Stimulation of c-myc Oncogene Expression Associated with Estrogen-induced Proliferation of Human Breast Cancer Cells

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ABSTRACT

Regulation of c-myc expression is known to be sensitive to a variety of mitogenic stimuli in various cell types. Since estrogen is a well-documented mitogen of estrogen-responsive human breast cancer (HBC) cells, we studied the influence of estradiol and its antagonist tamoxifen on the expression of c-myc in HBC cell lines. Using Northern hybridization analysis, we monitored the accumulation of c-myc mRNA in a number of HBC cell lines. The cell lines studied included the estrogen-responsive, estrogen receptor positive (ER+) MCF-7, T-47D, the nonresponsive, ER+ T-211, and a nontumorous breast cell line, HBL-100. The effects of endogenous estrogen were minimized by culturing the cells in medium containing 10% (v/v) charcoal-treated fetal bovine serum and tamoxifen (10^-6 M) for at least 15 min prior to estradiol treatment. In the ER+ cell lines the addition of estradiol resulted in a noticeable increase in c-myc expression after 15 min with a maximal (2-10-fold) induction in 1-2 h. In the ER- cell lines the level of c-myc mRNA was high and was unaffected by estrogen or tamoxifen; in the ER- cancer cell lines, no amplification nor rearrangement of the c-myc gene was observed. In contrast, the expression of another oncogene, c-H-ras, was not affected in both ER+ and ER- cell lines and was insensitive to estrogen and antiestrogen. These results suggest that regulation of c-myc expression may be an important step in estrogen-induced proliferation of HBC cells.

INTRODUCTION

A group of genes, known as protooncogenes, has been recognized as being important in cancerous proliferation and transformation (1-3). Of these protooncogenes the importance and function of c-myc is among the most studied. This is due to the fact that amplification, rearrangement, and translocation of the c-myc gene or variations in c-myc mRNA levels have repeatedly been observed in various tumors, differentiating cell lines, and mitogenically stimulated cell lines (4-9). In breast carcinomas amplification and rearrangement of c-myc has been recognized (5). In some of these carcinomas these changes have been associated with enhanced c-myc mRNA expression. Clinically, about one-third of all breast tumors are estrogen responsive, in that estrogen stimulates their proliferation (10). To better understand the action of estrogen in HBC we therefore chose to investigate the effects of this steroid hormone on c-myc mRNA expression in several HBC cell lines.

MATERIALS AND METHODS

Tissue Culture. The sources of all HBC cell lines were described previously (11). Stock cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 1-glutamine (4 mM), glucose (4.5 g/liter), penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively), bovine insulin (10 µg/ml), and 10% (v/v) fetal bovine serum. Trypsin/EDTA in Hanks' balanced salt solution was used for cell passage. All reagents were obtained from Flow or Gibco Laboratories. The cells were kept in a humidified atmosphere of 95% air:5% CO2 at 37°C. Hormones. 17β-Estradiol was purchased from Sigma Chemical Co., St. Louis, MO. Tamoxifen base was generously provided by Imperial Chemical Industries Pharma, Missassauga, Ontario. Stock solutions in ethanol were prepared each month for both hormones. Aqueous solution prepared from these stocks were used to treat the cells. The stock solutions were stored in the dark at -20°C.

Growth Response Studies. For growth studies, cells were plated at a density of 1 x 10^5 cells/cm^2 in insulin-free medium containing FBS (C) and left for 48 h. The medium was then changed to either C' (containing cFBS) (12), or T' (C' with 10^-6 M tamoxifen). After 48 h, estradiol (final concentration, 10^-7 M) was added to C' and T'. These conditions were then denoted as E and TE, respectively. This concentration of tamoxifen (10^-6 M) has previously been shown to achieve inhibition of cell growth without cytotoxicity, and the concentration of estradiol (10^-7 M) was used as a surrogate for the stimulation of cell growth in the presence of tamoxifen (13). A corresponding volume of ethanol was added to dishes not receiving estradiol; the final concentration of ethanol was 0.007% in all dishes. The cells were then allowed to grow for a further 8-10 days without medium change. After this time the cells were trypsinized, dispersed through an 18-gauge needle, and the cell number determined using an electronic cell (Coulter) counter.

Preparation of Cells for RNA Extraction. Stock cells were plated at 0.7 x 10^6 cells/cm^2 in medium C and were left to attach for 48 h. This medium was then replaced with either insulin-free medium containing 10% (v/v) cFBS with (T') or without (C') 10^-6 M tamoxifen for a further 48 h. Estradiol (10^-7 M) was then added and at various times after estradiol addition, cells were harvested for RNA isolation.

RNA Isolation and Northern Hybridization Analysis. Total RNA was isolated by the guanidinium isothiocyanate/cesium chloride method (14). For Northern blot analysis this RNA was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, fractionated on a 1% (w/v) agarose-2.2 M formaldehyde denaturing gel, and then transferred onto nitrocellulose paper as described by Maniatis et al. (15). The RNA was fixed onto the nitrocellulose by baking for 2 h at 80°C. The blot was then prehybridized in hybridization solution containing 50% (v/v) formalamide (16) at 42°C for at least 3 h. Nick-translated cDNA probe (1-5 x 10^6 cpm/µg DNA) was then added. Hybridization was carried out at 42°C for 16-20 h. Following hybridization the blots were washed twice in 1 x standard saline citrate-0.1% sodium dodecyl sulfate for 15 min and then once in 0.1 x standard saline citrate-0.1% sodium dodecyl sulfate at 65°C for 1 h. Blots were then exposed to Kodak XAR film at -70°C with an intensifying screen. When nitrocellulose blots were reused, they were first boiled in water for 3 min.

For dot blot analysis RNA isolated as described above was denatured according to the protocol of White and Bancroft (17) and spotted onto nitrocellulose filters using a BRL dot-blot manifold. Filters were then hybridized as previously described.

Quantitation of the relative amounts of specific RNA transcripts was performed by densitometric scanning of the hybridization signals. 

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RESULTS

Cell Response to Estrogen and Tamoxifen. In order to maximize the effects of estradiol on HBC cells it was necessary to deplete the endogenous levels of estrogen present in both cells and growth medium. This was done by using cFBS in the medium and by adding a growth inhibitory, nontoxic, concentration of tamoxifen (10^{-6} M) to offset the effects of any residual estrogens. The growth response data for T-47D and MCF-7 are summarized in Fig. 1. Partial growth inhibition was observed in estrogen-depleted medium (C') and was further suppressed on addition of tamoxifen (T') when compared with cells growing in the presence of untreated FBS (C) which contains endogenous estrogens. This growth inhibition was reversed by estradiol (10^{-7} M). Although Fig. 1 shows cell growth 10 days after various treatments, we have also measured the growth response in the first 48 h, the time interval where the fold increase in cell number for the five groups was C, 2.0; C', 1.3; T', 1.2; E', 1.8; and TE', 1.6. These data correspond well with the results in Fig. 1.

Using dot blot analysis, the estrogen nonresponsive BT-20 tumor line and the nontumorous HBL-100 line showed no regulation of c-myc expression by either estradiol or tamoxifen in contrast to MCF-7 and T-47D cells (Fig. 5). These estrogen nonresponsive cell lines, similar to the MDA-MB-231 cell line, expressed a higher level of c-H-ras mRNA which was unaffected by tamoxifen and estradiol.

Comparison of c-myc and c-H-ras Expression in ER+ and ER- HBC Cells. The patterns of expression of c-myc and c-H-ras, 60 min after estradiol rescue, in MCF-7 (ER+) and MDA-MB-231 (ER-) cells are depicted in Fig. 4. Whereas tamoxifen inhibited and estradiol stimulated c-myc expression in MCF-7, they had no effect on c-myc expression in MDA-MB-231 cells. It is of interest to note that the MDA-MB-231 cell line expressed a higher level of c-H-ras mRNA which was unaffected by tamoxifen and estradiol.

 Estradiol-Induced c-myc Expression

Fig. 2 depicts the time course of c-myc and c-H-ras mRNA accumulation in tamoxifen-inhibited MCF-7 cells "rescued" by estradiol. A significant increase in c-myc mRNA accumulation was observed after 15 min of estradiol rescue, with maximal levels (>10-fold) achieved between 60 and 90 min. The level of c-myc expression gradually declined for the next 10 h and remained at a level 3-fold above tamoxifen-treated cells for at least 54 h thereafter (Fig. 3). Fig. 3 also reveals a similar pattern of induction in another ER+ cell line, T-47D, although the maximal accumulation of c-myc mRNA occurred around 2 h after estradiol rescue. In contrast, the expression of c-H-ras was unaffected by either estradiol or tamoxifen (Fig. 2c).

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Fig. 3. Time course of the effect of estradiol on the accumulation of c-myc mRNA in ER+ MCF-7 and T-47D cells. Results are normalized in relation to tamoxifen growth-arrested cells prior to addition of estradiol (t = 0).

Fig. 4. Comparison of c-myc and c-H-ras expression in ER+ MCF-7 and ER- MDA-MB-231 cells. Total RNA (50 μg) was applied to each lane. Conditions C, C', T', E', and TE' are as described in the legend to Fig. 2. Exposure to estradiol (10^{-7} M) was 1 h. The blot was hybridized to c-myc and v-H-ras probes and exposed to film as described in the legend to Fig. 2. Also shown is the quantitation of the relative amounts of c-myc and c-H-ras, normalized to levels present in tamoxifen-inhibited cells (T').

Fig. 5. Dot blot analysis of regulation of c-myc mRNA expression in human breast cell lines. Cells were grown and RNA isolated as described in "Materials and Methods." Growth conditions and estradiol treatment were identical to those in Fig. 4. The blot was hybridized to c-myc insert probe (1 x 10^{6} cpm/ml; film exposure was 14 h). For clarity, hybridizations to only one concentration of RNA (0.025 μg) is shown.

also exhibited high, estrogen-independent, constitutive expression of c-myc mRNA. To determine whether this high level of c-myc expression in the estrogen nonresponsive cells was a result of gene rearrangement or amplification, Southern analysis of the c-myc gene was performed. The result of this study, shown in Fig. 6, indicates no rearrangement or amplification of the c-myc gene in any of the breast cancer cell lines. However, the nontumorous breast cell line HBL-100 has approximately 4-fold amplification, but no rearrangement of the c-myc gene.

DISCUSSION

We have shown that an induction (>10-fold) of c-myc mRNA occurs between 1 and 2 h after addition of estradiol to ER+ HBC cells growth retarded by the use of tamoxifen and cFBS. This time course is similar to c-myc induction by estradiol in the rat uterus (21) or by serum in hamster lung fibroblasts (4) and aortic smooth muscle cells (22). The higher level of c-myc expression in cells grown in medium containing untreated FBS (C, Figs. 2 and 3) is due to endogenous estrogens present in the FBS. Besides the estrogens present in FBS, phenol red, a pH indicator used in the growth medium, has been reported to have a weak estrogenic effect on ER+ cells (23). To offset the effects of phenol red and the residual estrogens associated with the cells, we added tamoxifen to our culture medium. This resulted in a 30–50% decrease in cell growth, when compared to that of cells grown in medium supplemented with cFBS alone (Fig. 1). We had also observed that c-myc induction was less marked if the cells were plated at more than 2.1 x 10^{4} cells/cm^2 (data not shown). One possible explanation is that ER+ HBC cells normally, or as a result of cell-cell contact, produce internal or external factors that when present and at a sufficiently high concentration can overcome the effects of estrogen depletion. Therefore each dish was plated with no more than 0.7 x 10^{4} cells/cm^2.
The results of our studies together with previously reported data offer a probable role for the enhanced expression of c-myc in estradiol-stimulated growth of HBC cells. Recently Studzinski et al. (27) have reported that the c-myc gene product regulates cell proliferation at the level of DNA synthesis, affecting the activity of DNA polymerase α. Also Edwards et al. (28) showed that antiestrogens decreased DNA polymerase activity in MCF-7 while addition of estradiol restored the activity. These findings together with ours would indicate that estradiol increases the accumulation of the c-myc mRNA the product of which in turn interacts with DNA polymerase α to activate DNA synthesis.

Two interesting observations were made by comparing c-myc expression in ER+ versus ER− cell lines (Figs. 4–6): (a) neither the removal nor addition of estradiol nor the presence of tamoxifen has an effect on c-myc expression in ER− cells; (b) high constitutive expression of c-myc mRNA occurred in all three ER− cell lines. In BT-20 and MDA-MB-231, this expression is not due to amplification of the c-myc gene, whereas gene amplification appears to be able to account for the increased c-myc mRNA in the HBL-100 cells. These observations suggest the possibility that in HBC cells the ability to express unregulated high levels of c-myc may be a prerequisite for estrogen-independent cell growth. Thus, estradiol regulation of c-myc expression may represent a necessary step in the mechanism by which estrogen enhances the growth of ER+ human breast cancer cells.

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