Expression of a Repressor of Estrogen Receptor Activity in Human Breast Tumors: Relationship to Some Known Prognostic Markers

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Abstract

The expression of a specific repressor of estrogen receptor activity (REA) was investigated by a semiquantitative reverse transcription-PCR assay in 40 human breast tumor biopsy samples with respect to steroid hormone receptor status and other known prognostic variables. The data showed that REA expression was positively correlated with estrogen receptor (ER) levels as defined by ligand-binding assays (Spearman $r = 0.3231; P = 0.0424$) and that the median level of REA mRNA was significantly (Mann-Whitney two-tailed test, $P = 0.0424$) higher in ER$^+$ tumors (median $= 94.5; n = 30$) compared with ER$^-$ tumors (median $= 64.5; n = 10$), with no significant differences ($P = 0.4998$) associated with progesterone receptor status alone. In addition, REA expression was inversely correlated with tumor grade (Spearman $r = -0.4375; P = 0.0054$). When the tumors were divided into two groups based on grade, REA expression was significantly (Mann-Whitney two-tailed test, $P = 0.0024$) higher in low-grade (median $= 97; n = 16$) compared with high-grade (median $= 76; n = 23$) tumors. These results provide preliminary data suggesting that the expression of REA varies among breast tumors and is correlated with known treatment response markers and inversely correlated with a marker of breast cancer progression. REA together with ER status may be an improved marker of endocrine therapy responsiveness in human breast cancer.

Introduction

Estrogens have important roles in both normal and neoplastic mammmary tissues; however, marked changes occur in estrogen action during both breast tumorigenesis and breast cancer progression (1). The mechanisms underlying altered estrogen signal transduction in target tissues is the focus of much research at present. Current concepts of estrogen action include cofactors that can either enhance or repress the transcriptional activity of the ER$^+$ (2). Recently, a highly specific repressor of the transcriptional activity of ligand-occupied ERs (ER-$\alpha$ and ER-$\beta$ but not other steroid hormone receptors such as PR or type II nuclear receptors) was identified and characterized using a yeast two-hybrid system (3). Furthermore, part of its mechanism of action appeared to involve functional competition with steroid hormone receptor coactivators such as SRC-1 (2). This repressor differed from previously identified corepressors such as nuclear receptor corepressor and silencing mediator for retinoid and thyroid hormone receptor; in that it was not structurally related to either of them, it showed great selectivity for ER as opposed to other steroid hormone or non-steroid-binding members of the nuclear receptor family, and it required ER to be bound to ligand with preferential effects being seen when the ligand was an antiestrogen (3). This repressor was therefore called REA. Because REA is selective for ER, it is highly relevant to investigate the expression of this gene in human breast tissues both normal and neoplastic.

Recently we demonstrated that REA is expressed in both normal and neoplastic human breast tissues, as measured by RT-PCR. Furthermore, the expression of REA was not significantly different between ER$^+$ breast tumors and their matched adjacent normal breast tissues (2). However, the tumor cohort in the previous study were all ER$^+$ as determined by ligand-binding assays and did not address the question of whether REA expression in breast tumors was correlated with known prognostic and endocrine treatment response markers. In this study, we investigated the relationship of REA expression in breast tumors to ER and PR status and other known prognostic variables.

Materials and Methods

Human Breast Tumors. Forty invasive ductal carcinomas were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status as determined by ligand-binding assays. The ER levels were 0–151 fmol/mg of protein, and 30 tumors were classified as ER$^+$ (defined as $>3$ fmol/mg of protein). PR levels were 0–285 fmol/mg of protein, and 20 tumors were classified as PR$^+$ (defined by $>10$ fmol/mg of protein). These tumors spanned a wide range of grade (grades 4–9), determined using the Nottingham grading system.

Cell Culture. T-47D human breast cancer cells were obtained from Dr. D. Edwards (Denver, CO), and MCF7 cells were obtained from the late Dr. W. McGuire (San Antonio, TX). T-47D cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 nm glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin as described previously (4). Cells were plated at $1 \times 10^6$ in 100-mm dishes and 2 days later treated with 10 nm medroxyprogesterone acetate and harvested at various times (1–48 h). MCF7 human breast cancer cells were depleted of estrogen by passaging stock cells twice in phenol red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum, 100 nm glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin (5% charcoal-stripped fetal bovine serum) as described previously (5). Cells were then plated as above in 5% charcoal-stripped fetal bovine serum and 2 days later treated with 10 nm estradiol-17$\beta$ and harvested for analysis at various times (1–48 h). The steroids were added directly from 1000$\times$ stock solutions in ethanol to achieve the required concentrations. The cells were harvested by scraping with a rubber policeman. After centrifugation, the cell pellet was frozen and stored at $-70^\circ$C until RNA was isolated.

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3. The abbreviations used are: ER, estrogen receptor; PR progesterone receptor; REA, repressor of estrogen receptor activity; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20-μm frozen tissue sections (20 sections per tumor) or cell pellets using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions and quantified spectrophotometrically. One μg of total RNA was reverse transcribed in a final volume of 25 μl as described previously (6).

Primers and PCR Conditions. The primers used were primer REAU (5’-CGA AAA ATC TCC CCT ACA-3’; sense; positions, GenBank Accession No. AF150962) and primer REAL (5’-CCT GCT TTT CTT TTT CTA CCA-3’; antisense; position, GenBank Accession No. AF150962). PCR amplifications were performed and PCR products analyzed as described previously (7) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 20 μl in the presence of 4 ng/μl of each primer and 0.3 μl of Taq DNA polymerase (Life Technologies). Each PCR consisted of 27 cycles (30 s at 57°C, 30 s at 72°C, and 30 s at 94°C) for measuring REA. PCR products were then separated on 1.8% agarose gels stained with ethidium bromide as described previously (7). Amplification of the ubiquitously expressed GAPDH cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (7). The identities of PCR products were confirmed by subcloning and sequencing, as reported previously (6).

Quantification and Statistical Analysis of REA Expression. After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst (Bio-Rad, Hercules, CA). At least three independent PCRs were performed. To control for variations between experiments, a value of 100% was arbitrarily assigned to the REA signal of one particular sample and all signals were expressed as a percentage of this signal. In parallel, GAPDH cDNA was amplified, and after analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst. Three independent PCRs were performed. Each GAPDH signal was also expressed as a percentage of the signal observed in the same tumor. For each sample, the average of REA signal was then expressed as a percentage of the GAPDH signal (arbitrary units).

Correlation between REA expression and tumor characteristics was tested by calculation of the Spearman coefficient, r. Differences between tumor subgroups were tested using the Mann-Whitney rank-sum test, two-sided.

Results

Measurement of REA mRNA Expression in Primary Human Breast Tumors with Different ER and PR Status. We previously developed a semiquantitative RT-PCR approach to measure REA mRNA in small amounts of human breast tissues (6). Cloning and sequencing confirmed the identity of the expected 397-bp PCR product as REA, and this PCR product was used to probe Northern blots of RNA extracted from human breast tumor biopsies as described previously (8). An ~1.5-kb transcript was detected, consistent with the previously described REA mRNA (Fig. 1). Varying levels of REA mRNA were detected in human breast tumor biopsy samples, which raised the question of whether the expression of REA in breast tumors was correlated with the known prognostic and treatment response variables, such as ER and PR status.

Tumors were identified according to their ER or PR status as described in “Materials and Methods.” After analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst. Ethidium bromide-stained gel of the RT-PCR analysis of some ER− and ER+ breast tumors is shown (top). The expected 397-bp REA PCR product (confirmed by sequence analysis) is shown.

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REA expression in human breast tumors

Fig. 3. For each tumor (n = 40), REA expression was quantified and expressed in arbitrary units corrected for GAPDH signal as described in “Materials and Methods.” The tumors were divided into low-grade (Nottingham grades 3–6; □) and high-grade (Nottingham grades 7–9; ○). The results are presented as a scatter graph. Arrows indicate the median value in each group. REA expression is significantly higher in low-grade tumors compared with high-grade tumors (Mann-Whitney two-tailed, P = 0.0024).

The current concept of the mechanism by which nuclear hormone receptors regulate gene transcription involves three main components as proposed by Katzenellenbogen et al. (9): the receptor, its ligands, and its coregulators. Coregulators appear to consist of at least two different groups of coregulators may underlie altered responses to steroid hormone agonists and antagonists (10–13). More recently, we and others have shown that the expression of the coactivators, steroid receptor RNA activator (16) and amplified in breast cancer-1 (15), were significantly increased in ER+ breast tumors and ER status alone, as defined by ligand-binding analysis, the level of REA mRNA was significantly (Mann-Whitney two-tailed test, P = 0.0424) higher in ER+ tumors (median, 94.5; n = 30) compared with ER- tumors (median, 64.5; n = 10), with no significant differences (P = 0.4988) associated with PR status alone (PR+ median, 91.5; n = 20; PR- median, 87.5; n = 20).

The relationship of the level of REA mRNA levels with ER status in human breast tumor biopsies suggested the hypothesis that REA expression may be regulated by estrogens and/or progestins. However, no effect of estrogen (10 nM estradiol-17β) on the steady-state REA mRNA levels in estrogen-depleted MCF7 cells was observed over a 48-h time span (data not shown). In addition, no effect of progesterin (10 nM medroxyprogesterone acetate) treatment on REA mRNA in T-47D cells was observed over a similar time span (data not shown). It was concluded that the expression of REA mRNA was not regulated by estrogens or progestins in human breast cancer cell lines.

Correlation of REA Expression with Tumor Characteristics.

Spearman analysis showed a significant correlation of the level of REA mRNA in the tumors with the level of ER as measured by ligand-binding assays (Spearman r = 0.3231; P = 0.042) but no significant correlation with the level of PR as measured by ligand-binding assays (Spearman r = 0.2777; P = 0.0841). These data are consistent with the data analyzed using clinically relevant cutoff values for ER (ER+ >3 fmol/mg of protein) and PR (PR+ >10 fmol/mg of protein) status as shown above. However, statistical significance of the correlation of REA mRNA and ER binding was lost when Spearman analysis was applied only to those tumors that were ER+ (>3 fmol/mg of protein). The level of REA mRNA was also found to be inversely correlated with tumor grade (Spearman r = -0.4375; P = 0.0054). When the tumors were divided into two groups based on grade (low, Nottingham grades 3–6; high, Nottingham grades 7–9), the level of REA mRNA (Fig. 3) was significantly (Mann-Whitney two-tailed test, P = 0.0024) higher in low-grade (median, 97; n = 16) compared with high-grade (median, 76; n = 23) tumors, which is consistent with the Spearman correlation analysis.

No significant correlations were found between the level of REA mRNA and age, nodal status, percentage of normal duct and lobular epithelium, or percentage of stromal or fat cell content within the tumor sections analyzed.

Discussion

Our data show that the level of REA mRNA in human breast tumors is significantly correlated with ER status and inversely correlated with grade. These data are the first to identify a correlation between REA mRNA expression and known prognostic and treatment response markers in human breast cancer biopsies. The positive correlation of REA and ER expression (a good prognostic variable and a marker of response to endocrine therapies) together with inverse correlation of REA expression and grade suggests that REA expression could also be a marker of good prognosis and likelihood of response to endocrine therapies such as antiestrogens. The loss of statistical significance of the correlation between ER levels and REA mRNA when only ER+ breast tumors were analyzed may be due to the reduced numbers of observations in that analysis (n = 30 compared with n = 40 for total tumor cohort) or may indicate the existence of some threshold effect associated with expression of ER and REA. This latter suggestion together with the lack of correlation of absolute ER levels and REA mRNA in ER+ tumors would be consistent with our observation that REA expression, at least at the RNA level, was found not to be regulated by estrogen.

REA has been identified as a protein that interacts in a yeast-two hybrid system with a dominant negative mutant ERα (3). It was shown to be a selective repressor of ER (both ERα and ERβ) transcriptional activity as determined in transient transfection assays using several estrogen-responsive element-containing promoters regulating a chloramphenicol acetyltransferase reporter gene. Cotransfection of a REA expression vector enhanced the potency of antiestrogens such as 4-hydroxytamoxifen and ICI 182780. Furthermore, REA competitively reversed coactivator, i.e., SRC-1, transcriptional enhancement of ER activity. Together these data suggest that REA is a corepressor of ER transcriptional activity.

Our data showed that the levels of expression of the two ER coactivators, steroid receptor RNA activator (14) and amplified in breast cancer-1 (15), were significantly increased in ER+ breast tumors compared with their normal adjacent breast tissues, whereas the level of REA, a repressor of ER activity, was not significantly different between the tumors and normal breast tissues in the same patient cohort. However, this investigation used only ER+ breast tumors and could not address the question of REA expression in relation to steroid receptor status and other prognostic variables in breast tumors. In addition, we and others have shown that the expression of the coactivators, steroid receptor RNA activator (16) and amplified in breast cancer-1 (17), varies among breast tumors and can be correlated with steroid receptor status in some cases. ER status itself is associated with grade, with most ER+ breast tumors being low grade and having low tumor proliferation rates,
defined by the percentage of S-phase cells (18), and this may contribute to the inverse relationship of REA with grade observed in this study. However, REA expression is more strongly inversely correlated with grade than positively with ER status; therefore, it is possible that a repressor of ER activity that can contribute to the proliferative activity of breast tumor cells could have a significant negative effect on breast cancer progression and thus functionally influence breast cancer progression. It is speculated that the coexpression of ER and REA may therefore provide better prognostic information than either alone.

ER status is also an important treatment response marker in human breast cancer (18) where the presence of ER in breast tumors increases the likelihood of response to endocrine therapies such as antiestrogens. However, a significant portion of ER+ tumors will not respond to tamoxifen initially, and of those tumors that do respond, many eventually will develop resistance to tamoxifen and other endocrine therapies (18). It has been speculated that altered relative ratios of coactivators and corepressors of ER may in part be a mechanism underlying such endocrine resistance. Direct proof of this hypothesis in vivo remains to be provided by measuring expression of the relevant genes in human breast tumors that are known to be clinically sensitive or resistant to tamoxifen and/or other endocrine therapies. However, the data presented here provide preliminary information that the expression of a specific repressor of ER activity varies among breast tumors and that expression is correlated with known treatment response markers and inversely correlated with a marker of breast cancer progression.

References

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