ethanol vehicle only. D: Cells were treated with 1×10^{-6} M TAM for 5 days. The in situ detection of DNA fragmentation was performed as described by Gavrieli et al. [1992].

transferase was omitted from the assay. Figure 3C is MCF-7 cells without TAM treatment (0.1% ethanol vehicle only). Figure 3D is a sample of MCF-7 cells treated with 1×10^{-6} M TAM for 5 days. Though some baseline of staining in nuclei is observed in control cells, there is a marked increase in the number of positively stained nuclei following TAM treatment which correlated well with the increased observance of apoptotic cells seen morphologically (Fig 1).

A recent report [Wärri et al., 1993] demonstrated that elevated expression of TGF-β1 and TRPM-2 genes are associated with apoptosis in breast cancer cells induced by antiestrogen toremifene. To determine whether TAM similarly induces the expression of TGF-β1 and TRPM-2 genes in MCF-7 cells, total RNA was isolated from MCF-7 cells following treatment with or without 1×10^{-6} M TAM for 3 or 6 days, and then analyzed by Northern blotting. As shown in Figure 4, a substantial increase in the amount of TGF-β1 and TRPM-2 mRNA was observed in MCF-7 cells after TAM treatment for 3 days. However, thereafter, the level of TGF-β1 mRNA dropped to control levels while TRPM-2 mRNA continued to increase up to 6 days. In direct contrast, the mRNA level of estrogen-induced pS2 gene was strongly suppressed.

Knabbe et al. [1987] have demonstrated that secretion of active TGF-β is increased 8–27-fold following treatment of MCF-7 cells with growth-inhibitory concentrations of antiestrogens. To determine whether TAM also induced TGF-β1 secretion in our experiments with MCF-7 cells, we assayed MCF-7-conditioned media in the Mv1Lu cell-based bioassay for detecting TGF-β1 activity [Absher et al., 1991]. The amount of biologically active TGF-β1 produced by MCF-7 cells treated with or without 1×10^{-6} M TAM was presented in Figure 5. It increased more than 4-fold in the media from MCF-7 cells treated with TAM for 3 days (closed circle). This strong temporal relationship between induction of the TGF-β1 gene expression, secretion of active prod-

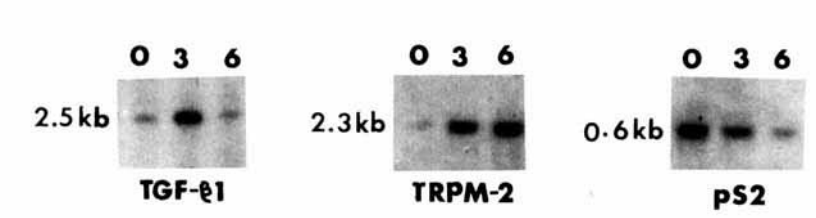

Fig. 4. The effect of tamoxifen on the expression of TGF-β1, TRPM-2, and pS2 genes in MCF-2 cells. Total RNA was isolated from MCF-7 cells after treatment with 1×10^{-6} M tamoxifen for 0, 3, or 6 days and analyzed by Northern blotting with ^{32}P-labeled TGF-β1, TRPM-2, and pS2 cDNAs.

uct, down-regulation of PS2, and MCF-7 cell apoptosis suggests that this growth factor may play a role in regulating cell death. Further study of the effects of exogenous TGF-β1 on MCF-7 cell death is required to more clearly delineate this possibility. To begin to address this, the effect of TGF-β1 on proliferation of MCF-7 breast cancer cells was studied. The inhibitory effects by TGF-β1 on MCF-7 breast cancer cell proliferation were dose-dependent on the concentration of TGF-β1 used. The relative adherent cell number (compared with that of control cultures) after a 3-day incubation in the presence of maximal inhibitory concentrations of TGF-β1 (10 ng/ml) was approximately 53% for MCF-7 cells (Fig. 6). At concentrations of 0.1 ng/ml or lower, TGF-β1 showed little growth-inhibitory effect on these breast cancer cells. Our data using the TUNEL assay clearly demonstrated that TGF-β1 can induce MCF-7 breast cancer cell apoptosis (Fig. 7). Thus, at least one

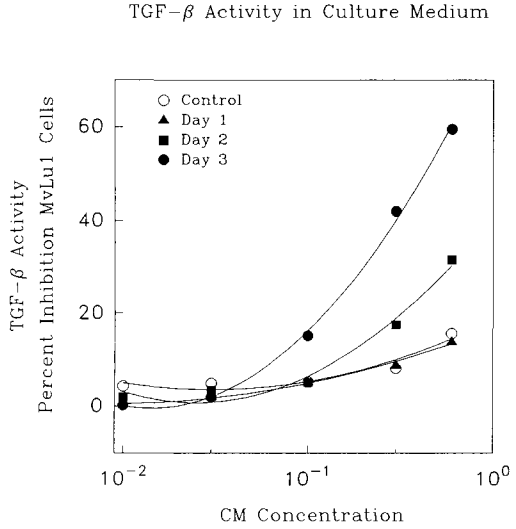

Fig. 5. Detection of TGF-β1 activity in serum-free conditioned media from MCF-7 cells treated with TAM for various days. TGF-β1 biological activity was assayed in 96-well microtiter plates of Mv1Lu cells as described [Absher et al., 1991].

mechanism by which TGFβ1 arrests growth is by the induction of apoptosis.

DISCUSSION

Many of the current anti-cancer drugs in use have been shown to ultimately cause apoptosis [Kaufmann, 1989; Eastman, 1990; Sen and D'Incalci, 1992]. Since apoptosis represents an autonomous suicide pathway that helps restrict cell numbers, a number of studies have explored the very attractive idea that tumor cells can be eliminated by artificially triggering death through apoptosis. Recently, Wärri et al [1993] demonstrated apoptosis in human breast cancer cells after treatment with the antiestrogen toremifene both in vivo and in vitro. In the present study, we have shown that TAM also causes growth inhibition and apoptosis of cultured MCF-7 breast cancer cells. Apoptosis was demonstrated by a classic morphologic appearance (condensation of chromatin: Fig. 1). Based on this observation, we then investigated this apoptotic progression induced by TAM by the 3'-end labeling TUNEL [Gavrieli et al., 1992], which detects internucleosomal DNA-breaks, thus allowing the in situ visualization of programmed cell death at the single-cell level. As shown in Figure 3, there was a marked increase in the number of stained nuclei in MCF-7 cells after treatment of TAM compared to control cells. The extent of programmed cell death in TAM-treated MCF-7 cells revealed by TUNEL is considerably greater than that detected by nuclear morphology, as shown in Figure 1. Thus TUNEL appears to be a sensitive method to detect apoptosis. In agreement with previous reports [Oberhammer et al., 1993], we also demonstrated that MCF-7 breast cancer cells exhibit a distinctive pattern of larger DNA fragments on agarose gel (Fig. 2).

In this study, we examined the temporal relationship of expression of genes reflecting either

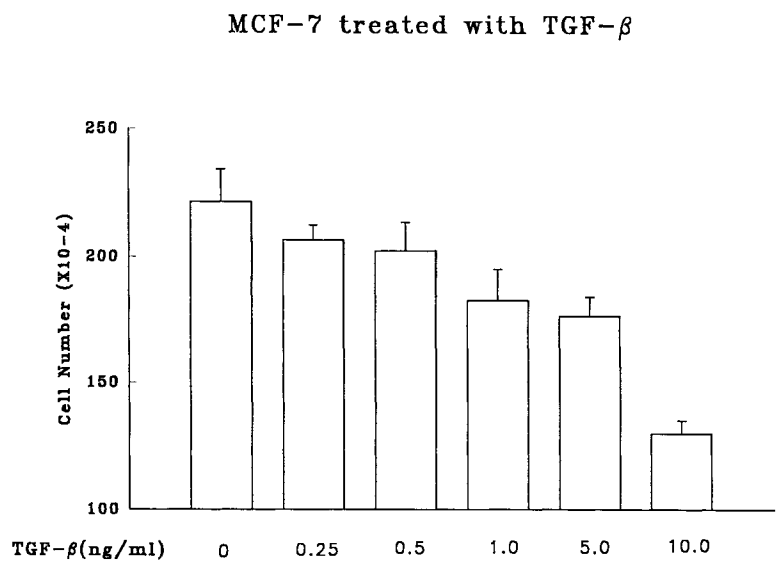

Fig. 6. The effect of TGF-β1 on proliferation of MCF-7 breast cancer cells. Breast cancer cells were incubated for 3 days in media containing various amounts of TGF-β1. The number of adherent cells in each culture dish was counted under hemacytometer.

"life" (pS2) or "death" (TRPM-2) and a potential indicator of these events (TGFβ1) during TAM treatment of MCF-7 cells. Testosterone-repressed prostate message-2 gene (TRPM-2) was originally cloned from regressing rat ventral prostate tissue [Leger et al., 1987] and has been shown to be induced during apoptosis in a large variety of other tissues, particularly of an endocrine nature [Buttyan et al., 1989]. An excellent temporal correlation between the expression of the TRPM-2 gene and activation of programmed cell death in human breast cancer cells and rat ventral prostate following hormone ablation was recently demonstrated by Kyprianou et al. [1990, 1991]. Although the function of TRPM-2 gene product remains unclear, it provides a remarkable and early indicator of programmed cell death in many types of mammalian cells. As shown in Figure 4, this also now seems to be true for TAM-induced apoptosis in MCF-7 cells. Interestingly, expression of estrogen-induced pS2 gene was concomitantly inhibited (Fig. 4). Similar results have also been reported by Wärri et al. [1993] in human breast cancer cells after treatment with toremifene.

TGF-β1 is a negative regulator of normal and tumoral mammary epithelial cells [Valverius et al., 1989]. There is accumulating evidence suggesting that increased expression of this gene is associated with programmed cell death in a variety of systems [Kyprianou and Issacs, 1989; Kyprianou et al., 1990, 1991; Rotello et al., 1991]. For example, the level of TGF-β1 mRNA increases during castration-induced apoptosis of rat ventral prostate [Kyprianou and Issacs, 1989]. Androgen-responsive prostatic cancer cells [Kyprianou et al., 1990] and estrogen-responsive breast cancer cells [Kyprianou et al., 1991] were also shown to have increased TGF-β1 expression during apoptosis following hormone deprivation. Secretion of active TGF-β1 was strongly induced in MCF-7 cells treated with a growth inhibitory concentration of the antiestrogen—LY117018 [Knabbe et al., 1987]. Collectively, these reports suggest an autocrine effect of TGF-β1 on the apoptotic process. Exogenous TGF-β1 has also been shown to induce apoptosis in cultured uterine epithelial cells [Rotello et al., 1991] and in the rat hepatoma cell line McA-RH7777 [Fukuda et al., 1993b], possibly indicating paracrine effects of TGF-β1.

Previously, Knabbe et al. [1987] have shown that TAM can induce autocrine secretion of TGF-β1 in human breast cancer cells, in which it inhibits breast cancer cell growth. TAM has also been shown to induce the secretion of active TGF-β1 from human fetal fibroblasts [Colletta et al., 1990] and from breast peritumoral stromal fibroblasts [Butta et al., 1992] despite an absence of ER in these cells. Our present data (Fig. 5) are in agreement with the results of Knabbe et al. that secretion of active TGF-β1 is induced following treatment of MCF-7 cells with antiestrogens. The steady-state level of TGF-β1

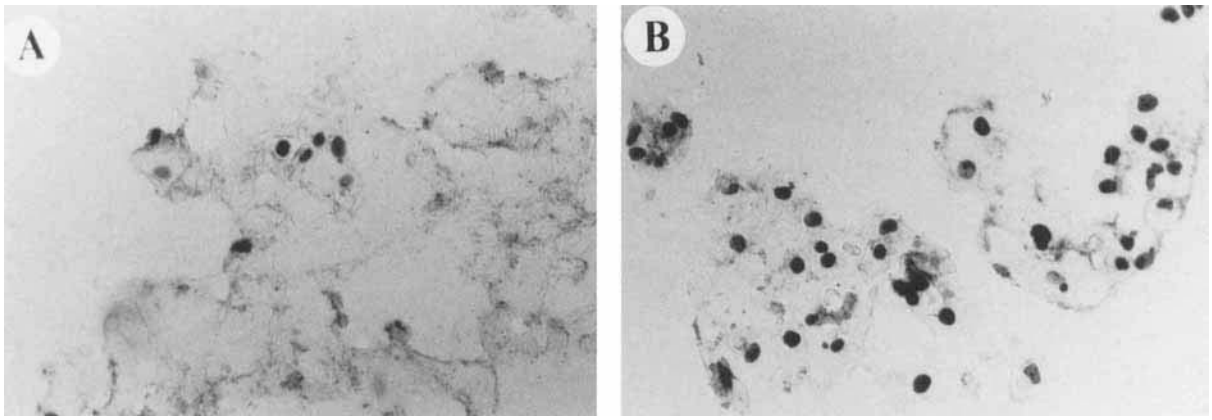

Fig. 7. In situ detection by TUNEL of DNA fragmentation in MCF-7 cells after treatment with (**B**) or without (**A**) TGF-β1 (0.5 ng/ml) for 3 days. Apoptotic cells were strongly stained.

mRNA was also enhanced by antiestrogens at early stage of the treatment (Fig. 4). Our results, however, contradict the data of Wärri et al. [1993], who reported that TAM decreased the steady-state level of TGF-β1 mRNA in MCF-7 cells. The reason(s) underlying this discrepancy are unclear at this time.

In summary, the results of the present study strongly suggest that programmed cell death is one of the mechanisms to cause growth inhibition of MCF-7 breast cancer cells induced by the anti-estrogen tamoxifen, and that this may be mediated by the secretion of active TGF-β1.

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