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 17β-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 17β-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 μg/ml streptomycin] with 5% fetal bovine serum in 75-cm2 flasks (Falcon, Lincoln Park, NJ) and grown in a 30% 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 μg/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 × 105 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 MIMIC 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 directions. 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 μg of RNA for cDNA synthesis (Clontech). After first-strand CDNA synthesis reaction, the cDNA was made into 100 μl final volume, and 4 μl was used for PCR. A typical PCR consisted of 0.2 μM dNTP, 2 μM MIMIC, 4 μl cDNA, 0.2 μM 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 μg/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.

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 × 104 cells) 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 the 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 A540 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 estrogen for 24 h. We found that the bcl-2 mRNA levels in the cells exposed to 17β-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 17β-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.
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 estrogen-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...
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**

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

Thomas T. Y. Wang and James M. Phang