

Effects of Estrogen on Apoptotic Pathways in Human Breast Cancer Cell Line MCF-7

Thomas T. Y. Wang¹ and James M. Phang

Laboratory of Nutritional and Molecular Regulation, National Cancer Institute-Frederick Cancer Research and Development Center, NIH, Frederick, Maryland 21702-1201

Abstract

We utilized a reverse transcription-PCR method to examine the effect of estrogen on the expression of mRNA for Bcl-2 and Bax, two modulatory proteins in the apoptotic pathway, in human breast cancer cell line MCF-7. We found that the *bcl-2* mRNA levels in the cells exposed to 17 β -estradiol were higher than those of control cells. Although the relative *bax* mRNA levels remained unchanged, the changes in *bcl-2* mRNA level occurred in a time- and concentration-dependent fashion. In addition, pretreatment with 17 β -estradiol protected MCF-7 cells from apoptosis. Our study provides evidence that responses of breast epithelial cells toward a steroid sex hormone involve regulation of the apoptotic pathway.

Introduction

Apoptosis (programmed cell death) is a normal physiological phenomenon that can be observed in various tissues. Cells undergoing apoptosis are characterized by distinct biochemical and morphological changes (1). It is known that apoptosis plays a role in differentiation processes, such as formation of digits during embryogenesis and selection of lymphocyte populations. In addition, perturbation of the apoptotic pathway has been found to be associated with tumorigenesis (2, 3). The protein Bcl-2 has been shown to be a component of the apoptotic pathway. Overproduction of Bcl-2 protein resulted in blockage of apoptosis and increased survival upon external "death" stimuli (4). The antiapoptotic activity of Bcl-2 is thought to be modulated by the protein Bax, and it has been proposed that the Bcl-2:Bax protein ratio may determine whether a cell would undergo apoptosis (5, 6). Breast epithelium undergoes cyclic apoptosis and fluctuation in Bcl-2 protein level during the menstrual cycle (7). Clinical studies showed a relationship between estrogen receptor expression and Bcl-2 protein level (8). Thus, steroid sex hormones such as estrogen may regulate Bcl-2, Bax levels, and, consequently, apoptosis. Exposure to estrogen has been associated with increased incidence of breast cancer (9); hence, a better understanding of its role in apoptosis could be important in the prevention and treatment of breast cancer. In the present study, we utilized estrogen receptor-positive human breast cancer MCF-7 cells as a model to address two questions: (a) Is *bcl-2/bax* mRNA expression regulated via estrogen's action? and (b) Does estrogen regulate the apoptotic pathways?

Materials and Methods

Cell Culture and Total RNA Isolation. MCF-7 cells were maintained in medium A [RPMI 1640 with 2 mM L-glutamine, 3.5 ng/ml hydrocortisone, 1.5 ng/ml insulin, 100 units/ml penicillin, and 100 μ g/ml streptomycin] with 5% fetal bovine serum in 75-cm² flasks (Falcon, Lincoln Park, NJ) and grown in the presence of 5% CO₂ in air at 37°C. One week before initiation of

experiments, cells were switched to medium A supplemented with 5% charcoal dextran-treated fetal bovine serum. Cells were grown to confluence and passaged with the use of trypsin-EDTA. Viable cells were quantitated by trypan blue exclusion. One day before treatment, cells were plated in medium B [phenol red-free RPMI 1640 containing 2 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 5% charcoal dextran-treated fetal bovine serum]. Total RNA was isolated from MCF-7 cells grown in 6-well Costar plates (5×10^5 cells/well in 3 ml of medium B) by a method described previously (10).

Quantitation of *bcl-2* and *bax* mRNA Levels. We utilized a competitive reverse transcription-PCR method to quantitate *bcl-2* and *bax* mRNA levels (11). The procedure used one set of primers to amplify both target cDNA and an externally added MIMIC² of known concentration. MIMICs for both *bcl-2* and *bax* quantitation were constructed with the use of the PCR-MIMIC construction kit (Clontech, Palo Alto, CA) following the manufacturer's direction. The primer pairs used for amplification of *bcl-2* and *bax* mRNA and construction of MIMIC fragments are listed in Table 1. We routinely used 1 μ g of RNA for cDNA synthesis (Clontech). After first-strand cDNA synthesis reaction, the cDNA was made into 100 μ l final volume, and 4 μ l were used for PCR. A typical PCR consisted of 0.2 μ M dNTP, 2 μ l MIMIC, 4 μ l cDNA, 0.2 μ M of each primer, PCR buffer, and Taq polymerase. The PCR profile was 95°C for 45 s, 65°C for 45 s, and 72°C for 2 min for 35 cycles, followed by 72°C for 7 min. After PCR, aliquots of the reaction were analyzed on 1.8% agarose gel (0.50 μ g/ml ethidium bromide). The amount of mRNA was quantitated by comparing relative intensities of the amplified MIMIC and specific message bands. All results were normalized against G3PDH mRNA quantitated with the use of identical PCR conditions. Results are expressed as level of expression relative to G3PDH. The primer pairs and competitor for G3PDH were purchased from Clontech.

Apoptotic Death Assay. We used DNA fragmentation as the criteria for apoptotic cell death. DNA fragmentation was measured with the use of the cell death ELISA (Boehringer Mannheim, Indianapolis, IN). MCF-7 cells (1×10^4 cell) were plated in each well of 24-well plates. After appropriate treatment, the cells were washed once with PBS, and 0.5 ml lysis buffer was added. After a 30-min incubation, the supernatant was recovered and assayed for DNA fragments according to manufacturer's protocol. Each treatment was performed in triplicate. Additional plates identically treated as above were analyzed for cell number with the use of the sulforhodamine assay (12). The A₄₀₅ obtained from the DNA fragmentation assay was then normalized for cell number, and the results are expressed relative to untreated control.

Results

Effects of Estrogen on *bcl-2* and *bax* mRNA Level. To examine the effects of estrogen on the apoptotic pathway, we used the human breast cancer cell line MCF-7 and monitored mRNA expression of apoptosis-related proteins Bcl-2 and Bax. The MCF-7 cells are a good model because they expressed estrogen receptor and, as in our initial study, also expressed both *bcl-2* and *bax* mRNA (data not shown). A competitive reverse transcription-PCR protocol was used to determine *bcl-2* and *bax* mRNA level. The primer for synthesis of MIMIC and the expected size for the target and the MIMIC are listed in Table 1. In an initial experiment, MCF-7 cells were treated with 10⁻⁹ M

Received 3/20/95; accepted 5/5/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed, at Laboratory of Nutritional and Molecular Regulation, Building 434/2, National Cancer Institute-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702-1201.

² The abbreviations used are: MIMIC, competitor DNA fragment; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

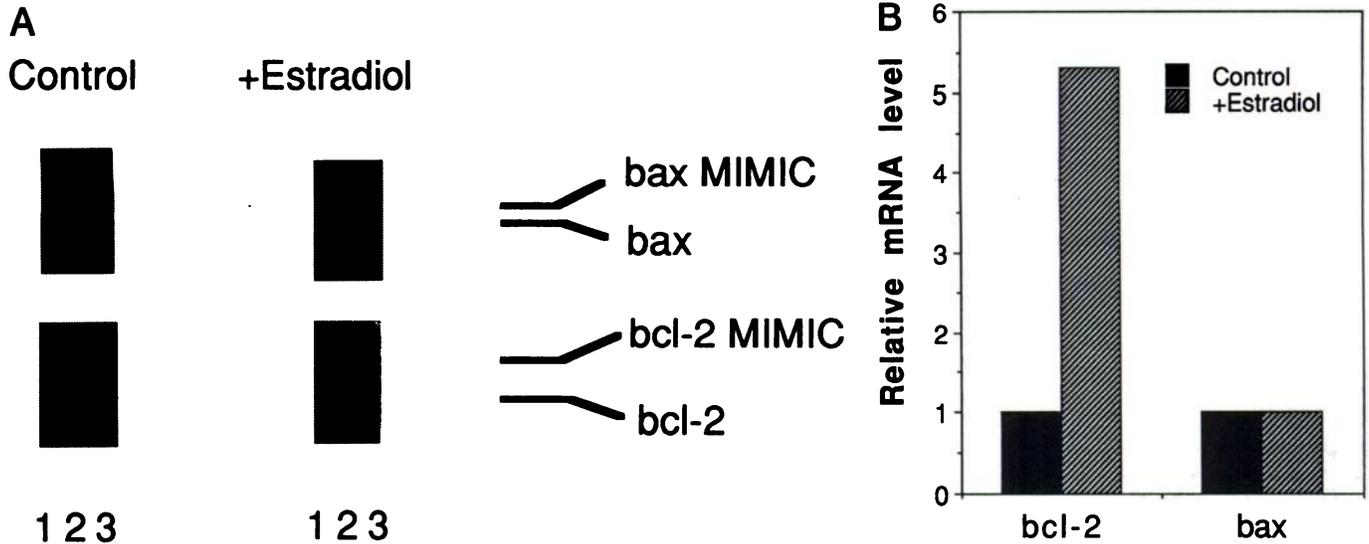


Fig. 1. Effects of 17β -estradiol on *bcl-2* and *bax* mRNA level. A, representative gel showing reverse transcription-PCR for *bcl-2* and *bax*. Lanes 1–3, 1:3 serial dilution of MIMIC. B, relative *bcl-2* and *bax* mRNA levels in control and 17β -estradiol (10^{-9} M)-treated MCF-7 cells. Cells were treated with or without estradiol for 48 h.

Table 1 Primer pairs for construction of MIMIC and amplification of *bcl-2* and *bax* mRNA

<i>bcl-2</i> MIMIC (620 bp)	
Forward	5'-ACTTGTGGCCAGATAGGCACCCAGTTGAGTCCATGGGGAGCTTT-3'
Reverse	5'-CAGCTTCGCCGAGATGTCCAGCCAGCGCAAGTCAAATCTCCTCCG-3'
<i>bax</i> MIMIC (620 bp)	
Forward	5'-CAGCTCTGAGCAGATCATGAAGACACGCAAGTCAAATCTCCTCCG-3'
Reverse	5'-GCCCATCTTCTCCAGATGGTGAGCTTGTAGTCCATGGGGAGCTTT-3'
<i>bcl-2</i> mRNA (385 bp)	
Forward	5'-ACTTGTGGCCAGATAGGCACCCAG-3'
Reverse	5'-CGACTTCGCCGAGATGTCCAGCCAG-3'
<i>bax</i> mRNA (538 bp)	
Forward	5'-CAGCTCTGAGCAGATCATGAAGACA-3'
Reverse	5'-GCCCATCTTCTCCAGATGGTGAGC-3'

17β -estradiol, and the relative level of *bcl-2* and *bax* mRNA was quantitated. Shown in Fig. 1A is a representative gel for analysis of PCR products. We found that treatment with 17β -estradiol resulted in an increase in *bcl-2* mRNA level, but produced no change in *bax* mRNA level (Fig. 1B). Observing a change in relative *bcl-2* mRNA level upon treatment with 17β -estradiol, we then further characterized this effect. We found that the increase in *bcl-2* mRNA level with 17β -estradiol was time dependent (Fig. 2A) for up to 48 h. In addition, the increase in *bcl-2* mRNA level accompanied the increase in estradiol concentration (Fig. 2B). The effect of 17β -estradiol on *bcl-2* mRNA levels was seen at estradiol concentrations as low as 10^{-11} M and plateaued at 10^{-10} M.

Effect of Tamoxifen on Estrogen-stimulated *bcl-2* mRNA Expression. To determine whether the increase in *bcl-2* mRNA was due to specific interaction between estradiol and estrogen receptor, we examined the effect of tamoxifen, an antiestrogen, on estradiol-stimulated *bcl-2* expression. We found that addition of tamoxifen (10^{-6} M) completely inhibited the stimulatory effects of estradiol, thus supporting the possibility that increased in *bcl-2* mRNA levels involve the interaction of estradiol with the estrogen receptor.

Effects of Estrogen on Apoptosis. The observation that estradiol increases *bcl-2* mRNA level would suggest that estradiol may inhibit

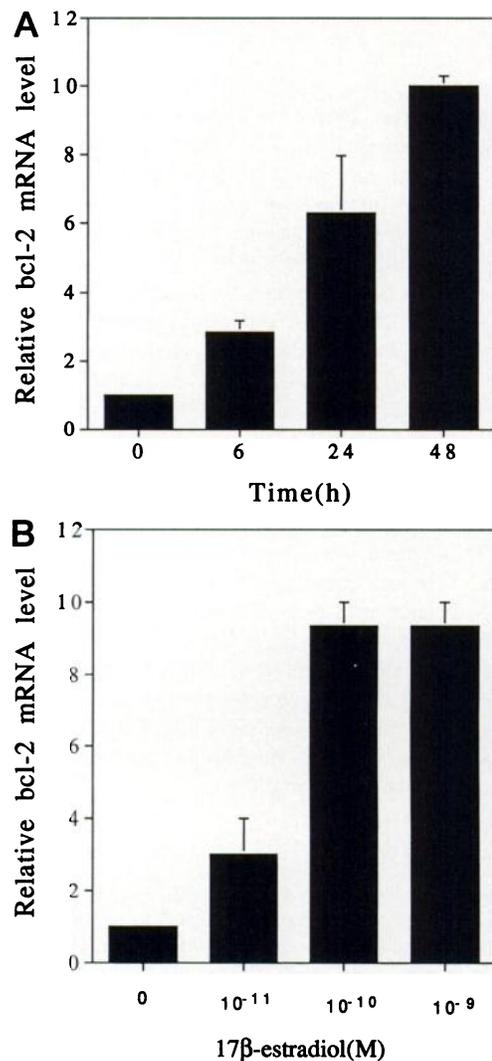


Fig. 2. Time- and concentration-dependent increase of *bcl-2* mRNA level. A, time-dependent increase of *bcl-2* mRNA level upon treatment with 17β -estradiol (10^{-9} M). Columns, mean; bars, SE ($n = 3$); $P < 0.0001$ (ANOVA). B, effects of various concentrations of 17β -estradiol on *bcl-2* mRNA level. Treatments were for 48 h. Columns, mean; bars, SE ($n = 3$); $P < 0.0001$ (ANOVA).

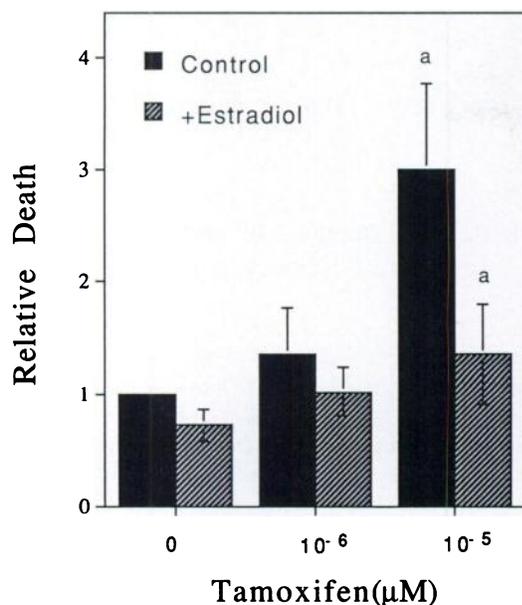


Fig. 3. Effect of 17β -estradiol pretreatment on tamoxifen-stimulated apoptosis. Cells were treated with 17β -estradiol (10^{-10} M) for 24 h, after which the media were removed and replaced with media containing tamoxifen (10^{-6} and 10^{-5} M) for an additional 24 h. Apoptotic death was determined as described in "Materials and Methods." Columns, mean; bars, SE ($n = 3$). a, $0.01 < P < 0.025$ (t test).

apoptotic pathways. We then examined the effect of pretreatment of cells with estradiol on apoptosis induced by tamoxifen. Cells were incubated with and without 17β -estradiol (10^{-10} M) for 24 h, by which time media were replaced with media containing tamoxifen. Apoptotic cell death was determined by monitoring DNA fragmentation. We found that cells pretreated with 17β -estradiol (10^{-10} M) appeared to be more resistant to tamoxifen-induced apoptosis (Fig. 3).

Discussion

In summary, we have demonstrated that treatment of MCF-7 cells with 17β -estradiol resulted in increased *bcl-2* mRNA levels but did not affect *bax* mRNA levels. Estradiol stimulated a time- and concentration-dependent increase in *bcl-2* mRNA. The estradiol-stimulated increase could be blocked by tamoxifen, suggesting that it was mediated by an interaction between estradiol and estrogen receptor. Moreover, pretreatment of MCF-7 cells with estradiol appeared to protect cells from apoptosis.

Our *in vitro* study using MCF-7 cells as a model provides direct evidence that steroid sex hormones such as estrogen can inhibit apoptosis by increasing the production of Bcl-2, an antiapoptotic protein. Our finding is consistent with clinical studies that correlated detection of Bcl-2 protein with expression of estrogen receptor (7, 8). These findings also support the hypothesis (7) that modulation of apoptosis may be a mechanism by which estrogen influences breast cancer risk. In addition, additional characterization of the role of estrogen in apoptotic pathways could lead to design of antitumor drug(s) that target this pathway.

The differential regulation of *bcl-2* and *bax* mRNA by estrogen is interesting. One may hypothesize that, at least in the case of breast epithelial cells, cell survival depends on external mitogenic signal. The survival of cell is enhanced by an increase in the production of antiapoptotic protein Bcl-2 to counteract death signal by Bax.

References

- Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, 26: 239–257, 1972.
- Carson, D. A., and Ribeiro, J. M. Apoptosis and disease. *Lancet*, 341: 1251–1254, 1993.
- Mikulski, S. M. Pathogenesis of cancer in view of mutually opposing apoptotic and anti-apoptotic growth signals (Review). *Intl. J. Onc.*, 4: 1257–1263, 1994.
- Reed, J. C. Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.*, 124: 1–6, 1994.
- Oltvai, Z. N., Millman, C. L., and Korsmeyer, S. J. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, 74: 609–619, 1993.
- Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L., Wang, H-G., and Reed, J. C. Interaction among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. *Proc. Natl. Acad. Sci. USA*, 91: 9238–9242, 1994.
- Sabourin, J. C., Martin, A., Baruch, J., Truc, J. B., Gompel, A., and Poitout, P. *bcl-2* expression in normal breast tissue during the menstrual cycle. *Int. J. Cancer*, 59: 1–6, 1994.
- Gee, J. M. W., Robertson, J. F. R., Ellis, I. O., Willsher, P., McClelland, R. A., Hoyle, H. B., Kyme, S. R., Finlay, P., Blamey, R. W., and Nicholson, R. I. Immunocytochemical localization of Bcl-2 protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. *Int. J. Cancer*, 59: 619–628, 1994.
- Nenci, I., Marchetti, E., and Querzoli, P. Commentary on human mammary preneoplasia. The estrogen receptor-promotion hypothesis. *J. Steroid Biochem.*, 30: 105–106, 1988.
- Sathyamoorthy N., Wang, T. T. Y., and Phang, J. M. Stimulation of pS2 expression by diet-derived compounds. *Cancer Res.*, 54: 957–961, 1994.
- Wang, A. M., Doyle, M. V., and Mark, D. F. Quantitation of mRNA by polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, 86: 9717–9721, 1989.
- Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.*, 82: 1107–1112, 1990.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Effects of Estrogen on Apoptotic Pathways in Human Breast Cancer Cell Line MCF-7

Thomas T. Y. Wang and James M. Phang

Cancer Res 1995;55:2487-2489.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/55/12/2487>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/55/12/2487>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.