The cis Decoy against the Estrogen Response Element Suppresses Breast Cancer Cells via Target Disrupting c-fos not Mitogen-activated Protein Kinase Activity

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ABSTRACT

Breast cancer, the most common malignancy in women, has been demonstrated to be associated with the steroid hormone estrogen and its receptor (ER), a ligand-activated transcription factor. Therefore, we developed a phosphorothiolate cis-element decoy against the estrogen response element (ERE decoy) to target disruption of ER DNA binding and transcriptional activity. Here, we showed that the ERE decoy potently ablated the 17β-estradiol-inducible cell proliferation and induced apoptosis of human breast carcinoma cells by functionally affecting expression of c-fos gene and AP-1 luciferase gene reporter activity. Specificity of the decoy was demonstrated by its ability to directly block ER binding to a cis-element probe and transactivation. Moreover, the decoy failed to inhibit ER-mediated mitogen-activated protein kinase signaling pathways and cell growth of ER-negative breast cancer cells. Taken together, these data suggest that estrogen-mediated cell growth of breast cancer cells can be preferentially restricted via targeted disruption of ER at the level of DNA binding by a novel and specific decoy strategy applied to steroid nuclear receptors.

INTRODUCTION

Breast cancer accounts for more deaths of American women than any other malignancy (1). Current therapy for primary breast cancer includes surgical resection with or without radiation or chemotherapy. Conventional adjuvant chemotherapy is suboptimal because it is associated with significant toxicity, and it may benefit only 20–25% of patients. This has motivated considerable effort toward finding novel therapeutic approaches for breast cancer (2–5).

ER, which belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors (6–8), plays a predominant role in estrogen-dependent breast cancer development and progression so that clinical targeting of the receptor has become an important treatment strategy (9, 10). ER can regulate gene transcription either by binding directly to the promoter of target genes or by binding indirectly through a mechanism involving other transcription factors (8, 11–13). Once the ER has bound estrogen and dimerized, it binds to ERE with a consensus sequence of 5'-GGTCAnmTACC-3' in the promoter region of genes. The ERE functions by initiating or enhancing the transcription potential of genes. Thus, disrupting DNA binding of ER could have a critical impact on the transcriptional role of ER.

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Materials and methods

Materials. ODNs were synthesized, and sequence targets were selected. The ERE decoy is a double-stranded phosphorothiolate 55-mer that exhibits a cis-element probe and transactivation of the p53 signaling pathway. However, CRE binding protein is a member of the CRE binding protein/activating transcription factor family that can activate CRE-transcription in response to cyclic AMP, Ca²⁺, and growth factor stimulation, which can occur in both normal and cancer cells. Piva et al. (23–25) studied modulation of ER gene expression using the decoy with specific PCR-generated DNA fragment or a decoy against the upstream promoter of the ER gene. In the case of breast cancer, the steroid hormone estrogen and its receptor ER are specifically associated with development and progression. After ER binding, the liganded ER activates transcription by an as yet unknown mechanism(s), resulting in stimulation of proliferation (26). It is reasonable to hypothesize that cis-element decoy against the ER may functionally interfere with ER DNA binding, subsequently suppressing breast cancer cells.

In this study, we developed a phosphorothiolate cis-element decoy against the ERE to target disruption of ER DNA binding and transcriptional activity, thus influencing estrogen-responsive gene expression and cell proliferation or apoptosis of human breast cancer cells. Moreover, the ERE decoy is proving to be an effective molecular tool to directly assess genes that could link ER/DNA interactions with cell growth.

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Cell Culture. The human breast carcinoma cell lines MCF-7 and MDAMB-231 were obtained from American Type Culture Collection and maintained in RPMI 1640 containing 10% FCS, 2 mm l-glutamine, penicillin, streptomycin (50 IU/ml and 50 μg/ml, respectively). The cells were deprived of serum for 24 h before stimulation. In experiments with E2, cells were cultured in phenol red-free RPMI 1640 (27).

Proliferation Assays. Cell proliferation was examined by measuring DNA synthesis using tritiated thymidine (3H-dThd) uptake (28). After transfection with the ERE or scrambled decoy ODN, the MCF-7 cells (50 × 10^3/well) were cultured in an 96-well microtiter plate in 200 μl of growth media using 5% FCS in the presence or absence of E2 (50 nM) for 16 h, pulsed for the remaining 4 h with [3H]thymidine (0.5 μCi/200 μl). [3H]Thymidine incorporation was analyzed by liquid scintillation counting.

ELISA to Detect Apoptosis. The cell death detection ELISA kit (Roche Diagnostics, Mannheim, Germany) was used to measure cytoplasmic histone-associated DNA fragment that result from the induction of apoptosis (29), according to the manufacturer’s instructions.

EMSAs. The end-labeled [32P] ODN probes correspond to the ERE consensus sequence: 5'-GATCCGTTAGCTACACC-TGACC-TGATGGAGCT-3' (27) or the RXRE consensus sequence: 5'-AGTTAGCTACAGGTCAGGT-3', respectively. The probe was incubated with 5 μg of nuclear extracted proteins in 15 μl of binding mixture [50 mM Tris-Cl (pH 7.4), 25 mM MgCl2, 0.5 mM DTT, and 50% glycerol] at 4°C for 15 min. For supershift assay, the nuclear extracts were preincubated with 1 μg of either normal rabbit serum or the specific antibodies to ER or RXRβ at 4°C for 30 min. The DNA-protein complexes were resolved on a 5% polyacrylamide gel. The dried gels were exposed to X-ray film (19).

Transfection of Luciferase Reporter Plasmids. Using the manufacturer’s instructions, FuGene-6 (Roche Diagnostics) was used to cotransfect the ERE-TK or RXRE-TK reporter plasmid and ERE decoy or scramble into cells (27). After transfection for 6 h, cells were followed by incubation in phenol red-free medium with or without 50 nM E2 for 16 h. Cell extracts were prepared using the reporter lysis buffer and measured by a luminometer (Monolight 3010; Pharmingen, San Diego, CA). All of the experiments were carried out in quadruplicate. The firefly luciferase activity was normalized with the Renilla activity (transfection efficiency) and with the protein content.

RPAss. Total RNA was isolated using TRIZol from treated and control cells. The mRNA was examined by RPA (27) using 20 μg of total RNA hybridized to 2 × 10^6 cpm of 32P-labeled probe corresponding to the multiprobe template sets hStress-1 overnight at 56°C. Unhybridized RNA was digested with RNase T1 and RNase A for 45 min at 30°C, then digested with proteinase K for 15 min at 37°C. After phenol/chloroform extraction and sodium acetate/ethanol precipitation, hybridized RNA probes were denatured at 90°C for 3 min and electrophoresed on a 5% polyacrylamide gel. The dried gels were exposed to X-ray film.

Western Blot Analysis. The cells (10^6 cells/ml) treated with the ERE or scrambled decoy ODN were solubilized in lysis buffer. Cell lysates were boiled in SDS sample buffer and subjected to 7.5% SDS-PAGE. All proteins were transferred to Immobilon-P (polyvinylidiene difluoride) membrane. Western blotting was performed by the mouse anti-human monoclonal c-Fos, antiactin, or ERK1/2 antibody that was diluted 1:1000 in blocking buffer (28, 30).

RESULTS

The ERE Decoy Inhibits E2-mediated Cell Proliferation and Induces Apoptosis of Human Breast Cancer Cells. The ER mediates breast cell proliferation and is the principal target for chemotherapy of breast carcinoma (31–33). Therefore, we first explored whether the ERE decoy could block estrogen-mediated MCF-7 cell growth. For this assay, the MCF-7 cells were treated with ERE decoy or scramble and stimulated by 50 nM E2. As presented in Fig. 1A, the ERE decoy inhibited the E2-stimulated [3H]thymidine incorporation of MCF-7 cells. In contrast, the scramble did not inhibit this cell growth stimulated by E2. Moreover, fluorescence-activated cell sorting showed the ERE decoy, but not the scramble ODN, also significantly inhibits BrdUrd incorporation and cell cycle progression (data not shown). These findings suggest that the ERE decoy effectively inhibits the E2-stimulated cell proliferation of breast cancer.

To identify if such inhibition of the ERE decoy on the human breast cancer cells may be dependent on the expression of ER, we also tested the effect of the ERE decoy on the ER-negative MDA-MB-231 breast cancer cells. Fig. 1B illustrated the ER ligand E2 could not stimulate [3H]thymidine incorporation of this cell line. In addition, the ERE decoy could not affect [3H]thymidine incorporation. These data indicate such inhibition of the ERE decoy on the human breast cancer cells is dependent on the expression of ER.

To further evaluate whether these cells treated by ERE decoy for longer time periods eventually undergo apoptosis, we used a cell death detection ELISA to assay cytoplasmic histone-associated DNA fragments. Fig. 1C showed treatment of ERE decoy, but not scramble ODNs, for 48 h could induce MCF-7 cell apoptosis in a dose-dependent manner even in the presence of E2, which is consistent with previous observations (34–36) on MCF-7 cells undergoing apoptosis after estrogen withdrawal.
ERE Decoy Specifically Binds to ER in Vitro. The ER typically activates gene transcription by binding to ERE (37, 38). To examine whether the interaction of the ER with ERE decoy can result in loss of specific binding to ERE, we carried out an EMSA using the recombinant ERα protein. Fig. 2A illustrates formation of an ER protein:ERE DNA probe complex (Lane a), which could be partially supershifted with anti-ER (Lane b) and 100-fold of the cold probe (Lane f) but not by the normal rabbit serum (Lane c) confirming its identity, indicating that recombinant ER can actively bind to the specific ERE sequences. Notably, incubation of recombinant ER with ERE decoy (Lane d), but not scramble (Lane e), results in substantial decrease in the ability of ER binding to ERE.

To additionally confirm the specificity of ERE decoy, we also performed an EMMA assay to test if the ERE decoy can affect RXR binding to its response element. Fig. 2B illustrates that recombinant RXRβ can specifically bind to the DNA probe containing specific response element sequences (Lane a), which could be partially supershifted with anti-RXRβ (Lane b) and 100-fold of the cold probe (Lane f), but not by the normal rabbit serum (Lane c), confirming its identity. Neither an ERE decoy (Lane d), nor a scramble (Lane e) affected the recombinant RXRβ protein binding to RXRE. Thus, the double-stranded phosphorothioate ERE decoy has unique ability to specifically bind to ER rather than other nuclear receptors such as RXR.

The ERE Decoy Specifically Blocks E2-induced ER DNA Binding Activity in Breast Cancer Cells in Vivo. DNA binding and transactivation of ER is critical for ligand-activated ER to regulate gene expression of breast cancer cells. Therefore, to clarify if the ERE decoy alters ER ability to bind to its cognate DNA ERE, we performed EMSA assays to test the effect of ERE decoy on the DNA binding activity of ER in breast cancer cell. Nuclear extracts prepared from MCF-7 cells treated with the ERE decoy in the presence of E2 or control cells were tested for their ability to bind a radiolabeled ERE ODN probe. As shown in Fig. 3A, nuclear extracts obtained from E2-stimulated MCF-7 cells displayed considerable ERE DNA binding activity (Lane b) as compared with equivalent protein samples obtained from non-E2 treated cells (Lane a). These E2-inducible DNA-protein complexes could be partially supershifted with anti-ER (Lane c), but not normal rabbit serum (Lane d), confirming its identity. The E2-induced ERE DNA binding was significantly decreased by the treatment of the ERE decoy (Lane e) but not by the scramble (Lane f). Therefore, the ERE decoy specifically blocks ER DNA binding activity in MCF-7 breast cancer cells.

ERE Decoy Specifically Blocks E2-induced ER Transactivation in Breast Cancer Cells. To assess whether the ERE decoy blocks the transactivation potential of ER in the human breast cancer cells, we transfected the ERE-luciferase reporter gene construct into MCF-7 cells to quantitatively analyze the effect of the ERE decoy on the E2-stimulated ER transactivation. As shown in Fig. 3B, E2 induced high ER luciferase activity in MCF-7 cells. ERE decoy substantially reduced transcription activity of ERE stimulated by E2. In contrast, the scramble ODN failed to show the inhibitory effect on transactivation of ERE. These observations suggest that the ERE decoy inhibits ERE DNA binding and subsequent transcriptional activity.

To additionally confirm the specificity of the ERE decoy on the ER transactivation, we also transfected another nuclear receptor, RXR luciferase reporter, into the MCF-7 cells. As shown in Fig. 3C, 9-cis-RA, a RXR-specific ligand, could markedly activate the transcription of RXR gene reporter. However, after treatment of the ERE decoy, inhibition of transactivation of RXR luciferase reporter was not observed. These data suggest the ERE decoy specifically targets inhibition of ER transcription activity.
c-fos is an estrogen-responsive gene target for ERE decoy inhibition of MCF-7 cell proliferation. It has been reported that ERE, a minimum length sequence, has two essential qualities, the ability to bind in a stable complex with ER and to mediate the induction of estrogen-responsive genes (38, 39). Several lines of evidence indicate that c-fos proto-oncogene induction by estrogens is a direct effect and, therefore, most likely mediated by the ER. An ERE that binds the ER and confers transcriptional activation to a heterologous promoter has been reported in the 5-flanking region of the human c-fos gene (40, 41). Therefore, we investigated the effect of ERE decoy on AP-1 promoter activity and c-fos gene expression. As shown in Fig. 4A, E2 markedly stimulated the c-fos mRNA expression, which is consistent with previous observations (42–44). Transfection of ERE decoy resulted in significant inhibition of the c-fos mRNA expression stimulated by estrogen compared with treatment with the scramble ODN. Similarly, decrease in c-Fos protein expression resulting from transfection with ERE decoy is displayed in Fig. 4B. Moreover, we cotransfected ERE decoy or scramble and AP-1 luciferase reporter into MCF-7 cells. As expected, transfection of ERE decoy, but not scramble, significantly reduced estrogen-induced AP-1 luciferase activity (Fig. 4C). These data suggested c-fos is a target estrogen-responsive gene for ERE decoy suppression of MCF-7 cell proliferation.

Fig. 4. c-Fos is a target for ERE decoy inhibition of MCF-7 cell proliferation. A. ERE decoy decreases the gene expression of c-fos. RPA analysis of mRNA was obtained from MCF-7 cells that were transfected with ERE decoy or control scramble at 37°C for 6 h and then stimulated with medium or 50 nM E2 for 1 h. MCF-7 cells mRNA were then hybridized with [32P]labeled RNA probes corresponding to transscripts for individual human c-fos (HSTRESS-1) according to PharnMingen protocol (see “Materials and Methods”). The autoradiograph of the RNase protected fragments were separated on 5% PAGE is shown. B. MCF-7 cells were transfected with ERE decoy or control scramble at 37°C for 6 h and then stimulated with medium or 50 nM E2 for 6 h. Cells were lysed and Western blotted with anti-c-fos or antiactin. Arrows indicate location of c-fos. C. ERE decoy inhibits AP-1 luciferase gene reporter activity. MCF-7 cells were cotransfected with a 3× AP-1 binding element–pGL3 promoter luciferase construct and an ERE decoy or control scramble. Cells were then stimulated with or without E2 (50 nM) for 16 h. Luciferase activity of lysed cells was measured and normalized.

ERE decoy targeted inhibits breast cancer cell growth

ERE Decoy Does not Affect Activation of MAPK. Estrogen activation of the MAPKs (45), Erk1 and Erk2, occurs in a variety of cell types. These studies indicate an involvement of the MAPK cascade in the proliferative response to E2 stimulation. Activation of MAPK family members Erk1 and Erk2 is triggered by the phosphorylation of a threonine and a tyrosine residue in their regulatory site. To determine whether MAPK inactivation may account for ERE inhibition of the proliferation regulated by E2, we assayed the effect of ERE decoy on the phosphorylation of Erk1 and Erk2 in the MCF-7 cells. As shown in Fig. 5, top panel, ERE decoy does not affect the phosphorylation of Erk1 and Erk2, although estrogen (50 nM) could up-regulate phosphorylation of these kinases. The blot was stripped and total level of Erk1 and Erk2 were detected, which displayed same loading levels (Fig. 5, bottom panel). These results indicate the inhibitory effect of ERE decoy on estrogen-mediated cell proliferation may not be mediated through inactivation of MAPK pathway.

DISCUSSION

The ERE Decoy Provides a Novel Molecular Intervention Approach to Estrogen-mediated Cell Growth and Apoptosis of Breast Cancer. Synthetic ODNs with high affinity for a target transcription factor can be introduced into target cells as decoy cis-elements to bind the factor and alter gene transcription (14–17, 46–48). Morishita et al. (49, 50) demonstrated the usefulness of the decoy approach in a rat model of restenosis. ER is a typical steroid nuclear receptor and functions as a ligand-activated transcriptional factor. Penolazzi et al. (23, 24) and Piva et al. (25) analyzed putative regulatory sequences localized inside P1 canonical promoter and P3 upstream promoter of ER gene by using the decoy strategy. Using the human breast cancer cell line ER-positive MCF-7, we first identified the effect of a cis-element decoy against the DNA binding element of ER on estrogen-mediated cell growth or apoptosis of human breast cancer cells. The specificity of ERE decoy on ER DNA binding and transactivation was characterized by using gel shift analysis and reporter gene assays. ERE decoy, but not scramble ODN, blocked the purified recombinant ER protein specifically bind to the ERE sequence in vitro. This inhibitory effect of ERE decoy on ER DNA binding and transactivation was also confirmed in the human breast cancer cells in vivo. However, ERE decoy failed to show significant inhibition on DNA binding and transactivation of the different class of nuclear receptor RXR. More importantly, specificity of ERE decoy on ER DNA binding domain was supported by its functional inability to affect cell growth of MDA-MB-231, an ER-negative cell line. Therefore, we believe that the ERE decoy may specifically react with the ERE sequences of target genes, leading to the significant loss of DNA binding and transactivation of ER. This event subsequently inhibits the ER-mediated gene expression and cell growth of the human breast...
cancer. The effectiveness of ERE decoy in breast cancer cells prompted the consideration of its use in therapy. The duration of TFD action is dependent, to a large extent, on the intracellular stability of the decoy ODN (14). The ERE decoy we used here is a double-stranded linear DNA with phosphothioation, which increases resistance to nuclease digestion. Moreover, the decoy at high concentration after direct exposure indeed inhibited MCF-7 cell proliferation (data not shown). Careful and systemic planning and improvement of this technology by addressing the method and timing of delivery, ODN stability and specificity of action (14, 46, 48) will facilitate the development of the ERE decoy as an important therapeutic modality for human breast cancer.

ERE Decoy Inhibits Breast Cancer Cell Proliferation via Down-Regulating c-fos Gene Expression but not Inactivation of MAPK.

EREs have been identified in hormone-responsive genes such as c-fos (41, 42) and c-jun (51, 52) but not c-myc (53), which bind the ER and are well suited to amplify tissue responses emanating from the initial ER:ERE interactions (54). Here, we provide the direct evidence that expression of c-fos gene is linked with ERE transcription by specifically blocking ERE DNA binding. Moreover, ERE decoy significantly reduced AP-1 luciferase activity. c-fos plays a key role in regulating cell proliferation and cell cycle entry through activation of cyclin D1. Crowe et al. (55) reported that c-fos function (i.e., cell cycle progression) is mediated at least, in part, by transcriptional down-regulation of p21Cip1/Waf1 using a c-fos/ER fusion construct in which this transcription factor is conditionally activated by E2.

Recently, several studies have indicated an involvement of the MAPK cascade in the proliferative response to E2 stimulation (56, 57). However, Lobenhofer and Marks (58) demonstrated that E2 treatment of MCF-7 cells stimulates cell cycle progression in the absence of detectable MAPK activation regardless of E2 or serum concentrations, cell density, or the method of hormone delivery. We have here found the ERE decoy does not affect activation of MAPK signal pathway, although estrogen (50 nM) markedly stimulates phospho-torbin of MAPK (Fig. 5). Our observations are in support of the concept that ERE decoy inhibition of E2-induced proliferation is based on the ability of this specific transcriptional factor decoy to attenuate ER binding to estrogen-responsive genes (c-fos) essential for mitogenesis rather than inactivation of the established mitogenic signaling pathway.

It is worthwhile to mention that PPARγ may also be involved in the effect of ERE decoy on breast cancer cells because the PPRE also contains an AGGTCA half-site. The ERE and PPRE differ in the topological arrangement of these half-sites. The ERE is a palindromic, whereas the PPRE is a direct repeat. Moreover, the affinity of the PPAR/HR heterodimer for the ERE is lower than for the PPRE (59). Wahl (59, 60) and Wang (61) observed signalizing cross-talk between PPAR/HRX and ER through ERE. More importantly, PPAR activation significantly inhibits cell growth of breast cancer cells (62). Therefore, whether the ERE decoy holds up against PPARγ in breast cancer cells awaits additional investigation.

Taken together, this is the first demonstration that transcription factor decoy may affect steroid-dependent cell growth or apoptosis of the human breast cancer and provides the proof of principle for a possible molecular intervention.

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