Hormones, estrogen and prolactin, transforming growth factor-beta (TGF-β), and matrix metalloproteinases (MMPs) may modulate breast cancer progression. The goal of this research was to examine the regulation of expression of TGF-β and MMPs (MMP-1, 2, 9) by estrogen and prolactin, independently and in combination, at physiological doses, and at doses stimulating cancer cell (T47D) proliferation in vitro. Prolactin, and estrogen synergistically, and similarly, inhibited the expression of TGF-β and MMPs at physiological concentrations without altering cell proliferation, indicating a beneficial role of the hormones. The growth stimulating concentration of prolactin, but not estrogen, also inhibited the TGF-β and MMP expression.

**Keywords:** T47D cells; Hormones; Gene expression; Prolactin; Estrogen; Breast cancer

**1. Introduction**

Genetic factors in breast cancer etiology encompass mutations in BRCA, Atm, c-erbB2, c-myc, p53, and rb-1 genes [1–3]. In addition, increased levels of estrogen and prolactin have been implicated in breast cancer etiology, and antiestrogens, such as tamoxifen, form effective treatments for breast cancer [1]. Estrogen stimulates expression of prolactin and its receptor, and prolactin in turn can regulate the expression of estrogen receptors [4–6]. Estrogen and prolactin can simultaneously induce tumors in nude mice, and act as promoters or inhibitors of carcinogen-induced neoplasia based on host factors or hormone concentrations, in rat models [7,8].

The matrix metalloproteinases (MMPs) are among the proteases involved in the metastasis of tumors. MMPs can degrade the basement membrane and stroma. The MMPs include the collagenases (MMP-1), and gelatinases (MMP-2, MMP-9). The breast tumors and adjacent stromal cells secrete collagenases [1,9,10]. MMP-2 and MMP-9 are co-upregulated in breast cancer cells, with serum MMP-2 levels correlating with tumor advancement [10–13]. Estrogen is reported to induce MMPs or to not alter the expression of MMPs [14]. The role of prolactin, and the synergistic/additive effect of prolactin and estrogen on MMP expression in breast cancer cells have not been reported.

Transforming growth factor-beta (TGF-β) is the primary regulator of the MMPs, in addition to cell
proliferation. The effect of TGF-β on MMP expression is cell specific, and can be inhibitory or stimulatory [15]. Prolactin and estrogen, respectively, stimulate cell proliferation, and inhibit TGF-β expression in breast cancer cells [16–21]. TGF-β can be procarcinogenic or anticarcinogenic, independent of its regulation of cell proliferation or expression of MMPs. TGF-β is reported to enhance invasiveness of a mammary cancer cell line by up-regulating urokinase, but without altering cell proliferation and inhibiting MMPs [22]. The combination effect of prolactin and estrogen on breast cancer cell proliferation, and TGF-β expression has not been reported.

TGF-β and MMPs are among the critical factors mediating tumorigenesis, and hormones may play a role in breast cancer etiology. The purpose of this study was to determine the effects of prolactin, estrogen, and a combination of estrogen and prolactin on the expression of TGF-β and MMPs in a breast cancer cell line (T47D) at physiological concentrations, and at concentrations that stimulate cell proliferation in vitro.

2. Methods

2.1. Cell culture

T47-D (ATCC) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 0.1% insulin. The cells were maintained in a humidified incubator with 5% carbon dioxide at 37 °C. Subconfluent cells were subcultured, by trypsinization, for routine maintenance treatment. Cells were seeded at 10^5 cells/mm² overnight, prior to stimulation with ovine prolactin (Sigma) or β-estradiol (Sigma) or a combination of prolactin and estrogen (physiological hormone doses, and higher doses stimulating cell proliferation in vitro) in media lacking phenol red and supplemented with 0.5% serum (physiological hormone concentrations), or without serum (higher doses).

2.2. Cell viability

Several independent experiments were performed to determine dose response effects of the hormones on cell viability, following which (a) physiologically relevant doses, and (b) doses allowing for maximal cellular proliferation were chosen for further studies. To determine optimal doses, cell viability was measured using MTT (Sigma) or MTS assay (Promega). These assays measure the cleavage of a tetrazolium ring by viable cells to yield formazan, measured spectrophotometrically.

2.3. Indirect ELISA

Hundred milli litre aliquots of the media were added to a 96 well plate and incubated overnight. The media were discarded, the wells blocked, and incubated with 100 μl of appropriate primary antibody (TGF-β, MMPs) overnight. The wells were washed extensively and then incubated with a secondary antibody linked to peroxidase for 1 h at room temperature. The wells were rewashed extensively and 100 μl aliquots of peroxidase substrate were added until color development. The plates were read spectrophotometrically at 405 nm.

2.4. MMP-1 promoter activity

MMP-1 Promoter activity was determined by transfecting cells in 35-mm dishes with 10 μg of MMP-1 promoter linked to the chloramphenicol acetyl transferase (CAT) plasmid or MMP-1 promoter-CAT plasmid DNA where the AP-1 site had been replaced by a Sma I restriction site (plasmids were gifts from Dr William Parks, Washington University School of Medicine, St Louis, Missouri 63110). The transfection was performed with fugene or escort transfection reagent, (Sigma, and Boehringer Mannheim respectively) for 24 h prior to treatment with 10 nM prolactin, 0.5 mM estrogen or a combination of each for 24 h. CAT expression in cells was then determined via an indirect ELISA with a primary antibody against CAT.

2.5. Statistical analysis

The cell proliferation data were analyzed using a 2-tailed, paired student T-test at 95% confidence interval. All other data were statistically analyzed using a 2-tailed ANOVA and the Tukey HSD method at 95% confidence interval.
3. Results

3.1. Cell viability/proliferation

Cell viability/proliferation of T47D cells was not significantly altered by the lower/physiological concentrations of prolactin (0.1, 0.4, 1.2 nM) or estrogen (15, 50, 150 nM) (data not shown). The effects of these concentrations on the expression of TGF-β, and MMPs were similar, and hence the data corresponding to the middle dose of estrogen (50 nM) and prolactin (0.4 nM) are presented in this paper.

Examination of cell proliferation by prolactin (3–100 nM), and estrogen (0.1–11 mM) indicated maximal stimulation of cell proliferation at 10 nM prolactin (186% of control), and at 0.5 mM estrogen (171% of control) (data not shown). The effects of these cell proliferation stimulatory doses of prolactin (10 nM) and estrogen (0.5 mM) on the expression of TGF-β, and MMPs are also presented in this paper.

3.2. TGF-β expression

Prolactin caused a significant decrease in the level of TGF-β to 33% of control at 0.4 nM, and 64% of control at 10 nM (P < 0.05) (Fig. 1(a) and (b)). Treatment with estrogen significantly inhibited TGF-β expression to 33% of control at 50 nM (P < 0.05), but not at 0.5 mM (Fig. 1(a) and (b)). The combination of 0.4 nM prolactin and 50 nM estrogen inhibited TGF-β expression to 22% of control (P < 0.05), like estrogen or prolactin alone (Fig. 1a). The combination of 10 nM prolactin and 0.5 mM estrogen inhibited TGF-β expression to 76% of control (P < 0.05), similar to the effect of prolactin alone (Fig. 1b).

3.3. MMP-1 expression

Prolactin treatment significantly inhibited MMP-1 expression to 50% of control at 0.4 nM prolactin, and to 53% of control at 10 nM (P < 0.05) (Fig. 1(c) and (d)). Estrogen treatment significantly inhibited MMP-1 protein level to 36% of control (P < 0.05) at 50 nM, but caused an insignificant change at 0.5 mM (Fig. 1(c) and (d)). Treatment with both 50 nM estrogen and 0.4 nM prolactin significantly inhibited MMP-1 expression to 40% of control (P < 0.05), similar to the effects of estrogen or prolactin alone (Fig. 1(c)). A combination of 0.5 mM estrogen and 10 nM prolactin significantly inhibited MMP-1 expression to 59% of control levels (P < 0.05), similar to the effect of prolactin alone (Fig. 1(d)).

3.4. MMP-2, and MMP-9 expression

Treatment with 0.4 nM prolactin significantly inhibited MMP-2, and MMP-9 expression to 15, and 13% of control, respectively (P < 0.05) (Fig. 1(e)). Fifty nano meter estrogen significantly inhibited MMP-2, and MMP-9 expression to 53, and 42% of control, respectively (P < 0.05) (Fig. 1(e)). The combination of 0.4 nM prolactin and 0.5 mM estrogen caused a significant inhibition in MMP-2 protein levels to 29% of control (P < 0.05), intermediate in inhibition to that by estrogen, and prolactin alone (Fig. 1(e)). Treatment with 0.4 nM prolactin and 0.5 mM estrogen caused a significant inhibition in MMP-9 expression to 11% of control (P < 0.05), similar to the effect of prolactin alone (Fig. 1(e)).

3.5. MMP-1 promoter activity

Prolactin (10 nM), estrogen (0.5 mM), and a combination of estrogen and prolactin at these concentrations significantly and similarly inhibited MMP-1 promoter activity to 82, 80, and 83% of control, respectively (P < 0.05) (Fig. 1f). None of these hormonal treatments had a significant impact on MMP-1 mutant promoter activity (Fig. 1(f)).

4. Discussion

Our studies indicate synergistic, and similar, inhibition of TGF-β and MMPs by physiological doses of estrogen (50 nM), prolactin (0.4, 10 nM), and the combination of estrogen (50 nM) and prolactin (0.4 nM). The higher/non-physiological dose of estrogen (0.5 mM) did not alter TGF-β and MMP expression, and did not modulate the inhibitory effects of prolactin (10 nM), in combination with it. Simultaneous inhibition of TGF-β and MMPs, may indicate the positive regulation of MMPs by TGF-β in breast cancer cells. Further, the inhibition of expression of MMPs by the physiological concentrations of
the hormones suggests the inhibition of breast cancer metastasis.

Prolactin and estrogen inhibited MMP-1 promoter activity via the AP-1 sequence in the promoter, suggesting the involvement of the AP-1 transcription factor. Prolactin has been reported to activate AP-1 transcription factor [23,24]. Prolactin activates the stat transcription factors via Jak kinase, and estrogen regulates genes containing the estrogen response element via binding to its receptor/transcription factor, and both can cross-regulate gene expression [25,26].

Breast cancer progression is associated with the loss of steroid regulated cell growth primarily via the loss of steroid receptors, enhanced serum and/or tissue levels of transforming growth factor-β (TGF-β), and enhanced secretion of MMPs [27–30]. Gelatinases activities have been found to be higher in estrogen receptor negative breast cancer cells than estrogen positive breast cancer cells [30]. It can therefore be
inferred that the hormones at physiological concentrations are beneficial in preventing breast cancer progression in hormone responsive cells, by inhibiting TGF-β and MMPs. Conversely, it can be inferred that the metastatic potential may be conferred by prolactin or/and estrogen on breast cancer cells by the induction of MMPs in adjacent stromal cells responsive to these hormones, or via the additional factors such as urokinase activation, or due to the lack of responsiveness of the breast cancer cells to the hormones, due to higher local non-physiological concentrations.

In conclusion, our studies indicate a beneficial role of prolactin and estrogen via the inhibition of the expression of TGF-β and MMPs.

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