Altered Estrogen Receptor α and β Messenger RNA Expression during Human Breast Tumorigenesis

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Abstract

Using a multiplex reverse transcription-PCR assay, we compared the relative expression of estrogen receptor (ER) α and ER-β mRNA between adjacent samples of normal breast tissue and matched primary breast tumors obtained from 18 different patients. Within this cohort, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. No differences in the ratio of ER-α:ER-β expression were observed in the ER-negative cohort. However, in the ER-positive cohort, a significantly (P < 0.02) higher ER-α:ER-β ratio was observed in the tumor compared with that of the normal tissue component. Our data revealed that the increase in the ER-α:ER-β ratio was due primarily to a significant (P < 0.005) increase in ER-α mRNA expression in conjunction with a lower ER-β mRNA expression in the tumor compared with that of the normal compartment in some, but not all, ER-positive cases. These results suggest that the role of ER-α and ER-β-driven pathways and/or their interaction change during breast tumorigenesis.

Introduction

Until recently, estrogen action was thought to be mediated principally through a single ER3, ER-α, a member of the steroid/thyroid/retinoic acid receptor superfamily (1). As with other members of the family, the ER-α protein consists of several structural and functional domains (A-F). The NH2-terminal transactivation function (AF-1) of the receptor is located within the A-B regions, whereas the DNA binding, the ligand-binding domain, and the second transactivation function (AF-2) reside in the C and E regions of the molecule, respectively (2). Upon ligand binding, conformational changes occur, and two ER-α molecules complexed with the hormone bind specifically to EREs located upstream of target genes. Interactions between ER-α and accessory proteins ultimately lead to the modification of the transcription of these genes (3). Similarly, the ER-α/estrogen complex can interact with c-fos/c-jun complexes to activate the transcription of target genes through activator protein 1 enhancer elements (4). Recently, evidence has been presented that ER-α and ER-β, although not identical, show some overlap. It has therefore been suggested (5) that the two receptors might interact and have different functional roles. In breast cancer, this interaction may be involved in the development of endocrine resistance. We have therefore compared the relative expression of ER-α and ER-β in normal and tumor tissues and the cell lines MCF-7 and MDA-MB-231.

Materials and Methods

Human Breast Tissues and Cell Line. Eighteen cases were selected from the National Cancer Institute of Canada- Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as described previously (17). The presence of normal ducts and lobules (median n = 6; range, 2-13) as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. Seven tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor values ranging from 2.2-11.2 fmol/mg protein, as measured by the ligand binding assay. Eleven tumors were ER positive (range, 3.5-159 fmol/mg protein), with progesterone receptor values ranging from 5.8-134 fmol/mg protein. These tumors spanned a wide range of grade (grade, 5-9), which was determined using the Nottingham grading system. For all normal and tumor samples, the percentage of epithelial cells, stromal component, and fat has been estimated by observation of the adjacent paraffin-embedded sections. For normal tissue, the median of the percentage of epithelial cells, stroma, and fat observed within the sections was 10 (range, 5–30%), 50 (range, 5–85%), and 40% (range, 5–90%), respectively. For tumor tissues, the median of the percentage of epithelial tumor cells, normal epithelial cells, stroma, and fat within the sections was 40 (range, 20–60%), 2.5 (range, 0–10%), 37.5 (range, 20–65%), and 20% (range, 10–50%), respectively. Three tumors (T1, T2, and T3) shown in a previous study (12) to express low ER-β/high ER-α, high ER-β/low ER-α, and high ER-β/high ER-α mRNA levels, respectively, were used to validate a multiplex RT-PCR that was designed to determine the relative expression of ER-α:ER-β mRNA. MDA-MB-231 cells were grown and harvested, and the cell pellets were stored at −70°C, as described previously (17). Total RNA was extracted from 20 μm of tissue sections (15- and 5-μm sections for normal and tumor breast tissue, respectively) or cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s instructions. One μg of total RNA was reverse-transcribed in a final volume of 25 μl as described previously (17).

Primer and PCR Conditions. The primers used consisted of ER-β-U primer (5'-GTCCATCGCCAGTTATCACATC-3' (sense), located in ER-β 130-151) and ER-β-L primer (5'-GCCTTACATCTTCACACGA-3' (anti-
sense), located in ER-ß 371–352. The nucleotide positions given correspond to published sequences of human ER-ß cDNA (5). The other pair of primers used consisted of ER-α-U primer [5′-TGTGCAAGACTATGTCTCA-3′ (sense), located in ER-α 792–811] and ER-α-L primer [5′-GCTCTCTCCTCCTGGTTTTA-3′ (antisense), located in ER-α 940–922]. The nucleotide positions given correspond to published sequences of human ER-α cDNA (1). PCR amplifications were performed, and PCR products were analyzed as described previously, with minor modifications (12). Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 15 µl in the presence of 1 µCi of [α-32P]dCTP (3000 Ci/mmol), 2 ng/µl ER-α-U/ER-α-L and/or 4 ng/µl ER-β-U/ER-β-L, and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. 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 (12). PCR products were subcloned and sequenced as described previously (12).

**Multiplex PCR Validation.** Total RNA was extracted from MDA-MB-231 cells previously shown to express very low ER-α but higher ER-ß mRNA levels (12) and from tumors T1, T2, and T3, and the characteristics of which are described above. In the first series of experiments, six cDNA preparations were prepared that contained varying percentages of MDA-MB-231 and tumor T1 cDNA by mixing 10, 8, 6, 4, and 0 µl of MDA-MB-231 cDNA with 0, 2, 4, 6, 8, and 10 µl of tumor T1 cDNA (0, 20, 40, 60, 80, and 100% T1 cDNA, respectively). The same experiment was reproduced using a 10-fold dilution of these six cDNA preparations. A second series of experiments was performed in which the six cDNA preparations contained a constant amount of MDA-MB-231 cDNA (5 µl) and 0, 1, 2, 3, 4, and 5 µl of T1 cDNA in a final volume of 10 µl (0, 10, 20, 30, 40, and 50% T1 cDNA, respectively). A third series of experiments contained six cDNA preparations in which the amount of T1 cDNA was held constant (5 µl) with increasing amounts of 0, 1, 2, 3, 4, and 5 µl of MDA-MB-231 cDNA in a final volume of 10 µl (0, 10, 20, 30, 40, and 50% MDA-MB-231 cDNA, respectively). Finally, 1 µl of T1, T2, and T3 cDNA was amplified independently for 22, 26, 30, and 34 cycles. In every case, PCR products were separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands, the addition of 5 ml of scintillant (ICN Pharmaceuticals, Inc., Irvine, CA), and counting in a scintillation counter (Beckman Instruments).

**Quantification and Statistical Analyses.** To quantitate ER-α mRNA expression relative to ER-ß mRNA expression, coamplification of ER-α and ER-ß cDNAs was performed using the multiplex PCR described above. Quantification of the signals was carried out by the exclusion of the bands corresponding to ER-α and ER-ß cDNAs, the addition of scintillant, and scintillation counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the highest signal measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. Indeed, the same tissue sample showed variations between experiments, a value of 100% was assigned to the highest signal observed in the experiment. The average of ER-α and ER-ß signals was plotted as a function of the percentage of T1 cDNA input (Fig. 1a). A direct relationship was found. Similar results were obtained using a 10-fold dilution of the cDNA preparations (data not shown). Using a constant amount of MDA-MB-231 cDNA plus or minus increasing amounts of T1 cDNA (containing primarily ER-α cDNA), a linear increase in the ER-α:ER-ß ratio with increasing ER-α input (T1 cDNA) was found (Fig. 2, A and B). An inverse but linear relationship was obtained using a constant amount of T1 cDNA and increasing amounts of MDA-MB-231 cDNA (data not shown). Finally, the rank of ER-α:ER-ß ratios in T1, T2, and T3 cDNA is expressed as a percentage of the GAPDH signal. Differences between averages of log(ER-α:ER-ß) obtained for matched normal and tumor compartments were tested using the two-tailed Wilcoxon signed rank test. Differences in expression levels were tested using the Wilcoxon signed rank test.

**Results**

**Multiplex PCR as an Approach to Determine the Relative Expression of ER-α and ER-ß.** To determine the relative expression of ER-α and ER-ß mRNA within any individual sample, we used a multiplex PCR assay. In this assay, two sets of primers are added to each individual PCR, thus allowing the coamplification of both ER-α and ER-ß cDNAs in a single tube and therefore eliminating variation introduced due to differences in cDNA loading. To determine whether the results obtained from the multiplex PCR assay directly reflected the initial ER-α:ER-ß cDNA ratio, a series of preliminary experiments was conducted. In these experiments, four different cDNA preparations were used. MDA-MB-231 cells, breast tumor T1, breast tumor T2, and breast tumor T3 had been previously shown to contain high ER-ß/low ER-α, very low ER-ß/high ER-α, high ER-β/low ER-α, and high ER-ß/high ER-α mRNA levels, respectively (12). The first experiment consisted of the multiplex amplification of spiked cDNA preparations containing various percentages of MDA-MB-231 and T1 cDNAs (Fig. 1). As shown in Fig. 1a, the PCR signal corresponding to ER-ß in MDA-MB-231 cells decreased with decreasing input of MDA-MB-231 cDNA, and the ER-α signal increased with increasing input of T1 cDNA. Quantification of the ER-α:ER-ß ratio signals was plotted as a function of the percentage of T1 cDNA input (Fig. 1b). A direct relationship was found. Similar results were obtained using a 10-fold dilution of the cDNA preparations (data not shown). Using a constant amount of MDA-MB-231 cDNA plus or minus increasing amounts of T1 cDNA (containing primarily ER-α cDNA), a linear increase in the ER-α:ER-ß ratio with increasing ER-α input (T1 cDNA) was found (Fig. 2, A and B). An inverse but linear relationship was obtained using a constant amount of T1 cDNA and increasing amounts of MDA-MB-231 cDNA (data not shown). Finally, the rank of ER-α:ER-ß ratios in T1, T2, and T3 cDNA is expressed as a percentage of the GAPDH signal. Differences between averages of log(ER-α:ER-ß) obtained for matched normal and tumor compartments were tested using the two-tailed Wilcoxon signed rank test. Correlations were tested by calculation of the Spearman coefficient (r).
ER-α AND -β mRNA IN HUMAN BREAST TISSUE

Fig. 2. Multiplex amplification of a constant amount of MDA-MB-231 (low ER-α/high ER-β content) cDNA and an increasing amount of tumor T1 (low ER-β/high ER-α content) cDNA. An aliquot of solutions containing a constant amount of MDA-MB-231 cDNA and an increasing amount of tumor T1 cDNA was prepared, amplified by multiplex PCR, and separated on an acrylamide gel as specified in “Materials and Methods.” A, autoradiograph of the gel. B, the ER-α:ER-β ratio signals is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

Comparison of the Relative Expression of ER-α:ER-β mRNA in Adjacent Normal Breast Tissue and Matched Primary Breast Tumors. To determine whether alterations may occur in the contribution of ER-α and ER-β signaling during breast tumorigenesis, the relative expression of ER-α and ER-β mRNA was measured in matched normal and primary tumor tissues from 18 different patients. Within the cohort of tumors studied, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. Total RNA was extracted from the frozen tissue sections and analyzed by multiplex RT-PCR. Examples of the results obtained are shown in Fig. 3A. In both normal and tumor compartments, two PCR products migrating at an apparent size of 242 and 149 bp were obtained. These PCR products were identified by cloning and sequencing to correspond to ER-β and ER-α cDNA, respectively. ER-α and ER-β signals obtained in three independent multiplex PCRs were quantified as described in “Materials and Methods.” The ER-α:ER-β ratio was calculated for each sample, and the results for each matched sample are presented in Fig. 4A. Considering all cases together, no significant change in the ER-α:ER-β ratio was observed between normal and tumor compartments. The cases were then divided into two groups based on the ER positivity of their tumors. Once again,

T3 using the multiplex PCR assay was not affected by the number of cycles used for the PCR over a range of 22–34 cycles. The ranking was similar to that deduced using several independent PCR determinations of the ER-α and ER-β mRNA levels, i.e. T1 ER-α:ER-β > T3 ER-α:ER-β > T2 ER-α:ER-β (data not shown). Multiplex PCR performed under the described conditions therefore seemed to be a reliable method with which to compare small tissue samples for their relative expression of ER-α and ER-β mRNA.

Fig. 3. RT-PCR analysis of ER-α and ER-β expression within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from matched normal (N) and tumor (T) compartments of ER-positive (ER+) or ER-negative (ER−) tumors, as determined by ligand binding assay. Corresponding cDNA was amplified, and PCR products were separated on an acrylamide gel, as described in “Materials and Methods.” PCR was performed using: A, ER-α and ER-β primer sets in a single tube (multiplex PCR); B, ER-β-specific primers only; C, ER-α-specific primers only. D, for each sample, GAPDH cDNA was amplified in parallel, and PCR products were separated on an agarose gel, as described in “Materials and Methods.” M, molecular weight marker (φX174 RF DNA/HaeIII fragments; Life Technologies, Inc.).
within the ER-negative cohort, no difference in the ER-α:ER-β ratio was seen between normal tissue and matched tumors. In contrast, in the ER-positive tumor group, a significant increase (two-tailed Wilcoxon signed rank test, \( P < 0.02 \)) in the ER-α:ER-β ratio was observed in the tumor compartment compared with that of the normal compartment. It should be stressed that a significant correlation was found between the ER-α:ER-β ratio observed in the tumor compartment and ER status by binding (Spearman \( r = 0.603; P < 0.01 \)).

**Independent Measurement of ER-α and ER-β mRNA Expression within Matched Normal and Tumor Compartments.** The observed increase in the ER-α:ER-β mRNA ratio of ER-positive breast tumors versus matched normal tissue could result from a decrease in the absolute levels of ER-β mRNA and/or an increase in the absolute ER-α mRNA levels within the tumor compartment relative to the matched normal tissue. To distinguish between these possibilities, the ER-β and ER-α mRNA levels were determined individually in each sample by RT-PCR, using either ER-β-specific primers or ER-α-specific primers. Examples of the results obtained are shown in Fig. 3, B and C, respectively. In parallel, amplification of the ubiquitously expressed GAPDH cDNA was also performed (Fig. 3D). For each sample, the ER-β and ER-α cDNA signals were quantified, and the average of signals obtained in two independent PCRs was normalized to the GAPDH signal, as described in “Materials and Methods.” The results are shown in Fig. 4, B and C, for ER-β and ER-α, respectively. No significant change in ER-β or ER-α mRNA expression was observed between the normal and tumor compartments within the ER-negative cases. Although the difference did not reach statistical significance \( (P > 0.05) \), ER-β mRNA expression was higher in the normal compartment versus the matched tumor component in 8 of 11 (72%) ER-positive cases. A significant (two-tailed Wilcoxon-signed rank test, \( P < 0.05 \)) increase in ER-α mRNA expression was measured in the tumor compartment of ER-positive tumors compared with that of the matched normal tissues. ER-α and ER-β signals observed in normal or tumor compartments did not correlate with the cellular composition of the section analyzed, i.e. percentage of normal epithelial cells, tumor epithelial cells, stroma, or fat (data not shown). One should note that although not statistically significant, trends toward an association between ER status by binding and ER-α (Spearman \( r = 0.397; P = 0.10 \)) and ER-β (Spearman \( r = -0.4254; P = 0.07 \)) have been observed.

**Discussion**

The discovery of the expression of a second ER in both normal and neoplastic human mammary tissues (12–15), together with the known perturbations of estrogen and antiestrogen sensitivity during breast tumorigenesis and breast cancer progression (16, 18–21), necessitates an investigation of the function of ER-β in human mammary tissue and a reevaluation of the estrogen signal transduction system in these tissues. We have used a multiplex assay in which ER-α and ER-β cDNA are amplified in the same reaction to investigate the relative expression of ER-α and ER-β mRNAs between adjacent samples of normal breast tissue and matched primary breast tumors. The choice of an RT-PCR-based approach to address the question of the relative expression of both receptors has been dictated by several parameters: (a) the absence of any publication to date using antibodies to detect ER-β protein in human breast tissue suggests that reliable antibodies are not yet available for this purpose; and (b) the expression of ER-β mRNA is relatively low in breast tissue, as demonstrated by the time needed to observe a signal in epithelial human breast cells in in situ hybridization studies (14) and by much weaker signals obtained, compared with ER-α, when analyzing breast tissue samples by RNase protection assay.4 The multiplex PCR assay developed here seems to be a reliable method with which to compare tissue samples for their relative expression of ER-α and ER-β mRNA. It should be stressed, however, that despite the good overall correlation coefficient observed, samples with an ER-α:ER-β ratio of <2 may be less reliably compared with each other (Fig. 2). This could possibly be a limitation of the multiplex approach, which would likely have a higher impact when comparing ER-negative tumors, in which ER-α is known to be weakly expressed. Such a limitation of multiplex PCR analysis of genes expressed at very low levels has previously been reported and may be circumvented by increasing cDNA input (22).

Our data show that in the cohort of patients whose tumors are ER positive by ligand binding, the ratio of ER-α:ER-β is significantly higher in breast tumors than it is in adjacent normal tissues. This difference seems mainly due to an up-regulation of ER-α mRNA levels within the tumor compartment. This observation is in agreement with previous published data showing a generally higher expression of ER-α detected immunohistochemically in ER-positive breast tumors than in normal breast tissue (see Ref. 23 and references herein). However, it is possible that down-regulation of ER-β expression in the tumor tissue may also contribute to the altered ratio in some tumors. Although the difference did not reach statistical significance, 72% of the ER-positive cohort showed a trend in which ER-β expression was lower in the tumor when compared with the normal compartment. The study of larger numbers of cases will be necessary to confirm this trend. Similarly, although no correlations have been

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4 Unpublished observation.
observed between the expression of ER-α and ER-β assessed by targeted PCR and the cellular composition of the sections analyzed, one cannot exclude the possibility that such relationships might exist. The study of a larger number of samples will also clarify this issue. If these RNA studies are paralleled at the protein level, then our data suggest that a significant change in the ratio of these two ERs occurs between normal and neoplastic breast tissues. This would further suggest that the contribution of ER-α- and ER-β-driven pathways and/or their interactions changes in conjunction with breast tumorigenesis. The hypothesis that such changes in ER-α and ER-β signaling pathways may occur during tumorigenesis is also supported by the recent observations of Brandenberger et al. (24). These authors showed that ER-α mRNA expression is equal or slightly higher in ovarian cancer tissues compared with normal ovary tissues, and ER-β mRNA expression is decreased in ovarian tumor tissue. The measurement of the ER-α:ER-β ratio correlated with ER status as assessed by ligand binding assay. Moreover, trends toward a positive correlation between ER-α and ER status and toward a negative correlation between ER-β and ER status were observed. Together, these data suggest that ligand binding is mainly due to the ER-α protein.

We have previously observed that the apparent ER-α:ER-β ratio in breast tumors varies widely (12). Our current results using the multiplex RT-PCR approach confirm and support these previous observations. Given the differential activity of tamoxifen-like antiestrogens and/or their interactions changes in conjunction with breast tumorigenesis. The hypothesis that such changes in ER-α and ER-β signalings pathways may occur during tumorigenesis is also supported by the recent observations of Brandenberger et al. (24). These authors showed that ER-α mRNA expression is equal or slightly higher in ovarian cancer tissues compared with normal ovary tissues, and ER-β mRNA expression is decreased in ovarian tumor tissue. The measurement of the ER-α:ER-β ratio correlated with ER status as assessed by ligand binding assay. Moreover, trends toward a positive correlation between ER-α and ER status and toward a negative correlation between ER-β and ER status were observed. Together, these data suggest that ligand binding is mainly due to the ER-α protein.

In conclusion, our results provide evidence to support the hypothesis that altered ER-α and ER-β expression may have a significant role in alterations of estrogen action that occur during human breast cancer. 

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References

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