Estrogen Regulation of Placental Alkaline Phosphatase Gene Expression in a Human Endometrial Adenocarcinoma Cell Line

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ABSTRACT

A human endometrial tumor (Ishikawa) cell line in culture responded to estradiol stimulation, as measured by growth and alkaline phosphatase activity. These effects were similar whether the medium was enriched with serum or was serum-free. Estradiol increased placental alkaline phosphatase activity 2–3-fold over control in these Ishikawa cells. The mechanism for this increase appeared to be at the level of transcription, at least in part, since there was an increase in the concentration of placental alkaline phosphatase mRNA. The administration of tamoxifen or 4-hydroxytamoxifen was unable to antagonize the estradiol-stimulated alkaline phosphatase enzyme activity or mRNA expression. The administration of tamoxifen alone had no effect on alkaline phosphatase enzyme activity, but tamoxifen did stimulate the steady state concentration of alkaline phosphatase mRNA. In contrast, a new antiestrogen, ICI 164,384, was able to antagonize both of these estradiol-stimulated effects.

INTRODUCTION

Human endometrial carcinomas have long been recognized as estrogen-responsive tumors that occur with greater frequency in women with prolonged exposures to unopposed estrogens, whether the exposure is endogenous, as in the case of women with chronic anovulation (1), or exogenous, as in the case of menopausal women on estrogen replacement therapy without progesterone replacement (2, 3). A spectrum of precancerous lesions exist in the endometrium which show hyperplastic tendencies and cellular atypia prior to malignant transformation (4, 5). The cellular and molecular basis for the role of estrogens in initiating these changes is not well characterized but is undoubtedly related to the known potential of steroids to initiate cell proliferation. Tumor cell lines in continuous culture have proved useful for elucidating the roles of steroid hormones in breast cancer and directing the various antiestrogen therapies for this disease (e.g., MCF7 cells) (6, 7). Unfortunately, human endometrial adenocarcinoma cells in continuous culture have proved inconsistent in their responses to steroid hormones, thus making it difficult to formulate an in vitro model for estrogen regulation of these tumors. The Ishikawa cell line was established by Nishida et al. (8), at the University of Tsukuba (Ibaraki, Japan), from a human endometrial adenocarcinoma. Previous studies with these cells have demonstrated their estrogen responsiveness with respect to cell growth and the activity of the enzyme alkaline phosphatase (9, 10). A subclone (3-H-4) of the original Ishikawa cells was obtained as a gift from Dr. Masato Nishida (Department of Obstetrics and Gynecology, University of Tsukuba, Japan), for further investigation of the response to physiological concentrations of estradiol. The present study demonstrates that this Ishikawa subline did indeed respond to estrogen administration with an increase in cell number, placental alkaline phosphatase activity, and mRNA concentration. Furthermore, the hormonal regulation of the cells was independent of the presence of serum, which may contain unquantified growth factors.

Estrogen is known to regulate protein synthesis in a variety of tissues and cell lines (11, 12), but the molecular aspects of the mechanism of the alkaline phosphatase response in the Ishikawa endometrial adenocarcinoma line have not previously been investigated. Previous biochemical studies demonstrated that the placental alkaline phosphatase isoenzyme is the primary alkaline phosphatase stimulated in the Ishikawa cells (9). The availability of a specific cDNA probe for placental alkaline phosphatase enabled us to examine the estrogenic and antiestrogenic regulation of placental alkaline phosphatase mRNA.

MATERIALS AND METHODS

Cell Culture. Ishikawa cells (subclone 3-H-4) were maintained in minimal essential medium with Earle's salts and 15% "stripped" fetal bovine serum (GIBCO, Grand Island, NY). Stripped serum was obtained by treating the serum with dextran-coated charcoal to remove steroid hormones (10) and was used throughout all experiments. L-Glutamine, penicillin, streptomycin, and fungizone (Flow Laboratories, Inc., McLean, VA) were added at final concentrations of 2 mM, 100 IU/ml, 100 μg/ml, and 2.5 μg/ml, respectively. The cells were maintained in 75-cm² culture flasks (Falcon Plastics, Los Angeles, CA) and were plated at low density in 25-cm² culture flasks or 6-well plates for growth curve analysis. Equal aliquots of cell suspension were plated in appropriate medium in flasks or culture dishes. After cell attachment overnight, day zero counts were obtained in triplicate to verify that plating densities were uniform. Medium was replenished every 3 days. Cells were harvested for counting and/or plating by removing the medium and incubating attached cells with trypsin-EDTA (GIBCO), for 20 min. The cells were pelleted and the trypsin was removed by washing.

Cell culture media were modified in several ways for experimental conditions. All experiments were carried out in medium prepared as above, without phenol red (13). Estradiol (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 10⁻⁸ M in ethanol. Medium for control cell cultures was prepared with 0.1% ethanol. Tamoxifen and 4-hydroxytamoxifen were gifts from Stuart Pharmaceuticals (Wilmington, DE). ICI 164,384 was a gift from Dr. A. E. Wakeling of ICI Pharmaceuticals (Cheshire, UK). Final concentrations of tamoxifen, 4-hydroxytamoxifen, and ICI 164,384 were 10⁻⁶ M. Cells cultured in the absence of serum were grown in medium composed of a 1:1 ratio of Dulbecco’s minimal essential medium and Ham’s F12 (Sigma), supplemented with ITS’ Premix (Collaborative Research, Bedford, MA) according to the manufacturer’s instructions. ITS’ Premix contains 12.5 mg insulin, 12.5 mg transferrin, 12.5 μg selenite sodium, 2.5 mg bovine serum albumin, and 10.7 mg linoleic acid, in 20 ml solution.

Cell Counting. After harvesting and resuspending cells as detailed above, cell aliquots were diluted in Count-a-Part cell diluent (Diagnostic Technology, Inc., Hauppauge, NY) and counted in a Coulter counter (Coulter Electronics, Hialeah, FL).

Alkaline Phosphatase Assay. Frozen cell pellets were thawed and homogenized on ice in 200 μl of 10 mM Tris-HCl buffer, pH 7.6. Alkaline phosphatase activity was then assayed by the method of Garcia-Rozas et al. (14), using p-nitrophenylphosphate as substrate.
Activity was expressed per cell number, per mg protein, or per μg DNA. Protein was measured according to the method of Bradford (15). DNA was measured in frozen cell pellets homogenized in 10 mM Tris-HCl buffer, pH 7.6, according to the method of LaBarca and Paigen (16), using a TKO 100 DNA fluorimeter (Hoefer Scientific Instruments, San Francisco, CA).

RNA Isolation. RNA was isolated as previously described and polyadenylated RNA was selected by polythymidylate-cellulose chromatography (17). RNAs were transferred electrophoretically to Nytran (18) adenylated RNA was selected by polythymidylate-cellulose chromatography (17). RNAs were transferred electrophoretically to Nytran (18) hybridized to nick-translated cDNA probes (19), washed, and detected by autoradiography. Autoradiograms were scanned using a video densitometer (Technology Resources, Inc., Nashville, TN).

cDNA Probes. The cDNA probe for placental alkaline phosphatase was generously provided by Dr. Harry Harris (Department of Human Genetics, University of Pennsylvania) (20). The 1A cDNA probe was obtained from a cDNA λ gt-10 library of rat uterine RNA and was previously shown not to be hormonally regulated (21).

RESULTS

Previous reports indicated that the Ishikawa cell line is estrogen responsive (9, 10). The results presented in Fig. 1A demonstrate that the difference between the number of cells grown in control or estrogen-treated medium was apparent after 5 days. Both control and estrogen-treated cells exhibited a plateau in cell density after 10 days. The increase in cell density was the greatest between 7 and 10 days following exposure to estradiol.

In order to exclude possible influences of growth factors present in serum, cells were cultured in serum-free medium. Again growth, as measured by cell number, was stimulated 2–3-fold in estrogen-treated cells over controls after 5–7 days in culture (Fig. 1B). The cell density increased the most between days 3 and 5 of estrogen treatment. A plateau in growth occurred in both control and treated cells after 5 days.

In previous studies, alkaline phosphatase activity has been used as a marker of estrogen responsiveness, and activity of the enzyme showed a 5–8-fold increase compared with nonstimulated controls (9). The results in Fig. 2 indicate that, following 5 days of stimulation, alkaline phosphatase activity increased in treated cells compared with control cells under the same experimental conditions as described for Fig. 1. The increase in enzyme activity was consistent up to 9 days of stimulation. These results were obtained whether the increase in alkaline phosphatase activity was expressed per cell number (Fig. 2) or per mg protein (Fig. 3). In addition, in the absence of serum, alkaline phosphatase activity was increased in estrogen-treated cells, compared to controls, after 8 days of stimulation (data not shown).

Biochemical studies have shown that alkaline phosphatase can exist as three isoenzymes: intestinal, placental, and liver-bone-kidney (22, 23). The particular isoenzyme stimulated by estradiol was determined using an immunological assay (22). The liver-bone-kidney and placental isoenzymes represented 77.9 and 21.7%, respectively, of the total alkaline phosphatase activity in control cells. In estradiol-stimulated cells, the enzyme activity attributed to placental alkaline phosphatase isoenzyme increased to 43.1%, while the liver-bone-kidney isoenzyme contribution fell to 55.1%. The alkaline phosphatase activity contributed by the intestinal isoenzyme was less than 1% in both cases. The placental isoenzyme specific activity expressed per μg protein exhibited a 3-fold increase compared with controls.

In order to determine if antiestrogens block the estradiol-induced increase in alkaline phosphatase activity, cells were grown in the presence of 10^-6 M tamoxifen or 10^-6 M tamoxifen plus 10^-8 M estradiol. The results presented in Fig. 3A show that tamoxifen alone had no effect on alkaline phosphatase activity and, furthermore, tamoxifen did not block the estradiol-stimulated increase in enzyme activity. Since these effects might occur as a result of the cells' inability to convert tamoxifen to its more active metabolite 4-hydroxytamoxifen, the studies were repeated using 4-hydroxytamoxifen (10^-6 M). Fig. 3B demon-

![Graph](https://example.com/graph1.png)

**Fig. 1.** Effect of estradiol (10^-8 M) on Ishikawa cell proliferation. *A,* medium containing stripped serum was replaced and cells were counted every 3 days. *B,* effect of estradiol (10^-8 M) on Ishikawa cell proliferation in serum-free medium. Cell number (millions of cells) represents the mean of three plates/treatment group ± SE. *E2,* estradiol; *Con,* control.

![Graph](https://example.com/graph2.png)

**Fig. 2.** Effect of estradiol (10^-8 M) on alkaline phosphatase activity. Medium was replaced every 3 days. Cells were harvested on the 5th and 7th day in culture and alkaline phosphatase activity was determined. Alkaline phosphatase activity was expressed per cell number. The values represent the mean activity of three plates/treatment group ± SD.
The compound has been shown to have a much higher affinity for estradiol (10^{-8} M) (E2), 4-hydroxytamoxifen (10^{-6} M) (OH-T), or estradiol and 4-hydroxytamoxifen. B, cells were treated with both the rat uterus and another human breast cancer cell line to demonstrate dose-dependent pure estrogen antagonist effects on rat uterine estrogen receptors than does tamoxifen and demethoxytamoxifen. This new antiestrogen functions as a pure antiestrogen in MCF-7 cells (24). This new enzyme activity prevents the estradiol-stimulated increase in alkaline phosphatase activity (Fig. 3). The increase in the concentration of mRNA recognized by the placental alkaline phosphatase probe was similar in all treatment groups.

mRNA isolated from cells grown in the presence of estradiol, ICI 164,384, or ICI 164,384 plus estradiol was also hybridized with the radiolabeled human placental alkaline phosphatase cDNA probe. The results show that the 2.7-kilobase placental alkaline phosphatase mRNA was not detected in control cells or in cells grown in ICI 164,384 alone, whereas estradiol increased the concentration of this mRNA. Placental alkaline phosphatase mRNA was also not detected in cells grown in the presence of both estradiol and ICI 164,384. Again, the blot was stripped and rehybridized to 1A cDNA (Fig. 5B).

**DISCUSSION**

Previous studies demonstrated that the Ishikawa cell line is estrogen responsive (9, 10). Holinka et al. (10) showed that estradiol stimulates growth, when compared to the cell density achieved in control cultures. Furthermore, cells grown in the absence of estradiol which had reached maximal cell density could be stimulated further with the addition of estradiol (10). In the present study, estradiol also had a growth-promoting effect, as indicated by increased cell numbers compared to control cultures. However, this increase can be accounted for by a transient response seen several days after plating. Longer periods of culture did not result in a further difference in cell density. In fact, extended periods of culture resulted in a plateau of growth regulatory factors, such as transforming growth factor β (27), or may be a function of the lack of a suitable matrix.

Our results also support those of Holinka et al. (28) demonstrating that Ishikawa cells are able to grow and remain estrogen responsive in serum-free medium, but the maximal cell density is lower than in complete medium. These results suggest that estradiol has a growth-promoting effect independent of serum growth factors. However, it is possible that estrogen stimulates the cells to produce growth-regulating proteins, which in turn mediate the increase in cell numbers. In MCF-7 cells, estradiol has been reported to increase transforming growth factor α, resulting in increased cell growth (29, 30). Similarly, such factors could also account for the observed plateaus in cell growth, again as antiestrogens modulate the growth of MCF-7.
 Estradiol caused a 2-3-fold increase in alkaline phosphatase activity, compared to control cells. However, the antiestrogen tamoxifen was unable to block this induction by estradiol. The lack of antagonism by tamoxifen was not due to the inability of Ishikawa cells to metabolize tamoxifen to its active metabolite 4-hydroxytamoxifen, since 4-hydroxytamoxifen also was unable to negate the estradiol-mediated increase in alkaline phosphatase activity. These results differ from those of Holinka and colleagues (9, 31) which show that 4-hydroxytamoxifen inhibits the estradiol-stimulated increase in alkaline phosphatase activity. This discrepancy may be due to variations in the responses of different sublines of Ishikawa cells.

The results in this study suggest that the mechanism of induction of alkaline phosphatase activity was at the level of transcription, since the concentration of placental alkaline phosphatase mRNA was increased in cells grown in the presence of estradiol, while mRNA levels were undetectable in control cells. Interestingly, the concentration of placental alkaline phosphatase mRNA isolated from cells grown in the presence of tamoxifen was elevated, compared to control, in spite of the fact that the enzyme activity remained unchanged. The work of Cox and Elson demonstrated that corticosteroids are able to increase the catalytic efficiency of alkaline phosphatase activity 5-20-fold in HeLa cells (32). They postulated the corticosteroid regulation of a modifier of enzyme activity. The increased synthesis of the modifier, requiring RNA and protein synthesis, resulted in greater catalytic activity of the enzyme over controls. In the case of the Ishikawa cells, estrogen stimulation of alkaline phosphatase activity may be the result of a two-step process involving both an increase in placental alkaline phosphatase messenger RNA and the synthesis of an uncharacterized modifier of enzyme activity. Tamoxifen might, therefore, act like estrogen to increase the mRNA for alkaline phosphatase activity through the production of transforming growth factor β (27).

Alkaline phosphatase activity in Ishikawa cells is estrogen responsive (9, 31). In the present study, alkaline phosphatase activity was also used to study estrogen regulation of these cells. Estradiol caused a 2-3-fold increase in alkaline phosphatase activity, compared to control cells. However, the antiestrogen tamoxifen was unable to block this induction by estradiol. The
phosphatase but fail to affect the modifier, resulting in an increased concentration of mRNA without the corresponding increase in enzyme activity. The existence and steroid regulation of such a modifier remains to be established.

While most antiestrogens have some agonist effects (26, 33), a new antiestrogen, ICI 164,384, has been developed and reported to be a pure antiestrogen which binds to the estrogen receptor with greater affinity than tamoxifen (25, 26). In contrast to tamoxifen, ICI 164,384 blocked both the estrogen-induced increases in alkaline phosphatase enzyme activity and the increase in mRNA concentrations (Fig. 5). These results are in agreement with our data regarding the regulation of C3 gene expression in the luminal epithelial cells of the rat uterus (34). In this model, tamoxifen stimulates the synthesis and secretion of C3 that is also induced by estrogens (35). In addition, ICI 164,384 blocked the estradiol-stimulated increases in both C3 synthesis and mRNA concentrations. Together, these data support the finding that ICI 164,384 is a pure antiestrogen in several systems. The differing abilities of the ligands estradiol, tamoxifen, and ICI 164,384 to regulate gene expression may be accounted for by differences in receptor affinities or possibly conformational changes in the receptor molecule (33). These ligand-dependent conformational changes could influence either receptor interaction with estrogen-responsive elements in the DNA or the interaction with cell-specific factors responsible for the stimulation of transcription (36, 37).

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