Featured Article

Regulating the Tumor Suppressor Gene Maspin in Breast Cancer Cells: A Potential Mechanism for the Anticancer Properties of Tamoxifen

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Abstract

Purpose: Mammary epithelial cells and the majority of breast cancer tumors require estrogen for continued growth. Antiestrogen therapy alone, or in combination with other drugs, has long been a common procedure for breast cancer treatment and prophylaxis. Thus, there is a critical need to elucidate the mechanism(s) of action of antiestrogen treatment, especially for patients who are at risk of breast cancer development or who are currently receiving hormone therapy. In this study, we examined the ability of hormones to regulate the expression of a tumor suppressor gene, maspin, which is a serine protease inhibitor (serpin) that plays an important role in mammary gland development and is silenced during breast cancer progression. Specifically, our hypothesis tested the clinical efficacy of tamoxifen to regulate maspin expression.

Experimental Design: We used maspin promoter luciferase reporter plasmids that were transfected into normal human mammary epithelial (HMEC1331) and MCF-7 breast cancer cells, followed by determination of the effect of hormones and their antagonists on maspin promoter activity. At the protein level, cytosolic fractions from both cell types before and after hormone treatment were subjected to Western blot analysis to determine maspin level.

Results and Conclusions: Our studies revealed that the antiestrogen tamoxifen induces maspin promoter activity. Interestingly, antiandrogen flutamide could also induce maspin in both cell lines tested. These observations were further confirmed in patient tissues. These novel findings provide a new mechanism of action for tamoxifen under normal and pathological conditions. More significantly, these findings could have a potential impact on future therapeutic intervention strategies for breast cancer.

Introduction

Maspin is present at high concentrations in normal mammary epithelial (and myoepithelial) cells, but its expression is down-regulated in primary breast cancer cell lines and lost in aggressive mammary carcinoma lines (1–3). Transfection of the mammary carcinoma cell line MDA-MB-435 with maspin cDNA significantly inhibited tumor growth and metastatic ability in nude mice (4), thus indicating a tumor suppressive activity for this protein. In addition, treatment of human breast and prostate cancer cells with recombinant maspin reduced cell motility (3–5).

Mammary epithelial cells and the majority of breast cancer tumors require estrogen for continued growth (6). Estrogen binds to estrogen receptor (ER), and the ensuing conformational change enables ER to recruit coactivators and induce estrogen-regulated gene expression. Antiestrogen therapy alone, or in combination with other drugs, has long been a common procedure for breast cancer treatment and prophylaxis (7). Tamoxifen (TAM) is a widely used nonsteroidal-selective ER modulator that has been shown to reduce the risk of invasive (and noninvasive) breast cancer in women (7, 8). In addition, TAM also reduces the occurrence of ER− invasive cancers by 69% and noninvasive cancers by 50% with no difference in the occurrence of ER− invasive cancer (7). TAM acts primarily by competing with 17β-estradiol (and other estrogens) in binding to ER. However, studies have unraveled non-ER-mediated mechanisms including signaling proteins, proto-oncogenes, and transforming growth factor β in TAM-mediated apoptosis (9–12). The clinical efficacy of TAM has been attributed to growth arrest and induction of apoptosis in breast cancer cells (9). However, prolonged TAM therapy could also lead to TAM resistance, thus requiring treatment cessation and/or alternative therapeutic approaches. As a prelude to understanding the mechanism of the action of TAM, we hypothesized that the antitumor action of tamoxifen could also be mediated, at least in part, through its ability to induce the tumor suppressor gene maspin. By using normal mammary epithelial and breast cancer cell lines, we tested the ability of tamoxifen to re-express maspin in maspin-deficient breast cancer cells.
Material and Methods

Tissue Specimens. Archival tissue, formalin-fixed paraffin-embedded specimens were obtained from the Department of Pathology at The University of Iowa. Patient 1 (43 years of age) had invasive ductal carcinoma [ER +, progesterone receptor (PR +)], whereas patient 2 (47 years of age) had ductal carcinoma in situ (ER −, PR +), they received 5 and 4 years of TAM therapy, respectively. Patients 3 (53 years of age) and 4 (56 years of age) had grade 3 invasive ductal carcinoma (ER −, PR −) and were also treated with TAM for 3 and 4 years, respectively.

Three primary tumor specimens were from patients who received no adjuvant hormone therapy (control group).

Tissue specimens were used in compliance with requirements of Institutional Review Board for the Protection of Human Subjects.

Cell Culture and Hormone Treatment. Normal human mammary epithelial cells (HMEC1331) were purchased from Biowhittaker, Inc. (Walkersville, MD) and maintained in defined mammary epithelial cell basal medium containing (5 mg/ml insulin, 10 μg/ml human epidermal growth factor, 0.5 mg/ml hydrocortisone, 52 mg/ml bovine pituitary extract and gentamicin; Bio- whittaker, Inc.). MCF-7 breast cancer cells were maintained in RPMI [containing 10% FCS and gentamicin (50 μg/ml)]. Cultures were determined to be Mycoplasma free using the Gen-Probe rapid detection system.

For hormone treatment studies, cultures were maintained in phenol red-free media 48 h before exposure to hormones at concentrations and times depicted in the figure legends.

Preparation of Cytosolic Fractions. Cytosolic fractions were prepared as described previously (13). Briefly, control and treated cells were lysed in buffer A [10 mM HEPES (pH 7.9) containing 10 mM NaCl, 1 mM DTT, 10% glycerol, 15 mM MgCl 2, 0.2 mM EDTA, 0.1% NP40, protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride], subjected to 3 freeze-thaw cycles and centrifuged (4500 × g, 10 min). The supernatant (cytosolic fraction) was removed, and the protein content was determined using bicinchoninic acid reagent. Equal amounts of cellular protein from various experimental treatments were subjected to SDS-PAGE and Western blot analysis using specific antibody to maspin (BD PharMingen, San Diego, CA). The reaction products were visualized using the enhanced chemiluminescence kit.

Hormone Regulation of Maspin Promoter. The maspin promoter luciferase-reporter assay was used to determine the effect of hormones on promoter activity. By using PCR and maspin promoter-specific primers, we generated the reporter constructs with amplicons spanning the maspin promoter region corresponding to −416 to +87, which contains the hormone-response element (HRE), proximal Ets and API1 sites, truncated promoter, and −956 to +87 (Fig. 1). By using site-directed mutagenesis, we generated a mutated promoter luciferase reporter construct (−416 to +87) with the HRE region AGATCAGA (−267 to −274), replaced by CCCCCCCCC (Fig. 1). These promoter regions were cloned into pGL3 basic vector (Promega, Madison, WI), and DNA sequences of the constructs were confirmed by DNA sequencing (The University of Iowa DNA Facility). HMEC1331 and MCF-7 cell lines were plated at 6 × 10 5 cells/well in 6-well dishes in hormone-depleted media. Cultures were transfected with 3–4 μg of DNA from the full-length, truncated, or the HRE-mutated maspin promoter reporter plasmids and an internal control plasmid (cytomegalovirus β-galactosidase expression plasmid to determine transfection efficiency) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). After 48 h, the cultures were treated with hormone supplemented media. We used 17β-estradiol, progesterone, and dihydrotestosterone at 10 −8 M, although TAM and flutamide (Sigma, St. Louis, MO) were used at 10 −7 M final concentration. Concentrations of TAM and flutamide were based on the reported concentrations found in tissues of patients receiving hormone therapy (14, 15). Following the hormone treatment, cell were harvested in reporter lysis buffer (Promega), and supernatant(s) were used to determine luciferase activity using the Luciferase Assay System (Promega) according to the manufacturer’s instruction. The results were expressed as the increased induction (or suppression) of the reporter plasmid after normalization against the internal control plasmid.

Immunohistochemical Analysis of Tissue Specimens. Archival tissues were sectioned at 4 μm, deparaffinized, and subjected to a water bath antigen-recovery protocol using citrate buffer (pH 6.0). Maspin expression was determined on the Dako Autostainer using the Vectastain universal Elite ABC Peroxidase kit (Vector Laboratories, Inc., Burlingame, CA). Monoclonal antibodies to maspin (BD PharMingen) were used at 1:200 dilution as described previously (16). All of the samples were reviewed by a board-certified pathologist (M. A. Vasef) who was blinded to the clinical outcome of these patients until the scoring had been completed. Maspin expression was determined by semiquantitative assessment of the stained tumor cells and the staining intensity.

Results

Previous studies have indicated the presence of HRE and API1 sites in the maspin promoter (17). By using the maspin promoter luciferase reporter construct, we were able to examine the direct effect of hormones on maspin promoter activity. The data indicate that 17β-estradiol, progesterone, and dihydrotestosterone had minimal effect on maspin promoter-derived luciferase activity in both cell lines tested (Fig. 1B). Flutamide exerted no significant changes in HMEC1331 cells but had some inductive effect in MCF-7 cells compared with untreated controls (Fig. 1B). The ER modulator TAM exerted an inductive effect on the promoter-driven luciferase activity, up to ∼10-fold in MCF-7 and ∼2.5 in HMEC1331 cells, as shown in Fig. 1B. This inductive effect was concentration and time dependent (data not shown). In addition, the effect of the hormones on both MCF-7 and HMEC1331 cells was mediated through the HRE in the maspin promoter, because the mutation of this site completely abolished the response to hormones (data not shown).

The promoter data were further supported by Western blot analysis of cell lysates collected from both HMEC1331 and MCF-7 cells, with and without hormone treatment. In the control primary HMEC1331 epithelial cells, both TAM and flutamide induced the expression of maspin (Fig. 2A). In MCF-7 cells, there was a very low level of cytosolic maspin under control conditions (hormone-depleted media), and this was fur-
ther induced in response to TAM and flutamide (Fig. 2B). In addition, the induction of maspin in response to TAM was time dependent (Fig. 2C). The stability of TAM-induced maspin expression was examined by treating the cultures with TAM (10^{-7} M) for 48 h. The media was removed; the cultures were rinsed with PBS and then maintained in TAM-free media for a further 48 h. Western blot analysis of cytosolic fractions from these cultures indicated that TAM induction was minimally altered upon removal of TAM and during the used time course (data not shown).

It was interesting to note that 17β-estradiol exerted some inhibitory effect on maspin levels in HMEC1331, although it had minimal to no effect on MCF-7 cells (Fig. 2).

Immunohistochemical analysis of tissue specimens from control individuals indicated the presence of maspin in myoepithelial and epithelial cells of the duct (Fig. 3A), although primary tumors presented with reduced levels of maspin (Fig. 3B). These observations were further confirmation of the vast body of literature already published on maspin expression in normal and primary tumor specimens (1–3, 18). In tissue from patients on TAM therapy, a very strong maspin staining intensity was observed in all four tissue specimens (Fig. 3C). Maspin was mostly in the cytoplasmic compartment with occasional nuclear association.

Discussion

Our studies indicate for the first time the ability of the widely used nonsteroidal-selective ER modulator, TAM, to induce the tumor suppressor gene maspin in MCF-7 cells, thus identifying a new mechanism (and benefit) associated with the clinical efficacy of this compound. The therapeutic effect of TAM (and its active metabolite 4-hydroxy tamoxifen) in hormone responsive breast cancers is believed to be because of its ability to compete with estrogen(s) for binding to ER (19). Both MCF-7 and normal mammary epithelial cells express ER-α and ER-β and thus could respond to estrogen(s) through transcriptional regulation of estrogen-regulated genes (20, 21). Interestingly, nonsteroidal antiandrogen, flutamide also exerted some inductive effect on the maspin promoter. However, this inductive effect of flutamide was more evident in MCF-7 compared with HMEC1331 cells. By using site-directed mutagenesis, we have demonstrated that TAM (and flutamide) act mostly through the HRE (and possibly the AP1) site(s) in the maspin promoter.
promoter, because the promoter luciferase construct with the HRE site AGATCAGA mutated to CCCCCCCC failed to respond analogously to hormone treatment.

Further corroboration of the promoter data was provided by Western blot analysis of cell lysates from control- and hormone-treated MCF-7 and HMEC1331 cells, which indicated induction of maspin protein after TAM treatment. The induction of maspin in response to TAM in MCF-7 cells could be indicative of a role for maspin in TAM-induced apoptosis. Studies from our laboratory and others have indicated a role for maspin in apoptosis in MCF-7 cell (13) and in animal models of breast cancer metastasis (22). However, it is also possible that TAM acting through the induction of a tumor suppressor gene (such as maspin) reduces the metastatic ability of breast cancer. This speculation is supported by studies indicating an inverse correlation between intracellular maspin levels and the invasive/metastatic ability of the breast cancer (1–3). It is of interest to note that TAM also induced myoepithelial cell expression of maspin, and this process was mediated through ER-β (myoepithelial cell lack ER-α; Ref. 23). These findings indicate that TAM could exert similar effect on two distinct breast cell types. The strong expression of maspin in the tumors of patients on TAM therapy lends further credence to the *in vitro* data. However, although produced from a small patient group, our data raises the concern that current treatment regimen for TAM although effective, is not sufficient in fully eradicating tumor recurrence. Therefore, it is imperative that a larger patient study is conducted to generate statistically relevant data to better understand the mechanism of TAM action and that the present clinical protocol(s) for TAM usage be re-evaluated and tightly monitored to improve drug efficacy and minimize induction of resistance and disease recurrence. In addition, it is important to consider that in some breast cancers that are maspin positive, this tumor suppressor may not be functional, thus its up-regulation by TAM in this context would be ineffective. Therefore, the status of other hormone-responsive key genes such as p53 and *Her-2/neu* has to be determined to further stratify these observations.

**Fig. 2** Western blot analysis of cytosolic fractions from control- and hormone-treated HMEC1331 (A) and MCF-7 cells (B) for maspin expression. HMEC1331 and MCF-7 cells plated in hormone-depleted media for 48 h were treated with different hormones for 48 h. Cytosolic proteins were extracted and subjected to SDS-PAGE and Western blot analysis. Coomassie Blue-stained section of the gels is depicted for loading control. C, time-dependent induction of maspin in response to tamoxifen (TAM) treatment. MCF-7 cells plated in hormone-depleted media were treated with TAM (at $10^{-7}$M) for 7 and 14 days, respectively. Cytosolic extracts were prepared and tested by SDS-PAGE and Western blot analysis for maspin. β actin was used for loading control. DHT, dihydrotestosterone; P, progesterone.
Our studies also reveal that 17β-estradiol treatment of HMEC1331 cells, which express a high basal maspin level, was associated with a decrease in maspin protein levels. On the basis of the published data, normal mammary epithelial cells express both ER-α and ER-β (20, 21). It remains to be determined whether in our system, the action of 17β-estradiol is mediated through ER-α or ER-β. The effect of flutamide on maspin induction in breast cancer cells simulates that of androgen withdrawal in prostate cancer, because recent studies have identified maspin induction after androgen ablation and/or radical prostatectomy (24). It is well documented that contrary to normal breast epithelial cells, breast cancer has a high level of androgen receptor (AR; also observed by us in the archival tissues tested), and the presence of this receptor is associated with high-grade and/or metastatic disease (25, 26). By using reverse transcription-PCR analysis, we have also confirmed that MCF-7 cells have a high level of AR with no detectable levels in HMEC1331 cells.5 This could indicate that the effect of flutamide on MCF-7 might be mostly mediated via AR. The induction of maspin by flutamide and the presence of AR in breast tumors could prompt the use of alternative approaches such as combined protocols to block both ER and AR in breast cancer.

Collectively, our data indicate that TAM is capable of regulating the expression of the tumor suppressor gene maspin in breast cancer cells. This observation could prompt the development of analogous compounds with directed and improved therapeutic efficacy and reduced adverse effects for breast cancer treatment.

References

5 Z. Khalkali-Ellis, unpublished observations.


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